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**Synthesis and physicochemical analysis of pyrazinib and
quininib prodrugs and discovery of novel cysteinyl
leukotriene receptor 1 antagonists for cancer treatment**

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Declaration

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Abstract

Gastrointestinal cancers, particularly oesophageal adenocarcinoma (OAC), are aggressive diseases with a poor 5-year survival rate of less than 20%. Current treatment for OAC is neoadjuvant therapy, including chemotherapy and chemoradiation which achieve a complete pathological response in only 30% of patients. The lack of validated biomarkers to predict therapy response and the absence of approved radiosensitisers for preoperative use highlight the need for novel treatment strategies. Similarly, uveal melanoma (UM), a malignancy of the eye prone to metastasis, requires innovative therapeutic approaches. A significant challenge in developing treatments for these cancers is the poor water solubility of many promising drug candidates, which limits their bioavailability and efficacy.

This thesis focuses on improving the water solubility and chemical stability of two promising anti-cancer agents, pyrazinib and quininib, which are under development as adjunct treatments for OAC and metastatic uveal melanoma (MUM), respectively. Both compounds suffer from suboptimal physicochemical properties, particularly poor water solubility, which restricts their *in vivo* preclinical testing and clinical delivery.

Pyrazinib was identified through a phenotype-based screen *in vivo* in zebrafish and discovered as a promising radiosensitiser *in vitro* in an isogenic OAC radioresistance model. While pyrazinib demonstrates good stability over a range of pH values, it has poor aqueous solubility. Chapter 2 focuses on the enhancement of water solubility of pyrazinib by designing and synthesising prodrugs of pyrazinib aimed at improving aqueous solubility. Various synthetic routes were explored, including Perkin condensation and metal-catalysed reactions, to optimize the yield and efficiency of pyrazinib synthesis. This chapter also details the development of amino acid prodrugs of pyrazinib, investigating their chemical stability, enzymatic activation, and water solubility. Moreover, the synthesised prodrugs were investigated for their radiosensitising activity *in vitro* in isogenic OAC radio-resistance cell line.

Furthermore, in this thesis, identification of novel cysteinyl leukotriene receptor 1 (CysLT₁) antagonists were carried out by utilising a ligand-based virtual screening (LBVS) technique. Chapter 3 outlines the computational methodologies employed, including protein preparation, water analysis using 3D RISM, and ligand-protein interaction mapping. LBVS led to the discovery of potential CysLT₁ antagonists which were subsequently evaluated for their binding affinity and antagonist activity through *in vitro* assays. This chapter provides a comprehensive analysis of the interaction dynamics between the identified hits and the CysLT₁ receptor, offering insights into their therapeutic potential.

Quininib (Q1) was identified through a phenotype-based screen *in vivo* in zebrafish and discovered as an inhibitor of LTD₄-induced activation of the human CysLT₁ receptor but suffers from poor water solubility and stability. Chapter 4 covers the synthesis of quininib and its analogues (Q series: Q7, Q28 and Q29), focusing on improving water solubility and chemical stability. Biological evaluations, including cytotoxicity and antiproliferative assays, assessed the anticancer efficacy of these analogues in MUM cancer cell lines, demonstrating their potential as effective anticancer agents. Furthermore, the *in vivo* toxicity and anti-angiogenic activity of quininib analogues, pyrazinib amino acid prodrugs and selected CysLT₁ potent hit antagonists were evaluated using a zebrafish model. Chapter 5 highlights the suitability of the zebrafish model for preclinical drug screening, detailing the experimental protocols for assessing *in vivo* toxicity and inhibition of angiogenesis. The findings from these studies provide valuable insights into the safety and efficacy of the synthesised compounds, supporting their potential use in cancer treatment.

Overall, this thesis presents novel research concerning the synthesis, physicochemical, and biochemical evaluation of pyrazinib and quininib analogues, aiming to improve water solubility and offer new therapeutic options for OAC and MUM. The findings support the potential use of these compounds in cancer treatment, addressing critical challenges in drug development for these formidable cancers.

List of Abbreviations

MeCN	Acetonitrile
ABC	ATP binding cassette
ADME	Absorption, Distribution, Metabolism, and Excretion
ACE	Angiotensin converting enzyme
API	Active Pharmaceutical Ingredient
BCS	Biopharmaceutical Classification System
BP	British Pharmacopeia
Boc	Di-tert-butyl decarbonate
^{13}C	Carbon 13
CADD	Computer aided drug design
CES	Carboxylesterases
CNS	Central nervous system
CysLT ₁	Cysteinyl leukotriene receptor 1
DA	Dorsal aorta
DCC	N, N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DLAV	Dorsal longitudinal anastomotic vessel
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO- <i>d</i> ₆	Deuterated Dimethyl Sulfoxide
EC	Electrostatic Complementarity
ECAR	Extracellular acidification rate
EC ₅₀	Half Maximal Effective Concentration
ED ₅₀	Median Effective Dose
EGFP	Enhanced green fluorescent proteins
E3	Embryo growth medium
5-FU	5-fluorouracil
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FGFs	Fibroblast growth factors
Fmoc	Fluorenylmethyloxycarbonyl

FT-IR	Fourier-transform infrared
GI	Gastrointestinal cancers
GI ₅₀	Growth Inhibition of 50%
GERD	Gastroesophageal reflux disease
GPCR	G protein coupled receptors
Gy	Gray (unit of radiation dose)
¹ H	Proton
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
hpf	Hours post fertilisation
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HTS	High throughput screening
HRMS	High resolution mass spectrometry
Hz	Hertz
IC ₅₀	Half Maximal Inhibitory Concentration
IOA	Investigational oncology agents
ISV	Intersegmental vessels
IV	Intravenous
<i>J</i>	Coupling constant
LBVS	Ligand based virtual screening
LC-MS	Liquid Chromatography-Mass Spectrometry
LC ₅₀	Lethal Concentration for 50% of the population
MAOS	Microwave-Assisted Organic Synthesis
MDR	Multidrug resistance protein-1
MeOH	Methanol
MP	Mobile phase
MP	Melting point
MS	Mass spectrometry
MW	Molecular Weight
MW	Microwave
MUM	Metastatic uveal melanoma
MR	Molar refractivity
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance

OAC	Oesophageal adenocarcinoma
OCR	Oxygen Consumption rate
OSCC	Oesophageal cell carcinoma
OE33P	OE33 parental
OE33R	OE33 radioresistant
OHSS	Ovarian hyperstimulation syndrome
PBS	Phosphate buffered saline
PDB	Protein data bank
PE	Plating efficiency
PSA	Polar surface area
PTU	Phenylthiourea
QSAR	Quantitative structure activity relationship analysis
R _f	Retention factor
RT	Room temperature
RBVS	Receptor-based virtual screening
RMSE	Root Mean Square Deviation
RPMI	Roswell Park Memorial Institute
RPS	Radial plot score
Ro5	Rule of five
ROS	Reactive oxygen species
RO	Reverse osmosis
SAR	Structure activity relationship
SBVS	Structure based virtual screening
SIVs	Sub-intestinal Vessels
SM	Starting material
TEA	Triethylamine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TPSA	Topological Polar Surface Area
UM	Uveal Melanoma
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VS	Virtual screening
WHO	World Health Organisation

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Chapter 1: General Introduction

1. Introduction to the thesis

The introductory chapter establishes the context and motivation for this research, providing a detailed overview of gastrointestinal (GI) and uveal melanoma (UM) cancers. It examines the current landscape of treatment strategies, highlighting the limitations of existing therapies and the urgent need for novel drug candidates. This chapter also outlines the importance of drug aqueous solubility and novel strategies to improve the poor aqueous solubility of potent anti-cancer drugs.

1.1 Gastrointestinal and ocular cancers: Incidence

Cancer is a significant cause of mortality, accounting for approximately 13% of deaths worldwide, and is a leading cause of death in Ireland.¹ It is a complex disease that results from the interaction of multiple genes and environmental factors and accounts for approximately 30% of deaths every year in Ireland.² According to the World Health Organisation (WHO), there were an estimated 20 million new cancer cases and 9.7 million deaths globally in 2022.³ Additionally, a 2021 analysis showed that the number of cancer cases has risen by 1% among people younger than 50 years old globally since the mid-1990s.⁴

There are over 100 types of cancers and GI cancers are the 5th most common type worldwide.⁵ GI cancers affect the organs of the digestive system and include liver, stomach, colorectal, small intestine, pancreatic, and oesophageal cancers.⁵ A study reported that there were approximately 5 million new cases of GI cancers per year and 3.4 million related deaths worldwide in 2018.⁶ GI cancers account for 26% of all cancer cases and 35% of related deaths, indicating a high global incidence and mortality rate.⁷ Additionally, the Irish Cancer Society revealed in its statistical cancer report that there are approximately 42,000 cases of cancer in Ireland each year, of which > 2,800 cases are diagnosed as GI cancers.² Oesophageal cancer is a cancer of the food pipe and almost 520 people are diagnosed with it in Ireland annually. Males have a higher incidence rate of oesophageal cancer. It is classified into two major subtypes, oesophageal cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC).⁸ Both subtypes differ mainly in terms of tumour location. The focus of this study is on OAC, an aggressive disease with poor 5-year survival rates of < 20%.⁹ This type of cancer is associated with risk factors such as chronic gastroesophageal reflux disease (GERD), obesity, and smoking.⁹ A high rate of incidence for OAC in men has been observed in western industrialised countries including USA, Canada, Australia and in the European Union (**Figure 1.1**).⁸

In addition to GI cancers, ocular cancer is another type of cancer with a significant incidence. The global incidence of ocular cancer varies, with estimates suggesting approximately 6 to 7 cases per million people annually.¹⁰ In Ireland, there are approximately 70 new cases of ocular cancer each year.¹¹ Ocular cancer affects the eye and its surrounding tissues, including various types such as

veal melanoma (UM), retinoblastoma, and squamous cell carcinoma of the conjunctiva.¹² UM is a primary ocular tumour in adults and it is a rare cancer with a 5-year survival rate of > 95%.¹³ Ireland has one of the highest incidences of UM in the world. The incidence rate in Ireland is reported to be 9.5 cases per million per year, which is significantly higher than the European average of 6 cases per million and the US average of 5 cases per million.¹² Metastatic uveal melanoma (MUM) occurs when the cancer spreads beyond the eye to the distant body parts. Liver is commonly the site of metastasis for UM and the survival rate substantially drops once metastasis occurs.¹⁴ Overall, cancer continues to be a significant health challenge around the globe, with different types of cancers contributing to morbidity and mortality. The incidence and survival rates of different cancers highlight the need for continuous research, early detection, and advancements in treatment to enhance patient outcomes.

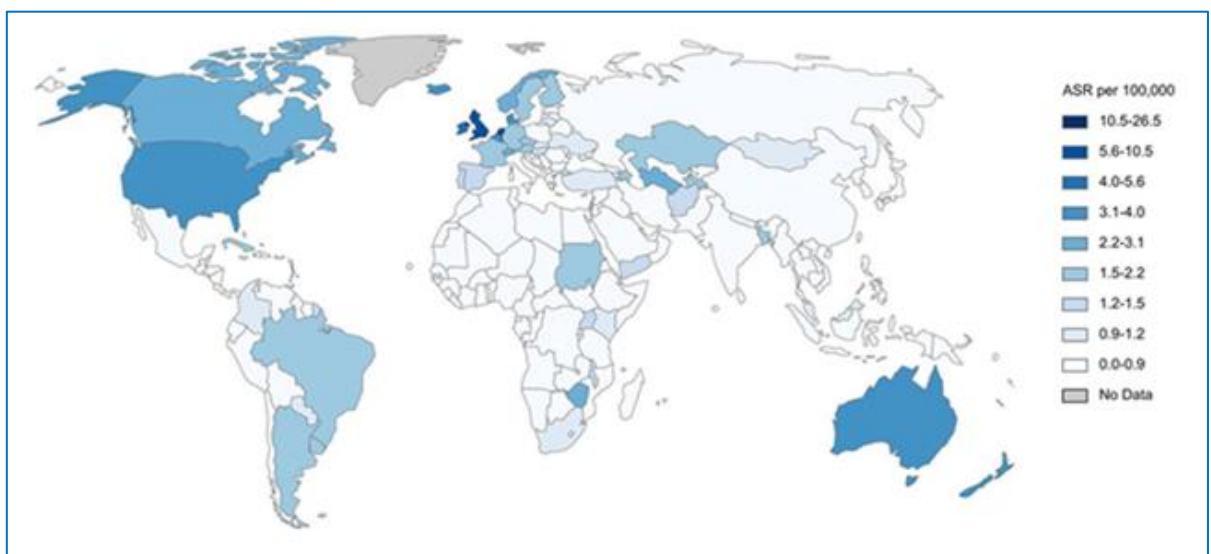


Figure 1.1: Annual incidence rate of OAC disease globally in males. Figure demonstrates the age standardised incidence rate per 100,000 men.

Image is taken from Smyth *et al.*⁸

1.1.1 Current treatment strategies

Cancer treatment often requires a multifaceted approach that combines surgery, chemotherapy, and radiation therapy to maximise therapeutic efficacy.¹⁵ This combination is essential for addressing the complexity of cancer, particularly in advanced stages, where monotherapy is frequently insufficient.¹⁶ Surgery is typically employed to remove the primary tumour, providing immediate and often curative intervention, especially in early-stage cancers.¹⁷ However, the invasive nature of surgery, along with its associated operative risks and extended recovery periods, necessitates the use of adjunct therapies such as chemotherapy and radiation therapy to target residual cancer cells and reduce the risk of recurrence. Chemotherapy is a systemic therapy which

utilises anti-cancer (cytotoxic) drugs or the combination of 2 or more drugs in a regimen to destroy cancer cells. One common type of regimen includes cisplatin and 5-fluorouracil, two chemotherapeutic drugs, discussed in Section 1.1.1.1.1. Additionally, chemotherapy can be used in combination with radiation therapy.

The standard treatment for OAC focuses on chemotherapy prior to surgery called neoadjuvant therapy, administering chemotherapy in combination with radiation. Surgery can be curative especially at early stages of OAC and offers immediate removal of tumour.¹⁸ However, surgery carries operative risks and lengthy recovery time. In contrast, neoadjuvant therapy aims to improve surgical outcomes by reducing the tumour size but its effectiveness varies among patients. Currently, there are no approved radiosensitisers for the treatment of OAC. Studies has shown that less than one third of patients show a beneficial response to neoadjuvant treatment, meaning 70% patients are unnecessarily exposed to toxicity and deconditioning.⁹ Hence, there is an urgent need for biomarkers to predict the response of neoadjuvant therapy.⁹ Similarly, the treatment of primary UM involves a multidisciplinary approach, with several advanced options available to patients such as chemotherapy, radiation therapy and local resection.¹⁹ Additionally, local resection can be performed in isolation or in combination with radiation therapy.¹⁹ For MUM, in clinical practice the mainstay treatments typically include systemic therapies such as chemotherapy, radiation therapy and immunotherapy. Emerging novel therapeutic approaches like targeting ferroptosis (cell death that involves iron-dependent lipid peroxidation) are areas of active research but not yet commonly used treatments in clinical practice for MUM.²⁰ Despite these advancements, systemic therapies remain crucial components in cancer treatment strategies.

1.1.1.1 Radiation therapy

Radiation therapy is a critical part of treating different types of cancer, such as GI and ocular cancers.²⁰ Radiation therapy can be used as the main treatment or in addition to surgery and chemotherapy. Its ability to specifically target cancer cells while protecting healthy tissue makes it an important tool in the fight against cancer. It is a treatment that uses powerful X-rays to penetrate the tumour tissue, destroying cancer cells and shrinking tumours through direct and indirect mechanisms (**Figure 1.2**).²⁰ In the direct mechanism, the radiation interacts directly with the DNA within the cell nucleus. In the indirect mechanism, radiation does not interact directly with the DNA but through the reaction of reactive oxygen species (ROS) within the cell.²¹ When radiation interacts with water molecules within the cells, it produces ROS including hydroxyl radicals. These ROS are highly reactive and can diffuse to DNA and cause similar types of damage as direct radiation (**Figure**

1.2).²² The effectiveness of radiation therapy can be significantly enhanced by the use of radiosensitisers.

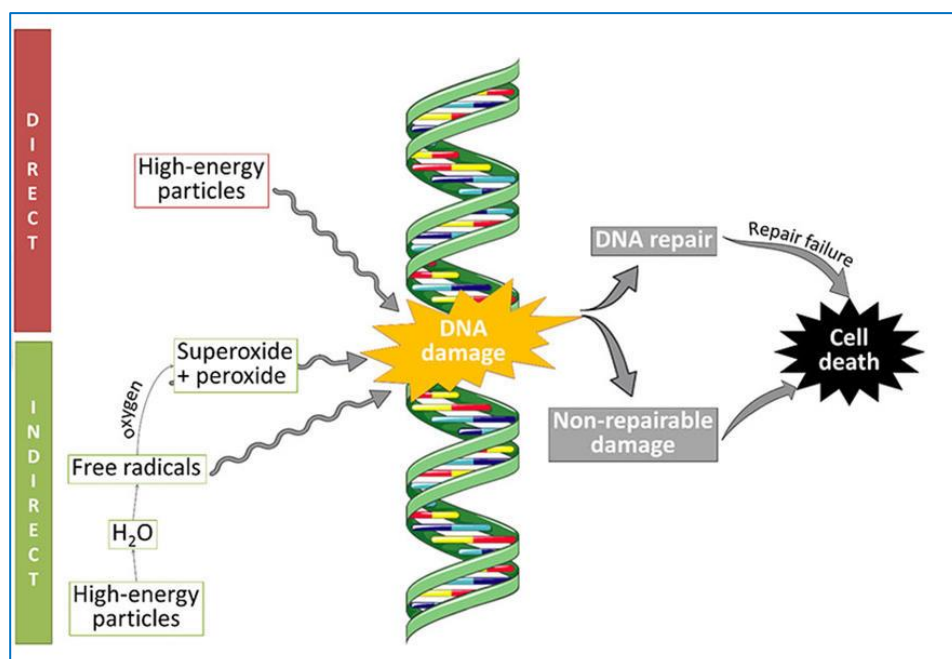


Figure 1.2: Schematic diagram of the mechanism of radiation therapy.

Image is taken from National Cancer Institute website.²¹

1.1.1.1.1 Role of radiosensitisers in cancer treatment

One of the critical advancements in enhancing the efficacy of radiation therapy is the use of radiosensitisers. Radiosensitisers are agents that make cancer cells more susceptible to the effects of radiation.²² They work by various mechanisms, such as altering the DNA repair processes of cancer cells, increasing oxygen levels within tumours, or modulating cellular pathways that influence radiation response.²² By increasing the sensitivity of cancer cells to radiation, radiosensitisers can improve therapeutic outcomes, allowing for lower doses of radiation to achieve even better results, thereby reducing the side effects and damage to healthy tissues.²² There are several marketed chemotherapeutic agents that also act as radiosensitisers and have shown promise in treatment of breast, ovarian, lung and GI cancer. For instance, cisplatin (a platinum-based drug), 5-fluorouracil (5-FU), paclitaxel, vincristine and temozolomide (**Figure 1.3**), act as radiosensitisers by enhancing DNA damage within cancer cells and inducing apoptosis.²³ Moreover, they are commonly used in chemoradiation regimens to shrink tumours before surgery, thereby improving surgical outcomes and overall survival rates.²³ In general, the integration of radiation therapy and radiosensitisers represents a significant advancement in the treatment of cancers. However,

poor water solubility can be a challenge with radiosensitisers, impacting their effectiveness and bioavailability in cancer therapy. Radiosensitisers mentioned, such as paclitaxel and vincristine are highly limited by poor water solubility.²⁴ This limitation can affect their delivery and distribution within the body, potentially influencing their radiosensitising effects.²⁴ Section 1.2 discusses the importance of drug aqueous solubility and various approaches to overcome poor water solubility.

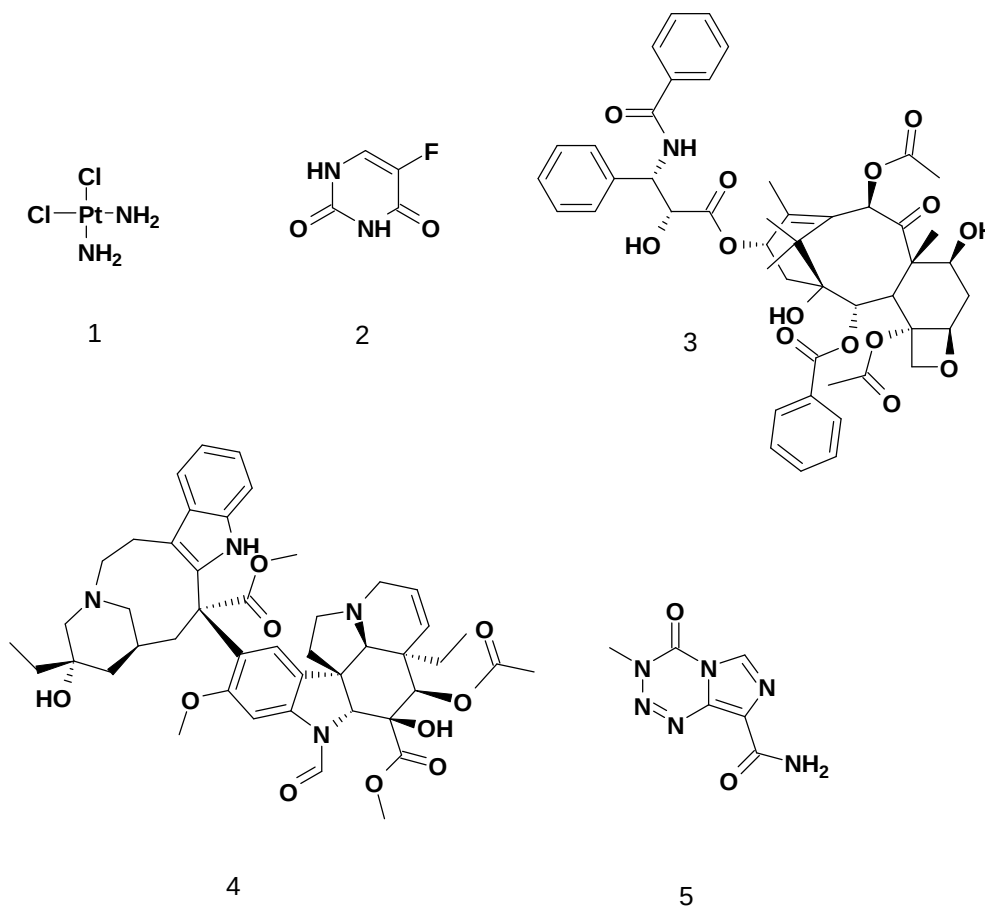


**Figure 1.3: Anticancer agents that enhance the effect of radiation during radiation therapy:
Cisplatin (1), 5-fluorouracil (2), Paclitaxel (3), Vincristine (4) and Temozolomide (5)**

1.2 Importance of drug aqueous solubility and membrane permeability

Drug absorption is a vital pharmacokinetics principle which involves the transportation of a drug from the site of administration to the site of action, for example to target cells or to cross several semipermeable cell membranes.²⁵ The most favourable and commonly employed route of drug intake is by oral ingestion of a drug due to convenient administration, cost-effectiveness, high patient compliance and flexibility in design of drug dosage form.²⁵ However, there are many hurdles that a drug needs to clear before it is deemed suitable for patient use and one of a major challenges in designing oral dosage drugs is their poor bioavailability.²⁶

The oral bioavailability of drugs depends on factors such as aqueous solubility, membrane permeability, pre-systemic metabolism and dissolution rate. However, the most common cause of poor oral bioavailability is the inadequate aqueous solubility and membrane permeability of a drug. Solubility and membrane permeability are the two major biopharmaceutical parameters of oral drug absorption.²⁶ Solubility can be defined in quantitative terms (**Table 1.1**) and in qualitative terms, referring to the ability of a substance to be dissolved in aqueous medium to form a homogenous solution. A drug with limited dissolution under these conditions is considered to have low aqueous solubility.²⁷ The solubility of drugs is affected by various factors, including molecular structure, particle size, crystal form, solvent, pH, temperature, and the inclusion of co-solvents and surfactants. These factors interact dynamically and require careful consideration during drug development to optimize solubility and enhance oral bioavailability of poorly water-soluble drugs.

Table 1.1 Solubility description and ranges according to British Pharmacopeia (BP).²⁸

Descriptive term	Solubility range (mg/mL)
Very soluble	>1000
Freely soluble	100-1000
Soluble	33-100
Sparingly soluble	10-33
Slightly soluble	1-10
Very slightly soluble	0.1 -1
Practically insoluble	< 0.1

A poorly water-soluble drug is likely to have slower absorption and may cause acute systemic toxicity. However, high local drug concentrations at the site of administration could result in precipitation, which may contribute to local toxicity effects.²⁹ Poorly soluble drugs are not a new phenomenon. Digoxin, known for its poor water-solubility and griseofulvin one of the earliest synthetic poorly water-soluble

drugs discovered in 1930, exemplify early challenges in drug solubility that spurred advancement in pharmaceutical development.^{30, 31} The solubility challenge is highlighted in the biopharmaceutical classification system (BCS). The BCS was developed in the 1990s as a scientific model to correlate *in vitro* solubility and permeability with potential *in vivo* performance of drug molecules (**Figure 1.4**).^{32, 33} The model was originally used by US Food and Drug Administration (FDA).³³

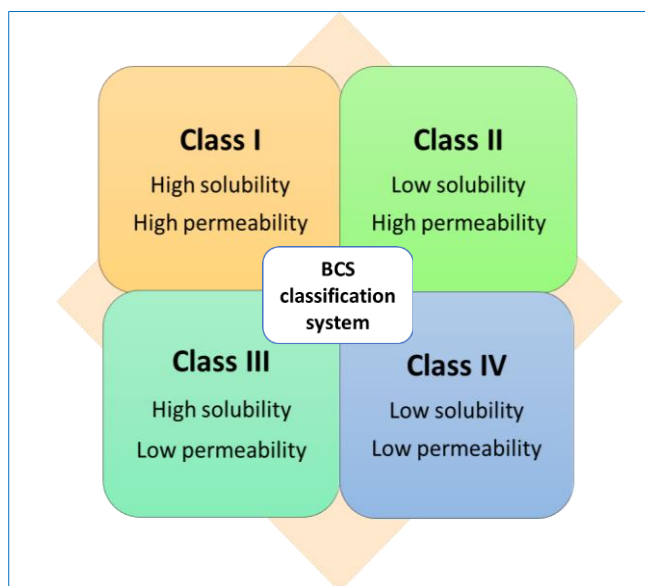


Figure 1.4: Biopharmaceutical classification system (BCS) model for the classification of APIs based on solubility and permeability.

Drugs that belong to BCS class I are characterised by their ability to easily dissolve in the gastrointestinal tract and efficiently cross cell membranes, leading to excellent bioavailability when administered orally. Conversely, class II drugs have low aqueous solubility but high permeability, making them suitable candidates for improving solubility. Class III drugs, while readily soluble in gastrointestinal fluids, face challenges in permeating intestinal membranes, necessitating enhancements in permeability to improve oral bioavailability. Meanwhile, class IV drugs exhibit both low aqueous solubility and permeability, often requiring non-oral dosage forms for effective delivery. Enhancing the solubility and permeability of class IV drugs is crucial to achieving therapeutic effects. Anticancer drugs can be categorised into various BCS classes. For instance, imatinib (**6**) and erlotinib (**7**) used in treating GI cancers belong to Class I and Class II, respectively. Imatinib is freely soluble in water, while erlotinib is practically insoluble in water but exhibits high cell permeability. In contrast, both doxorubicin (**8**) and paclitaxel (**3**, discussed in Section 1.1.1.1.1), both are chemotherapeutic agents. However, doxorubicin is categorised as a Class III due to its high solubility and low permeability while paclitaxel is classified as class IV due to its practical insolubility in water.³⁴ Figure 1.5 depicts the structures of imatinib, erlotinib and doxorubicin. Given that around 70–90% of drugs currently in the developmental pipeline

are categorised as having poor water solubility (BCS Class II and IV), there is a notable emphasis on enhancing solubility and permeability in pharmaceutical research.³⁵

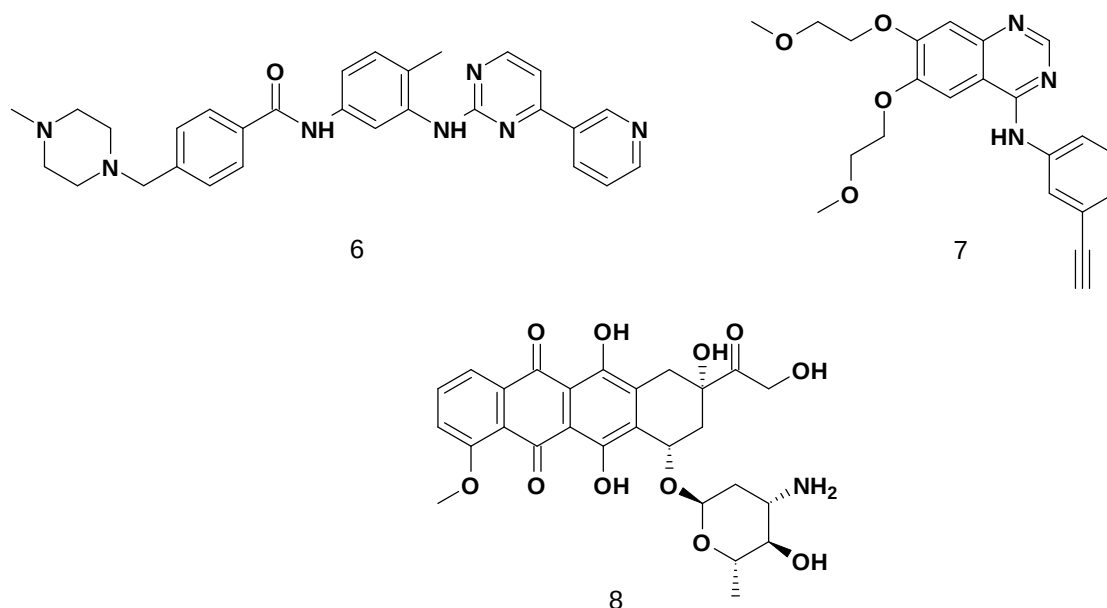


Figure 1.5: Chemical structures of anticancer agents; Imatinib (6), Erlotinib (7) and Doxorubicin (8)

As explained above, the cell permeability of a drug is a crucial parameter to be taken into account in development of effective drugs. The ability of a drug to cross the cell membrane is typically assessed based on its lipophilicity; generally, the more lipophilic the drug, the higher its likelihood of crossing the cell membrane. However, drugs that are more hydrophobic may become insoluble in body fluids potentially resulting in poor absorption.³⁶ Therefore, there needs to be an appropriate balance between water solubility (hydrophilicity) and membrane permeability (lipophilicity) to achieve a pharmacological response.³⁶ The aqueous solubility of a drug candidate is correlated to its lipophilicity which is estimated from its partition coefficient (LogP value). The LogP value is the logarithm of the partition of a drug into a two-phase system: hydrophilic (water) and lipophilic (octanol). There are different ways to measure drug solubility and cell permeability. For solubility, a shake-flask technique is commonly used. To predict cell permeability, computational methods can be used intensively which calculate a polar surface area (PSA) of a drug candidate.³⁷ Other popular methods to determine cell permeability include quantitative structure activity relationship analysis (QSAR), partial least square, support vector machines and Lipinski's rule of five (Ro5).³⁸ However, there are other rule based filters, such as Veber's and Ghose's rules. Medicinal chemists use Ro5 because it provides a clear, reliable framework for predicting a drug's oral bioavailability based on well-established criteria. Ro5 provides a

guide to a drug candidate's ability to cross the cell membrane and absorption at the site of action based on the criteria: no more than 5 hydrogen bond donors (e.g. OH and NH groups in drug molecule), molecular weight less than 500, LogP value lower than 5 and hydrogen bond acceptors less than 10 (oxygen, nitrogen, sulfur and fluorine atoms in a drug molecule).³⁸ There are several drug design approaches that have been introduced to improve low aqueous solubility of drug candidates. These strategies will be discussed in Sections 1.2.1 and 1.3.

1.2.1 Novel strategies of improving drug's aqueous solubility before formulation

Developing effective pharmaceutical formulations hinges on enhancing a drug's aqueous solubility, a critical step that ensures improved bioavailability and therapeutic efficacy. There are two routes available to address poor aqueous solubility of a drug candidate; improving the drug's characteristics in early development stage (API processing) through chemical approaches and optimising the formulation later in the developmental stage through physical approaches (**Figure 1.6**).³⁹ While it is possible to address solubility issues during formulation, addressing them early on eliminates surprises.³⁹ Occasionally, early drug development techniques such as reducing particle size, usage of dissolution enhancers, incorporating complexing agents, and utilising surfactants fail to enhance water solubility and decrease toxicity to acceptable levels. For instance, certain surfactants used in injectable formulations often have adverse effects, such as causing anaphylactic reactions. For these reasons, the salt formation and prodrug approach offer a safe and effective strategy to enhance drug solubility (discussed in Sections 1.2.1.1 and 1.3). Both of these strategies aim to overcome solubility challenges and pave the way for the development of more effective and readily accessible medications.⁴⁰

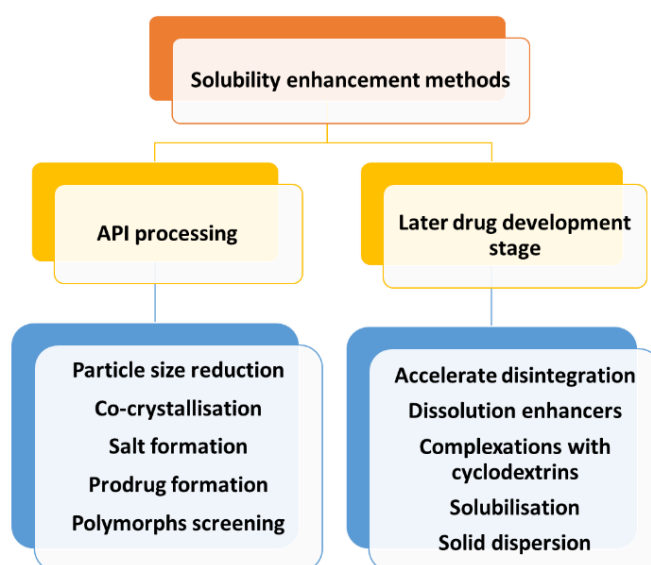


Figure 1.6: Methods to enhance drug aqueous solubility at API processing or optimising in a later drug development stage.

1.2.1.1 Salt formation technique

Salt formation is an effective strategy in drug development to enhance solubility. In addition, other parameters such as stability and bioavailability of poorly water soluble drugs can be positively influenced by this method.²⁸ It is a simple and well-established technique that is accepted by regulatory agencies to influence the solid-state properties of drugs.²⁸

The salt formation process involves reacting the drug/API with an appropriate acid or base to form a salt.⁴¹ For example, an acidic drug can be reacted with a base to form a salt, and vice versa for basic drugs. The resultant salt form often exhibits superior physicochemical properties compared to the parent compound, such as improved dissolution rate and enhanced stability. The choice of counterion (acid or base) is crucial as it influences the drug's properties.⁴¹ Common acids used as salt formers include hydrochloric acid, sulfuric acid, citric acid, fumaric acid, and tartaric acid. Common bases include sodium hydroxide, potassium hydroxide, and calcium hydroxide.

Salt formation is particularly significant with approximately 50% of all currently marketed small molecule APIs administered in their salt form.⁴² Several anticancer drugs benefit from salt formation to overcome solubility challenges. For instance, methotrexate sodium (**9**) improves the solubility of methotrexate, making it more effective for cancer treatment.⁴³ Similarly, imatinib mesylate (**10**) is used to improve the solubility and bioavailability of imatinib, enhancing its effectiveness in treating cancers like chronic myelogenous leukaemia.⁴⁴ Vincristine sulfate (**11**) is often administered in its sulfate salt form to improve solubility and bioavailability, enhancing its therapeutic action. These examples illustrate how converting anticancer drugs into their salt forms can effectively address solubility challenges, thereby improving their therapeutic efficacy and patient outcomes. These examples illustrate how converting anticancer drugs into their salt forms can effectively address solubility challenges, thereby improving their therapeutic efficacy and patient outcomes.

Salt formation is a widely accepted and effective strategy for improving physicochemical properties of drugs. One significant limitation is that this approach is only applicable to ionisable drugs. Additionally, the choice of counterion is crucial and can significantly impact the properties of the resultant salt. Poor selection of counterions can lead to undesirable effects such as instability or toxicity. To overcome this limitation and further optimise drug efficacy, an alternative prodrug approach can be employed. The prodrug approach is discussed in detail in Section 1.3.

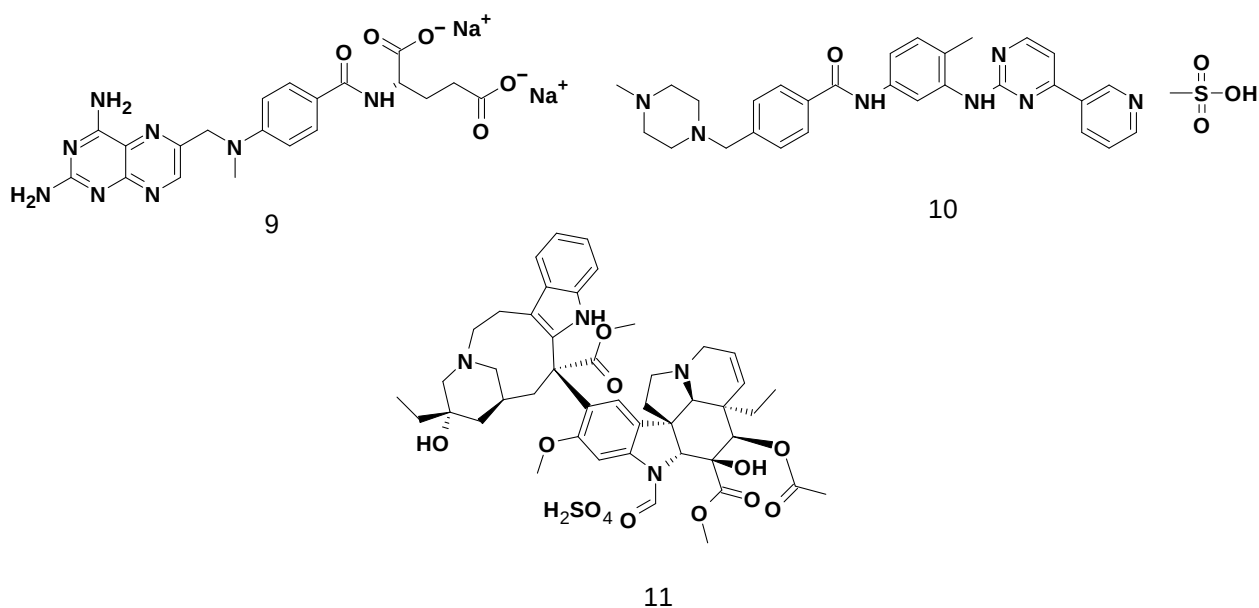


Figure 1.7: Chemical structures of anticancer drugs in their salt form: methotrexate sodium (9), imatinib mesylate (10) and vincristine sulfate (11).

1.3 The prodrug design and approach for improving drug aqueous solubility

The prodrug approach is a chemical approach which offers to decrease undesirable biopharmaceutical and physicochemical properties of pharmacologically active drugs. Introduced in the 1950's, the prodrug concept has long been successful due to its ability to chemically modify the active drug to enhance aqueous solubility and lipophilicity.⁴⁵ The prodrug approach has achieved tremendous success globally in the recent years. Over a past decade, the FDA has approved more than 12% of new drugs as prodrugs.⁴⁶ In the past, the prodrug approach was considered as the temporary solution for improvement of physicochemical properties of an active drug but this is not the case now. Implementation of the prodrug approach at the initial stages of drug research and development is growing.⁴⁷ The enhancement of biopharmaceutical and pharmaceutical properties of the active drug promotes the prodrug approach in the drug development process as it leads to various benefits such as reduction in cost, time and efforts.⁴⁸ There are three fundamental overlapping objectives of the prodrug approach which include pharmaceutical, pharmacokinetics and pharmacodynamic objectives. The pharmaceutical objective aims to enhance oral bioavailability, water solubility, stability and permeability. Pharmacokinetics and pharmacodynamics focus on improving oral absorption, achieving tissue selective delivery of active drugs, reducing toxicity and improving therapeutic index.

The term prodrugs relates to pharmacologically inactive or weakly active derivatives of an active drug designed to be able to bio-converted to the active parent drug within the human body.⁴⁷ A prodrug must undergo an activation process following intake in which it is converted to the active drug and pharmacological effects can then be exerted (**Figure 1.8**).⁴⁵ Prodrug activation will be discussed in detail in Section 1.3.2. Prodrugs can be delivered by many routes such as oral, intravenous, intramuscular, inhalation and transdermal. However, the most desirable route is oral administration due to the range of advantages such as high aqueous solubility, chemical stability and targeted delivery. For example, site specific delivery to the GI tract for the treatment of inflammatory bowel disease offers better dosing by avoiding degradation in stomach acid, potentially allowing for higher doses with fewer side effects.⁴⁹

In prodrug design, carrier linked prodrugs are the traditional approach and can be classified as bipartite and tripartite prodrugs.⁵⁰ Bipartite prodrugs are simple prodrugs in which the carrier is directly attached to the parent drug, and tripartite prodrugs have a spacer which connects the carrier to the parent drug. Common chemical moieties used as carriers include esters, amides, carbamates, carbonates, ethers, imines, and phosphates (**Figure 1.8**).⁵⁰ A special subtype of carrier-linked prodrugs are mutual prodrugs, which involve two active compounds, each acting as a carrier for the other, thereby enhancing effectiveness through synergistic action. Another variant are macromolecular prodrugs, which use polymeric backbones as carriers.⁵¹ These are commonly used in the development of prodrugs that are designed to be cleaved within a cell and in targeted drug delivery systems. This approach improves drug solubility, stability, release, and pharmacokinetics. Furthermore, it helps in the accumulation of the drug at the site of action, thus improving safety.⁵¹

In addition to these conventional methods, modern prodrug approaches have emerged, leveraging advances in molecular biology and nanotechnology.⁵² These approaches include the use of enzyme-responsive prodrugs that are activated in specific biological environments as well as prodrugs that utilise nanocarriers for targeted delivery and controlled release.⁵² These innovative strategies further enhance the solubility, bioavailability, and therapeutic index of drugs, providing a versatile toolkit for drug development.⁵² The primary emphasis of this research is on ester-linked prodrugs. As we transition into discussing ester prodrugs, it is essential to note their substantial role within carrier-linked prodrugs, highlighting their significance in altering drug properties.

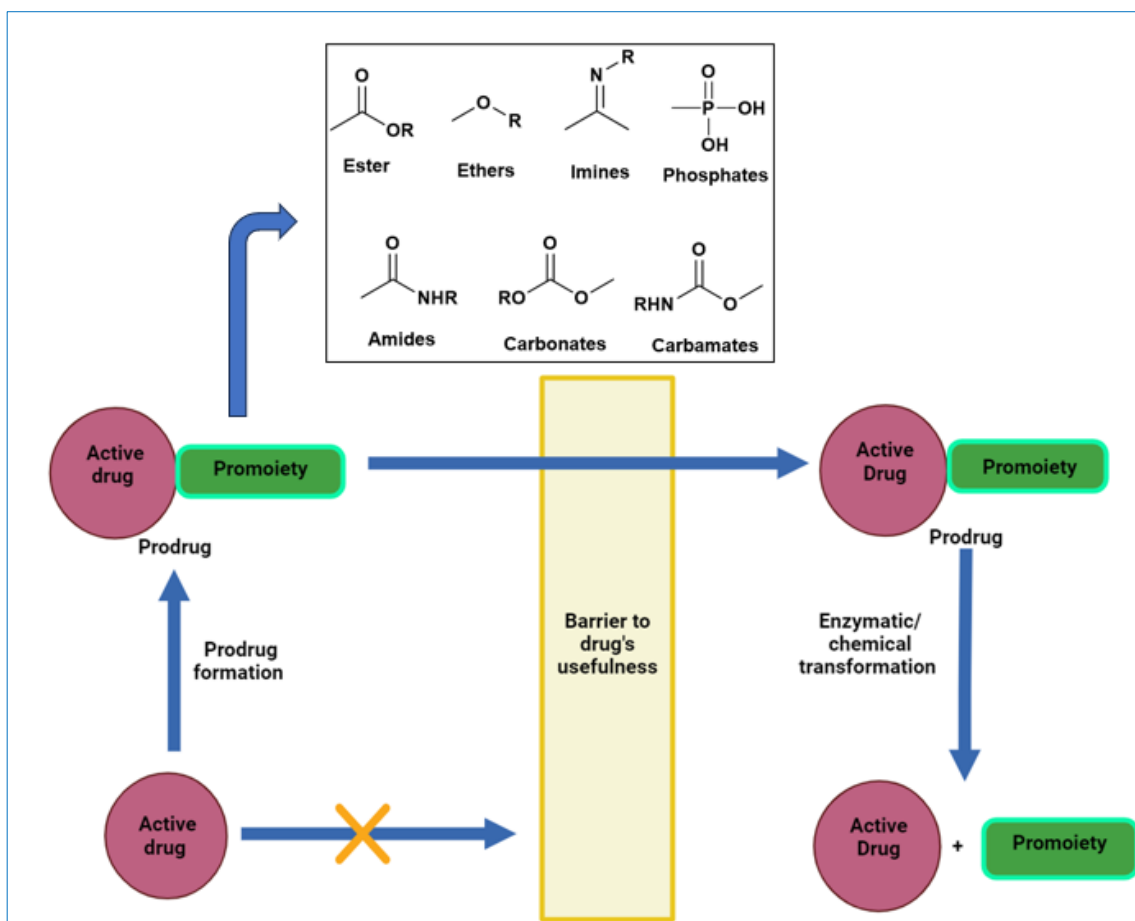


Figure 1.8: The basic concept of prodrug design to improve the biopharmaceutical and physicochemical properties of pharmacologically active drugs.

1.3.1 Ester prodrugs

When designing a prodrug, it is crucial to ensure that the prodrug effectively converts to the active drug once it is absorbed in the bloodstream and that any protecting groups cleaved from the molecule are non-toxic.⁵³ Various chemical moieties can be added to the active drug with ester bonds being the most commonly used.⁵³ The classic approach to designing an ester prodrug involves esterifying hydroxy or carboxylic acid functional groups on the compound of interest.⁵⁴ These types of strategies are effective, synthetic and highly prudent. Typically, ester prodrugs increase the water solubility and improve the lipophilicity of drug candidates. Many medications contain a carboxylic acid functional group essential for their pharmacological activity.⁵⁵ However, presence of high polarity hinders oral administration, as these drugs are largely ionised in the small intestine (pH 5-7) preventing passive diffusion through membranes.⁵⁵ For example, angiotensin converting enzyme (ACE) inhibitors are formulated as ester prodrugs which enhance the water solubility and increase lipophilicity, hence improving oral absorption. These include ethyl ester prodrugs such as enalapril (**12**), trandolapril (**13**),

quinapril (**14**) and benazepril (**15**) which are FDA and European Medicine Agency (EMA) approved for use with in European Union (**Figure 1.9**).⁵⁶

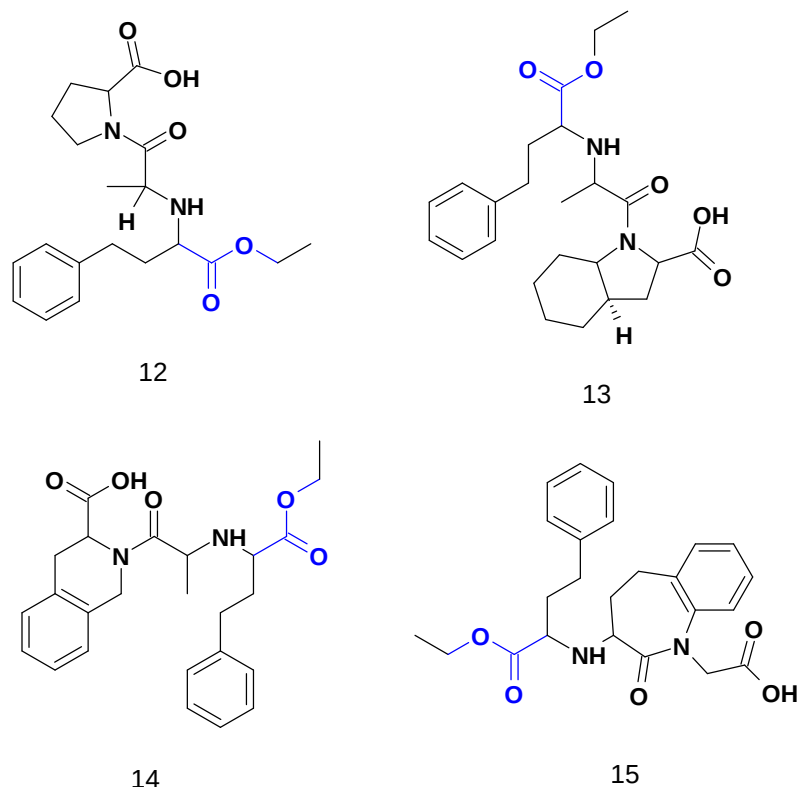


Figure 1.9: Example of ACE inhibitors ethyl ester prodrugs: Enalapril (12), Trandolapril (13), Quinapril (14) and Benazepril (15). Ester bond is coloured in blue.

SN38 (7-ethyl-10-hydroxy camptothecin, **16**), a potent anticancer drug used in the treatment of colorectal, lung, cervical and ovarian cancers is limited in clinical applications due to its poor aqueous solubility (0.1-0.38 mg/mL) and low satbility.⁵⁷ To overcome these hurdles, irinotecan (**17**) a carbamate prodrug of SN38 was developed to modestly improve solubility (0.12-2.8 mg/mL) with its hydrochloride salt (**18**) achieving 25 mg/mL (**Figure 1.10**) with.^{58,59} However, despite these improvements irinotecan is limited by side effects and suboptimal pharmacokinetics, driving research towards alternative strategies. Recent research efforts have focused on ester-based prodrugs of SN38 which offer significantly, improved solubility and bioavailability. Recently, Bala *et al.* designed and synthesised a series of lipophilic prodrugs through esterification, in which long-chain triglycerides were attached to the hydroxy group of SN38 at position 10 and 20 respectively (**Figure 1.10**). The solubility of these novel prodrugs was increased up to 444-fold and they exhibited enhanced stability in gastric fluid. Moreover, the prodrugs showed significantly reduced cytotoxicity as compared to SN38 (IC₅₀: 0.0053 μ M). Additionally, a predictable reconversion of prodrugs to SN38 was observed in the presence of plasma.

In particular, the utilisation of prodrugs containing esters *in vivo* can enhance bioavailability, as the ester groups are eliminated through enzyme catalysis or spontaneous hydrolysis which is discussed in Section 1.3.2.

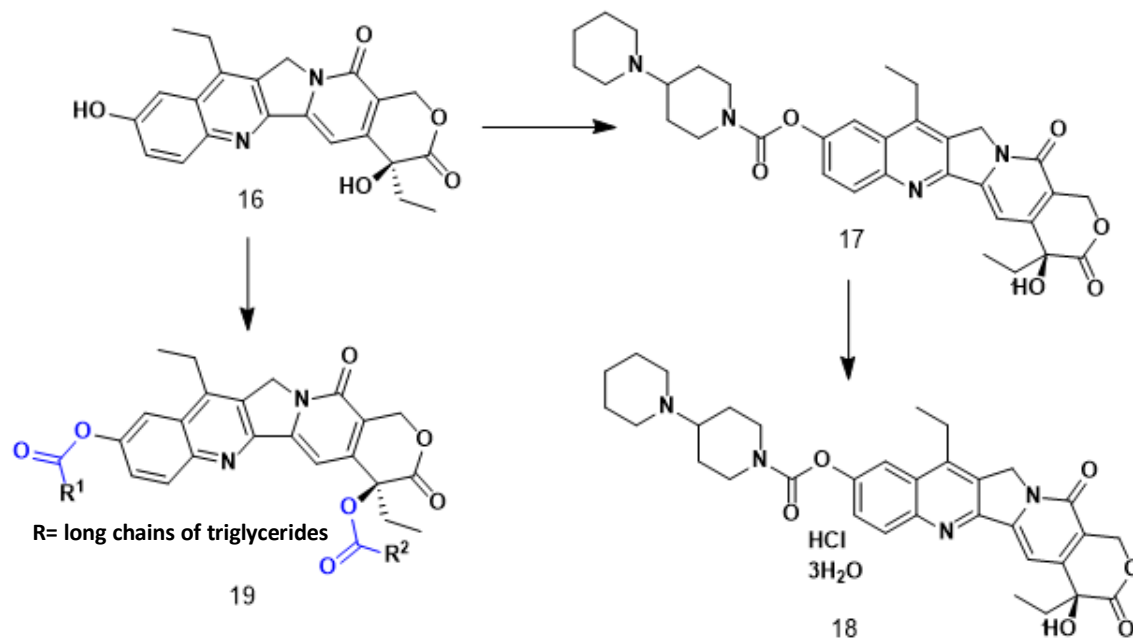


Figure 1.10: Prodrugs of SN38 (16): irinotecan (17), irinotecan hydrochloride trihydrate salt (18) and lipophilic ester prodrugs (19). Ester bond is coloured in blue.

1.3.1.1 Amino acid ester prodrugs

Amino acid ester prodrugs are a distinct category of ester prodrugs that aim to improve water solubility by incorporating amino acids as the carrier moiety. Amino acids are important building blocks of cells, but they require specific transport systems to cross the plasma membrane.⁶⁰ Furthermore, amino acids are highly water soluble and have pharmacological activity with minimal toxicity, making them ideal carriers for developing prodrugs of therapeutic agents that have limited absorption. This approach involves linking an active drug molecule with an amino acid through an ester bond. By utilising the natural transport and metabolic pathways of amino acids, this strategy aims to improve the delivery and efficacy of the drug.⁶¹ Amino acid prodrugs are widely used in clinical practice. For instance, a potent and selective antagonist of adenosine receptors, MSX-2 (**20**) shows effective *in vitro* activity, but this molecule exhibits low water solubility hindering *in vivo* studies. Two approaches were selected for resolving this issue: introduction of a phosphate group, which resulted in high water solubility but was not readily absorbed by the oral route. In contrast, the second approach involved the incorporation of the amino acid *L*-valine. The water solubility of the valine prodrug (**21**) was evaluated and found to be 7.3 mg/mL which is superior to that of the parent drug. Moreover, the valine prodrug (**21**) meets the

requirements for an oral prodrug, it shows high water solubility, high stability towards gastrointestinal fluids and rapid enzymatic hydrolyses by esterase (**Section 1.3.2**). The chemical structure of MSX-2 (**20**) and its valine prodrug (**21**) is shown in Figure 1.11. Another example is the antiviral drug, penciclovir (**22**), which is a potent and selective inhibitor of herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) but is limited by its poor oral bioavailability.⁶² In order to overcome this obstacle, amino acid ester prodrugs were synthesised by coupling the amino acids, *L*-valyl and *L*-isoleucyl with the two hydroxyl groups of penciclovir (**23 a and b, Figure 1.11**). All the amino acid ester prodrugs were highly soluble (>100 mg/mL) and sufficiently stable in aqueous medium, compared with penciclovir (1.7 mg/mL).^{62, 63} In addition, the oral bioavailability of the prodrugs was evaluated in mice and it was found that the *L*-valyl prodrug (**23 a**) displayed approximately 4-fold higher bioavailability than that of penciclovir.⁶² As previously discussed in Section 1.3, prodrugs are designed not only to improve oral bioavailability but also to improve the pharmacokinetic profile of the drug molecule. This includes enhancing oral absorption, controlling rapid drug metabolism and reducing drug toxicity. One notable example is the antiviral drug acyclovir (**24**), which has a commercially successful *L*-valyl ester prodrug known as valaciclovir (**25, Figure 1.11**). Valaciclovir enhances intestinal membrane permeability and increases aqueous solubility, facilitating improved delivery at higher oral doses.⁶⁴ In addition, the *L*-valyl group enables the drug to be absorbed by di- and tripeptide transporters in intestinal epithelial cells.⁶⁴ This leads to enhanced intestinal permeation and increased bioavailability of valaciclovir, which is 54% compared to only 20% for acyclovir.⁶⁴ As a result, there is a significant improvement in clinical benefit due to the higher concentrations of acyclovir being delivered.

Although there are not many commercially available amino acid ester prodrugs specifically designed for anticancer purposes, research continues into developing amino acid ester prodrugs for cancer treatment, focusing on improving water solubility and pharmacokinetic profile. For instance, floxuridine (**26, Figure 1.11**) is a potent chemotherapeutic drug that has demonstrated 5000-fold higher antiproliferative activity compared to its metabolite 5-FU *in vitro* in various cancer cell lines. However, its rapid metabolism to 5-FU *in vitro* and *in vivo* remains a major challenge in delivering floxuridine to cancer cells for improved cytotoxic action. Thus, to control rapid metabolism and to improve the oral bioavailability of floxuridine, Landowski *et al* designed amino acid ester prodrugs of floxuridine (**27, Figure 1.11**). The combined results of *in vitro* studies suggested that amino acid ester prodrugs may be potential candidates for improving oral bioavailability *in vivo*, due to their 8-to-11-fold greater cell permeability and high resistance to metabolism, compared to floxuridine.⁶²

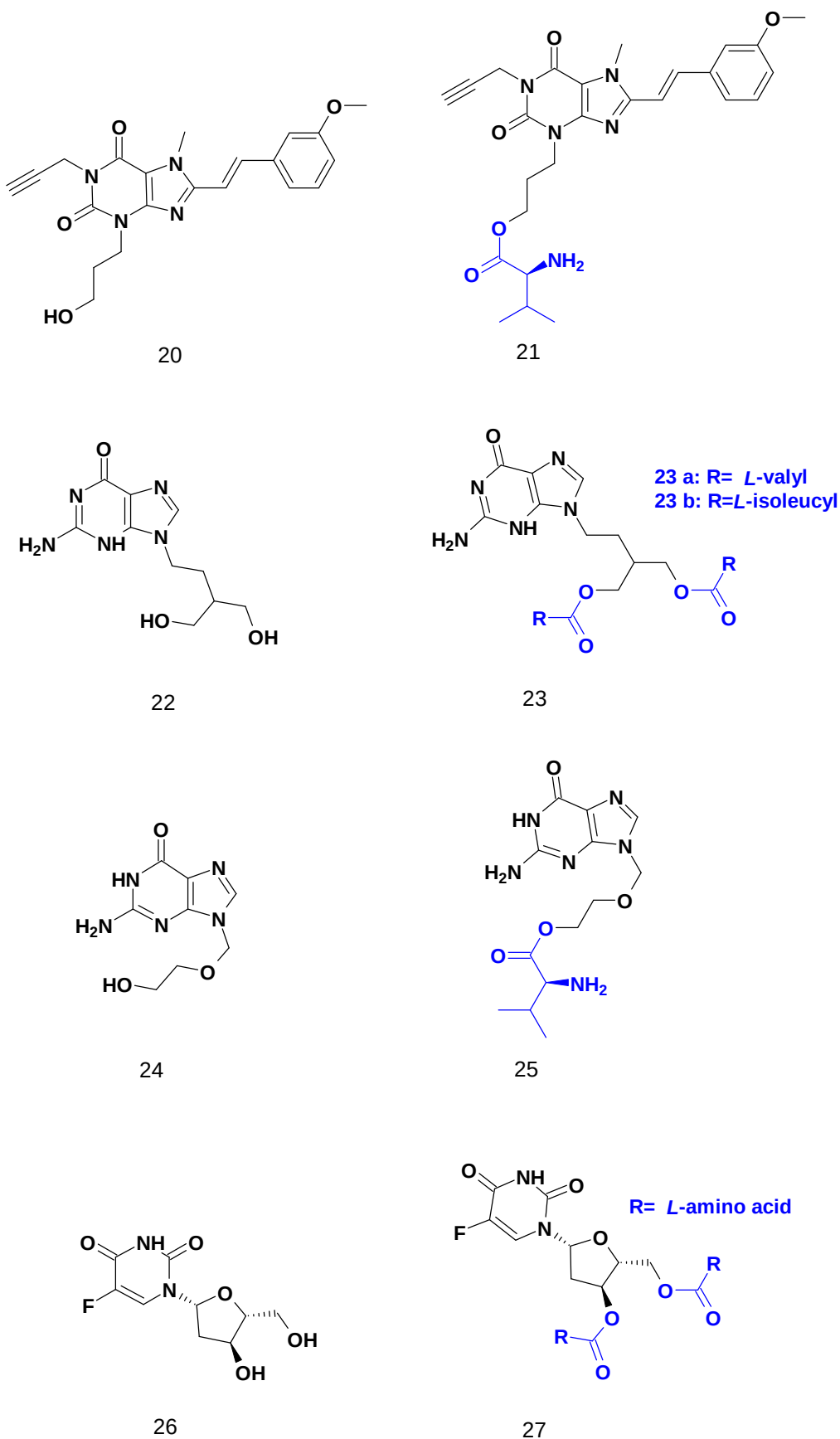


Figure 1.11: Chemical structure of MSX-2 (20), penciclovir (22), acyclovir (24) and floxuridine (26) and their amino acid ester prodrugs (21), (23 a and b), (25) and (27). Amino acid ester moiety attached to the drugs is highlighted in blue.

1.3.2 Activation of prodrugs

The activation of prodrugs is a crucial step in prodrug design, as it ensures the effectiveness of the drug, minimises side effects, and optimises drug delivery.⁶⁵ As mentioned earlier, regardless of the specific approach, the prodrug must undergo an activation step following intake in which the active drug is freely liberated and thus its pharmacological activity can then be exerted.⁶⁵ The prodrug activation can be based on chemical process or enzyme mediated conversion.

1.3.2.1 Enzyme-mediated activation

The activation of prodrugs through enzymes plays a crucial role, as it enables the controlled release of active drugs inside the body.⁶⁶ Enzymes commonly involved in the activation process include hydrolytic enzymes (esterases, phosphatases, proteases), oxidoreductases [cytochrome P450 enzymes (CYP 450)], transferases, lyases and isomerases.⁶⁶ Among these, esterases are particularly important for the activation of ester prodrugs due to their ability to hydrolyse ester bonds, leading to the release of the active drug. These enzymes are widely distributed throughout the body and can be found in both intracellular and extracellular environments. Based on their activation mechanism, prodrugs are classified into two types, type 1 and 2 prodrugs, based on the enzymes involved and their specificity (Table 1.2).

Table 1.2: Classification of prodrugs based on their mechanism of activation and their site of action.

Prodrugs type	Site of activation	Subtypes	Location of activation
1	Intracellular	1A	Therapeutic target tissue/cells
		1B	Liver cells, GI mucosal and lung cells
2	Extracellular	2A	GI fluids
		2B	Systemic circulation: blood plasma

Type 1 prodrugs are designed to be activated intracellularly, typically by enzymes that are naturally present in the target cells. For ester prodrugs, intracellular esterases such as carboxylesterase, play a critical role. These enzymes are abundant in therapeutic target tissues/cells (Type 1A) and in metabolically active cells such as liver cells, GI mucosal cells, and lung cells (Type 1B). The specificity of these enzymes ensures that the prodrug is converted to its active form only within the target cells, reducing systemic side effects and enhancing therapeutic efficacy.

Carboxylesterases (CES) are a group of enzymes that play a crucial role in the metabolism of ester-containing compounds, including many prodrugs.⁶⁷ They hydrolyse ester bonds to release active drugs from their prodrug forms. CES are widely distributed in various tissues, with high concentrations found

in the liver, intestines, and blood plasma.⁶⁷ Examples of type 1A and 1B prodrugs activated by CES are irinotecan (**17**, **Figure 1.10**) and enalapril (**12**, **Figure 1.9**). Irinotecan (**17**) is classic example of a type 1A prodrug, entering the target colorectal tumour cells and being hydrolysed by CES to release the active metabolite, SN38 (**16**).⁶⁸ Upon activation SN38 inhibits topoisomerase I, an enzyme essential for DNA replication and transcription in cancer cells, leading to DNA damage and cell death.⁶⁸ Moreover, type 1A prodrugs include many antiviral and chemotherapeutic agents. Enalapril (**12**) is a type 1B prodrug which is hydrolysed by hepatic CES to enalaprilat (**28**), a potent ACE inhibitor (**Figure 1.12**).⁶⁹

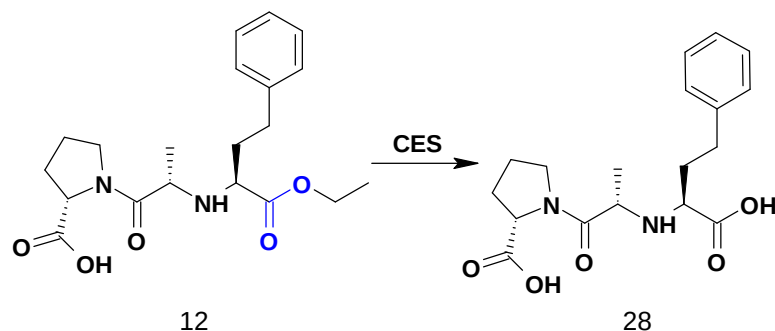


Figure 1.12: Example of type 1B prodrug activation of Enalapril to its parent drug Enalaprilat by liver carboxylesterases.

Type 2 prodrugs are activated extracellularly, either in the circulatory system or at the site of administration. These prodrugs rely on enzymes that are found in the extracellular environment i.e. GI fluids and blood plasma.⁷⁰ Esterases present in the GI fluids (Type 2A) and in the systemic circulation, such as blood plasma esterase (Type 2B), are responsible for the hydrolysis of ester bond containing prodrugs. The activation of ester prodrugs by this extracellular esterase allows for a controlled release of the active drug at the site of absorption or in the bloodstream, facilitating systemic drug delivery. An example of a type 2A prodrug is oxyphenisatin diacetate (**29**) used in the treatment of triple negative breast cancer which is activated to oxyphenisatin (**30**) in the GI tract by carboxylesterase present in the GI fluids (**Figure 1.13**).⁷¹ A common example of a type 2B prodrug is acetylsalicylic acid (**31**), an ester prodrug which gets converted to salicylic acid (**32**) by plasma esterase (**Figure 1.13**).⁷²

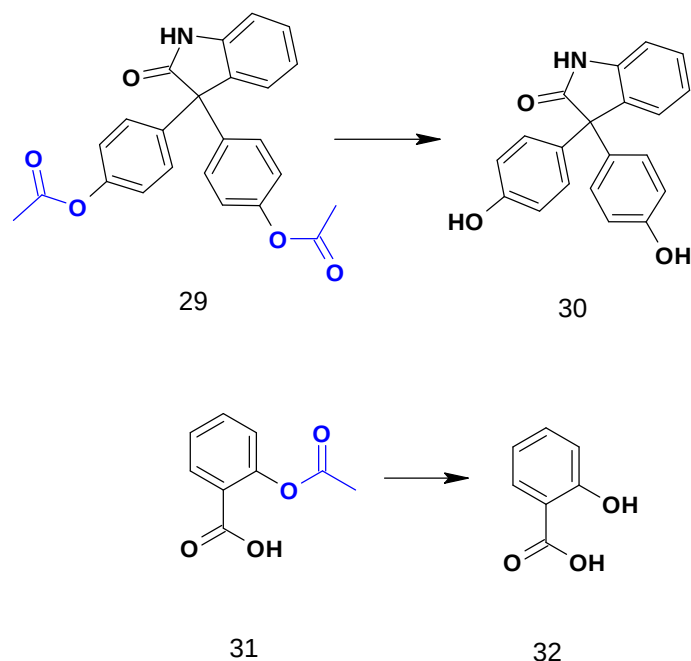


Figure 1.13: Example of type 2A and B prodrugs: activation of oxyphenisatin diacetate (29) to oxyphenisatin (30) in the GI tract (type 2A); acetylsalicylic acid (31) to salicylic acid (32) in blood plasma (type 2B).

1.3.2.2 Non-enzymatic activation: chemical activation

Activation of prodrugs via chemical processes does not require enzymes and is an alternative strategy in drug design. Its aim is to ensure the controlled release and activation of the active drug in specific environments.⁷³ This approach takes advantage of the prodrug's intrinsic chemical properties and the environmental conditions to achieve the desired therapeutic effects.⁷³ Examples of chemical processes that are involved in prodrug activation are pH, temperature and light sensitive activation.⁷⁴

Prodrugs can be designed to be stable at a neutral pH but undergo activation in environments with different pH levels, such as the acidic environment of the stomach or tumour tissues. In addition, prodrugs can be activated by exposure to specific wavelengths of light. This allows for precise spatial and temporal control over drug activation. An example of non-enzymatic prodrug activation includes sulfasalazine which is pH sensitive and can be cleaved by the mildly acidic environment in the colon to release the active anti-inflammatory agent, mesalamine. The principles of pH, temperature, and light sensitivity can be incorporated into their design for specific applications. For example, Saha *et al* synthesised a pH responsive auto-fluorescent polymeric nano-carrier drug (**33**) for the administration of chlorambucil (**34**) into the cancer cells.⁷⁵ They utilised pH induced cleavage of the aliphatic ester linkage connecting chlorambucil (CBL) with a maleimide moiety which can self-organise in aqueous medium (**Figure 1.14**).⁷⁵ The *in vitro* drug release studies revealed that 90% of **33** was converted to **34** at pH 5, resulting in higher toxicity and effective killing capability toward tumour cells.⁷⁵

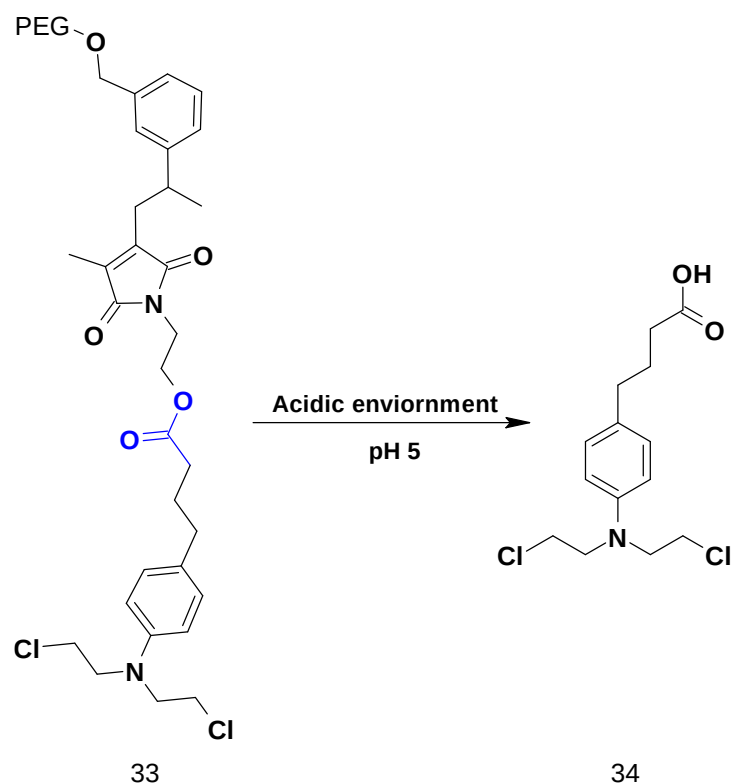


Figure 1.14: pH induced release of CBL (34) from nanocarrier (33) under acidic conditions at pH 5.

In general, non-enzymatic chemical activation of prodrugs offers a robust and versatile approach for targeted drug delivery and activation. By leveraging specific environmental conditions, this strategy can enhance the therapeutic index of drugs, providing effective treatment with reduced side effects. However, there are some challenges associated with this approach such as stability; balancing the stability of the prodrug in the bloodstream or during storage with its ability to activate under specific conditions.

The majority of anticancer drugs and prodrugs discussed in this study have nitrogen containing heterocycles within their chemical structures. The next section looks at nitrogen containing anticancer agents.

1.4 Nitrogen containing heterocyclics as promising biological agents

Nitrogen-containing heterocyclic compounds are common in clinical drugs used to treat diseases and have demonstrated significant potential in the development of anticancer agents. A recent study found that 82% of newly approved small-molecule drugs include a nitrogen heterocycle.⁷⁶ Their distinct structures enable their interaction with various biological targets.⁷⁶ Among the diverse range of these compounds are indole (35), pyridine (36), purine (37), pyrrole (38), imidazole (39), pyrazole (40), pyrimidine (41), quinoline (42) and pyrazine (43) heterocycles (**Figure 1.15**). This study focuses on quinoline and pyrazine derivatives due to their potent chemical and biological properties (**Sections 1.4.1 and 1.4.2**).

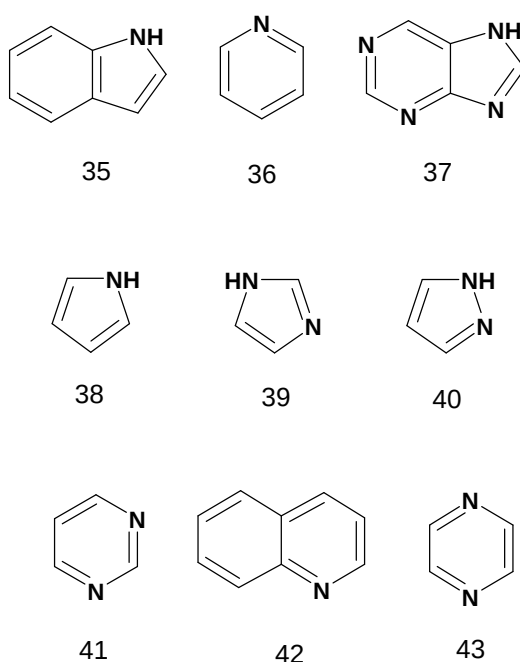


Figure 1.15: Chemical structures of nitrogen containing heterocyclic compounds including indole (35), pyridine (36), purine (37), pyrrole (38), imidazole (39), pyrazole (40), pyrimidine (41), quinoline (42) and pyrazine (43).

1.4.1 Quinoline and its derivatives

Quinolines are class of heterocyclic compound that feature a benzene ring fused with a pyridine moiety, with the molecular formula of C_9H_7N (**Figure 1.16**).⁷⁷ The quinoline ring is a crucial structural component in both natural and synthetic compounds, serving as the foundational framework for numerous pharmacological applications (**Figure 1.16**). The pharmacological uses of quinoline are further explained in this section, highlighting their roles in various therapeutic areas.

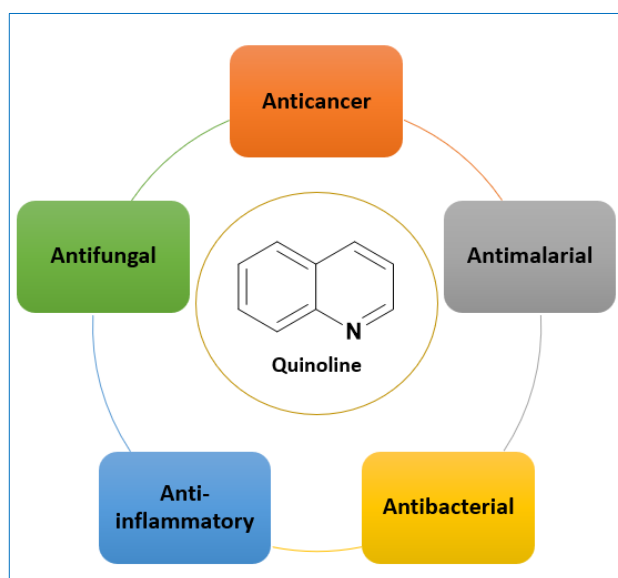
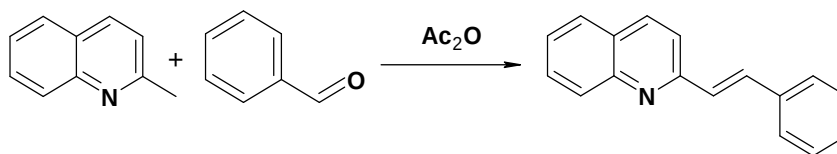


Figure 1.16: Overview of the diverse pharmacological applications of the quinoline ring including anticancer, antimalarial, antibacterial, anti-inflammatory and antifungal.

Quinoline is a weak tertiary base and can form salt with acids.⁷⁷ To prepare the quinoline scaffold, numerous traditional synthetic routes have been reported. These include Gould–Jacob, Friedländer, Pfitzinger, Skraup, Doebner–von Miller, and Conrad–Limpach reactions, transition metal catalysed reactions, transition metal free ionic liquid mediated reactions, ultrasound irradiation reactions, and green chemical processes.⁷⁷ However, many of these methods suffer from problems such as low yields, harsh reaction conditions, longer reaction times and use of toxic solvents.⁷⁷ A notable derivative of quinoline is 2-methylquinoline, also known as quinaldine. It is an important intermediate in organic synthesis and is used in the synthesis of various compounds, including anticancer, antimalarial, antibacterial and antifungal agents.⁷⁸ Moreover, 2-methylquinoline is synthesised by Skraup reaction, using aniline and glycerol in the presence of acetic acid.⁷⁸ One of the interesting applications of 2-methylquinolines is in the formation of (*E*) styryl quinoline derivatives. The traditional method is a Perkin condensation reaction (discussed in Chapter 2) of 2-methylquinoline with various aromatic aldehydes as shown in Scheme 1.1. Styryl derivatives are a class of organic compounds characterized by the presence of a styryl group, typically consisting of an ethylene-vinyl moiety.⁷⁹ This structural motif

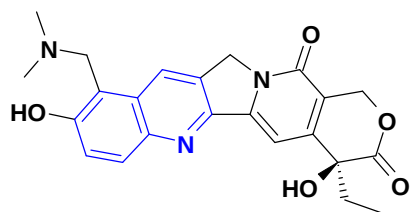
contributes to their unique chemical and biological properties.⁷⁹ Quinoline derivatives are a subset of styryl compounds where the styryl group is attached to a quinoline ring system (**Scheme 1.1**).



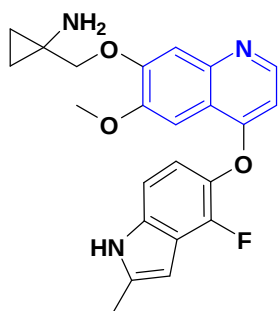
Scheme 1.1: Synthesis of 2-styrylquinolines

Quinoline based compounds represent a significant class of heterocyclic molecules renowned for their diverse chemical and biological activities.⁸⁰ These compounds are pivotal in various industries, including dye and paint manufacturing, as well as the production of insecticides and antifungal agents.⁸⁰ The versatility of the quinoline ring system is further exemplified by its widespread use in pharmaceuticals. Quinoline derivatives are prominently featured in various therapeutic agents, serving as essential component in the treatment of various diseases. Quinoline bearing structures are present in many biologically active drugs.⁸¹ For instance, quinoline-based antimalarial drugs such as chloroquine and mefloquine have been instrumental in combating malaria.⁸¹ Similarly, antibacterial agents like ciprofloxacin and sparfloxacin are widely used to treat bacterial infections. In recent years, six quinoline containing drugs have been approved by FDA.⁷⁶ These approvals span multiple areas, including anticancer, antibacterial and antimalarial therapies, showcasing the versatility of quinoline derivatives.⁷⁶

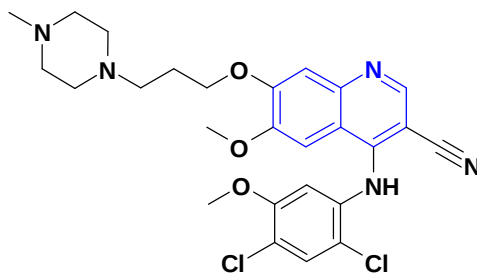
The quinoline ring is a key feature in several anticancer drugs which have demonstrated remarkable effectiveness in suppressing cell proliferation through a variety of mechanisms. These mechanisms include inducing cell cycle arrest, promoting cell death, inhibiting angiogenesis, disrupting cell migration, and modulating other essential biological pathways. Examples of quinoline containing anticancer agents which are vital in cancer therapy are irinotecan (**17**) and topotecan (**44**, **Figure 1.17**) which is another derivative of SN38 (**16**).⁸² Moreover, quinoline based protein kinase inhibitors such as anlotinib (**45**) and bosutinib (**46**) have been approved for cancer treatment (**Figure 1.17**).⁸² Li *et al* reported the discovery of novel quinoline-chalcone derivatives as inhibitors of tubulin, showing incredible effectiveness against cancer with IC₅₀ values in the nanomolar range.⁸³ Additionally, quinoline chalcone (**47**, **Figure 1.17**) effectively stopped cell division at the G₂/M phase, induces apoptosis, disrupts the mitochondrial membrane potential, and stimulates the generation of ROS in K562 cells.⁸³ Similarly, quinoline chalcone (**48**, **Figure 1.17**) exhibits significant cytotoxic activity against HL60 cells, with an IC₅₀ value of 0.59 μ M.⁸³



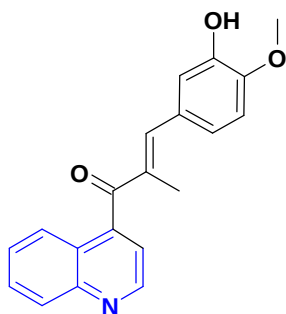
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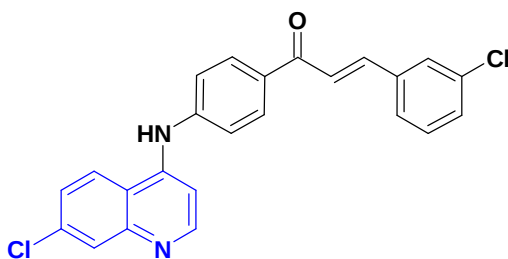
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47



48

Figure 1.17: Chemical structure of quinoline based drug molecules: Topotecan (44), Protein kinase inhibitors such as Anlotinib (45), Bosutinib (46), Quinoline chalcone derivatives (47 and 48). The quinoline structure is highlighted in blue.

1.4.2 Pyrazine and its derivatives

Pyrazine is a six-membered heterocyclic organic ring that contains two nitrogen atoms at positions 1 and 4 (**Figure 1.18**). It is a colourless water soluble solid with a chemical formula of $C_4H_4N_2$. This structural motif is relatively simple but provides a versatile platform for the creation of wide variety of compounds with potent biological activities. Pyrazine is a very weak base (pK_a of 0.6) due to the inductive and electron withdrawing effect of second nitrogen atom, as illustrated in Figure 1.18.⁸⁴

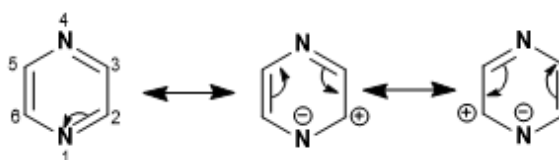
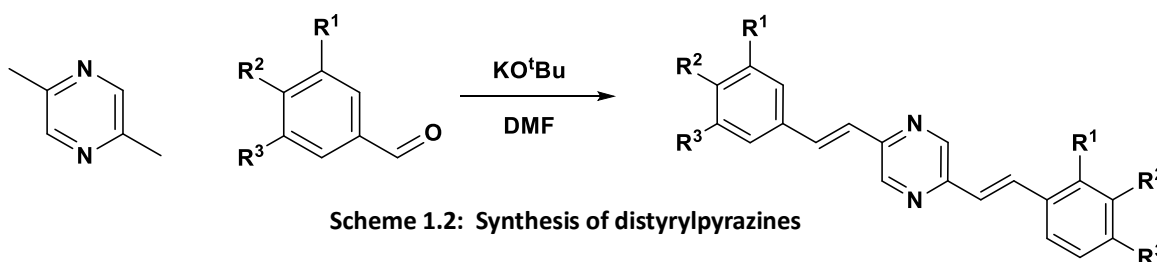


Figure 1.18: Inductive effect of pyrazine.

The synthesis of pyrazine is limited to several methods. It can be efficiently prepared by using α -halo ketones or through the condensation reaction diamines and epoxides.⁸⁵ Another approach involves the dehydrogenation of piperazines. Commercially, pyrazine is typically synthesised *via* dehydrogenative coupling of α -amino carbonyl or α -diketones with vicinal diamines.⁸⁵

A pyrazine derivative, 2-methylpyrazine can be used to synthesise styryl pyrazine compounds by reacting with wide range of benzaldehydes (**Section 1.4.3**). Schmitt *et al* demonstrated a synthesis of series of distyrylpyrazines by aldol condensation of aromatic aldehydes with 2,5-dimethylpyrazine as shown in Scheme 1.2.⁵⁰ The aldol condensation is a fast access to distyrylpyrazines, however the yields were achieved in a moderate range (4-49%).



Scheme 1.2: Synthesis of distyrylpyrazines

Pyrazine is a prominent topic in pharmaceutical chemistry research due to its extensive use in the synthesis of biologically active ingredients and catalysts.⁸⁴ Its wide range of pharmacological effects make it a valuable building block for development of anti-inflammatory, anticancer, antibacterial and anti-diabetic agents. Figure 1.19 illustrates example of pyrazine derivatives in marketed anticancer drugs.⁸⁴ These compounds highlight the significance of pyrazine derivatives in the development of effective treatments for cancer.

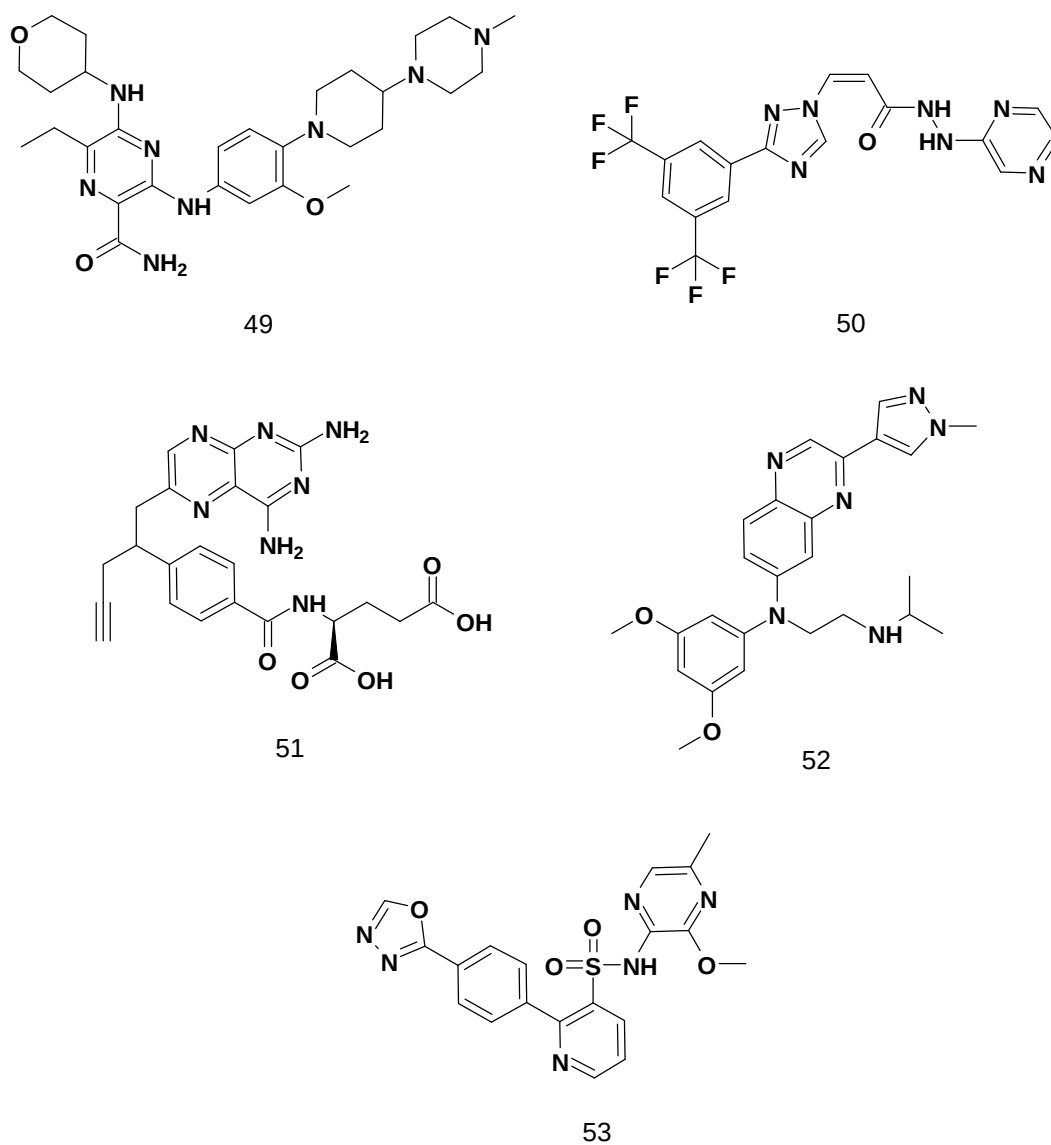


Figure 1.19: Chemical structures of pyrazine derivatives in marketed anticancer drugs Gilteritinib (49), Selinexor (50), Pralatrexate (51), Erdafitinib (52) and Zibotentan (53)

1.5 Aims and objectives of the project

1.5.1 Overall hypothesis

Synthesis of prodrugs of pyrazinib, quininib and 1,4-dihydroxyquininib could significantly enhance their physicochemical properties including water solubility and chemical stability. This could potentially improve their therapeutic efficacy and further utilisation in the *in vivo* pre-clinical studies.

1.5.2 Overall aim

The overall aim of this thesis is to enhance the water solubility and chemical stability of potent anti-cancer drugs, pyrazinib and quininib analogues through the design and synthesis of their prodrugs and salt formation. This thesis also aims to identify and evaluate new cysteinyl leukotriene receptor 1 (CysLT₁) antagonists using computational methods.

1.5.3 Specific aims of thesis

Pyrazinib and quininib analogues (Q series)

1- Synthesis

- To synthesise pyrazinib, quininib and 1,4-dihydroxyquininib as synthetic precursors.

2- Physicochemical properties improvement

- To develop a series of pyrazinib amino acid prodrugs, quininib acetyl ester prodrug and its hydrochloride salt to improve water solubility and chemical stability of pyrazinib and quininib.

3- Measurement of physicochemical properties: water solubility and chemical stability

- To measure the water solubility and chemical stability of prodrugs and compare with the parent drugs.

4- Investigating *in vitro* hydrolysis of prodrugs

- To measure the *in vitro* enzyme and plasma mediated hydrolysis of pyrazinib amino acid prodrugs and quininib ester prodrug.

5- Evaluating the *in vitro* and *in vivo* biological activity of the prodrugs

In vitro studies using cancer cell lines model

- To determine the effect of radiosensitivity of pyrazinib amino acid prodrugs and compare with pyrazinib.
- To evaluate the antiproliferative activity and cytotoxicity of quininib and its ester prodrug and hydrochloride salt.

In vivo studies using zebrafish model (These studies were performed in Bioreperia, Linköping, Sweden)

- To evaluate the *in vivo* toxicity and anti-angiogenic activity of pyrazinib and its amino acid prodrugs using zebrafish model.
- To evaluate the *in vivo* toxicity of quininib and its ester prodrug using zebrafish model.

Identification of cysteinyl leukotriene receptor 1 (CysLT₁) antagonists

1- Identification of potential hits of CysLT₁

- To Identify potential hits for the further development of CysLT₁ selective antagonists by ligand based virtual screening (LBVS) (The LBVS study was performed in Cresset, Cambridge, UK).

2- Evaluating the *in vitro* and *in vivo* biological activity of potential hits

- To Investigate the *in vitro* antagonist effect of the selected hits on human CysLT₁ receptor
- To evaluate the *in vivo* toxicity and antiangiogenic effect of selected hits using zebrafish model.

Achieving these aims and objectives will significantly improve the therapeutic utility of pyrazinib and quininib analogues by overcoming their water solubility and stability limitations, potentially paving the way for their future clinical development. Moreover, the identification of novel CysLT₁ antagonists offers new possibilities for cancer therapies.

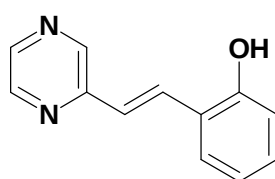
Chapter 2: Design, synthesis and physicochemical analysis of radiosensitiser pyrazinib and its prodrugs

2.1: Introduction

2.1.1 Pyrazinib as a promising radiosensitiser

Pyrazinib (**54**), [P3, IUPAC name: (2-[(*E*)-2-(pyrazine-2-yl)vinyl]phenol)] is a small molecule containing a pyrazine ring and a phenol (**Figure 2.1**). Previous studies by Buckley *et al.* identified anti-angiogenic and anti-metabolic activity *in vivo* in zebrafish and *in vitro* in isogenic models of oesophageal adenocarcinoma (OAC) radioresistance.⁸⁶ Hence, Buckley *et al.* carried out investigations with pyrazinib to determine its effect on three aspects: angiogenesis, metabolism and cancer-related inflammation. Inflammation forms an inter dependent network with angiogenesis and metabolism which plays an important role in radioresistance.⁸⁶ In an *in vivo* zebrafish model, measurement of oxygen consumption rate (OCR), indicative of oxidative phosphorylation and extracellular acidification (ECAR) reflective of glycolysis, demonstrated that pyrazinib caused simultaneous reductions in both OCR and ECAR. In addition, investigation in an *in vitro* OAC radio-resistance cell line (OE33R) revealed that pyrazinib significantly reduced OCR and ECAR. Moreover, pyrazinib was observed to enhance radio-resistance by reducing the survival fraction of OE33R cells. Further investigations revealed that pyrazinib enhanced radiosensitivity of OE33R cell lines under hypoxic conditions. Pyrazinib also showed a reduction in inflammatory secretions in OE33R cells. In general, these findings further support the development of pyrazinib as a promising radiosensitiser in OAC.⁸⁶

In a more recent study by Buckley *et al.*, radiosensitiser pyrazinib was tested for the first time *ex-vivo* in OAC tumours.⁸⁷ The study showed that pyrazinib significantly reduced OCR and ECAR in OAC biopsies.⁸⁷ Additionally, it inhibited the real-time metabolic rate in OAC biopsies under hypoxia. However, it does not reduce T-cell viability.⁸⁷



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Figure 2.1: Chemical structure of pyrazinib (**54**)

2.2.2 Pyrazinib solubility problems during *in vivo* testing

The physicochemical properties of pyrazinib were never evaluated before, and despite being a promising radiosensitiser, pyrazinib was noted for its poor aqueous solubility during *in vivo* testing carried out in a xenograft model by Kennedy *et al.* During that *in vivo* testing, pyrazinib was added to RPMI media, FBS and various solvents including 100% water but resulted in precipitation as shown in Figure 2.2. Pyrazinib was insoluble in RPMI and solvents used for *in vivo* testing and its poor aqueous solubility limits further pre-clinical *in vivo* testing. Previously, the O'Boyle group studied the chemical stability and aqueous solubility of pyrazinib. The chemical stability was measured at three pH values (4, 7.4 and 9) over a period of 24 hours and pyrazinib observed to have good stability at all three pH values (Figure 2.3).⁸⁸ The aqueous solubility of pyrazinib was measured by the shake-flask method and displayed a value of 0.00005 mg/mL.⁸⁸ Thus, it was important to modify the structure of pyrazinib to achieve the required aqueous solubility for pre-clinical *in vivo* testing. To achieve this, a prodrug approach was pursued.



Figure 2.2: Solubility problems: precipitation of Pyrazinib (54) in solutions for *in vivo* testing. (Left) vial 1: 10% DMSO, 30% ethanol, 60% phosphate buffered saline; vials 2 – 4: 10% DMSO, 30% ethanol, 60% water; (Middle) 100% water; (Right) vial 1 RPMI, vial 2, RPMI + 10% FBS and vial 3 FBS only.

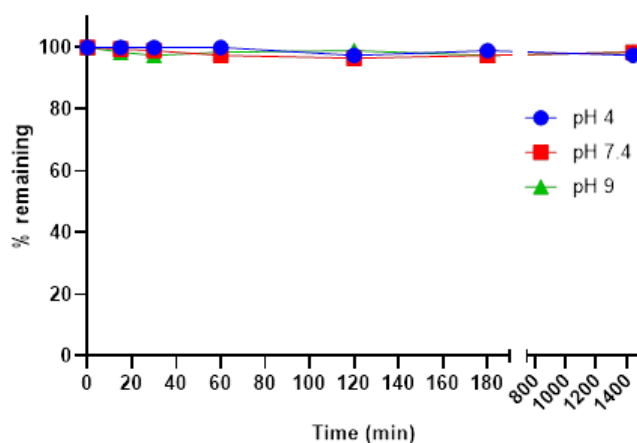


Figure 2.3: Chemical stability profile of pyrazinib (54) at three pH values (4, 7.4 and 9).

2.2 Specific aims of chapter 2

1. To optimise synthesis of pyrazinib (**54**)
2. To enhance the aqueous solubility of pyrazinib by synthesising its amino acid prodrugs (**63-69**).
3. To analyse the aqueous solubility and chemical stability of the prodrugs and comparing with the parent drug.
4. To investigate the metabolic stability of amino acid prodrugs
5. To investigate the *in vitro* bioactivity of the prodrugs and comparing with pyrazinib.

2.3 Pyrazinib: synthetic routes and chemistry

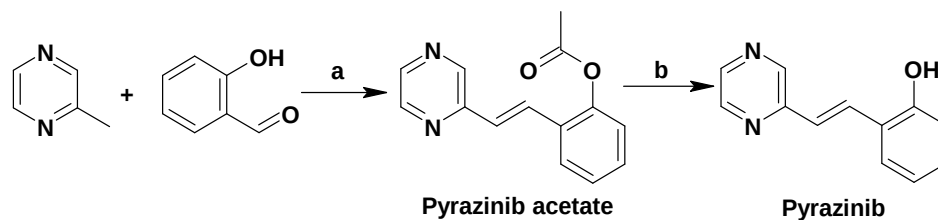
This section deals with the synthetic routes applied for the synthesis of pyrazinib. Initially, pyrazinib was synthesised by the Perkin method (**Section 2.3.1**). This method resulted in a longer reaction time and low yield of pyrazinib. As a result, it was decided to develop an improved synthetic method to address these issues. Therefore, we attempted the synthesis of pyrazinib using 4 different methods (**Section 2.3.2**).

2.3.1 Synthesis of pyrazinib by Perkin condensation reaction

The Perkin reaction is a useful method for synthesising α - β unsaturated aromatic acids. It involves the condensation of the aromatic aldehyde with acetic anhydride which acts as a base catalyst. Other bases can also be used such as triethylamine.⁸⁹ The first example of the Perkin reaction was proposed in 1868 by William Henry Perkin, involving the synthesis of coumarin by condensing the sodium salt of salicylaldehyde with acetic anhydride.⁹⁰ Typically, the reaction is initiated by heating the aldehyde with an excess of acetic anhydride at a high temperature of 180-200°C and requires a long reaction time. However, it is limited by low yields of the target compounds and the formation of by-products.

In a recent study, Orozco *et al.* reported the modified Perkin method to synthesise (*E*)-styryl quinoline. This method utilised methylquinoline and aromatic aldehyde as reactants in acetic anhydride as a solvent. Since pyrazinib shares a similar structure and stereochemistry as styryl quinoline, the modified Perkin condensation method was employed for its synthesis. The synthesis of pyrazinib is a two-step procedure as shown in Scheme 2.1. The first step involved the reaction of 2-methylpyrazine with salicylaldehyde in acetic anhydride under a conventional reflux condition at a high temperature of 180 °C for reaction time of 6 days to obtain pyrazinib acetate (**55**) in < 20% yield. Then, the second step involved the hydrolysis of pyrazinib acetate (**55**) under basic conditions, resulting in the formation of pyrazinib (**54**) with a poor yield of < 15%.

Scheme 2.1 Perkin condensation reaction for the synthesis of pyrazinib acetate followed by hydrolysis to afford pyrazinib.

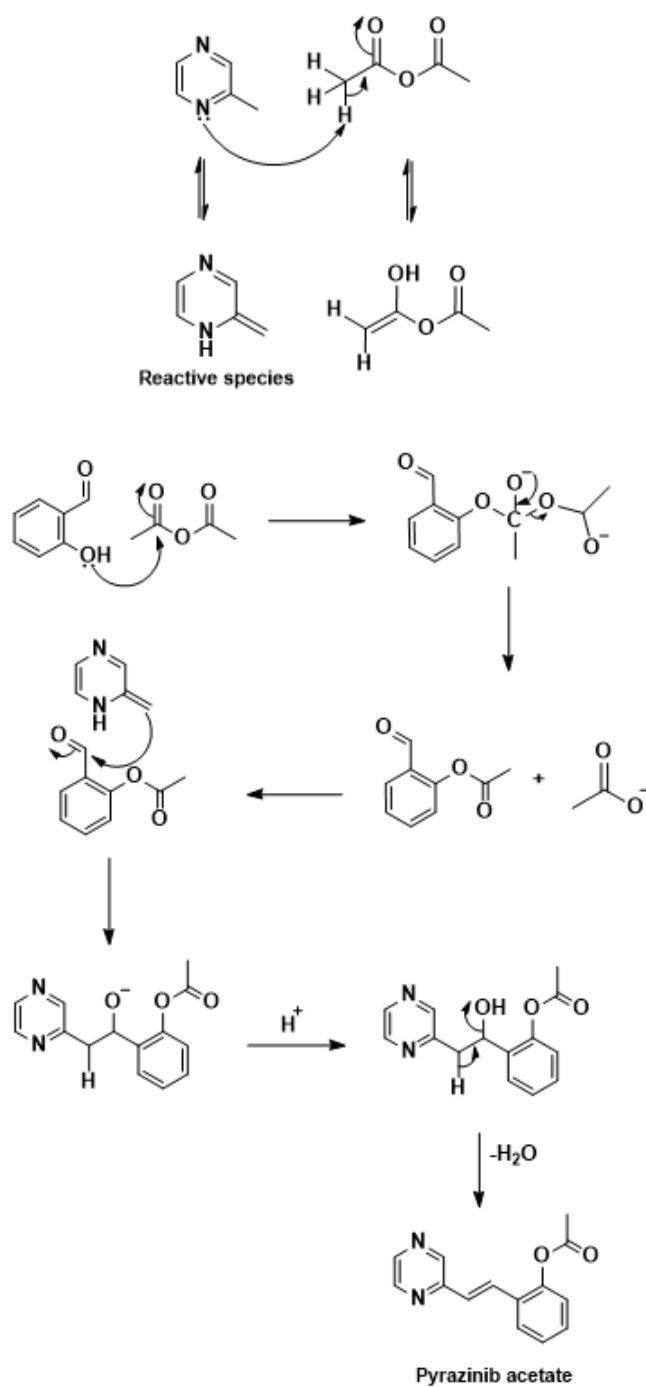


Reagents and conditions: (a): acetic anhydride, reflux at 180 °C for 6 days, 15.6% (b): NaOH, EtOH/EtOAc (1:1), reflux at 100°C, until complete as indicated by TLC, 12.5%

The plausible reaction mechanism and the tautomerisation for 2-methylpyrazine during the formation of pyrazinib acetate is illustrated in Scheme 2.2. The reaction mechanism for the synthesis of pyrazinib acetate *via* Perkin condensation begins with the nucleophilic attack of the nitrogen atom in the pyrazine ring on acetic anhydride. This attack results in the formation of a reactive species by tautomerisation. Then, 2-hydroxybenzaldehyde attacks the carbonyl carbon of acetic anhydride, leading to the formation of an ester and the departure of acetic acid. Following this, the reactive species formed attacks this ester intermediate, forming a new carbon-carbon bond. Finally, the reaction concludes with the elimination of water, resulting in the formation of the final product, pyrazinib acetate (**Scheme 2.2**).

The tautomerisation for 2-methylpyrazine is a limiting step and could be a potential cause for the poor yield. Tautomerisation involves the rearrangement of a molecule by transferring a proton, leading to an equilibrium between two constitutional isomers.⁹¹ In the case of 2-methylpyrazine, the tautomerisation process would involve shifting a proton within a pyrazine ring which is not straightforward due to the aromaticity and the placement of nitrogen atoms in the ring structure. Additionally, pyrazine has a low pKa (0.6), indicative of its weak basicity, making proton transfer less favourable and slower. The protonated form is relatively unstable, further hindering relative intermediate formation during tautomerisation. Consequently, the slow tautomerisation process results in a lower overall reaction yield than expected. This suggests that optimising the reaction conditions could overcome this limitation and improve the yield.

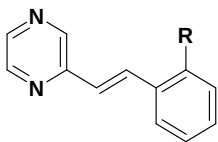
Scheme 2.2 Proposed reaction mechanism to afford pyrazinib acetate by Perkin condensation.



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The structure of pyrazinib acetate and pyrazinib were fully characterised by IR, ^1H and ^{13}C NMR spectroscopy. IR spectroscopy showed the presence of carbonyl group of pyrazinib acetate at 1758 cm^{-1} . A broad signal at 3000 to 3301 cm^{-1} was observed for pyrazinib due to the phenolic hydroxy (Table 2.1).

Table 2.1: % yield and IR cm^{-1} of pyrazinib acetate and pyrazinib.

Compound number	Structure	Yield (%) ^a	IR ν_{max} cm^{-1} (ATR)
			
55	R = OC(O)CH ₃	15.6	1758 (C=O)
54	R = OH	12.5	3301 (OH)

^a yield for single step

The ^1H and ^{13}C NMR spectra of pyrazinib acetate (**55**) is shown in Figures 2.4 and 2.5. All of the signals in the spectra were assigned and are shown beside the structure. The ^1H NMR spectrum of pyrazinib acetate (**Figure 2.4**) confirms the presence of the *trans* configuration of the double bond resonating at δ 7.75 and 7.41 ppm with a coupling constant of 16 Hz which is characteristic of *trans* bonds. A *cis* configuration would exhibit a lower coupling constant in the range of 6-8 Hz.⁹² The methyl group of the acetate group is observed at δ 2.39 ppm. The signals at δ 8.53, 8.65 and 8.84 ppm are attributable to the pyrazine ring protons. The phenyl shows chemical shift values at 7.2, 7.35, 7.43 and 7.92 ppm.

In the ^{13}C NMR of pyrazinib acetate (**Figure 2.5**), the signal at δ 169.7 ppm is assigned to the carbonyl group of the acetate group. The carbons of the *trans* double bond have chemical shift values of 127.4 and 127.3 ppm respectively. The carbon of the methyl group of the acetate group is observed at δ 21.2 ppm.

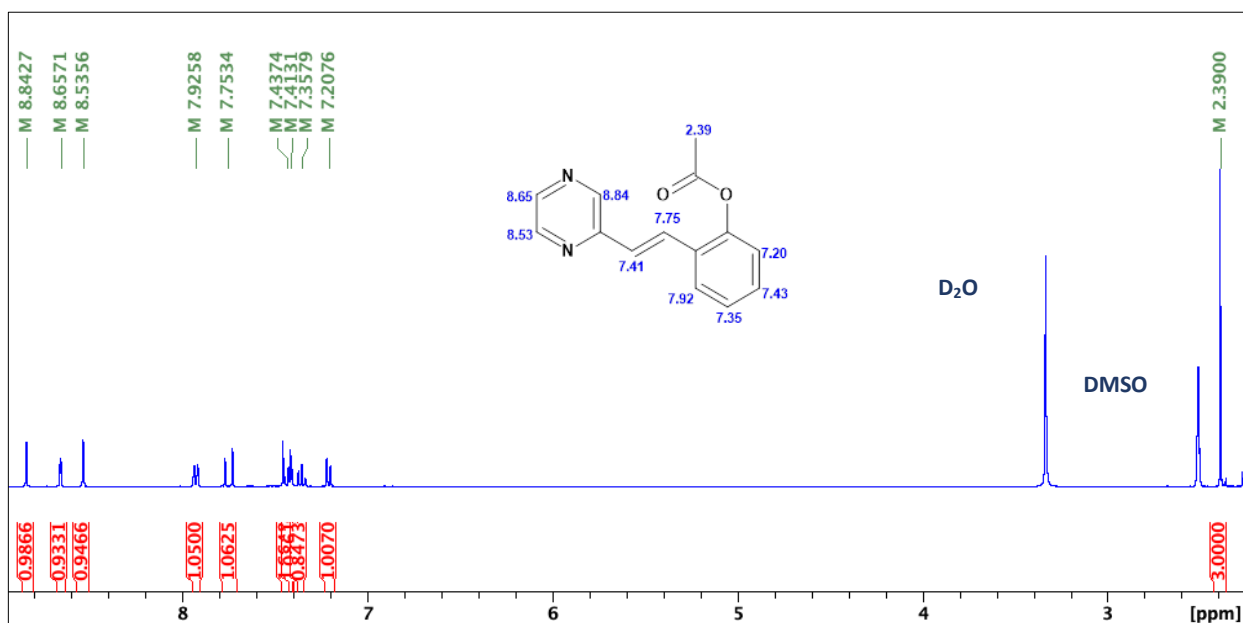


Figure 2.4: ^1H NMR of pyrazinib acetate (55) in $\text{DMSO}-d_6$ at 600 MHz

(Note: DMSO signal: 2.50 ppm and water signal: 3.3 ppm)

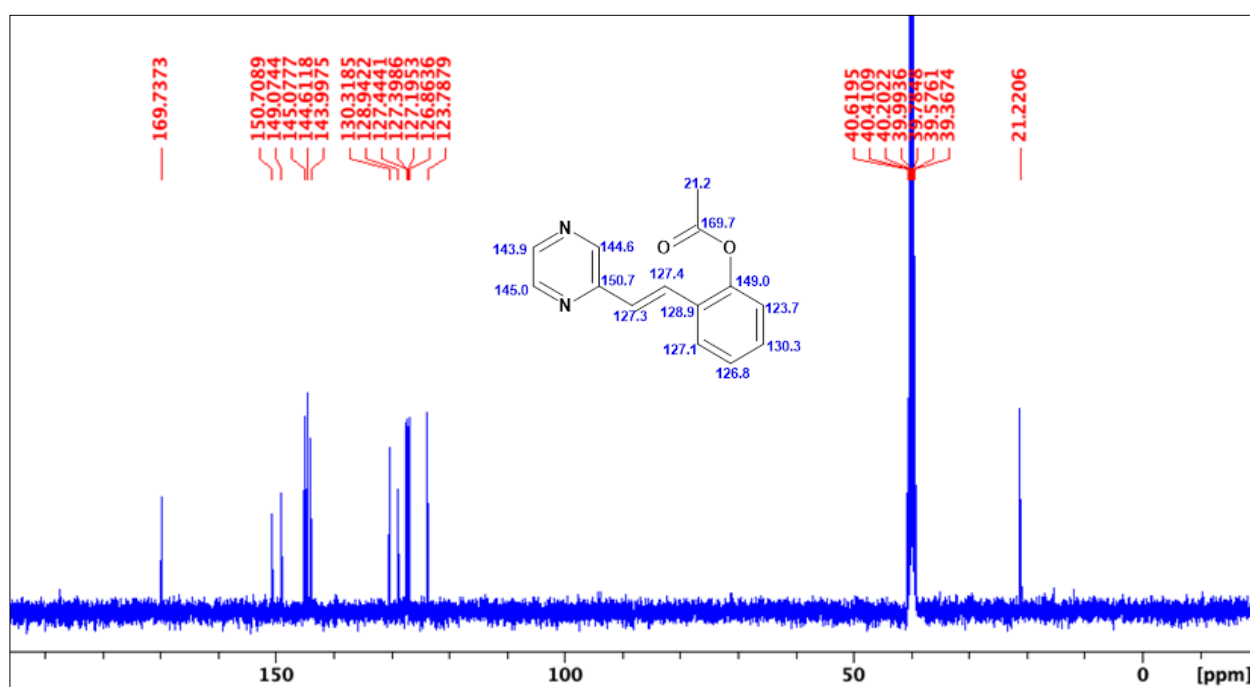


Figure 2.5: ^{13}C NMR of pyrazinib acetate (55) in $\text{DMSO}-d_6$ at 600 MHz

(Note: DMSO signal: 39.3 to 40.6 ppm)

The ^1H NMR spectrum of pyrazinib (**54**) (Figure 2.6) shows the presence of a broad singlet at δ 10.0 ppm, indicating the formation of an OH group. The ^{13}C NMR of pyrazinib shows no presence of the carbonyl group which indicating the successful hydrolysis of the acetate group to afford the phenolic moiety.

In the ^1H NMR of pyrazinib the *trans* configuration of the alkene bond is detected at δ 8.03 and 7.37 ppm. The signals at δ 8.46 ($J = 2.5$ Hz), 8.60 ($J = 2$ Hz) and to 8.73 ($J = 1.5$ Hz) ppm are attributable to the pyrazine ring protons. The phenol ring is *ortho* substituted. To understand the complicated aromatic systems, J values are helpful to assign the aromatic protons. The characteristic J values for *ortho* is 6 -9 Hz, while *meta* is smaller 2-3 Hz.

The protons at 6.92 and 6.86 ppm appear as an *ortho*-coupled doublet ($J = 7.8$ Hz) and an *ortho*-coupled triplet ($J = 7.6$ Hz), respectively. Furthermore, the proton at 7.18 ppm appears as a triplet of doublets (td, $J = 7.1$ and 1.6 Hz). The larger J value (7.1 Hz) indicates *ortho* coupling with the proton at 6.86 ppm, while the smaller J value (1.6 Hz) suggests *meta* coupling with the proton at 6.92 ppm. The proton at 7.64 ppm appears as a doublet of doublets (dd, $J = 8$ and 1.7 Hz) due to its coupling with two non-equivalent protons; the larger J value corresponds to *ortho* coupling with the proton 7.18 ppm, and the smaller J value corresponds to *meta* coupling with the proton 6.92 ppm.

Figure 2.7 shows the ^{13}C NMR of pyrazinib (**54**). The disappearance of the carbonyl signal at 169.7 ppm and the methyl group at the 21.2 ppm is evidence of the hydrolysis of the C-O of the ester bond. The carbon atom attached to the hydroxy group appears further downfield in the spectra at 156 ppm. Moreover, the carbon signals of pyrazine ring appear downfield from 151 to 143 ppm. The carbon signals at 130.3 and 124 ppm were attributed to the *trans* double bond of the molecule. Additionally, the carbon signals of phenol ring appear between 116 to 130.4 ppm. Full assignment of proton and carbon signals are shown in Figures 2.6 and 2.7.

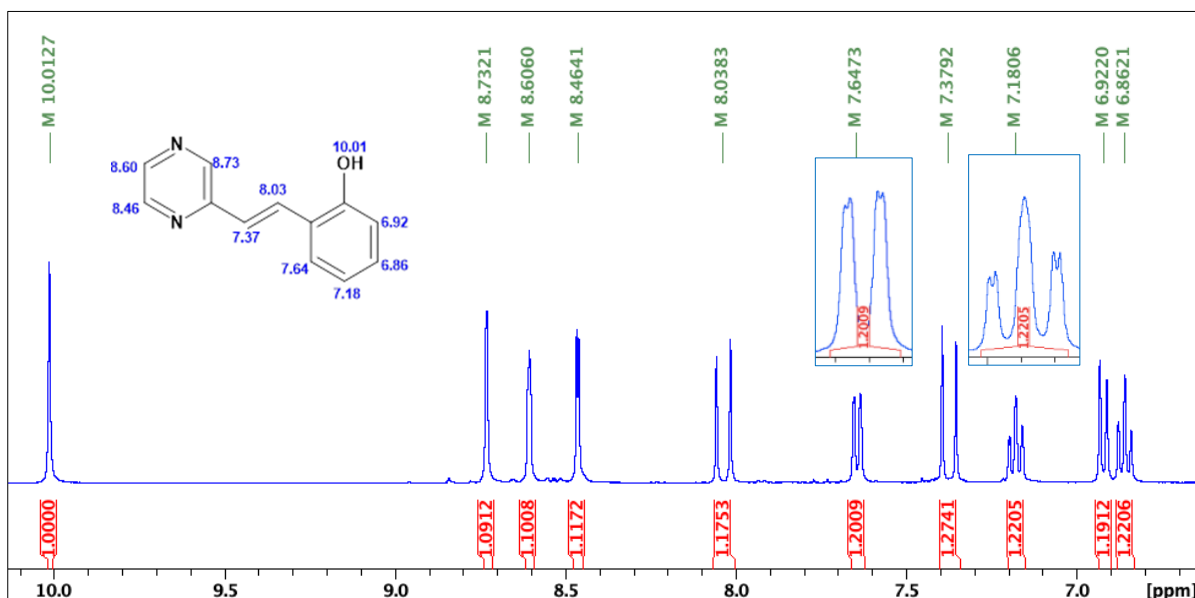


Figure 2.6: ^1H NMR of pyrazinib (54) in $\text{DMSO}-d_6$ at 600 MHz

(^1H NMR showing from 6.86 to 10.01 ppm and expanded signals 7.18 and 7.64 ppm).

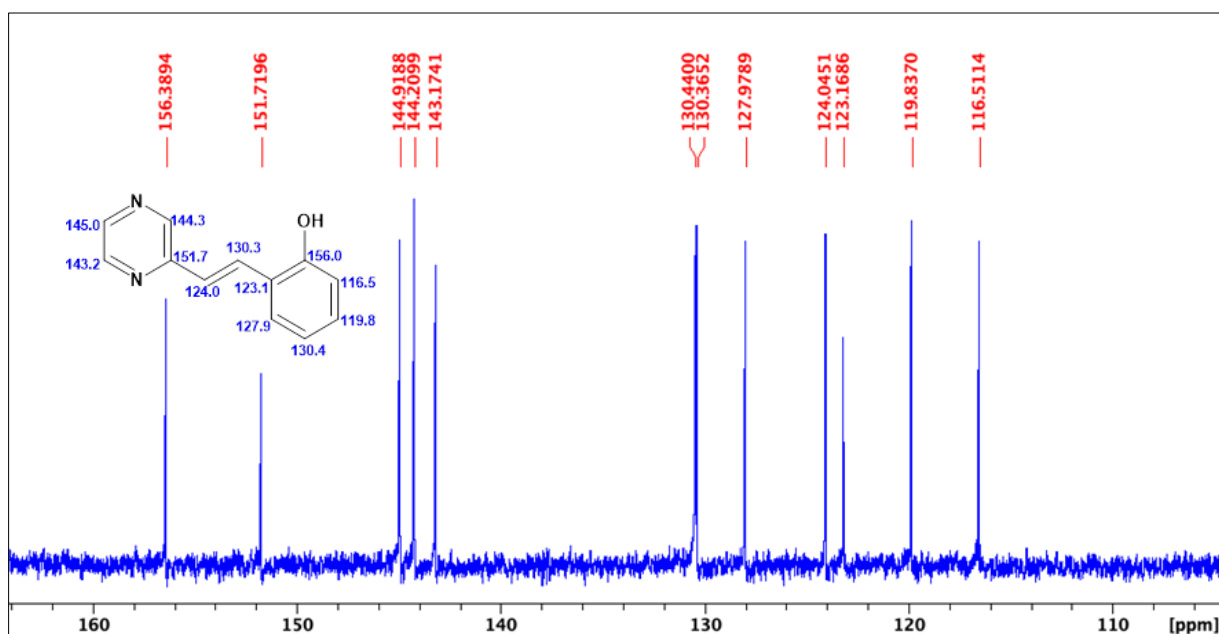


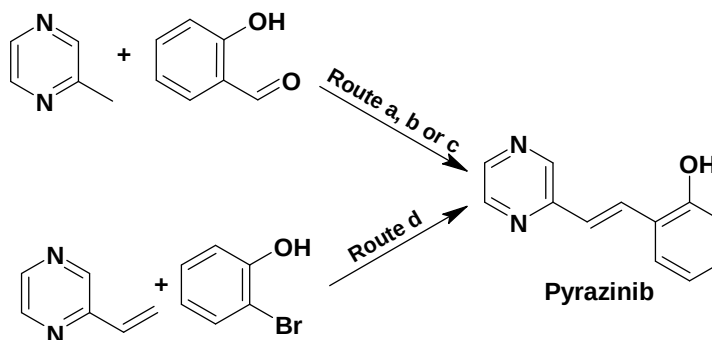
Figure 2.7: ^{13}C NMR of pyrazinib (54) in $\text{DMSO}-d_6$ at 600 MHz

(^{13}C NMR showing from 116 to 156 ppm).

2.2.2 Investigation of synthetic routes to improve the reaction time and yield of pyrazinib

To improve the yield and decrease the reaction time of pyrazinib (**54**) synthesis, 4 different synthetic routes were investigated (**Scheme 2.3**). Two routes (A and D) utilised microwave-assisted synthesis (MAOS) techniques.

Scheme 2.3: Synthesis of pyrazinib using 4 different synthetic methods.

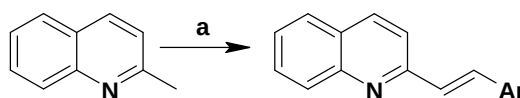


Reagents and conditions: (a) zinc chloride (0.45 mmol), MW at 140°C for 5 hours, 0.5%; (b) cobalt chloride (10 mol%), water, reflux at 120°C for 24 hours, 1%; (c) $\text{Ca}(\text{OTf})_2$ (5 mol%), Bu_4NPF_6 (5 mol%), under neat conditions at 130°C for 5 hours, 4%; (d) palladium (II)acetate, triethylamine, acetonitrile, MW at 160°C, for 15 mins, 36%

2.2.2.1 Route A: Zinc catalysed MAOS

MAOS is becoming increasingly important nowadays due to its advantages such as shorter reaction times, greater yields and solvent-free conditions. Li *et al.* reported the synthesis of (*E*)-2-styrylquinoline derivatives by using 2-methylquinolines with substituted benzaldehydes under microwave irradiation and solvent-free conditions in the presence of the catalyst, zinc chloride (**Scheme 2.4**).⁹³

Scheme 2.4: MAOS-zinc catalysed synthesis of (*E*)-2-styrylquinoline derivatives reported by Li *et al.*



Reagents and conditions: (a) ArCHO , zinc chloride (0.45 mmol), MW irradiation for 4-15 minutes

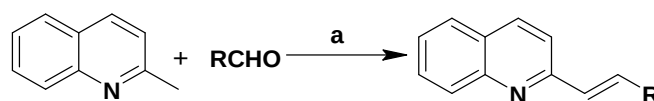
The MAOS of pyrazinib was carried out on a 10 mmol scale, in which 2-methylpyrazine was reacted with 2-hydroxybenzaldehyde using zinc chloride for 5 hours at 140 °C under solvent-free conditions (**Scheme 2.3, route A**). After 5 hours, TLC indicated the formation of pyrazinib with a bright yellow spot at R_f value of 0.35cm [mobile phase: hexane: ethyl acetate (1:1)]. The formation of pyrazinib was confirmed by ^1H NMR. The isolated, purified yield of pyrazinib was 0.5%. The key benefits of this method include being a one-step solvent free reaction, reducing the long reaction time of 6

days to 5 hours and ease of product purification due to fewer by-products. However, the yield was much lower compared to the conventional Perkin route.

2.2.2.2 Route B: Cobalt catalysed direct C(sp³)-H alkenylation

In 1959, Edward Barrall reported the first C(sp³) functionalisation of 2-methylquinolines.⁹⁴ Jamal *et al.* reported a simple and straightforward methodology to afford 2-alkynylated quinolines by direct alkenylation of 2-methylquinoline with a wide range of aldehydes including heteroaromatic aldehydes in the presence of a catalyst and water (**Scheme 2.5**).⁹⁴ This type of synthetic strategy may not only significantly simplify and shorten the reaction time but also allow the use of inexpensive and environmentally friendly starting materials. The key aspect of this type of reaction is the use of a Lewis acid to catalyse the reaction. Various transition metal and alkaline earth complexes can act as Lewis acids and be used as catalysts for these types of reactions.⁹⁴ Jamal *et al.* screened various cobalt salts and found CoCl₂ to be the most effective in promoting the reaction and improving the yield in the presence of water at 120°C as shown in Table 2.2.

Scheme 2.5: Synthesis of 2-alkynylated quinolines by cobalt catalysed direct alkenylation reported by Jamal *et al.*



Reagents and conditions: (a) cobalt chloride (10 mol%), water, reflux at 120°C for 24 hours

Table 2.2: Optimisation of the reaction conditions for the synthesis of 2-alkynylated quinolines by cobalt catalysed direct alkenylation.⁹⁴

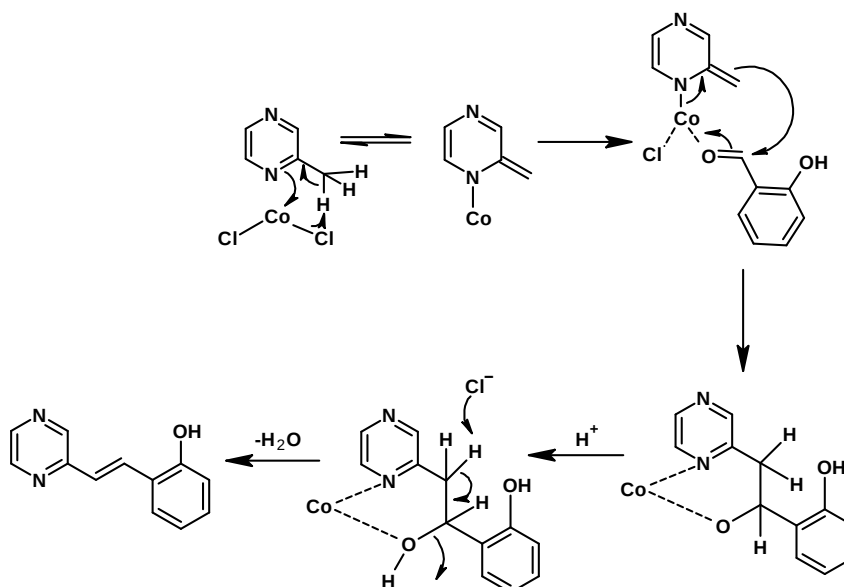
Cobalt salt (10 mol %)	Solvent	Temperature (°C)	Yield (%)
Co(ClO ₄) ₂ ·6H ₂ O	THF	140	53
CoC ₂ O ₄ ·2H ₂ O	THF	140	trace
Co(acac) ₂	THF	140	62
No catalyst	Water	120	30
CoCl ₂	THF	140	78
CoCl ₂	Water	120	90

Our approach towards route B, began with reacting 2-methylpyrazine with 2-hydroxybenzaldehyde in the presence of CoCl₂ and water under reflux at 120 °C for 24 hours to afford pyrazinib as shown in Scheme 2.3, route B. The reaction mixture was monitored by TLC, and the crude mixture was

purified by flash column chromatography to afford pyrazinib in a yield of < 3%. This indicates that the direct alkenylation of 2-methylpyrazine with 2-hydroxybenzaldehyde was not as successful as with 2-methylquinoline. The reason for the unsuccessful reaction could be acid-catalysed tautomerization of 2-methylpyrazine. Acid catalysed tautomerisation of 2-methylpyrazine introduces significant challenges to its chemical reactivity. The formation of tautomer not only alters the molecule's structure but also the reactivity profile. This can lead to low yield or potentially failure of the reaction.

The plausible reaction mechanism is shown in Scheme 2.6. The first step of the reaction mechanism involves the C-H bond activation of the 2-methylpyrazine ring facilitated by cobalt chloride (Lewis acid) that coordinates to the nitrogen atom, leading to the C(sp³)-H bond cleavage. The second step involves the benzylic addition to the carbonyl of 2-hydroxybenzaldehyde, leading to the formation of an alcohol intermediate. Finally, cobalt chloride is eliminated to afford pyrazinib.

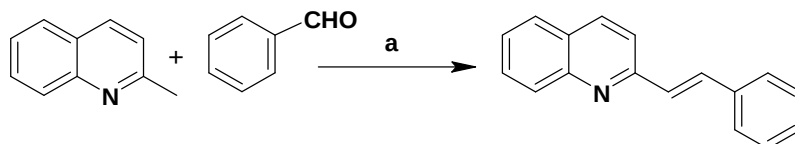
Scheme 2.6: Plausible cobalt catalysed reaction mechanism to afford pyrazinib.



2.2.2.3 Route C: Calcium catalysed direct C(sp₃)-H functionalisation.

After the unsuccessful cobalt catalysed C-H functionalisation of 2-methylpyrazine with 2-hydroxybenzaldehyde, we approached route C to improve the % yield of pyrazinib. This green synthetic methodology was reported by Yaragorla *et al.* for the synthesis of (*E*)-2-styrylazaarenes by utilising an alkaline earth metal, such as calcium, as a catalyst under solvent-free conditions (**Scheme 2.7**).⁹⁵ Ca (II) is a green Lewis acid and is highly attractive for use as a catalyst due to its low cost and stability towards moisture and water.⁹⁵ They optimised reaction conditions for the synthesis by using an additive, tetrabutylammonium hexafluorophosphate, changing solvent and temperature conditions to improve the % yield and shorten the reaction time as shown in Table 2.3. Additionally, using an additive with calcium triflate (Lewis acid catalyst) reduced the reaction time by 3 hours (from 8 to 5 hours) as well as marginally increasing the yield (**Table 2.3**).

Scheme 2.7: Synthesis of (*E*)-2-styrylazaarenes by calcium catalysed C-H functionalisation 2-methylquinolines



Reagents and conditions: (a) Ca(OTf)₂ (5 mol%), Bu₄NPF₆ (5 mol%), under neat conditions at 130°C for 5 hours.

Table 2.3: Optimisation studies for the synthesis of (*E*)-2-styrylazaarenes.⁹⁵

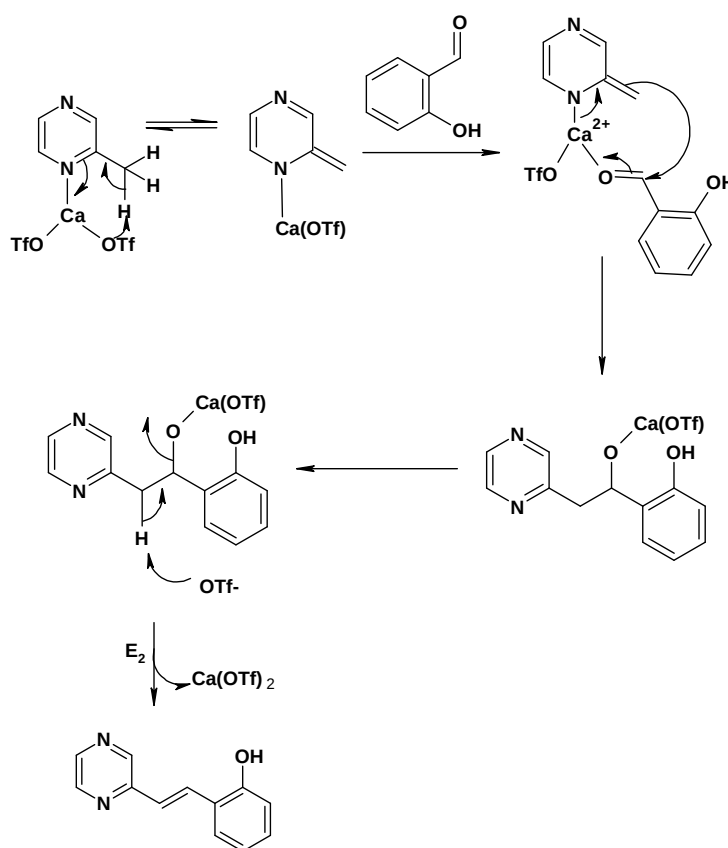
Ca(OTf) ₂ (mol%)	Bu ₄ NPF ₆ (mol%)	Solvent	Temperature (°C)	Time (hrs)	Yield (%)
5	5	water	100	24	40
5	0	neat	130	8	94
5	2	neat	130	5	96
5	5	neat	130	5	96
0	5	neat	130	24	40
0	0	neat	130	24	35

With the aim of improving the yield of pyrazinib, we began our approach towards route C by using calcium triflate (5 mol%) along with additive tetrabutylammonium hexafluorophosphate (5 mol%) under neat conditions at 130°C for 5 hours as shown in Scheme 2.3, route C. The reaction was monitored by TLC, and a yellow spot for pyrazinib appeared on the TLC after 60 min of starting the

reaction which confirmed the formation of pyrazinib. After 5 hours the crude mixture was purified by flash column chromatography to afford pyrazinib in 4% yield. Route C also failed to improve the % yield of pyrazinib but shortened the reaction time compared to the Perkin method.

A plausible reaction mechanism has been described in Scheme 2.8. The first step of the reaction mechanism involves the coordination of the calcium catalyst to the nitrogen of 2-methylpyrazine ring. This complex undergoes nucleophilic addition with 2-hydroxybenzaldehyde which results in the formation of an intermediate. In the final step, the intermediate undergoes E₂ elimination, where the triflate ion abstracts the β-hydrogen, leading to the formation of a double bond and affording the pyrazinib.

Scheme 2.8 Plausible calcium catalysed reaction mechanism to afford pyrazinib.



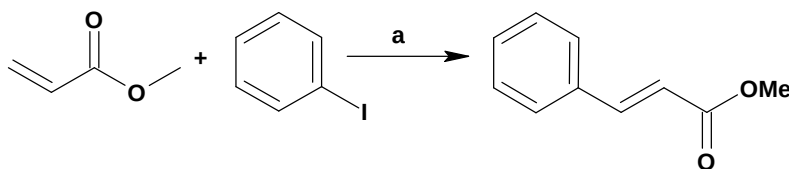
2.2.2.4 Route D: Heck reaction MAOS

With the aim of improving the % yield and shortening the reaction time for the synthesis of pyrazinib, the reaction time was successfully reduced from 6 days to 5 hours using route C. However, the aim of increasing % yield still was not achieved. Hence, route D was approached, a Heck reaction carried out under microwave irradiation. The Heck reaction was discovered by Mizoroki and Heck in the early 1970's.⁹⁶ It is a chemical reaction which involves C-C coupling between vinyl halides and an alkene in the presence of palladium catalyst and base.⁹⁷ The reaction requires palladium salts as catalysts such as palladium (II) acetate or palladium chloride along with typical bases (triethylamine, sodium acetate and potassium carbonate).⁹⁷ The Heck reaction has the advantages of proceeding under mild conditions, tolerates a variety of functional groups such as aldehydes, esters and nitro groups and often affords high yield of the desired products. It is limited by difficult separation. This synthetic methodology was taken from CEM microwave assisted Heck reaction protocols, in which they demonstrated a modification of a conventionally heated transformation to microwave heating (MAOS).⁹⁸ They carried out the conventional Heck reaction of iodobenzene with methyl acrylate at 80°C for 20 hours (**Scheme 2.9**).⁹⁸ However, after applying calculations based on the Equation 2.1, where, T= microwave heating temperature, T₀ = conventional heating temperature, x = temperature increase coefficient, they noticed that simply doubling the temperature to 160°C (x = 8) via microwave irradiation could lead to shorter reaction time of 10 minutes.⁹⁸

$$\text{Equation 2.1: } T = T_0 + (10^\circ\text{C})x, t \approx \frac{T_0}{2^x}$$

Furthermore, they carried out the microwave assisted Heck reaction at various reaction times such as 3, 5, 10 and 15 minutes and noticed that reaction time of 15 minutes increased the % yield by 7 % (from 78% for 3 mins to 85% for 15 mins).⁹⁸ Notably, the formation of by-products increased as the reaction time increased, hence the reaction time of 10 minutes met comparable level of yield and purity.

Scheme 2.9: MAOS-assisted Heck reaction of iodobenzene with methyl acrylate.



Reagents and conditions: (a) palladium (II)acetate, triethylamine, acetonitrile, MW at 160°C

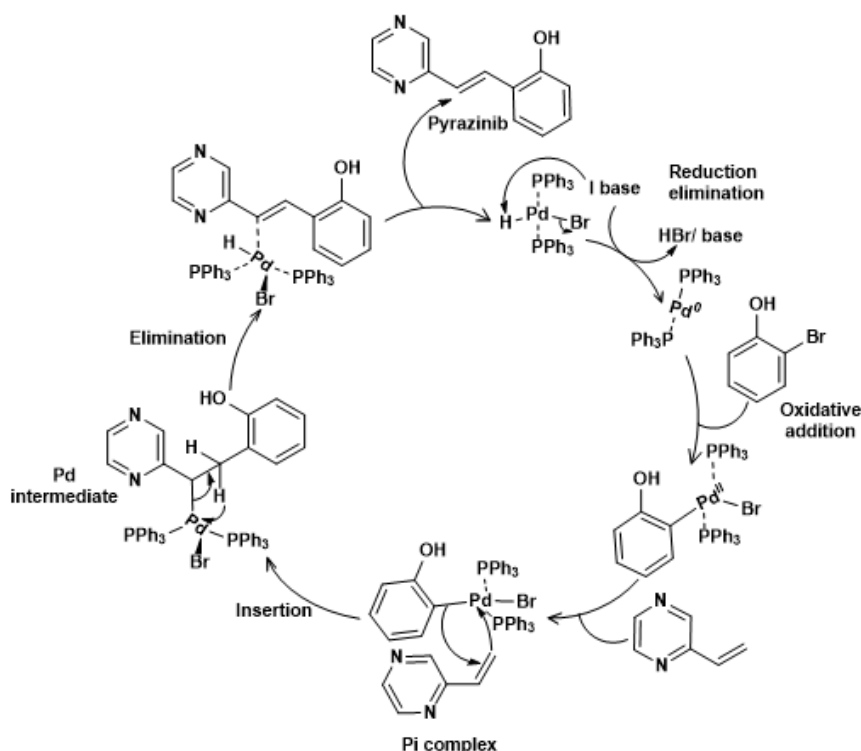
MAOS-assisted Heck coupling was investigated for the synthesis of pyrazinib. The reaction began by reacting 2-vinylpyrazine with 2-bromophenol in the presence of palladium (II) acetate, triethylamine and acetonitrile at 160°C (**Scheme 2.3, route D**). The reaction time was optimised and it was noticed that yield was increased as the reaction time increased (**Table 2.4**) but as stated before that increased reaction time resulted in increased formation of by-products. Hence, the best reaction time was found to be 15 minutes as the yield of pyrazinib was comparably higher than other reaction times shown in Table 2.4. The percentage yield mentioned in Table 2.4 was determined after isolating the pure pyrazinib product from reaction mixture through flash column chromatography. The plausible Heck reaction catalytic cycle is shown in Scheme 2.10. This Heck catalytic cycle illustrates a palladium-catalysed coupling reaction. Key steps include oxidative addition of the 2-bromophenol to Pd (0), insertion of 2-vinylpyrazine into the Pd (II) complex, and reductive elimination to release pyrazinib (**54**) while regenerating Pd (0).

Table 2.4: Optimisation of the reaction times for the synthesis of pyrazinib by MAOS-assisted Heck reaction.

Entry	Time (min)	Yield (%)
1	2	15
2	5	24
3	15	36
4	30	36

Note: Temperature was 160°C for all reactions.

Scheme 2.10: Plausible palladium catalysed Heck reaction mechanism to afford pyrazinib.



2.2.3 Comparison of the percentage % yield obtained for pyrazinib by Perkin method vs four attempted synthetic routes

This section compares the % yield and reaction time of the conventional and MAOS synthetic methods attempted for the synthesis of pyrazinib. Table 2.5 shows the reaction conditions, reaction time and % yield achieved for pyrazinib by all the synthetic methods including the Perkin reaction. Firstly, a conventional Perkin method was tried which is a two-step procedure and required a reaction time of 5-6 days with pyrazinib afforded in 12% yield. To improve this yield and to achieve a shorter reaction time, 4 different synthetic methods were investigated. All routes involved the use of different catalysts, as a catalyst leads to faster and more energy efficient chemical reactions. Routes A and D are MAOS assisted reactions, can lead to a shorter reaction time when accompanied by a catalyst, can improve yield for the desired product. However, route A resulted in an extremely poor yield of 0.5% for pyrazinib. In comparison, route D (Heck reaction by MAOS) afforded pyrazinib in 36% yield, a notable improvement in yield, and reduced the reaction time to 15 minutes. On the other hand, conventional synthetic routes B and C are one-step synthesis, but the yield was extremely poor (only 1% and 4% yields, respectively). One major advantage of routes A, B and C is that purification of pyrazinib was easier than conventional Perkin method due to the formation of fewer by-products. However, these routes are limited due to their extremely poor yield of pyrazinib. Of all four synthetic routes attempted in this investigation, route D successfully increased the yield of pyrazinib, shortened the reaction time, led to easier purification and reduction in number of steps compared to conventional Perkin reaction. The study to improve the yield of pyrazinib by different synthetic routes determined that the Heck reaction, facilitated by MAOS, can afford pyrazinib in an improved yield within just 15 minutes.

Table 2.5: Comparison of reaction conditions and % yield of pyrazinib obtained by Perkin reaction and four alternative synthetic routes.

Perkin reaction		Four alternative synthetic routes			
		A	B	C	D
Reaction conditions	Step 1: Acetic anhydride, reflux at 180°C Step 2: Sat. NaOH, EtOH/Ethyl acetate, reflux at 100°C	Zinc chloride, MW at 140°C	Cobalt chloride (10 mol%), H ₂ O, reflux at 120°C	Calcium Triflate (5 mol %), Bu ₄ NPF ₆ (2 mol %), heat at 130°C	Palladium (II)acetate, triethylamine, acetonitrile, MW at 160°C
Reaction time	5-6 days	5 hours	24 hours	5 hours	15 mins
Pyrazinib % yield	12	0.5	1	4	36

2.3 Improvement of aqueous solubility of pyrazinib

2.3.1 Design and synthesis of amino acid prodrugs of pyrazinib: HBTU coupling reactions

As discussed in section 2.1.2, due to poor aqueous solubility, pyrazinib cannot be further utilised in *in vivo* animal studies. Hence, to improve its aqueous solubility, it was decided to develop amino acid prodrugs of pyrazinib. Amino acids are biological agents with low toxicity levels, and have been shown to improve the water solubility of parent drugs.⁹⁹ In order to prepare amino acid prodrugs of pyrazinib with improved aqueous solubility, the phenolic group of pyrazinib was coupled to a variety of amino acids. In this study, a HBTU base catalysed coupling route was attempted (discussed in Section 2.3.1.2). HBTU is a standard coupling agent. During peptide synthesis, it activates the carboxylic acid by forming a stabilized *HoBt* leaving group.¹⁰⁰ It was introduced in 1978 and gained popularity in chemistry and industry due to its mild activating properties.¹⁰¹ In addition, it provides short reaction times with quantitative yields, and it is highly stable and soluble in organic solvents. Typically, amino acids prodrugs are either esters or amides, in which a carboxylic acid group or an amino group of an amino acid is coupled to the functional group of the parent drug such as hydroxyl, amine or carboxyl.⁹⁹ Moreover, in order to control the coupling process, it is important to use a protected amino acid.¹⁰² A short introduction to amino acid protecting groups is below in Section 2.3.1.1.

2.3.1.1 Amino acid protecting groups

The protection of a functional group is crucial in the chemistry of polyfunctionalized molecules when a reaction has to be conducted in a part of the molecule without affecting the rest of the molecule. The amino group is a key functional group which is present in several compounds such as natural products, peptides and amino acids. The protection of the amino (NH_2) functionality of amino acids is an important aspect of peptide chemistry to prevent the amino acid from polymerizing once activated. In peptide synthesis, both in solution or on solid phase, the process typically progresses from the C to N direction.¹⁰³ The amino protecting groups must exhibit solubility in common solvents and prevent epimerization during the coupling process. The removal of the protecting groups must be fast, efficient, free of side reactions, and should provide an easy elimination of by-products. Other desirable characteristics of amino-protected amino acids include their crystalline nature and stability. Among the large array of protecting groups, the two most widely used amino-protecting groups in organic chemistry are the 9-fluorenylmethoxycarbonyl (Fmoc) and the tert-butyloxycarbonyl (Boc) groups.

Initially, the coupling of pyrazinib with Fmoc-protected amino acid was attempted using HBTU and DIPEA base. However, the Fmoc group was cleaved during the coupling process, due to its sensitivity towards bases. Conversely, Boc fulfils the requirement of a good protecting group and is preferred

for amino protection due to its stability towards most nucleophiles and bases. Therefore, the Boc protecting group was deemed most suitable for HBTU base catalysed coupling process.

2.3.1.1.1 Boc protecting group

The Boc group is widely used in organic synthesis due to its chemical stability under basic and mildly acidic conditions (**Figure 2.8**).¹⁰⁴ The conditions for protecting a free amine are quite flexible, often resulting in high yields and fast conversions under mild conditions. The protection process is usually carried out in water, THF, or acetonitrile at room temperature with moderate heating (40°C), in the presence of a base.¹⁰⁵ The amino acids to be coupled to pyrazinib were commercially available as Boc protected carbamates.¹⁰⁶ The reaction mechanism for the formation of Boc protected amine involves the amine attacking a carbonyl group of di-*tert*-butyl dicarbonate, which leads to the departure of *tert*-butyl carbonate as a leaving group. Then, the *tert*-butyl carbonate accepts a proton from the protonated amine and the *tert*-butyl bicarbonate breaks down into CO₂ (gas) and *tert*-butanol. The Boc group is highly unstable under acidic conditions, and its cleavage largely relies on strong acids such as TFA. Methods used for Boc deprotection in this study is described in detail in Section 2.3.2.

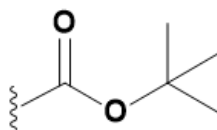


Figure 2.8: Structure of the Boc protecting group

2.3.1.2 Synthesis of pyrazinib Boc-amino acid conjugates

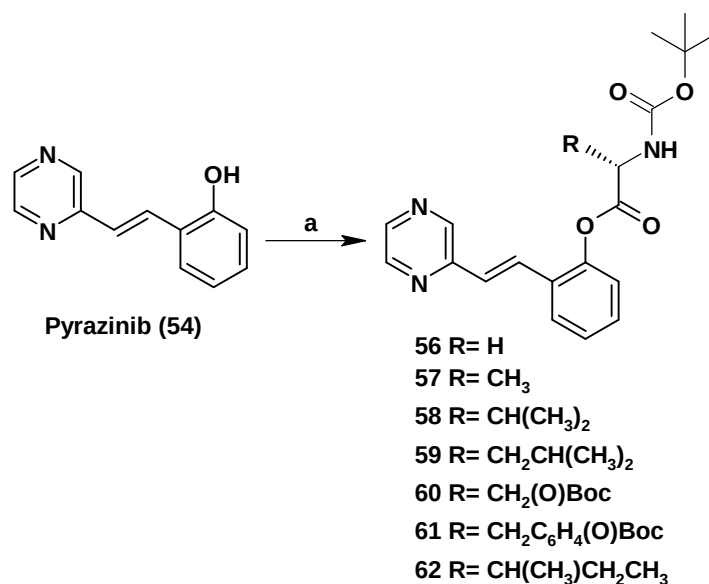
In this work, HBTU with diisopropylethylamine (DIPEA base or Hünig's base, a strong non-nucleophile base employed as a proton scavenger in peptide synthesis) were employed to facilitate the coupling of the hydroxyl group of pyrazinib with the carboxylate moiety of amino acids. A representative reaction scheme is shown in Scheme 2.11. This synthetic coupling route had been reported by Tripodi *et al* to couple *trans*-2-azetidinones with (*L*) *N*-Boc-proline amino acid.¹⁰⁷

Amino acids with their amine groups protected by a Boc group were used during the coupling reaction. This Boc protection ensured that only the carboxylate group of the amino acids could react with the hydroxyl group of pyrazinib preventing any unwanted reactions involving the amine group. Amino acids containing a hydroxyl group such as serine and tyrosine also required a Boc protection of their hydroxyl group (**Table 2.6**). All synthesised pyrazinib Boc-amino acid conjugates are listed in Table 2.6.

The rationale for the selection of valine, glycine, tyrosine, serine, alanine, isoleucine, and leucine

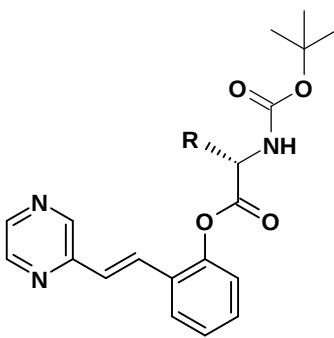
in the synthesis of pyrazinib Boc-amino acid conjugates is based on factors including their diverse structural features and functional groups. The chosen amino acids represent a diverse set in terms of side chain structure and functional groups, and serine and tyrosine both contain an extra OH. This diversity results in variations in the physicochemical properties of the conjugates, influencing their solubility and stability.

Scheme 2.11: Synthesis of pyrazinib Boc-amino acid conjugates by HBTU coupling reaction.



Reagents and conditions: (a): N-Boc amino acid (2.5 eq), anhydrous acetonitrile, HBTU (2.2 eq), DIPEA base (61 eq), stirring at room temperature for 24 hrs, 48-60%

Table 2.6: List of synthesised pyrazinib Boc-amino acid conjugates (56-62).

Compound number	Structure	Amino acid	Yield (%)	IR ν_{max} cm^{-1} (ATR)
				
	R =			
56	H	Glycine	48	3200 (NH), 1755 (C=O, ester linkage bond), 1709 (C=O Boc)
57	CH ₃	Alanine	52	3311 (NH), 1765 (C=O, ester linkage bond), 1701 (C=O, Boc)
58	CH(CH ₃) ₂	Valine	55	3333 (NH), 1754 (C=O, ester linkage bond), 1686 (C=O Boc)
59	CH ₂ CH(CH ₃) ₂	Leucine	57	3298 (NH), 1764 (C=O, ester linkage bond), 1702 (C=O, Boc)
60	CH ₂ (O)Boc	Serine	54	2975 (NH), 1769 (C=O, ester linkage bond), 1709 (C=O, Boc), 1635 (C=O, Boc)

61	CH ₂ C ₆ H ₄ (O)Boc	Tyrosine	60	3335 (NH), 1763 (C=O, ester linkage bond), 1705 (C=O Boc), 1663 (C=O, Boc)
62	CH(CH ₃)CH ₂ CH ₃	Isoleucine	60	3275 (NH), 1761, (C=O, ester linkage bond), 1703 (C=O, Boc)

All products were purified by flash column chromatography and obtained in the yield of 48-60%. All the conjugates were fully characterized by NMR spectroscopy, IR spectroscopy and mass spectrometry. The ¹H NMR spectrum of pyrazinib Boc valine derivative (**58**) is shown in Figure 2.9. The ¹H NMR shows three protons of the pyrazine ring at δ 8.96, 8.61 and 8.51 ppm. The coupling constant for the protons attached at the double bond is 16 Hz (δ 7.8 and 7.36 ppm respectively) indicating the *trans* geometry. The CH x 2 and the CH₃ x 2 of the valine amino acid appear at δ 4.15, 2.26, 1.04 ppm. Moreover, the CH₃ x 3 of the Boc group appears as a singlet at δ 1.38 in the ¹H NMR spectrum as indicated on Figure 2.9. The aromatic protons including pyrazine appears from 7.11 to 8.96 ppm.

The ^{13}C NMR of pyrazinib Boc valine (**58**, **Figure 2.10**), shows a signal for the carbonyl of the ester group at δ 171.6 ppm and carbonyl of Boc group at δ 156 ppm. Furthermore, the carbon signals at δ 28.5 and 79.0 ppm respectively, attributes to the Boc group and the carbon signals at δ 60.4, 29.9 as indicated on Figure 2.10 and 19 ppm correspond to the valine amino acid. The IR spectra of these compounds showed an additional signal at approximately $1686\text{--}1709\text{ cm}^{-1}$ due to the presence of an additional carbonyl group (**Table 2.6**).

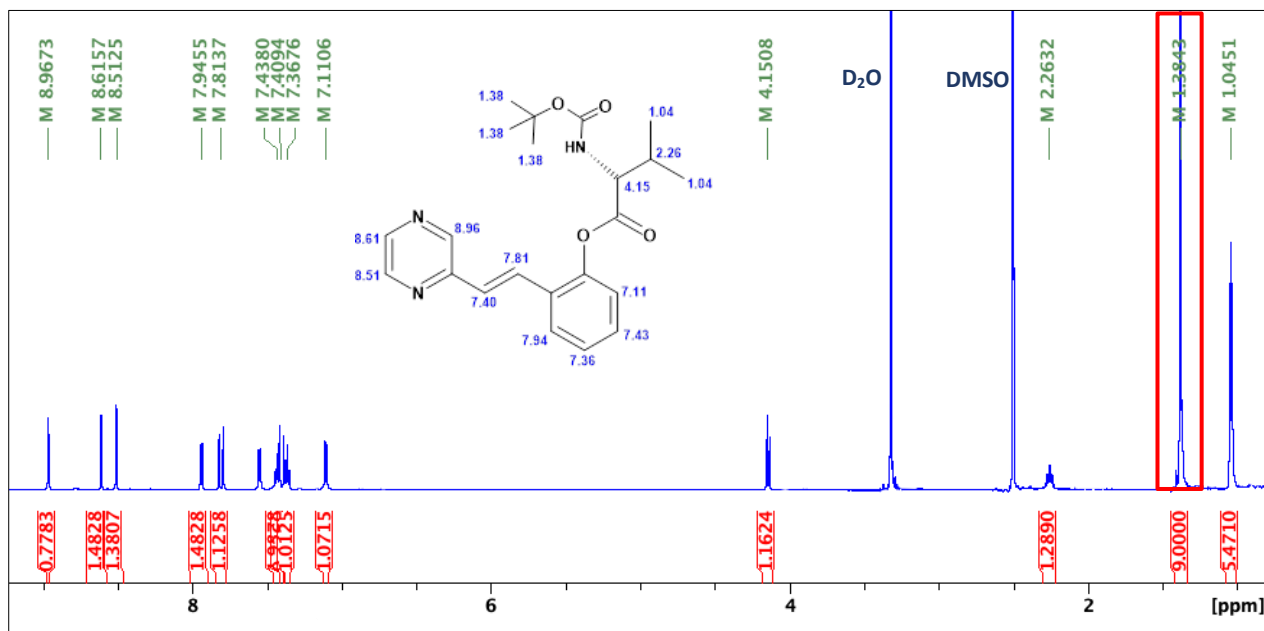


Figure 2.9: ^1H NMR of pyrazinib Boc valine (**58**) conjugate in $\text{DMSO-}d_6$ 400 MHz. Signal for Boc group highlighted in red box. (Note: DMSO signal: 2.50 ppm and water signal: 3.3 ppm)

2.3.2 Deprotection of pyrazinib amino acid prodrugs

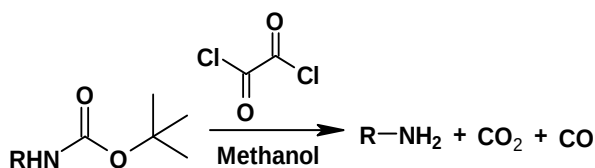
2.3.2.1 Deprotection of the Boc group

Deprotection of the Boc group from the pyrazinib Boc derivatives (**56-62**) synthesised above was accomplished under acidic conditions with a strong acid to yield the free amine. Previous studies on stability of pyrazinib indicated that it is stable at basic and acidic pH (**Figure 2.3**). The Boc group was cleaved using traditional acid mediated conditions to afford amino acid pyrazinib prodrugs (**63-69**) with yields ranging from 24 to 92% (**Table 2.7**).

The Boc-protected amino acid pyrazinib derivative was added to concentrated TFA. The reaction was rapid and monitored by TLC. According to TLC, the deprotection was achieved in less than 10 minutes. The ^1H NMR of the final product showed the removal of the Boc group but also showed traces of TFA, and difficulties arose in the complete removal of TFA from the amino acid pyrazinib conjugates. To overcome this difficulty, it was decided to attempt a new method for Boc deprotection which does not require a strong acid. A mild deprotection of the Boc group using oxalyl chloride was reported by George *et al.*¹⁰⁸ This method requires oxalyl chloride in methanol, where oxalyl chloride mainly acts as a catalyst with versatile applications. It is used for various purposes, such as synthesising acid chlorides and preparing dihydroquinolines.¹⁰⁹

To investigate if oxalyl chloride in methanol could deprotect Boc-amino acid substrates (**Scheme 2.12**), the reaction was conducted at room temperature and required over 24 hours to complete Boc deprotection. It was observed that the oxalyl chloride deprotection method required a longer duration for deprotection and resulted in a yield < 10%.

Scheme 2.12: Deprotection of Boc group using oxalyl chloride in methanol.¹⁰⁸



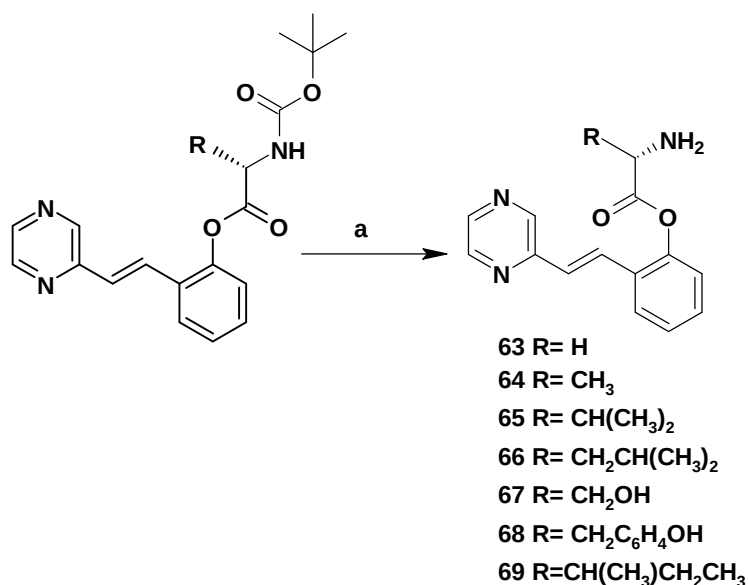
Further, it was decided to reattempt the acid deprotection method using TFA in DCM (1:1) as shown in Scheme 2.13. The reaction progressed rapidly, with TLC indicating deprotection in under 10 minutes with R_f value at 0.32 – 0.38 cm (mobile phase: n-hexane: ethyl acetate (1:1)). The crude mixture was then washed with water, and freeze-drying was employed to entirely eliminate water from the final product. This approach successfully removed the Boc group from amino acid pyrazinib derivatives, yielding purified products in less than 10 minutes with a yield 24-92%. The final product was stored at -20°C until further use.

The pyrazinib amino acid derivatives were fully characterised by ^1H NMR, ^{13}C NMR and FT-IR. The ^1H NMR of valine derivative is shown in Figure 2.11, and confirmed the disappearance of Boc group, as there is no peak present at 1.38 ppm. The ^1H NMR shows three protons of the pyrazine ring at δ 8.85, 8.65 and 8.54 ppm. The coupling constant for the protons attached at the double bond is 16 Hz (δ 7.8 and 7.47 ppm respectively) and indicates the *trans* stereochemistry. The $\text{CH} \times 2$ and the $\text{CH}_3 \times 2$ of the valine amino acid appear at δ 4.41, 1.25, 1.14 ppm. The aromatic protons including pyrazine appear from 7.24 to 8.85 ppm. The NH_2 signal is not visible due to rapid proton exchange with residual water in $\text{DMSO}-d_6$ which could suppresses the signal.

The ^{13}C NMR of the valine derivative shows a signal for the carbonyl of the ester group at δ 168 ppm (**Figure 2.12**). Furthermore, the ^{13}C NMR indicates the disappearance of Boc as there are no signals present at 156, 79 and 28 ppm. The carbon signals at δ 58, 30 and 18 ppm correspond to the valine amino acid. The carbon signals from 150 to 144 ppm (excluding 148 ppm, as it corresponds to the other aromatic ring) are attributable to the pyrazine ring.

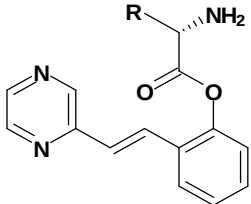
The IR spectra of these compounds showed the signals at approximately 3100 and 1769 cm^{-1} due to the presence of free amine and a carbonyl group (**Table 2.7**).

Scheme 2.13: Deprotection of pyrazinib amino acid derivatives.



Reagents and conditions: (a): TFA in DCM (1:1), room temperature, until complete as indicated by TLC, 24-92%

Table 2.7: List of pyrazinib amino acid conjugates (63-69) after deprotection of Boc group.

Compound number	Structure	Amino acid	Yield (%)	IR ν_{max} cm^{-1} (ATR)
				
	R =			
63	H	Glycine	46	3102 (NH ₂), 1751 (C=O ester linkage bond)
64	CH ₃	Alanine	62	3048 (NH ₂), 1754 (C=O ester linkage bond)
65	CH(CH ₃) ₂	Valine	92	3100 (NH ₂), 1769 (C=O ester linkage bond)
66	CH ₂ CH(CH ₃) ₂	Leucine	75	3100 (NH ₂), 1768 (C=O ester linkage bond)
67	CH ₂ OH	Serine	24	3442 (OH), 2979 (NH ₂), 1765 (C=O ester linkage bond)
68	CH ₂ C ₆ H ₄ OH	Tyrosine	75	3171 (OH), 2161 (NH ₂) 1741 (C=O ester linkage bond)
69	CH(CH ₃)CH ₂ CH ₃	Isoleucine	78	3359 (NH ₂), 1766 (C=O ester linkage bond)

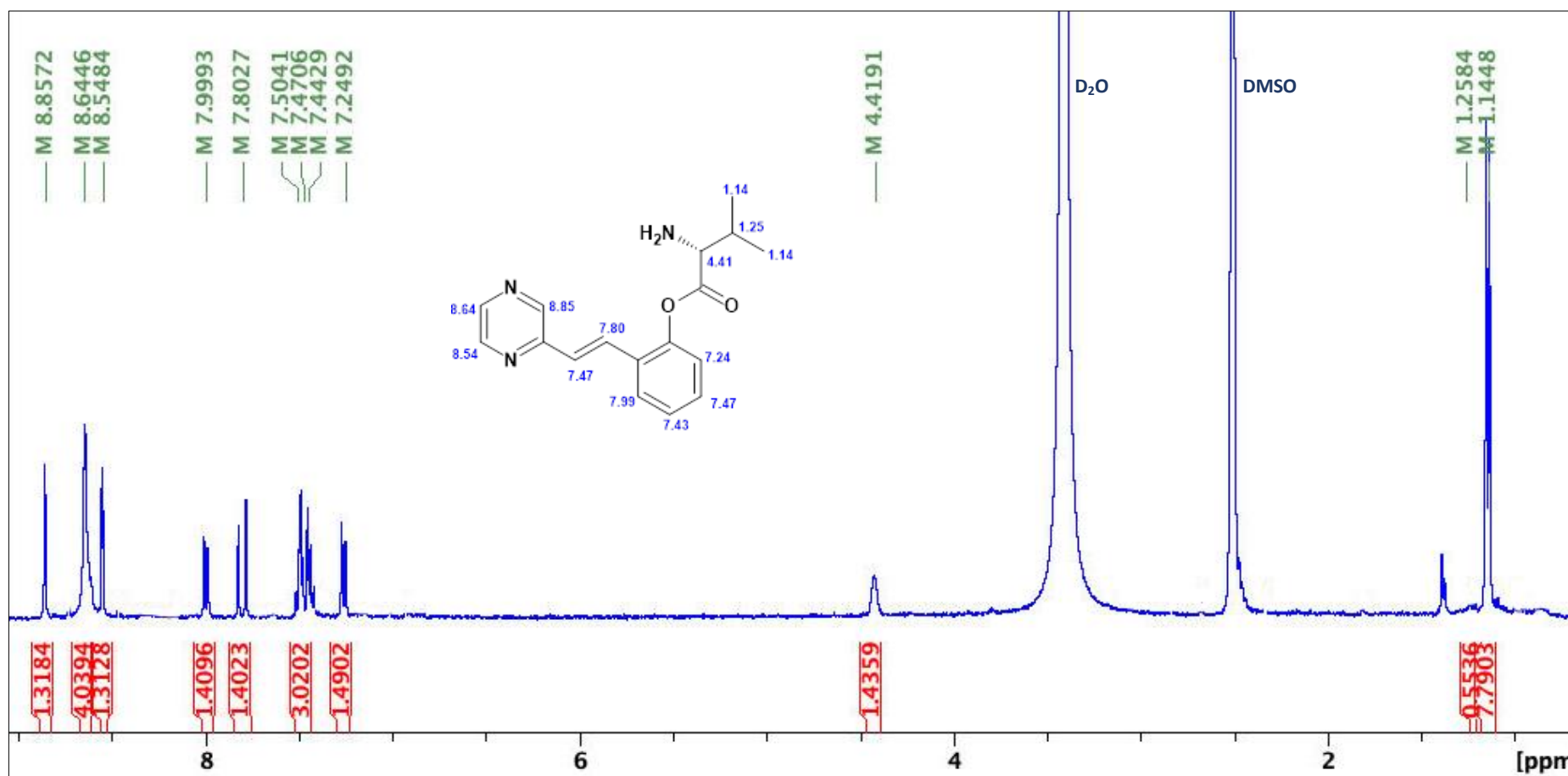


Figure 2.11: ¹H NMR of pyrazinib valine (65) in DMSO-*d*₆ at 400 MHz.

(Note: DMSO signal: 2.50 ppm and water signal: 3.3 ppm)

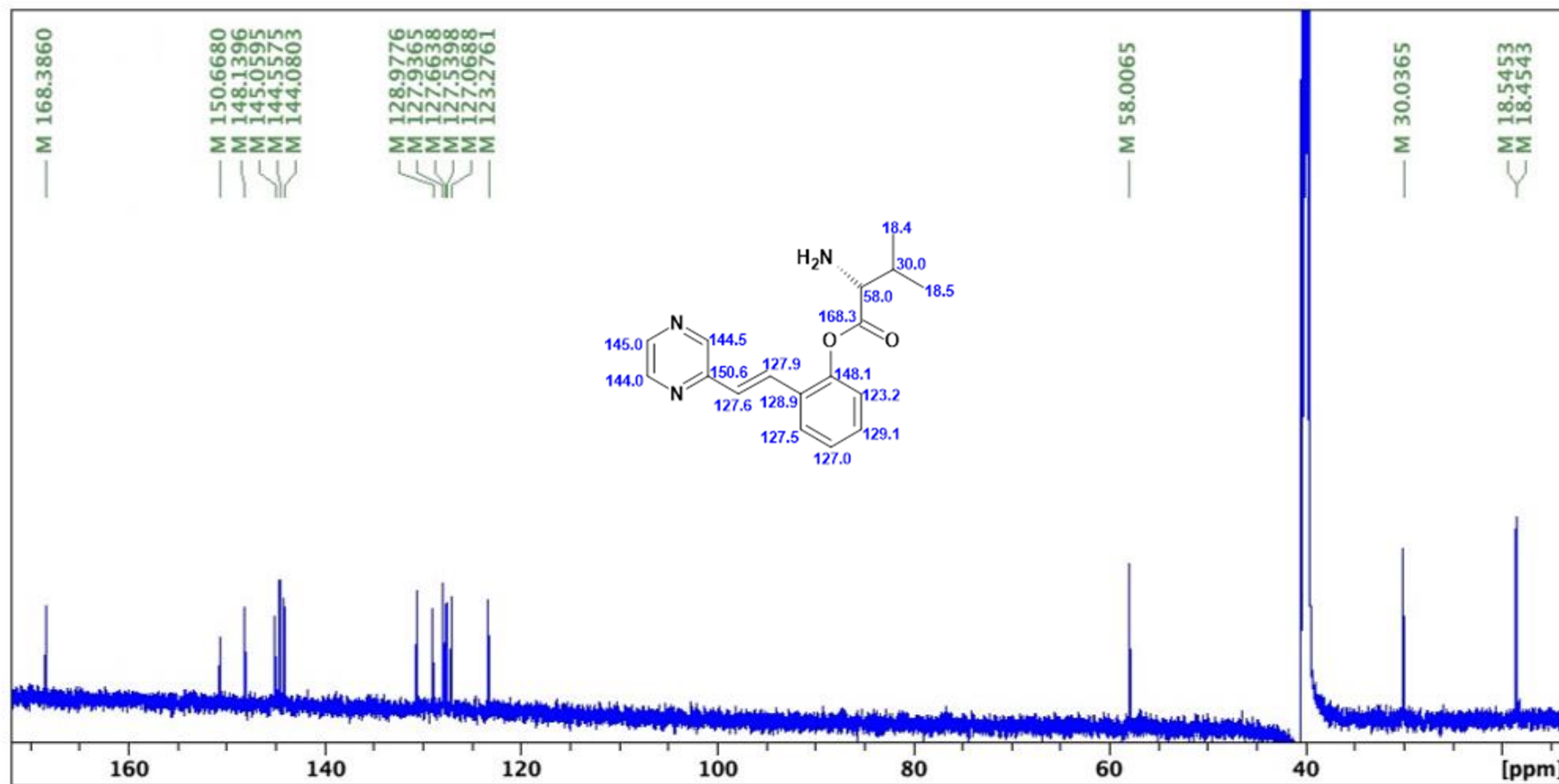


Figure 2.12: ^{13}C NMR of pyrazinib valine (65) in $\text{DMSO-}d_6$ at 400 MHz.

(Note: DMSO signal: 39.5 to 40.5ppm)

2.4 Physicochemical analysis of pyrazinib amino acid prodrugs

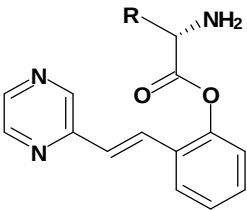
2.4.1 Aqueous solubility studies of pyrazinib amino acid prodrugs (63-69)

Determination of the aqueous solubility of a drug is essential during initial stages of the drug discovery process. Low solubility of drug can result in unreliable outcomes during *in vivo* testing. Aqueous solubility studies were performed according to the previously published protocol from our research group.⁸⁸ The aqueous solubility of the pyrazinib amino acid derivatives (**63-69**) was measured and compared to the parent compound, pyrazinib (**54**). The aqueous solubility was measured using the shake-flask method with HPLC analysis. The shake-flask method is considered to be a suitable method to determine solubility among other methods available.¹¹⁰ The shake flask technique can be applied to all types of compounds and is compatible with a wide range of media.¹¹⁰ In this study, a mechanical shaker (Mini gyro-rocker, SSM3, Stuart) was employed. Briefly, samples of pyrazinib amino acid derivatives (**63-69**) were freshly prepared in HPLC grade water and placed in the mechanical shaker for 24 hours at room temperature. After 24 hours, the suspensions were filtered through a 0.45-micron filter, further diluted appropriately with mobile phase quantified by HPLC and detected between 360-340 nm using a PDA detector. All measurements were made in triplicate and expressed in mg/mL. Standard serial dilutions (0.25 to 0.0039 mg/mL) were prepared for all the pyrazinib amino acid derivatives from a stock solution (0.5 mg/mL) in the mobile phase of ACN: water (70:30).

All 7 synthesised pyrazinib amino acid analogues (**63-69**) showed comparably higher aqueous solubility than 0.00005 mg/mL for pyrazinib (**Table 2.8**). Among these derivatives, highest solubility was obtained for isoleucine, leucine, alanine and serine derivatives (0.95, 0.79, 0.76, and 0.6 mg/mL in water respectively) which are greater than the recommended solubility value of 0.05 mg/mL for potential drug candidates.

Notably, the isoleucine analogue (**69**) demonstrated a remarkable 2375-fold increase in aqueous solubility compared to pyrazinib (**54**). The observed increase in the aqueous solubility of the prodrugs is attributed to the amino acid group which could also lead to improved absorption, distribution, metabolism, and elimination (ADME) properties. Consequently, the pharmacokinetic profile of pyrazinib could be improved but further testing would be needed.

Table 2.8: Aqueous solubility values for a series of pyrazinib amino acid prodrugs (63-69) and their fold increase relative to the parent compound. N=3.

Compound number	Structure	Amino acid	Solubility (mg/mL)	Fold increase
				
	R =			
63	H	Glycine	0.23	575
64	CH ₃	Alanine	0.76	1900
65	CH(CH ₃) ₂	Valine	0.51	1275
66	CH ₂ CH(CH ₃) ₂	Leucine	0.79	1975
67	CH ₂ OH	Serine	0.60	1500
68	CH ₂ C ₆ H ₄ OH	Tyrosine	0.11	275
69	CH(CH ₃)CH ₂ CH ₃	Isoleucine	0.95	2375
54	Pyrazinib	N/A	0.00005	N/A

N/A = not applicable

2.4.2 *In vitro* stability studies of pyrazinib amino acid prodrugs

2.4.2.1 Chemical stability studies

The chemical stability of the pyrazinib amino acid derivatives was assessed by HPLC in phosphate buffer at three pH values (acidic, neutral and basic) over a period of 24 hours. All the compounds were detected between 360-340 nm using a PDA detector. The chemical stability of the parent compound, pyrazinib was studied by O'Boyle group (discussed in Section 2.2.2) and was stable at all pH ranges over a period of 24 hours.⁸⁸ The chemical stability graphs of the pyrazinib amino acid prodrugs are displayed in Figure 2.13.

Amino acid derivatives including isoleucine, leucine and glycine dropped below 50% over a period of 24 hours at pH 4. In contrast, valine, tyrosine, serine and alanine derivatives-maintained levels above 50% in the same acidic conditions for up to 24 hours. At pH 7.4 most of the amino acid analogues demonstrated stability $\geq 50\%$ over 24 hours, with the exception of tyrosine derivative which was an exception and dropped to below 50%. Furthermore, the tyrosine analogue exhibited less than 50% stability at pH 7.4 also showed similar stability profiles under basic conditions (pH 9) over 24 hours.

Overall, alanine derivative remained $\geq 50\%$ at all pH ranges over a period of 1440 mins. The half-life of amino acid analogues (**63-69**) was calculated that remained approximately at 50% or below at all pH values at any time point is summarised in Table 2.9. Empirical half-life was used as a practical measure where reaction order could not be determined due to non-linearity or limited degradation. For compounds that degraded below 50%, reaction order was determined by comparing first and second half-lives. The isoleucine analogue (**69**) was selected for further progression due to its high-water solubility and stability profile. This implies that metabolic stability studies (enzymatic and plasma hydrolysis) can be conducted on **69** under physiological conditions.

Table 2.9: Half-lives ($t_{1/2}$) and kinetic reaction order of a series of pyrazinib amino acid prodrugs (63-69) in phosphate buffers (pH 4, 7.4 and 9) and analysed by HPLC, N=3.

Compound number	Amino acid derivative	pH 4	pH 7.4	pH 9
63	Glycine	First $t_{1/2}$: 30 mins Second $t_{1/2}$: 120 mins Reaction order: second	N/A	N/A
64	Alanine	N/A	N/A	N/A
65	Valine	N/A	N/A	N/A
66	Leucine	First $t_{1/2}$: 20 mins Second $t_{1/2}$: 100 mins Reaction order: second	N/A	N/A
67	Serine	N/A	N/A	N/A
68	Tyrosine	N/A	First $t_{1/2}$: 120 mins Second $t_{1/2}$: 1320 mins Reaction order: second	First $t_{1/2}$: 60 mins Second $t_{1/2}$: 800 mins Reaction order: second

69	Isoleucine	N/A	N/A	N/A

N/A = Not available for the compounds whose stability remained $\geq 50\%$ over the period of 24 hrs.

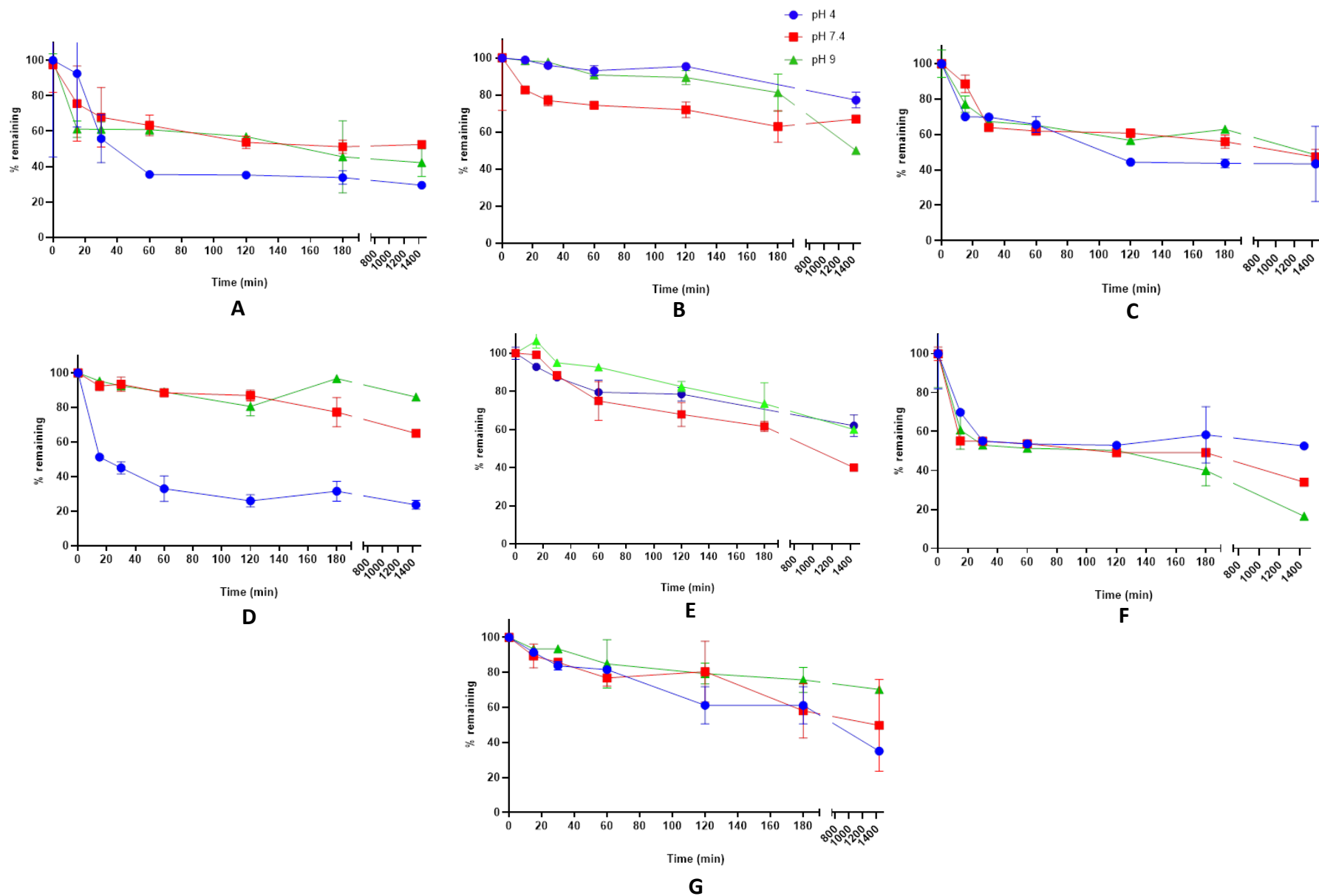


Figure 2.13: Chemical stability profile of a series of pyrazinib amino acid derivatives in PBS at pH 4, 7.4 and 9 analysed by HPLC. (A) Glycine (63), (B) Alanine (64), (C) Valine (65), (D) Leucine (66), (E) Serine (67), (F) Tyrosine (68) and (G) Isoleucine (69). Colour code: Blue= pH 4, Red= pH 7.4 and Green= pH 9

2.4.2.2 *In vitro* metabolic studies

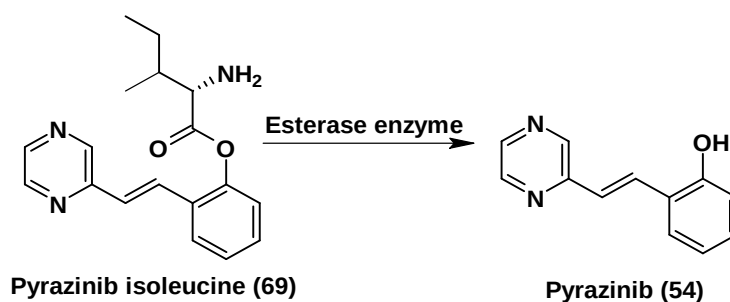
2.4.2.2.1 Enzymatic hydrolysis of pyrazinib isoleucine prodrug

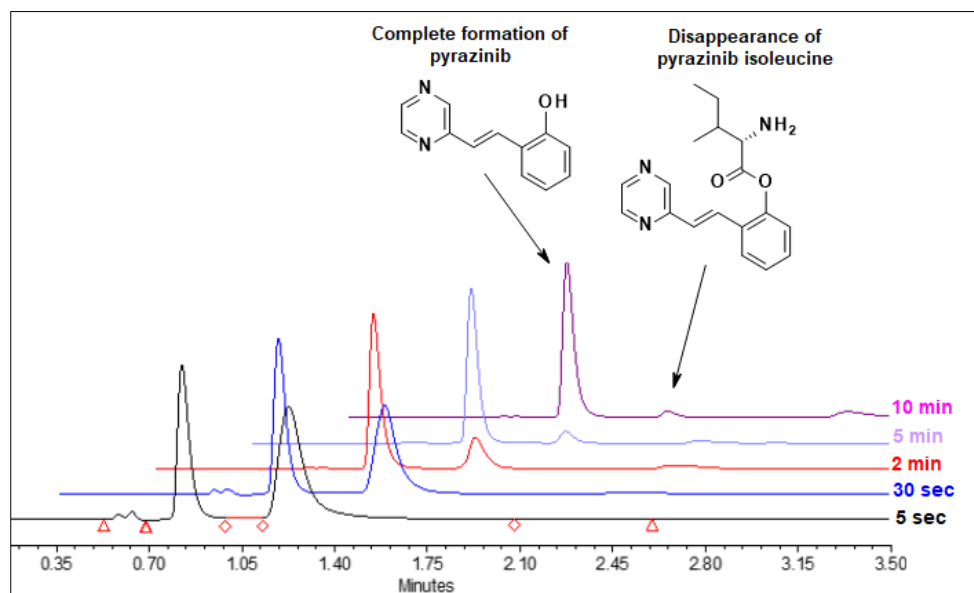
A major step in prodrug design involves the conversion of a prodrug to its parent drug in an efficient manner. There are different processes to achieve this conversion through enzyme mediated hydrolysis or non-enzyme chemical hydrolysis (discussed in section 1.3.2). An enzymatic stability/hydrolysis study was carried out on pyrazinib isoleucine derivative (**69**) due to its high aqueous solubility and chemical stability at physiological pH (7.4).

The enzymatic hydrolysis of **69** was carried out by using a commercially available porcine liver esterase (PLE, Merck, product no: E3019, specific activity 95.7 U/mg) as this enzyme efficiently hydrolyses ester bonds. The reaction mixture containing pyrazinib isoleucine (0.75 mg/mL) and PLE (0.024 mg/mL) was incubated under physiological conditions (PBS, pH 7.4 at 37°C) for 10 minutes. The reaction mixture was quenched with an ice-cold MeCN: MeOH (1:1) mixture before analysis by RP-HPLC (**Figure 2.14 A**). In addition, a control sample of **69** was used to ensure its stability (**Figure 2.14 C**).

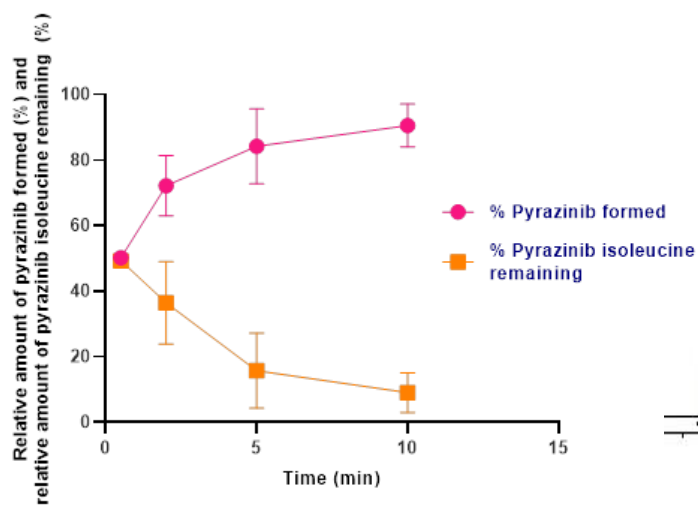
Notably, **69** readily converted to **54** within 5 minutes of incubation (**Scheme 2.14**), as low amounts of pyrazinib isoleucine were observed after 10 minutes. As shown in Figure 2.14 B, 15.7% of **69** remained and 84% of pyrazinib was generated within 5 minutes. The data shows conversion of prodrug to the parent drug at 5 minutes. These findings suggests that the pyrazinib prodrug approach may serve as an effective strategy, due to the rapid conversion of prodrug to its parent drug, in the presence of an esterase enzyme *in vivo*.

Scheme 2.14: Conversion of pyrazinib isoleucine (**69**) to pyrazinib (**54**) in the presence of esterase enzyme at 37 °C in PBS (pH 7.4).

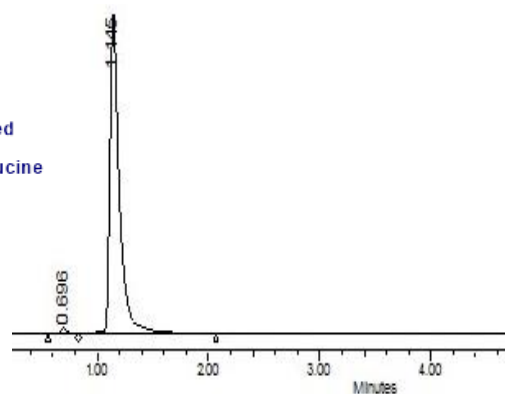




A



B



C

Figure 2.14: (A) Stack chromatograms of PLE esterase mediated hydrolysis of 69. Disappearance of 69 indicated at retention time of 1.22 min and formation of 54 indicated at retention time of 0.827 min; (B) Enzymatic hydrolysis profile of 69. N=3; (C) Sample of 69 incubated without PLE as a control indicated at a retention time of 1.4 minutes.

2.4.2.2.2 Human blood plasma hydrolysis of pyrazinib isoleucine prodrug

Ethical approval was obtained prior to conducting this assay, ethics number 2021A03.

To our knowledge, there are a variety of specified and non-specified esterase enzymes present in human plasma and liver being a most important site of synthesis.¹¹¹ Human plasma contains 4 types of esterase enzymes; butyrylcholine esterase, paraoxonase, acetylcholine esterase and albumin esterase.¹¹¹

As shown earlier in Section 2.4.2.2.1 esterases quickly cleaved the ester linker of **69**. Our aim is to identify prodrugs with excellent solubility, chemical stability and rapid conversion to the parent drug while minimizing unproductive metabolism. After observing the swift conversion of pyrazinib isoleucine prodrug (**69**) to pyrazinib (**54**) in the presence of porcine esterase, we proceeded to conduct a human plasma stability/hydrolysis study on pyrazinib isoleucine prodrug (**69**). The reaction mixture containing **69** (0.75 mg/mL) with blood plasma in PBS (1:1, pH 7.4) was incubated at 37°C, for 10 minutes. 100 % hydrolysis to pyrazinib was observed immediately (at t= 5 seconds) (data not shown). To assess the conversion rate at a slower pace, the plasma concentration was reduced to a ratio of 20:1 (PBS: plasma). Despite reduced concentration, immediate conversion to **54** was again observed at 5 seconds as shown in Figure 2.15. These findings highlight the high efficiency of the esterase enzymes present in human plasma for rapid hydrolysis of the **69** even at lower plasma concentrations. As a result, it is expected that **69** would be rapidly converted to **54** *in vivo*.

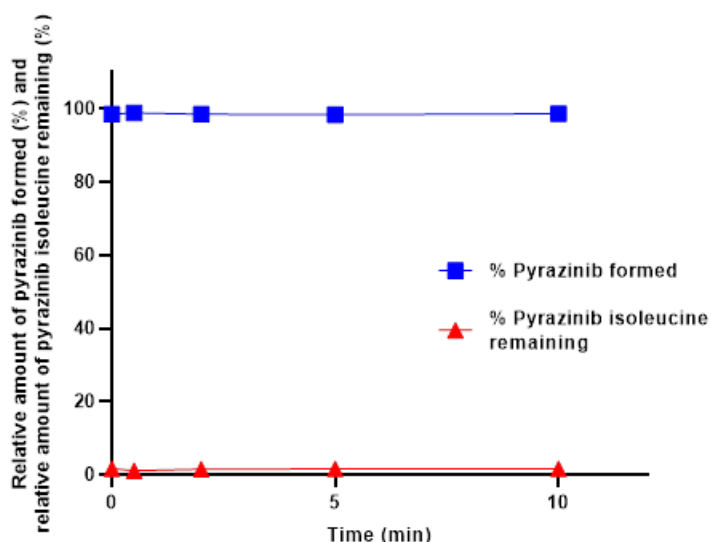


Figure 2.15: Plasma hydrolysis profile of pyrazinib isoleucine (**69**) in 20:1 PBS: plasma incubated at 37°C for 10 minutes. N=3.

2.5 Analysing the *in silico* physicochemical properties, pharmacokinetic and drug-likeness profile of pyrazinib and its amino acid prodrugs

It is essential to predict the ADME profile of drugs at an early stage which can lead to significant cost reduction in drug development. As an alternative to experimental procedures, computer models have been promoted for ADME prediction, especially at initial stages where the chemical structure of the drug is under investigation and compound availability is restricted. There are many online free portals available for ADME prediction of a drug by reducing the time and cost on drug candidate testing. SwissADME is a web tool run by the Swiss institute of Bioinformatics (SIB) that enables the calculation of physicochemical properties as well as evaluation of ADME properties, pharmacokinetic properties and drug likeness of small molecules.¹¹² SwissADME calculates a bioavailability radar, physicochemical properties, lipophilicity, water solubility, pharmacokinetics and drug-likeness of a small molecule.¹¹² In this section, SwissADME web tool was used to predict *in silico* physiochemical parameters, pharmacokinetics and drug likeness of pyrazinib (**54**) and its amino acid prodrugs (**63-69**).

2.5.1 Bioavailability radar of pyrazinib and its amino acid prodrugs

The first section of SwissADME deals with the 2D chemical structure, canonical SMILES and bioavailability radar of small molecules. A bioavailability radar is created for every molecule, providing an initial assessment of its “drug-likeness”. This radar takes into consideration six physicochemical properties: lipophilicity (LIPO), size (SIZE), polarity (POLAR), solubility (INSOLU), flexibility (FLEX), and saturation (INSATU).¹¹³ These properties contribute to creating the most favourable conditions for oral bioavailability. The pink region of the radar represents the optimal range for oral bioavailability. For a molecule to be considered drug-like, its physicochemical properties must fall entirely within this pink region. Figure 2.15 displays the bioavailability radars generated for pyrazinib and its amino acid prodrugs. The lipophilicity of pyrazinib and its amino acid analogues falls entirely in the pink region. However, pyrazinib is too unsaturated as it can be seen on the radar that its saturation property (INSATU) is outside the pink region (**Figure 2.16**). Conversely, amino acid analogues; leucine and isoleucine (**66** and **69**) contain long sp³ bonded carbon chains have a lower degree of unsaturation within the pink region (**Figure 2.16 A and B**). In contrast, other analogues glycine, alanine, valine, serine and tyrosine (**63, 64, 65, 67** and **68**) have higher degrees of unsaturation falling outside the pink region (**Figure 2.16 C-F**). Based on the bioavailability radars generated by SwissADME, it can be concluded that the leucine and isoleucine analogues (**66** and **69**) are predicted to be orally bioavailable. This prediction is based on the observation that their physicochemical properties fall within the optimal range, suggesting that the

analogues possess favourable characteristics for oral administration, such as appropriate solubility, permeability, and molecular size.

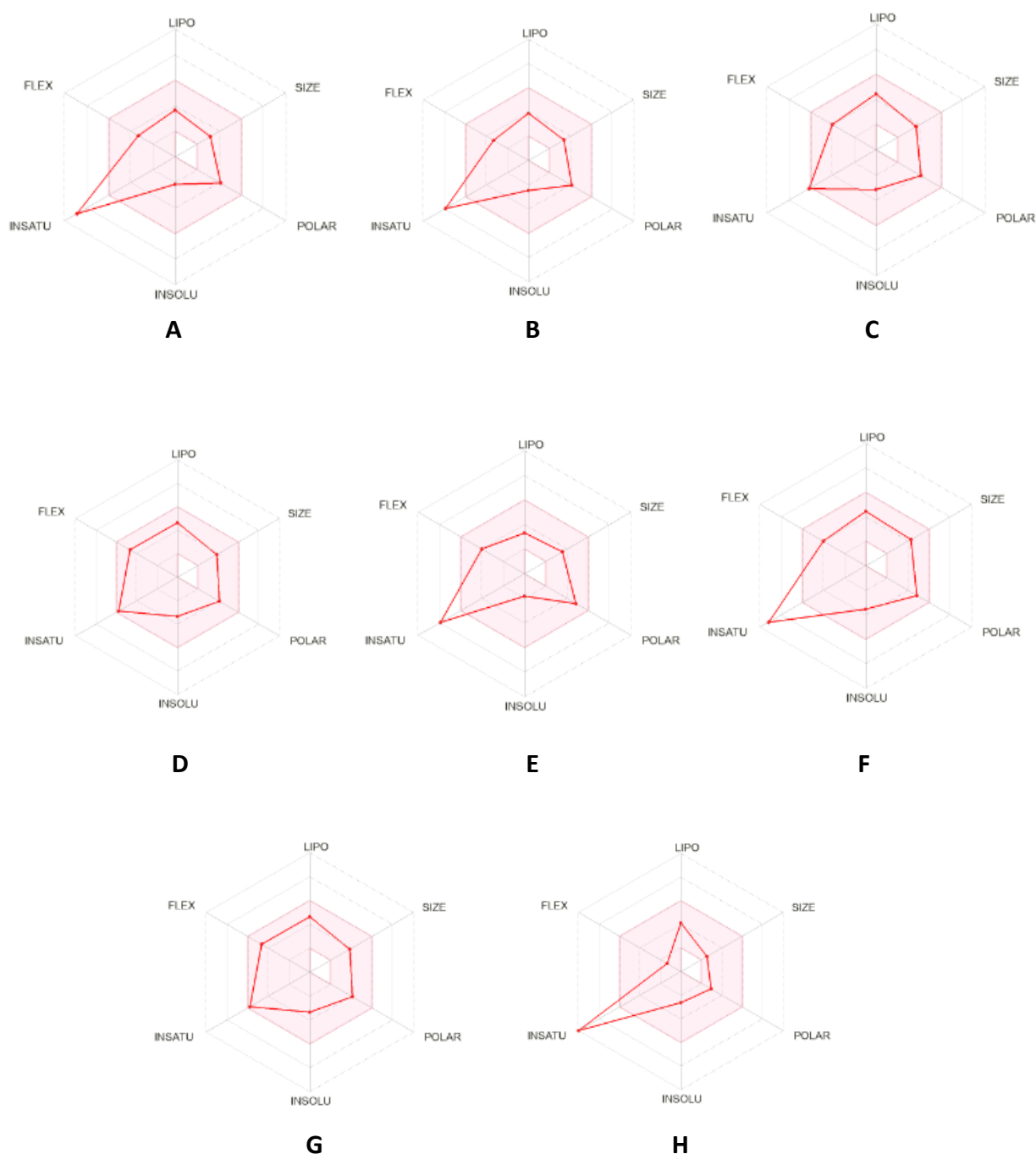


Figure 2.16: Bioavailability radars of pyrazinib and its amino acid analogues. (A) Glycine (63); (B) Alanine (64); (C) Valine (65); (D) Leucine (66); (E) Serine (67); (F) Tyrosine (68); (G) Isoleucine (69); (H) Pyrazinib (54). The pink area represents the range for each property: SIZE (MW 500-150 g/mol), POLAR (TPSA 120 -30), INSOLU (Log S), INSATU (not higher than 6, fraction of carbon not less than 0.25) and FLEX (no more than 9 rotatable bonds).

2.5.1.1 *In silico* physicochemical properties

Physicochemical properties are an important parameter of a molecule that influence stability, bioavailability, formulation, safety and metabolism. These properties can be predicted using 5 different rule-based filters: Lipinski's Ro5, Veber, Ghose, Egan and Muegge, along with a bioavailability score. Lipinski's rule outlines the criteria for determining the oral bioavailability of a molecule. The rule of five (Ro5) established by Lipinski specifies the following parameters: number of hydrogen bonds (HBA) ≤ 10 , number of hydrogen bond donors (HBD) ≤ 5 , molecular weight (MW) < 500 g/mol and log P value < 5 .¹¹⁴ Veber's filter includes the number of rotatable bonds ≤ 10 and TPSA ≤ 140 Å²; Ghose's filter includes the MW 160-480 g/mol, Log P ≤ 5.6 and molar refractivity (MR) 40-130 m³/mol; Egan's filter includes Log P ≤ 5.88 and TPSA ≤ 130 Å² and finally, Muegge's filter includes MW 200-600 g/mol, Log P -2 to 5, number of rotatable atoms ≤ 15 , HBA ≤ 10 and HBD ≤ 5 .¹¹⁵

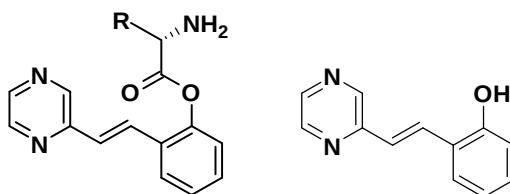
This section focuses on the SwissADME computed physicochemical/drug-likeness properties of pyrazinib and its amino acid prodrugs, as summarised in Table 2.10. Upon examination of table 2.10, pyrazinib and its amino acid analogues meet all criteria of Lipinski's Ro5, as their MW is < 500 , HBA < 10 , HBD < 5 and their LogP values < 5 (discussed in Section 2.5.3). Additionally, the molar refractivity (MR) parameter of a drug molecule is a measure of the overall polarity of a molecule and should not exceed 130 m³mol⁻¹ or be under 40 m³mol⁻¹.¹¹⁶ Both pyrazinib (**54**) and its amino acid analogues (**63-69**) are within the ideal range of MR (40-130 m³mol⁻¹). Another important parameter is the polar surface area (PSA), calculated using the fragmental technique called topological polar surface area (TPSA). A good drug candidate should have PSA in range of 140 Å² to 20 Å².¹¹⁷ A TPSA value increases as the number of polar groups within the drug structure increases.¹¹⁸ SwissADME computed TPSA values reveal that pyrazinib and its amino acid prodrugs (**63-69**) fall within the ideal range of TPSA of 140 -20 Å². Typically, a drug candidate with a TPSA value < 140 Å² indicates a good intestinal absorption and < 60 Å² indicates penetration through the blood brain barrier.¹¹⁹ Serine and tyrosine prodrugs (**67** and **68**) display a TPSA value > 60 Å², due to an extra OH along with a free amino group increasing their polarity and potentially indicating good intestinal absorption. Moreover, the number of rotatable bonds (< 9) and Csp³ saturation (0.25-1) are crucial for drug bioavailability, indicating the molecule's flexibility and saturation of a small molecule. Molecules with a higher fraction of sp³ carbons are more prone to metabolism and elimination from the body compared to those with lower values.¹²⁰

According to SwissADME, pyrazinib and its amino acid analogues fulfil the requirement of number of rotatable bonds. The Csp³ saturation of isoleucine and leucine analogues (**69** and **66**) falls within the ideal range of 0.25 to 1 (**Table 2.10**). In contrast, glycine, alanine, valine, serine and tyrosine (**63**,

64, 65, 67 and 68) as well as pyrazinib are predicted to have $C_{sp^3} < 0.25$. This suggests that these compounds are less likely to be metabolised and eliminated from the body, compared to isoleucine and leucine. Based on SwissADME computed physicochemical properties, isoleucine (**69**) and leucine (**66**) analogues are predicted to have good oral bioavailability. This is because they fall within the ideal ranges for all physicochemical properties. Other amino acid analogues (serine, tyrosine, alanine, valine and glycine) complied with Lipinski's Ro5, fell within the ideal range of molar refractivity, TPSA, number of rotatable bonds but showed the lowest saturation.

Interestingly, all seven amino acid analogues are in agreement with the other 4 rule-based filters (Veber, Ghose, Egan and Muegge) without any violations. However, it is noteworthy that pyrazinib (**54**) does not meet Muegge's MW criterion as its MW is slightly lower (198 g/mol). Additionally, the bioavailability score, a critical parameter for considering a molecule as a therapeutic candidate must be > 0.10 .¹²¹ Interestingly, all seven amino acid analogues and pyrazinib exhibited a predicted bioavailability score of 0.55. In summary, these results are significant as they suggest that pyrazinib and its amino acid analogues (**63-69**) possess favourable drug-likeness properties according to these filters.

Table 2.10: *In silico* physicochemical properties of pyrazinib and its amino acid analogues (63-69) using Swiss ADME.



Pyrazinib amino acid prodrugs Pyrazinib

- 63 R= H
 64 R= CH₃
 65 R= CH(CH₃)₂
 66 R= CH₂CH(CH₃)₂
 67 R= CH₂OH
 68 R= CH₂C₆H₄OH
 69 R= CH(CH₃)CH₂CH₃

Compound number	Chemical Formula	MW (g/mol)	HBA	HBD	MR (m ³ /mol)	TPSA (Å ²)	Rotatable bonds	Fraction Csp ³
63	C ₁₄ H ₁₃ N ₃ O ₂	255.27	5	1	71.61	78.1	5	0.07
64	C ₁₅ H ₁₅ N ₃ O ₂	269.30	5	1	76.42	78.1	5	0.13
65	C ₁₇ H ₁₉ N ₃ O ₂	297.35	5	1	86.03	78.1	6	0.24
66	C ₁₈ H ₂₁ N ₃ O ₂	311.38	5	1	90.84	78.1	7	0.28
67	C ₁₅ H ₁₅ N ₃ O ₃	285.30	6	2	77.58	98.33	6	0.13
68	C ₂₀ H ₁₇ N ₃ O ₃	347.37	6	2	98.12	98.33	6	0.05
69	C ₁₈ H ₂₁ N ₃ O ₂	311.38	5	1	90.84	78.1	7	0.28
54 (Pyrazinib)	C ₁₂ H ₁₀ N ₂ O	198.22	3	1	59.43	46.01	2	0.00

2.5.2 Water solubility and lipophilicity

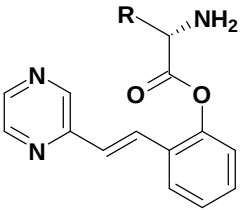
This section of Swiss ADME deals with the lipophilicity and water solubility of small molecules. In order to have good biological activity, a drug should possess strong lipophilic character, which enables it to easily pass through cell membranes. Swiss ADME predicts the lipophilicity of small molecules by calculating their LogP values. It utilises 5 methods (iLogP, XLogP, WLogP, MLogP and SILICO-SLogP) to determine lipophilicity (LogP). The LogP values of pyrazinib and its amino acid analogues are given in Table 2.11. These values are consensus LogP (average of all 5 LogP values). Pyrazinib (**54**) and its prodrugs (**63-69**) are predicted to have LogP values < 5. In addition, leucine and isoleucine (**66** and **69**) exhibit high LogP values of 2.50 whereas the serine analogue (**67**) displays the lowest LogP value of 0.82, indicating its high hydrophilicity.

A drug must have a strong hydrophilic character, as this property greatly affects its oral absorption. SwissADME predicts the water solubility by calculating the LogS value of small molecules by using various methods. These methods include, LogS (ESOL), LogS (ALI) and LogS (SILICOS-IT). The water solubility of the compounds ranges from poorly soluble to highly soluble. Poorly soluble values lie from -10 to -6, moderately soluble from -6 to -4, soluble from -4 to -2 and highly soluble are -2 to > 0.¹²² The LogS (ESOL) values of pyrazinib and its prodrugs are given in Table 2.11.

Based on Swiss ADME computed solubility values, pyrazinib and its amino acid prodrugs fall within the soluble range. The highest solubility predicted for the serine analogue (**67**) which is -1.81. Based on the computed Swiss ADME LogP and LogS values, it can be concluded that amino acid analogues and pyrazinib itself are water soluble and possess favourable LogP values. Some analogues have a LogP value of < 1.80, indicating potential limitations in permeability. Nonetheless, they are deemed suitable for both oral and intestinal absorption, which will be further discussed in Section 2.5.3.

SwissADME calculated LogS (ESOL) values were compared with experimentally determined values of all the compounds (**Table 2.11**). The comparison of solubility values from SwissADME predictions and experimental data reveals notable discrepancies. According to SwissADME all amino acid analogues are predicted to fall in the soluble (-4 to -2) range, except for serine analogue and pyrazinib which are predicted to be highly soluble (-2 to > 0). In contrast, experimental data indicates that all amino acid analogues exhibited reasonable experimental water solubility (0.95 to 0.11 mg/mL), whereas pyrazinib is practically insoluble (0.00005 mg/mL). This inconsistency highlights a limitation of computational model like SwissADME which relies on computational methods that may not account for the complexities of real experimental conditions such as solubility influenced by crystal structure or solvent interactions.

Table 2.11: Swiss ADME predicted LogP and LogS values of pyrazinib (54) and its amino acid analogues.

Compound number	Structure	Amino acid derivatives	Consensus LogP	LogS (ESOL)	Experimental solubility values (mg/mL)
					
	R =				
63	H	Glycine	1.29	-2.15	0.23
64	CH ₃	Alanine	1.64	-2.64	0.76
65	CH(CH ₃) ₂	Valine	2.26	-3.14	0.51
66	CH ₂ CH(CH ₃) ₂	Leucine	2.51	-3.36	0.79
67	CH ₂ OH	Serine	0.82	-1.81	0.60
68	CH ₂ C ₆ H ₄ OH	Tyrosine	2.15	-3.54	0.11
69	CH(CH ₃)CH ₂ CH ₃	Isoleucine	2.50	-3.36	0.95
54	Pyrazinib	N/A	1.80	-2.26	0.00005

N/A = not applicable

2.5.3 Pharmacokinetic profile

The pharmacokinetic profile of a drug defines its ADME properties. Bioavailability is further influenced by ADME properties including gastrointestinal (GI) absorption, blood-brain barrier (BBB) access, interaction of the molecule with CYP isoenzymes and P-glycoprotein (P-gp).

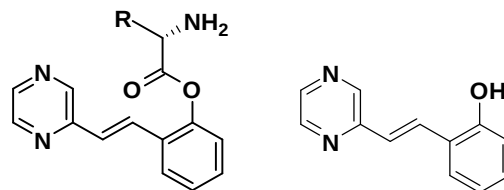
P-gp is an ATP binding cassette (ABC) transporter and functions as a biological barrier by extruding toxins and xenobiotics out of cells.¹²³ Moreover, it also plays a role in multidrug resistance.¹²³ CYP isoenzymes are basic enzymes involved in phase 1 biotransformation. The 5 major isoenzymes in biotransformation are CYP3A4, CYP2D6, CYP2C9, CYP2C19 and CYP1A2.¹²⁴ According to different authors, it is estimated that 50 to 90% of therapeutics are substrates of at least one of the 5 isoenzymes.¹²² When a drug inhibits the activity of these enzymes, it can alter the metabolism of other drugs, potentially leading to their accumulation or production of other metabolites which may lead to toxicity.¹²⁵

The SwissADME predicted ADME properties of pyrazinib and its amino acid prodrugs are shown in Table 2.13. The results are shown as 'Yes' or 'No' based on whether the molecule has high GI absorption, BBB permeation is a substrate for P-gp or inhibitor of CYP isoenzymes, respectively.

Interestingly, pyrazinib and its amino acid analogues are predicted to have a high degree of gastrointestinal (GI) absorption suggesting the high bioavailability of these molecules. It was observed that only leucine (**66**) and isoleucine (**69**) analogues, along with pyrazinib itself, are predicted to have favourable membrane permeability properties. Both **66** and **69** analogues have a LogP value > 1.80, Csp³ > 0.25 but their TPSA > 60 Å², whereas, pyrazinib has a LogP value of 1.80 with a TPSA value < 60 Å², supporting its membrane permeability. In contrast, other amino acid analogues (**63**, **64**, **65** and **67**) have LogP values < 1.80, Csp³ < 0.25 and TPSA values > 60 Å², indicating reduced membrane permeability (**Table 2.10** and **2.11**). Tyrosine analogue (**68**) despite having a LogP value of 2.15 has a high TPSA value (98.33 Å²) and lower Csp³ fraction (0.05) further influencing its membrane permeability.

Regarding drug metabolism, pyrazinib and its amino acid analogues are not predicted to be P-gp substrates. However, pyrazinib and glycine (**63**), leucine (**66**), tyrosine (**68**), isoleucine (**69**) analogues inhibit at least 1 out of 5 CYP isoenzymes. This can result in drug-drug interactions or the formation of toxic metabolites, potentially leading to adverse effects or toxicity. In contrast, alanine (**64**), valine (**65**) and serine (**67**) analogues are not predicted to act as inhibitors of any of the 5 isoenzymes which reduces the risk of drug-drug interactions and potential toxicity.

Table 2.12: Swiss ADME predicted pharmacokinetic parameters of pyrazinib (54) and its amino acid prodrugs (63-69).



Pyrazinib amino acid prodrugs Pyrazinib

63 R= H

64 R= CH₃

65 R= CH(CH₃)₂

66 R= CH₂CH(CH₃)₂

67 R= CH₂OH

68 R= CH₂C₆H₄OH

69 R= CH(CH₃)CH₂CH₃

Compound number	GI absorption	BBB Permeation	P-gp substrate	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4
63	High	No	No	Yes	No	No	No	No
64	High	No	No	No	No	No	No	No
65	High	No	No	No	No	No	No	No
66	High	Yes	No	Yes	No	No	No	No
67	High	No	No	No	No	No	No	No
68	High	No	No	No	No	Yes	Yes	No
69	High	Yes	No	No	Yes	No	No	No
54 (Pyrazinib)	High	Yes	No	Yes	No	No	No	No

2.6 Biochemical analysis of pyrazinib and its amino acid prodrugs

2.6.1 Evaluating the effect of pyrazinib and its amino acid prodrugs on radiosensitivity in on isogenic model of OAC radio-resistance

2.6.1.1 Introduction

Pyrazinib was evaluated for radiosensitivity on OE33R cells and identified as a radiosensitiser by reducing the survival fraction of OE33R cells.⁸⁷ OE33R cells are a type of cancer cell line derived from human oesophageal adenocarcinoma.

In this section, we sought to investigate the effect of pyrazinib amino acid prodrugs on radiosensitivity *in vitro* using OAC radiation resistant cell lines (OE33R). Four amino acid prodrugs (**64**, **67**, **68** and **69**) were selected based on their higher solubility or better chemical stability (**Figure 2.17**). Notably, despite **68** exhibiting lower solubility, it was included in the selection process due to its > 50% stability at pH 4 after 24 hours, demonstrating its potential as a viable candidate for further investigation.

The radiosensitivity of selected amino prodrugs was investigated by clonogenic assay. The clonogenic assay is a gold standard to evaluate the proliferative capacity of cancer cells and to assess the radiosensitivity. This is an *in vitro* cell survival assay that relies on the ability of a single cell to grow into a colony.¹²⁶ Subsequently, the number of colonies formed are quantified to assess the treatment's effectiveness: A lower count of colonies suggests a more successful treatment outcome, whereas a higher count indicates less effective treatment.¹²⁶ The cell survival rate (%) can be calculated based on the number of colonies formed. The OE33R colonies formed after treatment with amino acid prodrugs were irradiated with X-rays to assess their radiosensitivity. This assay was used to evaluate and compare the radiosensitivity of the amino acid prodrugs with that of the parent drug, providing insights into their potential impact on both cell proliferation and response to radiation.

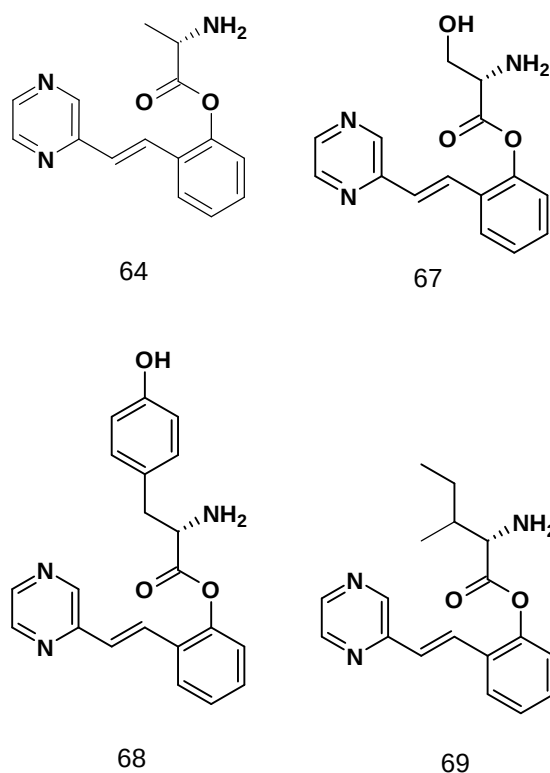


Figure 2.17: Selected pyrazinib amino acid prodrugs evaluated for their radiosensitivity. Alanine (64), Serine (67), Tyrosine (68) and Isoleucine (69) prodrugs.

2.6.1.2 Methods

2.6.1.2.1 Generation of radioresistant cell line (OE33R)

The human oesophageal adenocarcinoma OE33 cell line was obtained from the European Collection of Cell cultures. OE33R cells were generated as previously described by Lynam *et al.*¹²⁷

2.6.1.2.2 Storage of cells

When preserving cells, frozen cell stocks were prepared from cell lines that were growing in the exponential phase at 70-80% confluency. The cells in the flask were trypsinised, and 10 mL of complete medium was added to neutralize the trypsin. Then, the cells were centrifuged at 180 rpm for 3 minutes. After centrifugation, the supernatant was discarded, and the cell pellet was resuspended in FBS (3 mL) containing 10% DMSO. The cell suspension was aliquoted into 1 mL portions in cryovials, which were then stored at either -80 °C for a short term or under liquid nitrogen for long term.

2.6.1.2.3 Breaking out of cells

Frozen cell stocks were thawed at 37°C, added to Roswell Park Memorial Institute (RPMI) 1640 medium a nutritive liquid use in cell culture applications supplemented with 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland) and centrifuged at 180 rpm for 3 minutes. Then, the supernatant was decanted and the cells were resuspended in RPMI and transferred to 25 cm² culture flasks with RPMI and incubated at 37°C in a 5% CO₂/ 95% air. Once the cells reached 70-80% confluency, they were trypsinised and transferred to 75 cm² culture flasks.

2.6.1.2.4 Cell subculture

Cells were examined daily using an inverted phase contrast Nikon microscope (Nikon Corporation, Tokyo, Japan). OE33R cells were cultured in RPMI 1640 medium. When cells reach 70-80% confluency, they must be split to prevent cell death. The growth medium was removed, and cells were washed with 3 mL of PBS. To detach the cells, 2 mL of trypsin-EDTA (0.5% trypsin, 0.02% EDTA) was added to the flask, and the cells were incubated for 3-5 minutes at 37°C in a 5% CO₂/95% air environment. After detachment, 6 mL of complete medium was added to deactivate the trypsin. The cell pellets were collected in 15 mL tubes by centrifugation at 180 rpm for 3 minutes. Then, the cells were counted (described in next section) and a fraction of resuspended cells are transferred to T75 culture flask with 10-13 mL of complete growth medium.

2.6.1.2.5 Cell counting

A haemocytometer was used to count cells. It contains a 5 x 5 grid of squares and the cells were counted in the 4 corner squares (**Figure 2.18**). Cell pellets were collected as previously described, the supernatant was decanted and the cell pellet was suspended in 1 mL complete medium. The cell suspension (20 µL) was resuspended in 180 µL of trypan blue, mixed by pipetting, and 20 µL was added to the haemocytometer. The number of cells in each of the 4 corner squares were counted, excluding the cells touching the bottom and right-hand side edges of the grid. The number of cells per mL was counted using the following calculations:

$$(\text{Total number of cells} / 4) \times 10,000 \times 10 = \text{number of cells per mL}$$

Where; 4 = number of squares used, 10,000 = haemocytometer constant and 10 = trypan blue dilution factor

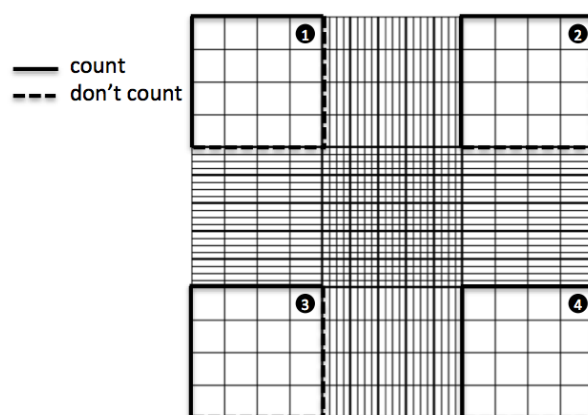


Figure 2.18: A representative image of haemocytometer grid. Cells are counted in the four corner squares.

2.6.1.2.6 Clonogenic assay

OE33R cells were trypsinised and seeded in 1.5 mL of RPMI-1640 in triplicate in 6 well plates and allowed to adhere overnight. After 24 hours cells were treated with 0.1% DMSO (control), 10 μ M pyrazinib, 0.1% water (control) or 10 μ M amino acid prodrugs (**64**, **67**, **68** and **69**) and incubated for 24 hours at 37°C in a 5% CO₂/95 % air environment. Following 24 hours of incubation, the treated cells were irradiated at 2 Gy, while another set of treated cells remained mock irradiated (0 Gy). Following irradiation, the colonies were allowed to grow for 5 to 7 days. Once the colonies were completely formed, they were stained and fixed with crystal violet (**Section 2.6.1.2.7**) and allowed to air dry. Irradiation was performed using a Gulmay Medical X-ray generator (RS225, Gulmay Medical) at a dose rate of 2 Gy per 1 minute and 12 seconds.

Plating efficacy (PE), fraction of colonies formed by untreated cells was calculated using the formula, $PE = \text{number of colonies formed} / \text{number of cells seeded}$. The surviving fraction (SF), the number of colonies formed by treated cells was calculated using the formula, $SF = \text{number of colonies formed} / (\text{number of cells seeded} \times PE)$.

2.6.1.2.7 Crystal violet assay

Cells were fixed with crystal violet (60% methanol, 0.05% crystal violet and non-sterile water). The cells were incubated with stain for 30 minutes at room temperature. The fixative was removed and cells were washed with water. Following the water wash, plates were left to air dry.

2.6.1.2.8 Statistical analysis

Statistical analysis was performed using unpaired t-test with in GraphPad Prism v7.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Error bars represent mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (N = 4-5).

2.6.1.3 Results and discussion

The ability of pyrazinib amino acid prodrugs to sensitise the OAC cells to radiation was investigated by clonogenic assay. OE33R cells were treated with pyrazinib and its amino acid prodrugs (**64**, **67**, **68** and **69**) at 10 μ M for 24 hours and irradiated at 2 Gy, while another set of treated cells remained mock irradiated (0 Gy), serving as control of radiation exposure. The radio-sensitising effect was evaluated based on the % survival fraction of the cells. A lower survival fraction of OE33R colonies indicated increased cell death in response to drug treatment and radiation.

Pyrazinib reduced the surviving fraction of OE33R cells at 2 Gy, compared to DMSO (vehicle control) at 2 Gy radiation (**Figure 2.19**). The surviving fraction of OE33R cells treated with pyrazinib at 2Gy radiation dose was reduced by 20%, indicating the enhanced radiosensitising effect of pyrazinib.

Conversely, the prodrugs were dissolved in water rather than DMSO, with water serving as the vehicle control. Interestingly, none of the amino acid prodrugs significantly reduced the surviving fraction of OE33R at 2 Gy, compared to water (**Figure 2.19**). The clonogenic assay results also reflects the proliferative activity of OE33R cells after treatment and irradiation. The significant reduction in survival fraction of OE33R cells with pyrazinib treatment indicates an inhibition of proliferation, whereas the lack of significant reduction with the amino acid prodrugs suggests that these compounds do not impact the proliferative capacity of the cells under the experimental conditions.

These findings confirm that pyrazinib significantly enhances the radiosensitivity of OE33R cells, while its amino acid prodrugs did not exhibit the same effect. This outcome was not expected as the prodrugs were designed to be cleaved within the cells to release pyrazinib. The lack of effect suggest that the prodrugs might not efficiently converted to pyrazinib within the cells. It is also possible that prodrugs were metabolised or degraded in a way that reduces their effectiveness and their stability in the cellular environment might differ from their stability in a more controlled environment.

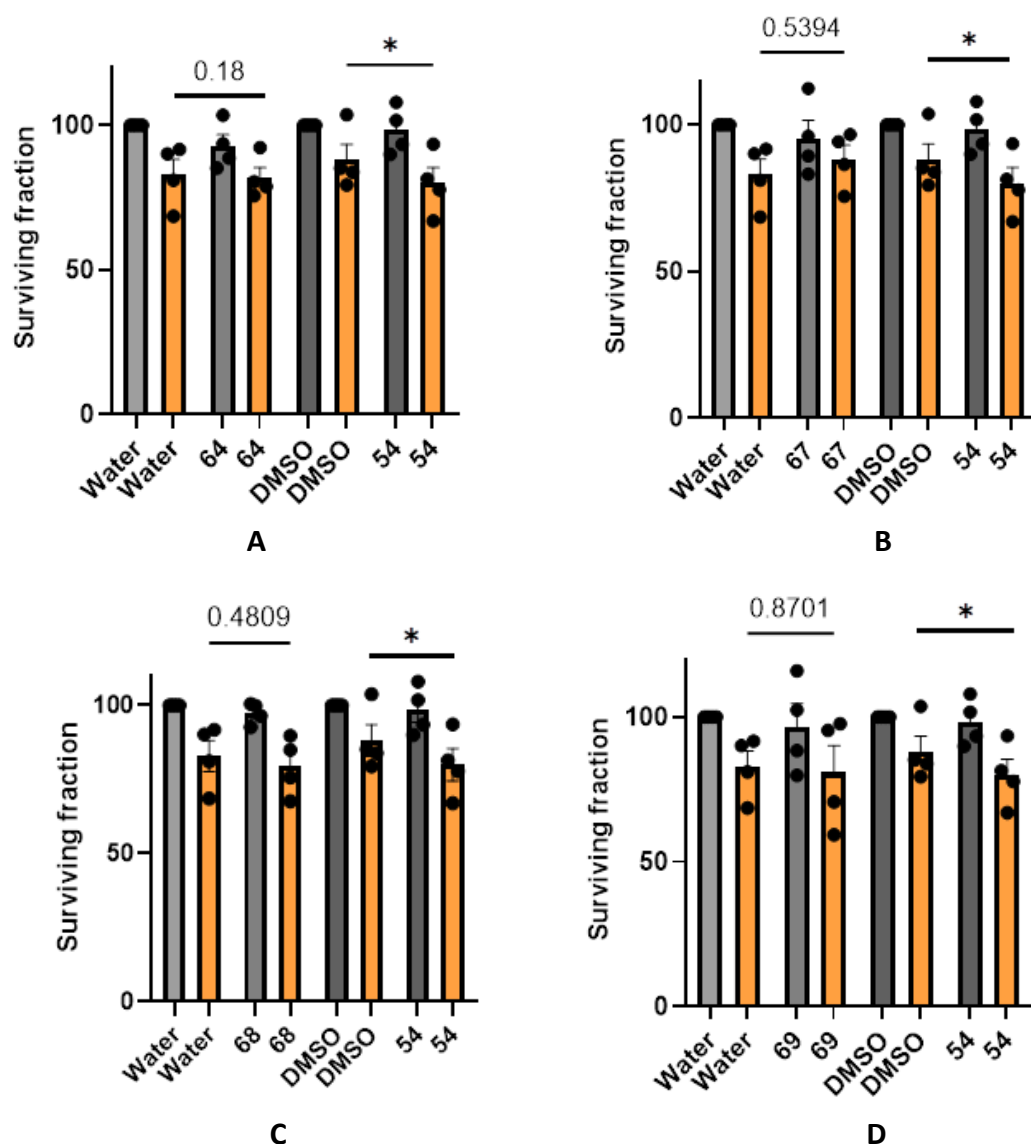


Figure 2.19: The effect of pyrazinib (54) and its amino acid prodrugs on radiosensitivity *in vitro* in radioresistant cell line (OE33R) following 24 hrs treatment assessed by clonogenic assay at 10 μ M concentration, irradiated at 2 Gy and control plates mock irradiated at 0 Gy. (A) Alanine analogue (64); (B) Serine analogue (67); (C) Tyrosine analogue (68); (D) Isoleucine analogue (69). N=4. Unpaired t-test was performed for statistical analysis. Error bars represent mean $\pm$ SEM, * $p < 0.05$ or ns: non-significant.

Colour code: Grey = 0 Gy and Orange = 2 Gy.

2.6.2 Anticancer activity of pyrazinib amino acid prodrugs in NCI-60 human tumour drug line screen

The NCI-60 screen is a comprehensive drug screening program established by the United States's National Cancer Institute. The NCI-60 Human Tumour Cell Lines Screen has been a valuable resource for the global cancer research community for over 20 years.¹²⁸ It was developed in late 1980's and fully implemented in 1990.¹²⁸ The screen uses 60 different human tumour cell lines to identify and study new compounds that can inhibit or kill tumour cells.¹²⁹ It can screen up to 7,000 small molecules (both synthetic and purified natural products) annually for potential anticancer activity.¹²⁹ The NCI-60 panel covers a wide range of cancer types, including leukaemia, melanoma, CNS, lung, colon, brain, ovary, breast, prostate, and renal cancers. This screen can produce intricate dose-response patterns. Moreover, it helps in early drug development by identifying compounds which show selective toxicity towards cancer cells.¹²⁹

In the one-dose assay, compounds are tested at a single high dose of 1×10^{-5} M (10 μ M) across the 60 cell lines. The resulting data is presented as a mean graph showing the percentage growth of treated cells relative to untreated controls. This method allows for the detection of both growth inhibition (values between 0 and 100) and cell lethality (values below 0). For example, a value of 100 indicates no growth inhibition, a value of 40 represents 60% growth inhibition, a value of 0 means no net growth, -40 signifies 40% lethality, and -100 indicates complete cell death. Compounds that show significant growth inhibition in this assay may advance to further testing, including 5-dose assays.

In this study, pyrazinib (**54**) and its six pyrazinib amino acid prodrugs (**63-66**, **68**, and **69**) were submitted for evaluation in the NCI-60 cell line screen. Due to a limited quantity, the serine analogue (**67**) could not be tested. Unfortunately, none of the six submitted prodrugs or pyrazinib progressed to the 5-dose testing stage. The results from the one-dose testing are presented as mean graphs showing the percentage growth of treated cells relative to untreated controls. The anticancer data revealed that the growth percentages for these compounds ranged from 85 to 137%. This range indicates varying effects on cell proliferation: values below 100% suggest growth inhibition, while values above 100% indicate potential growth promotion. The variability in these percentages highlights the diverse impact of these compounds on cancer cell proliferation, from minimal inhibition to potentially growth-promoting effects. In contrast, the parent drug pyrazinib itself showed the growth percentages from 96 to 177% (**Appendix 2**), indicating that that pyrazinib may promote cell growth or exhibit varying effects on different cell lines. This variability suggests that pyrazinib's impact on cancer cell proliferation is multifaceted, potentially enhancing growth in certain cancer cell lines. The mean growth graph for pyrazinib isoleucine prodrug (**69**) is shown in

Figure 2.20. The mean growth graphs for all other tested compounds, including pyrazinib, as generated by the NCI-60 screening, are available in the appendices section.

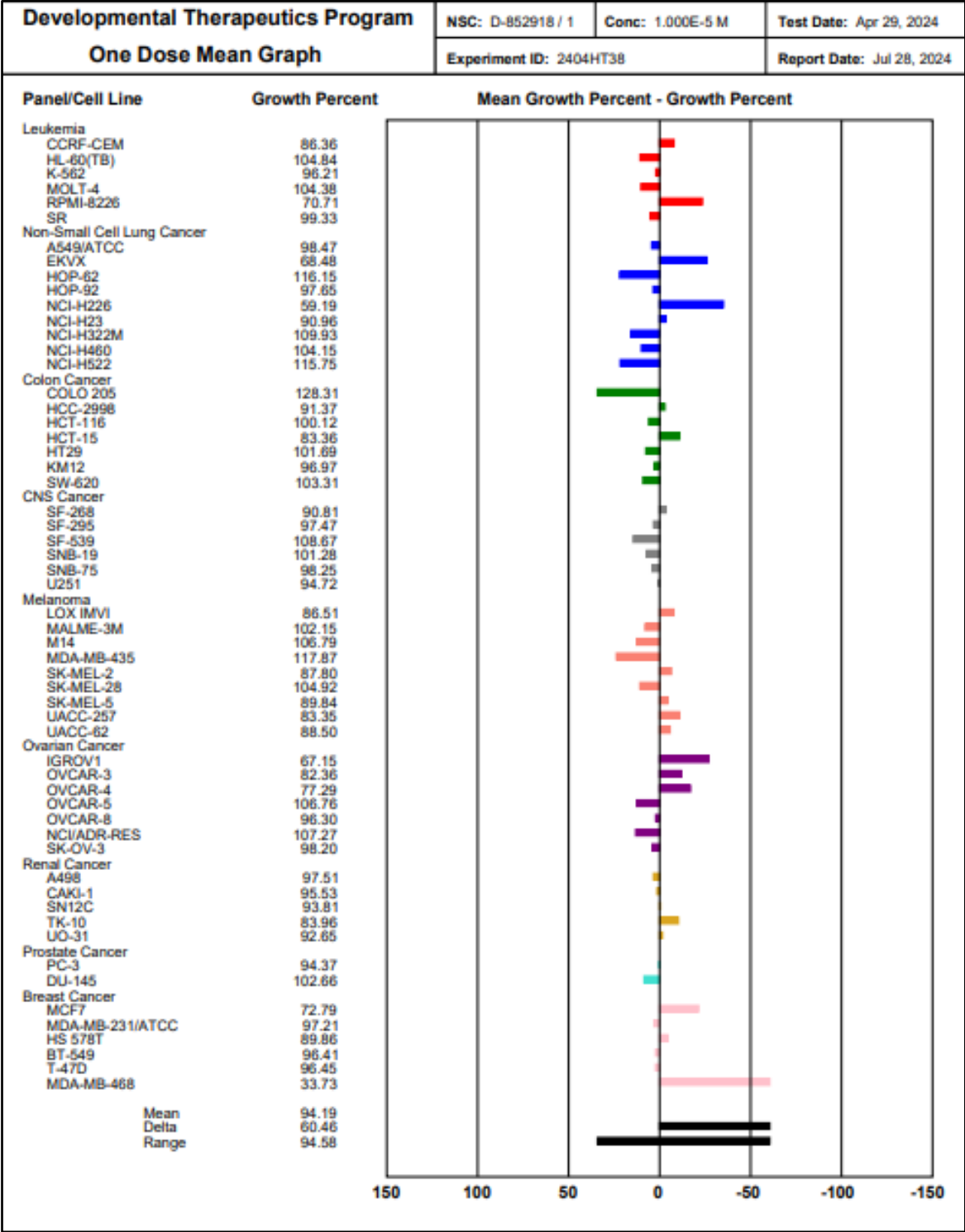


Figure 2.20: Mean growth graph displays of NCI-60 cell line screening data for pyrazinib isoleucine prodrug (69) (10 µM). The bars to right indicate growth promotion and bars to the left indicate growth inhibition.

2.7 Chapter summary

This chapter focused on enhancing the physicochemical properties of pyrazinib, a poorly water-soluble dual-action radiosensitiser small molecule with demonstrated anti-metabolic and anti-inflammatory actions in the OAC tumour model, both *in vivo* and *ex vivo*. Initially, pyrazinib was synthesized using the Perkin condensation method, which resulted in prolonged reaction times and lower yields. To address these limitations, the synthesis of pyrazinib was explored through four literature-based methods and the MAOS Heck reaction. The MAOS Heck reaction notably increased the yield and reduced the reaction time of pyrazinib synthesis. To further improve the solubility of pyrazinib, water-soluble amino acid prodrugs were synthesised by coupling various amino acids to pyrazinib *via* appropriate coupling reactions.

The water solubility and stability of these amino acid prodrugs were evaluated and compared to those of pyrazinib. The resulting prodrugs exhibited significantly enhanced solubility compared to pyrazinib itself, with pyrazinib isoleucine (**69**) showing the highest solubility at 0.95 mg/mL, representing a 2375-fold increase. However, upon evaluating the radiosensitising effects of the prodrugs, it was found that they did not demonstrate radiosensitising effects on the OAC radioresistant cancer cell line OE33R, in contrast to pyrazinib. Despite the lack of radiosensitising effects in the prodrugs, this study provides valuable insights into the synthesis and modification of pyrazinib. Amino acid prodrugs were also submitted to NCI and tested at the one-dose stage of the NCI-60 cell line screen. The successful enhancement of solubility through amino acid prodrugs highlights a potential strategy for improving the pharmacokinetic properties of pyrazinib and similar compounds. Future research could explore alternative modifications to retain or enhance the radiosensitivity. Furthermore, *in vivo* toxicity and anti-angiogenic activity of pyrazinib and its isoleucine prodrug (**69**) are discussed in Chapter 5.

Chapter 3: Synthesis, physicochemical and biochemical analysis of quininiib and its structural analogues

3.1 Introduction

3.1.1 Discovery of quininib and development of the quininib analogues (Q series)

Quininib (**70**) [Q1, (IUPAC name: 2-[(*E*)-2-(quinolin-2-yl)vinyl]phenol)], a small molecule containing a quinoline ring with an (*E*) alkene core structural motif (**Figure 3.1**) was discovered during an unbiased small molecule chemical screen for determinants of developmental angiogenesis in zebrafish.¹³⁰ Previous studies have found that it is well tolerated in *in vivo* and *in vitro* mammalian angiogenesis models. Prior to testing on animal models, Murphy *et al.* tested the safety and efficacy of **70** by using *in vitro* human microvascular endothelial cell lines (HMEC-1) which showed that **70** inhibits angiogenic endothelial tubule formation. Then, the anti-angiogenic activity of **70** was investigated using *in vivo* zebrafish intersegmental vessels (ISVs) and it was found that **70** significantly inhibits blood vessel development in zebrafish embryos.¹³¹ Additionally, Murphy *et al.* investigated the effect of **70** on tumour growth in a colorectal cancer mouse model. Treatment with 50 mg/kg of **70** resulted in significantly reduced tumour growth as compared to 25 mg/kg and the vehicle control.¹³¹ Further studies have revealed that during a target profiling study, **70** significantly inhibited LTD₄-induced activation of the human CysLT₁ receptor, with an IC₅₀ of 1.4 µM, demonstrating much greater potency against CysLT₁ compared to CysLT₂, which exhibited a higher IC₅₀ of 38.5 µM.¹³² This confirms that **70** acts as an antagonist of CysLT₁ and that it binds more potently to CysLT₁ than to CysLT₂. Moreover, **70** was tested in an *in vivo* ocular model and inhibited retinal revascularisation, whereas montelukast (a clinically known antagonist of CysLT₁, discussed in Chapter 4) had minimal effect on revascularisation.¹³⁰

To enhance drug efficacy, the Kennedy group (UCD) expanded upon the quinoline-containing library, by designing distinct structural analogues of **70** (Q series). These include Q22 (**71**) (IUPAC name: 2-[2-(2-quinolyl)ethynyl]phenol), Q8 (**72**) (IUPAC name: 2-[(*E*)-2-(2-quinolyl)vinyl]benzene-1,4-diol) containing an additional hydroxyl group on position 4 of phenol ring compared to **70** and Q18 (**73**) (IUPAC name: 2-[(*Z*)-2-(2-quinolyl)vinyl]phenol), the *cis* (*Z*) isomer of **70**, as hydrochloride salts (**Figure 3.1**). To identify more efficacious analogues of **70**, Mahon *et al.* investigated **71**, **72** and **73** for their anti-angiogenic activity using *in vivo* zebrafish ISVs and *in vitro* in HMEC-1 cell lines. All of these analogues showed significant inhibition of developmental angiogenesis but **72** showed increased potency due to its additional hydroxy group compared to **70**. Additionally, **72** showed significant inhibition of the CysLT₁ receptor.¹³⁰ Later, Butler *et al.* investigated the effect of **72** on tumour growth in a mouse colorectal xenograft model and noted that it reduced tumour volume as compared to the vehicle control.¹³³ As **70** showed inhibition in vascularisation of an ocular model, the effect of **70** and its potent analogue **72** was investigated in human metastatic uveal melanoma (MUM) cell lines and exerted anti-UM effects.¹⁵⁹ Furthermore, in a recently published article,

Tonelotto *et al.* investigated the mechanism of action of 1,4-dihydroxy quininib (Q7, **74**), parent drug of **72** and its potential application in treating MUM.¹³⁴ They discovered that **74** regulates ferroptosis markers and key proteins in MUM cell lines (OMM 2.5) and tissues, indicating that ferroptosis could serve as a promising biomarker and therapeutic target for MUM.¹³⁴

Collectively, these findings support the Q series as a novel collection of analogues of **70** with anti-angiogenic, anti-vascular and anti-proliferative activity. However, **70** and its analogues are limited by poor physicochemical properties.

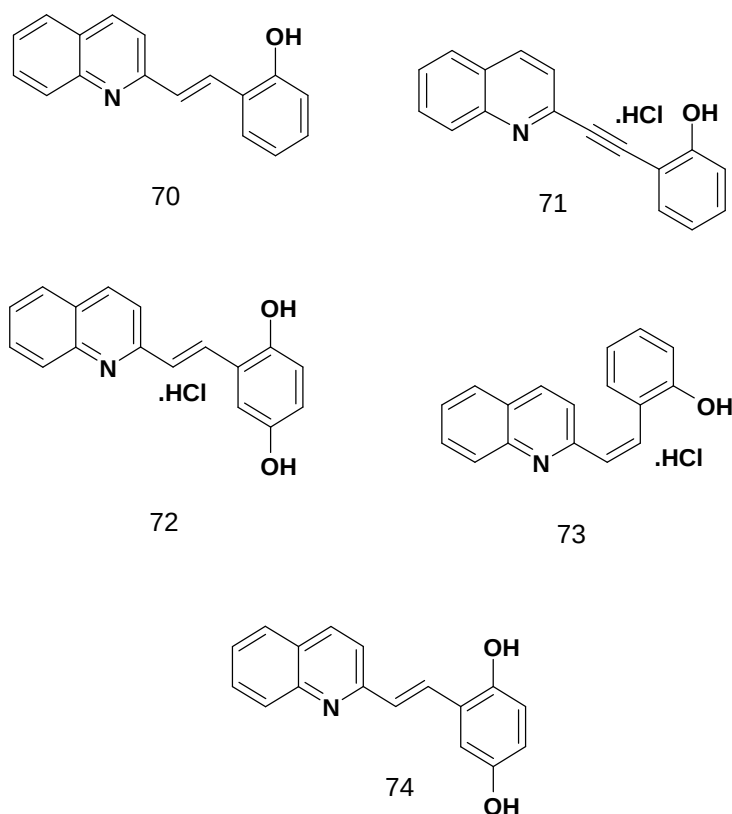


Figure 3.1: Structure of **70** and its structural analogues including **71**, **72**, **73** and **74**.

3.1.1.1 Physicochemical problems of **70**, **72** and **74**

Previously, the O'Boyle group (TCD) studied the stability and solubility of **70**, **72** (HCl salt of **74**) and **74**. Our studies revealed that **70** precipitated out in water. It has a water solubility of 0.00036 mg/mL suggesting that it is practically insoluble in water (**Figure 3.2 A**). Compound **74** exhibited limited solubility (0.0001 mg/mL) and was unstable across the range of pH values (4, 7.4, and 9) with less than 50% remaining after 15 minutes (**Figure 3.2 B**).¹³⁵ To improve the water solubility of **74**, hydrochloride salt **72** was formed. While **72** demonstrated excellent solubility (1.99 mg/mL) and

better stability at pH 4 and 7.4, compared to **74**, it exhibited poor stability at pH 9 (**Figure 3.2 C**).¹³⁶ Given the soluble but unstable nature of salt **72** and poor water solubility of **70** (0.00036 mg/mL), we proposed prodrug formation of **70** and **74** to address the solubility and stability problems. This includes synthesis of ester and amino acid prodrugs of **70** and **74**.

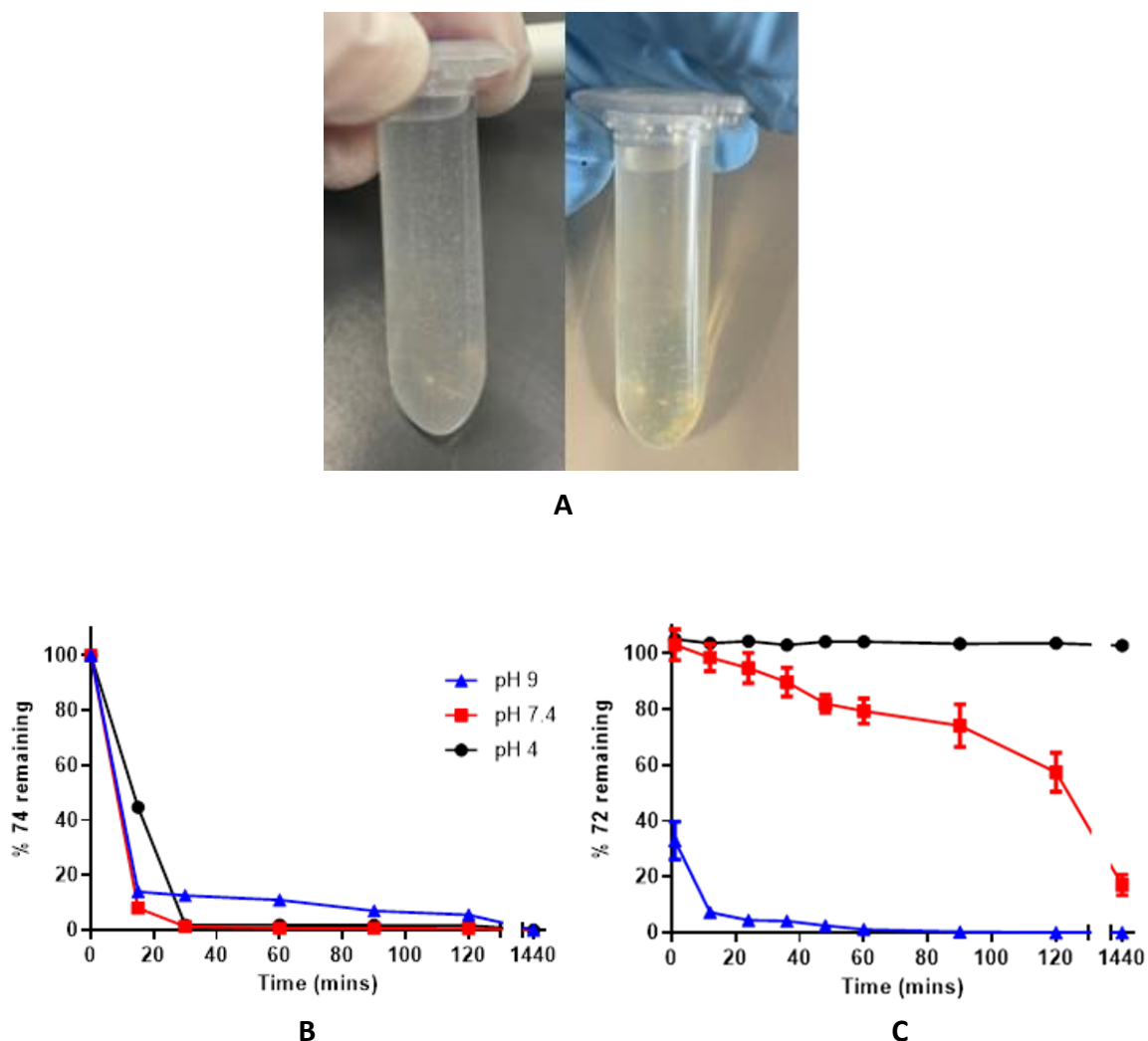


Figure 3.2: (A) Precipitation of **70** in PBS pH 7.4 (Left) and 100% water (Right); (B-C) Chemical stability of **74** and **72** determined by HPLC using detection at 280 nm at pH 4, 7.4 and 9. The stability profile at different pH values reflect the acidic, neutral and basic conditions on the degradation rates. This analysis provides insights into their degradation profiles and pH dependent stability.

3.2 Specific aims of chapter 3

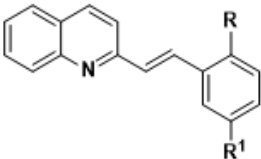
1. To synthesise **70**, **74**, their amino acid prodrugs, an ester prodrug of **70** (Q28, **76**), and salt formation of the ester prodrug, **76** (Q29, **77**).
2. To measure the aqueous solubility and stability of the synthesised analogues and comparison with the parent drugs.
3. To investigate the enzymatic hydrolysis of the ester analogue (**76**).
4. To investigate the biological activity of ester analogues (**76** and **73**) in comparison with the parent drugs.

3.3 Synthesis and characterisation of **70** and its analogues

3.3.1 Synthesis of acetyl ester quininib (**76**) by Perkin condensation reaction, the formation of HCl salt of **76** (**77**) and the synthesis of **70** and **74**.

Recent literature has focused on the synthesis and chemistry of new quinoline-based agents. Quinolines have structural derivatives including 2-methylquinolines and one of the interesting applications of 2-methylquinoline derivatives is their use as precursors in the synthesis of (*E*)-2-styrylquinolines.⁷⁷ Traditionally, (*E*)-2-styrylquinolines are prepared using the Perkin condensation method.⁷⁷ Compound **70** and the majority of its structural analogues including **74** belong to the category of (*E*)-2-styrylquinolines. As a result, structural analogues of **70** [acetyl ester quininib (**76**) and **75**] were synthesised using Perkin condensation method and their hydrolysis in basic conditions led to the formation of **70** and **74** (Table 3.1). Furthermore, Orozco *et al* outlined other synthetic methodologies for their synthesis such as MAOS catalysed reaction and conventional catalysed synthesis.⁷⁷

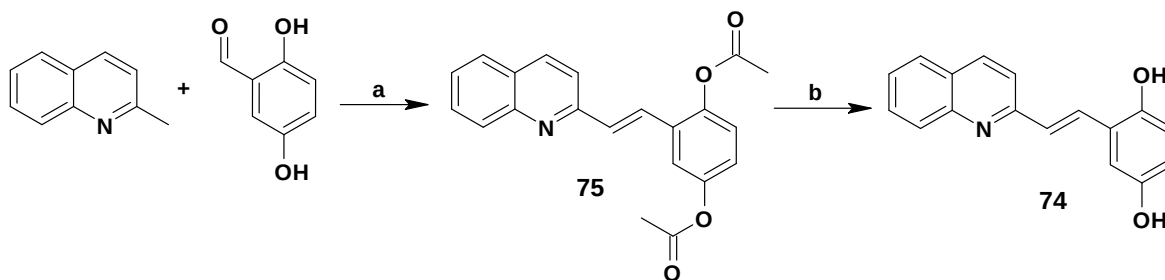
Table 3.1: 70 and its analogues prepared by Perkin condensation method.

Compound number	Structure	% Yield	Melting Point (°C)	IR ν_{max} cm^{-1} (ATR)
				
70	R= OH, R¹= H	92 ^b	216	3008 (OH)
74	R= OH, R¹= OH	85 ^b	200	3054 (OH)
75	R= OC(O)CH ₃ , R¹= OC(O)CH ₃	90 ^a	N/A	1758 (C=O)
76	R= OC(O)CH ₃ , R¹= H	95 ^a	160	1749 (C=O)
77	R= OC(O)CH ₃ , R¹= H .HCl	60 ^c	206	1754 (C=O)

N/A: compound was used further without purification. ^a yield for Perkin condensation (first step); ^b yield for hydrolysis (second step); ^c yield for salt formation (third step)

Accordingly, in the initial experimental phase, the synthesis of **74** followed a two-step procedure (**Scheme 3.1**). The first step involves the reaction of 2-methylquinoline with 2,5-dihydroxybenzaldehyde in acetic anhydride to obtain **75**. The reaction mixture was stirred at 140°C for 24 hours. The second step involves the hydrolysis of **75** using saturated sodium hydroxide to afford **74**. Compound **74** was purified by flash column chromatography and isolated as a brown/yellow solid in an overall yield of > 80%.

Scheme 3.1: Perkin condensation reaction for synthesis of 75 and base-mediated hydrolysis to form 74.

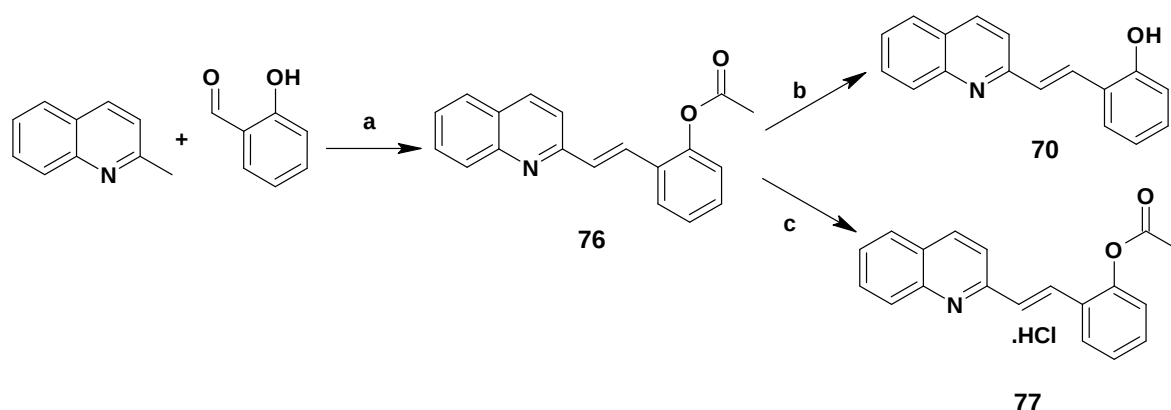


Reagents and conditions: (a) Acetic anhydride, reflux, 24 hr, 90%; (b) NaOH (aq.), EtOH/EtOAc (1:1), reflux at 100°C, until complete as indicated by TLC, 85%

Compound **74** was synthesised due to the potential coupling of its 1,4 dihydroxy groups with various amino acids in order to increase its water solubility. However, this was unsuccessful (discussed in Section 3.3.3). In an effort to increase the water solubility of Q compounds, we turned our attention to **70**. Previous studies have shown that **70** has poor water solubility but exhibited potency in various biological *in vitro* and *in vivo* investigations. On the basis of these observations, it was envisioned that **70** contains one hydroxy group which might undergo a coupling reaction. With this idea, we attempted to synthesise amino acid ester prodrugs of **70**. Unfortunately, this coupling reaction also failed (discussed in Section 3.3.3). After the failure of amino acid coupling to **70**, it was decided to synthesise an acetyl ester prodrug of **70** (**76**). A common example of an acetyl ester prodrug is aspirin which was synthesised to improve water solubility, lipophilicity and taste. In addition, the acetyl group is susceptible to enzymatic cleavage.¹³⁷ There are other examples of ester prodrugs as discussed in Chapter 1.

With this new strategy, we began our approach towards the synthesis of the acetyl ester of **70** (**76**) by reacting 2-methylquinoline with 2-hydroxybenzaldehyde in acetic anhydride at 140°C for 24 hours (**Scheme 3.2**). Compound **76** was obtained in an excellent yield > 95%. Compound **70** was synthesised by hydrolysis of the acetyl group of **76** under basic conditions. **70** was purified by flash column chromatography and isolated as a yellow solid in a yield of 90% (**Scheme 4.2**). In addition, a hydrochloride salt of **76** (**77**) was synthesised. Compound **77** was prepared using 2N HCl in ether under inert conditions (**Scheme 3.2**). The major advantage of synthesising Q analogues (**75** and **76**) by the Perkin method is high yield ($\geq 90\%$). We also attempted to synthesise **74** by MAOS in order to shorten the reaction time of 24 hours (discussed in Section 3.3.1.1).

Scheme 3.2: Synthesis of 76 by Perkin condensation method and base-mediated hydrolysis to form 70 and the HCl salt formation 77.



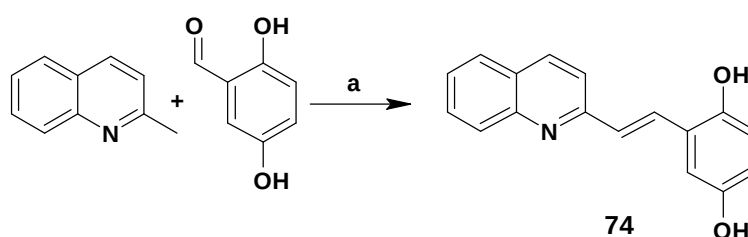
Reagents and conditions: (a) Acetic anhydride, reflux, 24 hr, 95%; (b) NaOH (aq.), EtOH/EtOAc (1:1), reflux, until complete as indicated by TLC, 92%; (c) 2N HCl in ether, RT, N₂, 5-10 min, 60%.

3.3.1.1 Synthesis of 74 by zinc catalysed MAOS

Following the successful synthesis of **74** by the Perkin method, we aimed to reduce the reaction time from 24 hours by employing zinc catalysed MAOS, while also decreasing the amount of solvent used in the process. In chapter 2 we discussed MAOS and its various advantages including shorter reaction time. A procedure reported by Li *et al.* described the synthesis of (*E*)-2-styrylquinoline derivatives.

The MAOS reaction of **74** proceeded by reacting 2-methylquinoline with 2,5-dihydroxybenzaldehyde under solvent free conditions in the presence of zinc chloride (**Scheme 3.3**). The reaction mixture was exposed to MW (CEM Discover SP microwave) irradiation for 15 mins. After 15 minutes TLC [mobile phase: hex: EtOAc (1:1)] indicated the formation of **74** with a bright yellow spot (*R_f*: 0.31 cm). The reaction mixture was treated with saturated potassium hydroxide and the product appeared as yellow solid. Compound **74** was recrystallised from methanol as a yellow solid. Additionally, ¹H NMR confirmed the formation and purity of **74**. The major advantages of this synthetic route are that it is a one-step procedure and has a shorter reaction time (15 mins vs 24 hrs). However, the yield was lower by zinc catalysed MAOS (60%) as compared to the conventional Perkin method.

Scheme 3.3: Synthesis of **74** by zinc catalysed MAOS.



Reagents and conditions: (a) zinc chloride (0.45 mmol), MW at 140 °C for 15 minutes, 60%

3.3.2 Chemical characterisation of **70**, **74** and **76**

Synthesised analogues (**70**, **74** and **76**) were fully characterised by spectroscopic techniques including ^1H and ^{13}C NMR. In addition, X-ray crystal structures for **70**, **74** and **76** were also determined.

3.3.2.1 Chemical characterisation of **76**

The ^1H and ^{13}C NMR of **76** is shown in Figures 4.3 and 4.5. The protons of the acetate group of **76** are indicated in the ^1H NMR at 2.42 ppm. The protons of the *trans* double bond have a chemical shift of 7.80 and 7.52 ppm with a *J* value of 16 Hz and appear as doublets. Moreover, the protons on the quinoline ring appear at 8.37, 8.02, 7.97, 7.91, 7.77 and 7.58 ppm. Furthermore, the protons attached to the aromatic ring with acetate group have chemical shifts of 7.21, 7.42, 7.36 and 7.98 ppm. The protons at 7.42 and 7.21 exhibit a doublet of doublet pattern (**Figure 3.4**), indicating that each proton is coupled to two neighbouring protons with different coupling constants; *ortho* coupling (*J*= 8 Hz) is stronger, compared to *meta* coupling (*J*= 2 Hz).

In the ^{13}C NMR of **76**, the carbon of the acetate appears at 20.8 ppm and the carbonyl carbon appears at 169.3 ppm. The carbons of the *trans* double bond appear downfield in the spectrum at 130.8 and 126.8 ppm respectively. The quaternary carbon attached to the nitrogen atom of the quinoline ring appears downfield in the spectrum at 155.2 ppm. Compound **77**, the HCl salt of **76** was also characterised by ^1H and ^{13}C NMR. Since the spectra were identical to those of **76**, they are not discussed further in this section.

Furthermore, **76** was crystallised as colourless crystals and its crystal structure was characterised by X-ray crystallography (**Figure 3.6**). The X-ray crystal structure of **76** provides insights into its molecular geometry and intermolecular interactions. The molecular structure of **76** features typical bond lengths and angles which are consistent with its chemical composition. Notably, the structure includes a C=C double bond (C11-C12) with a bond length of 1.33 Å which is consistent with the

expected range for C=C double bonds (1.33 Å) (**Figure 3.6**). The bond length for the double bond between the oxygen atom (O20) and the carbon atom (C21) is 1.1993 Å, typical for a carbon-oxygen double bond and indicative of a strong π -bonding interaction.¹³⁸

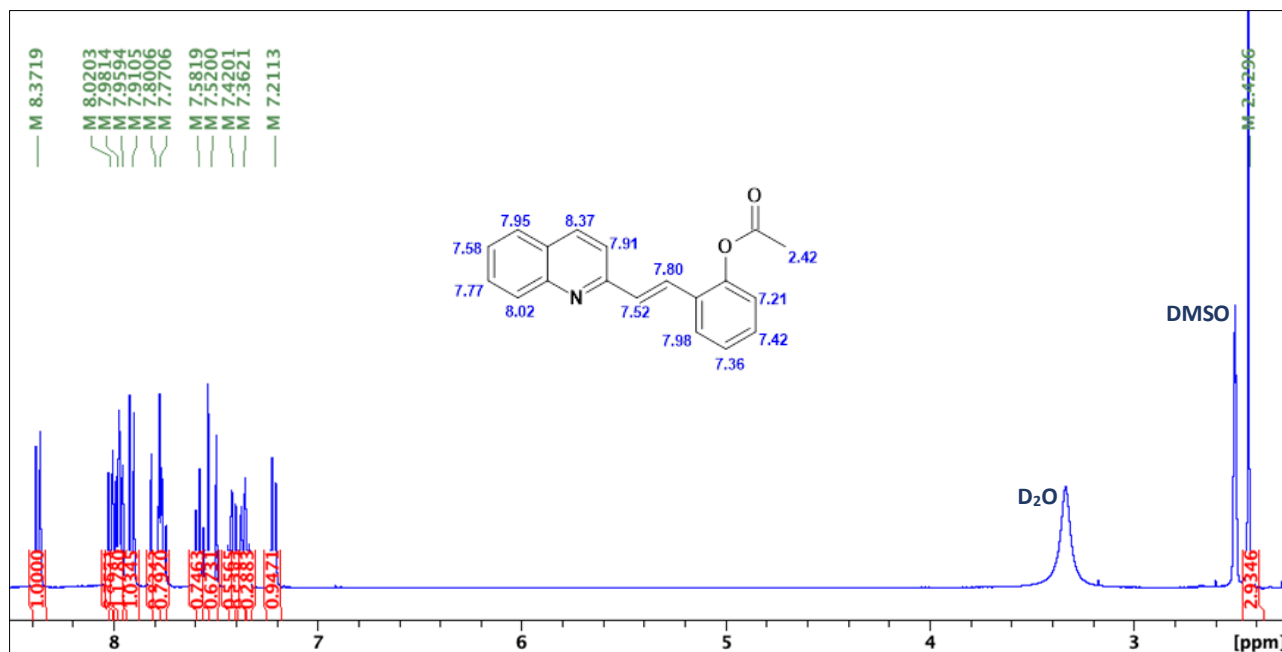


Figure 3.3: ¹H NMR spectrum of 76 in DMSO-*d*₆ at 400 MHz

(Note: DMSO signal: 2.50 ppm and water signal: 3.3 ppm)

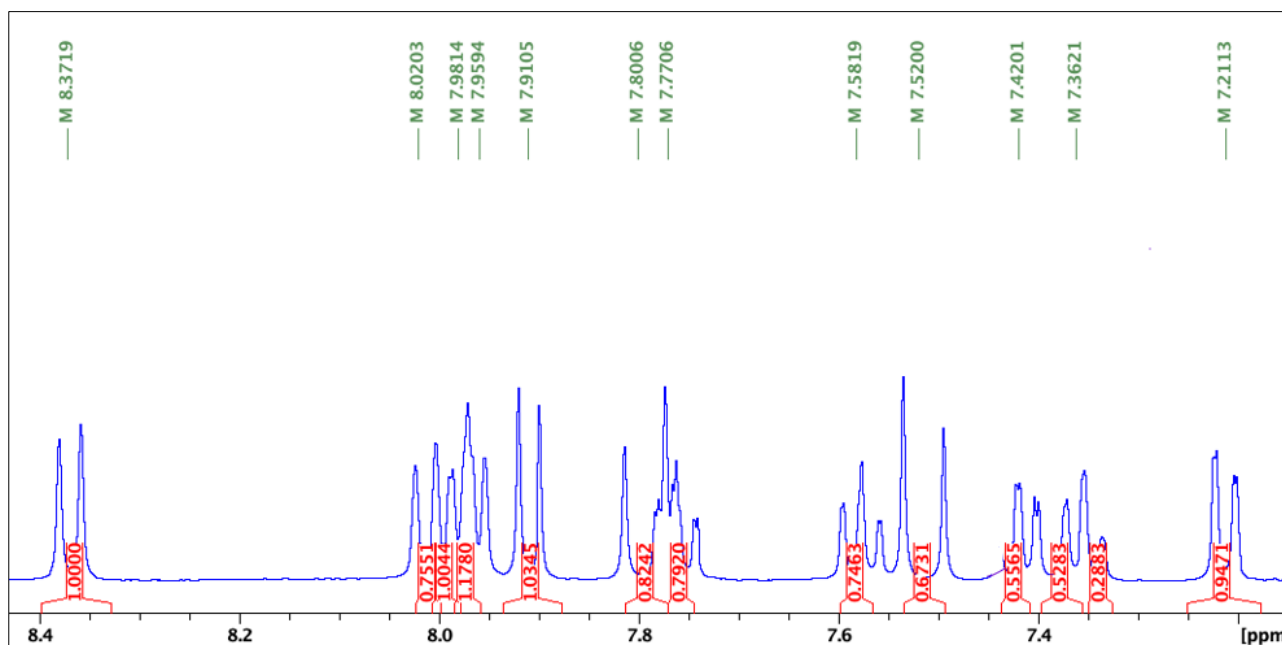


Figure 3.4: Zoomed version of ¹H NMR of 76 from 7.21 to 8.37ppm.

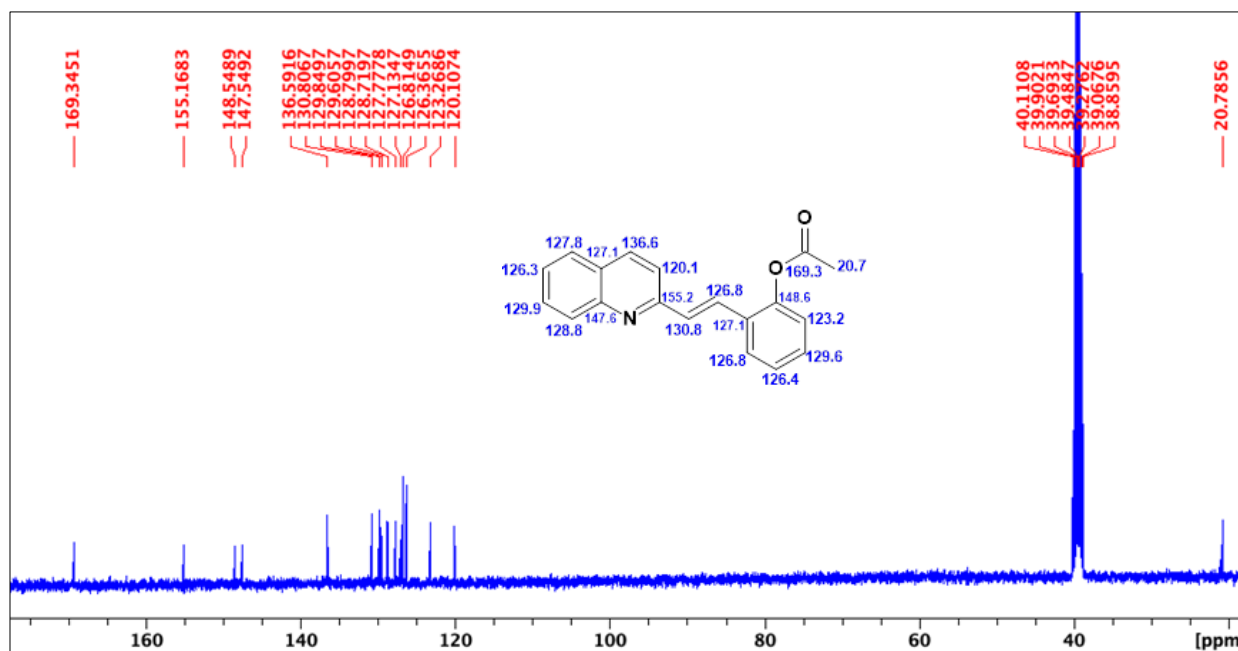


Figure 3.5: ^{13}C NMR spectrum of 76 in $\text{DMSO}-d_6$ at 400 MHz

(Note: DMSO signal: 38.8 to 40.1ppm)

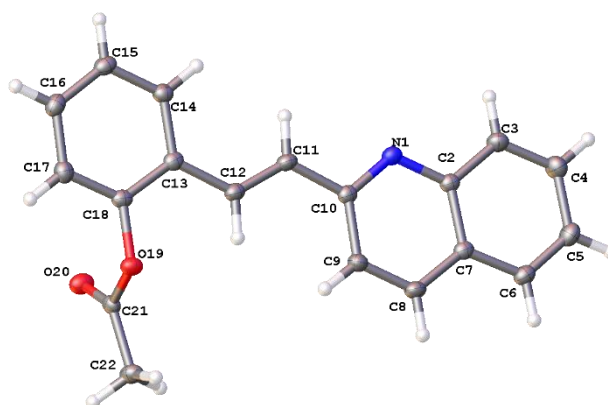


Figure 3.6: X-ray crystal structure of 76. Displacement parameters shown at 50% probability.

3.3.2.2 Chemical characterisation of **70**

Compound **70** was synthesised by basic hydrolysis of **76**. IR spectroscopy confirmed the hydrolysis by showing a broad signal at 3054 cm^{-1} due to the formation of phenolic hydroxy group (**Table 3.1**). **70** was characterised by ^1H and ^{13}C NMR (**Figures 3.7 and 3.8**) and its X-ray crystal structure is shown in Figure 3.8. The ^1H NMR confirms the hydrolysis of the acetate group by showing a broad peak at 10 ppm attributed to the hydroxy group proton. The characteristic doublets at 7.46 and 8.05 ppm with J value of 16 Hz are indicative of a *trans* double bond. Moreover, the protons attached to quinoline ring appear downfield at 8.31, 7.98, 7.92, 7.79, 7.73 and 7.53 ppm. The aromatic protons of the phenolic ring were appeared from 6.81 to 7.66 ppm.

The ^{13}C NMR also confirms the hydrolysis by disappearance of the carbonyl signal at 169 ppm. The carbon attached to the hydroxy group appears in the aromatic downfield region at 156.2 ppm. The carbons of *trans* double bond appear at 127.7 and 130.0 ppm respectively. Furthermore, the carbon attached to the nitrogen atom of the quinoline ring has a chemical shift of 155.3 ppm. The carbons attached to the phenolic ring appeared between 115.8 to 123.3 ppm. For the full assignment of signals, see Figure 3.7 and 3.8.

Compound **70** was crystallised from methanol as bright yellow crystals and its crystal structure was characterised by X-ray crystallography (**Figure 3.9**). The X-ray crystal structure of **70** offers valuable insights into its molecular geometry and intermolecular interactions. Its structure reveals typical bond lengths and angles that align with its chemical composition. A key feature is the C=C double bond between C8 and C9, with a bond length of $1.34\text{ \AA}$, which falls within the expected range for such bonds ($1.33\text{ \AA}$), as shown in Figure 3.9 A. Additionally, the crystal structure emphasizes the presence of an extensive hydrogen bonding network. In crystal structure of **70**, the hydrogen bonding network involves interactions between hydrogen atoms attached to nitrogen and oxygen atoms (**Figure 3.9 B**). The presence of weak hydrogen bonding exemplifies complexity and robustness of the molecular and crystal structure of **70**. This combination of interactions is essential for the compound's structural integrity and its physical properties.

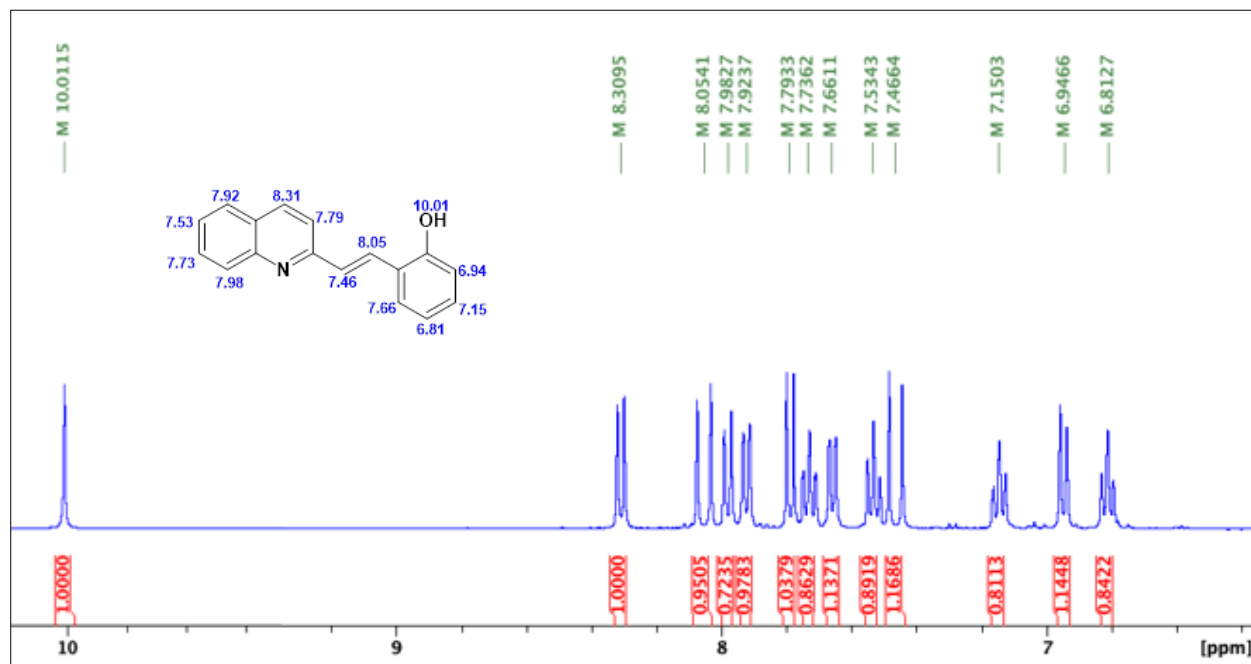


Figure 3.7: ¹H NMR spectrum of 70 in DMSO-*d*₆ at 600 MHz

(¹H NMR showing from 6.81 to 10.01 ppm, peaks < 6.81 ppm primarily attributed to NMR solvent (DMSO-*d*₆), have been excluded for clarity).

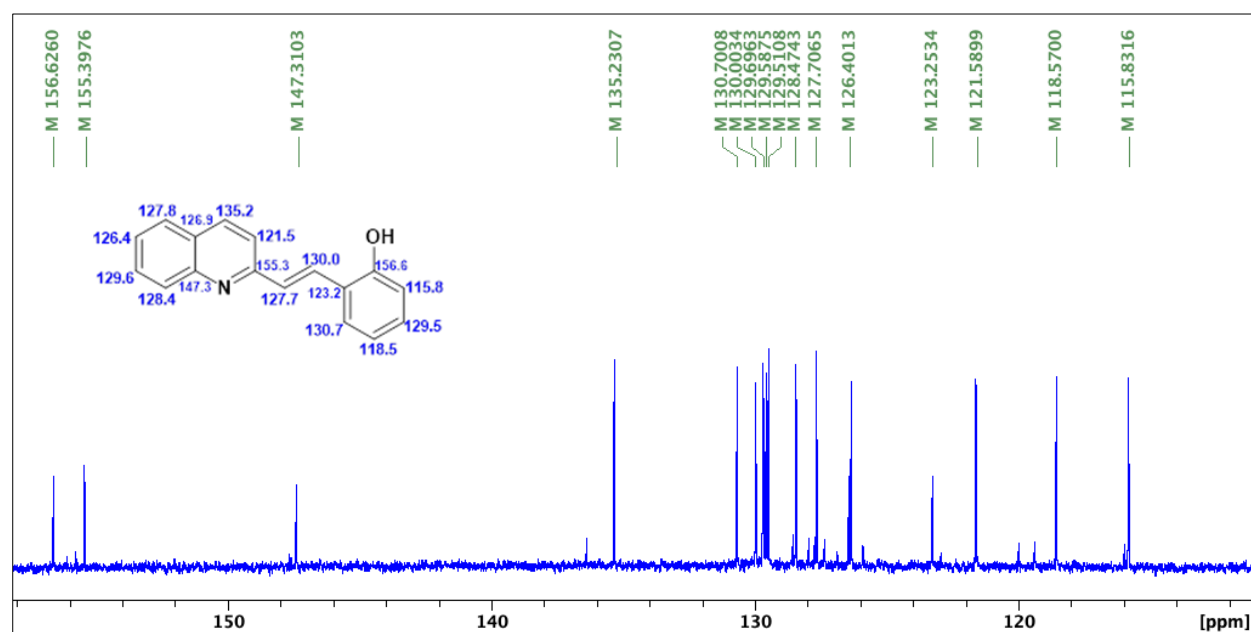


Figure 3.8: ¹³C NMR spectrum of 70 in DMSO-*d*₆ at 600 MHz

(¹³C NMR showing from 115 to 156 ppm peaks < 115 ppm primarily attributed to NMR solvent (DMSO-*d*₆), have been excluded for clarity).

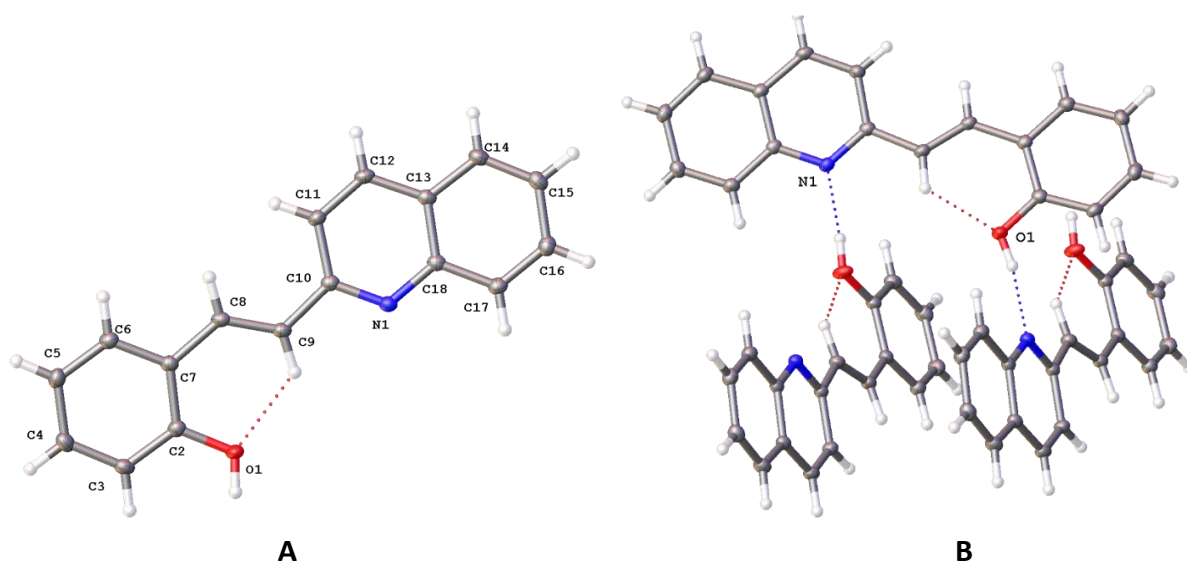


Figure 3.9: X-ray crystal structure of 70. (A) Molecular structure of 70. Displacement parameters shown at 50% probability. Dotted lines indicate the hydrogen bonding; (B) A section of the hydrogen bonding network seen between three molecules of 70, represented by dotted lines. Displacement parameters shown at 50% probability.

3.3.2.3 Characterisation of **74** by NMR spectroscopy

The synthesis of **74** was a two-step procedure, in which **75** was synthesised as an intermediate and further hydrolysed under basic conditions to afford **74**. The ^1H and ^{13}C NMR of **74** are shown in Figures 3.10 and 3.11 and its X-ray crystal structure is shown in Figure 3.12.

The ^1H NMR shows two broad peaks at 9.38 and 8.88 ppm indicating the presence of two hydroxy groups. The characteristic doublets at 7.37 and 8.03 ppm with J value of 16 Hz are attributable to the protons of the *trans* double bond. The protons of the aromatic region appeared downfield between 6.65–8.38 ppm. The diphenol is an *ortho*, *para* substituted ring. In aromatic compounds, splitting effect does not only come from *ortho* but also from *meta* protons due to conjugated bonds. Therefore, coupling constants (J values) are helpful in detecting the complicated aromatic regions.¹³⁹ The characteristic J values for *ortho*: 6–9 Hz, *meta*: 2–3 Hz and *para* 0–1 Hz.¹³⁹ The doublet at 6.76 ppm with a J value of Hz indicates that this proton is at *meta* position relative to the quinoline ring substituent. The proton at 6.65 ppm appears as doublet of doublet with J values of 9 and 3 Hz. This splitting pattern suggests that it is *meta* to proton at 7.05 ppm (2.8 Hz coupling) and *ortho* to the proton at 6.76 ppm (9 Hz coupling) (**Figure 3.10**). The signal at 7.05 ppm appears as doublet with 2.8 Hz coupling indicating it is in the *ortho* position relative to the nitrogen-containing substituent. Therefore, the proton at 7.05 ppm is *ortho* to the quinoline ring substituent, the proton at 6.76 ppm is *meta* to this substituent, and the proton at 6.65 ppm is *meta* to the proton at 7.05 ppm and *ortho* to the proton at 6.76 ppm.

In the ^{13}C NMR, carbon directly attached to the hydroxy groups appears in the aromatic region at 149.2 and 156.6 ppm. The carbons of *trans* double bond appear at 127.3 and 130 ppm respectively. Furthermore, the carbon attached next to the nitrogen atom of the quinoline ring has a chemical shift of 150.4 ppm. The aromatic carbons, other than those attached to hydroxy groups, appeared between 112.9 to 123.7 ppm.

Compound **74** was crystallised from methanol and its crystal structure was characterised by X-ray crystallography (**Figure 3.12**). The X-ray crystal structure of **74** offers important insights into its molecular geometry and intermolecular interactions. The structure exhibits typical bond lengths and angles that are in line with its chemical composition. A notable feature is the C=C double bond between C11 and C12, with a bond length of 1.33 Å, indicating conjugation with the aromatic ring. This bond length falls within the expected range for C=C double bonds (1.33 Å), as shown in Figure 3.12 A. The intermolecular interaction in the crystal structure is noteworthy. The OH bond length is determined to be 0.999 Å forming a weak CH...O interaction and a significant OH...N interaction is observed between the hydroxyl group and the nitrogen atom (**Figure 3.11**).

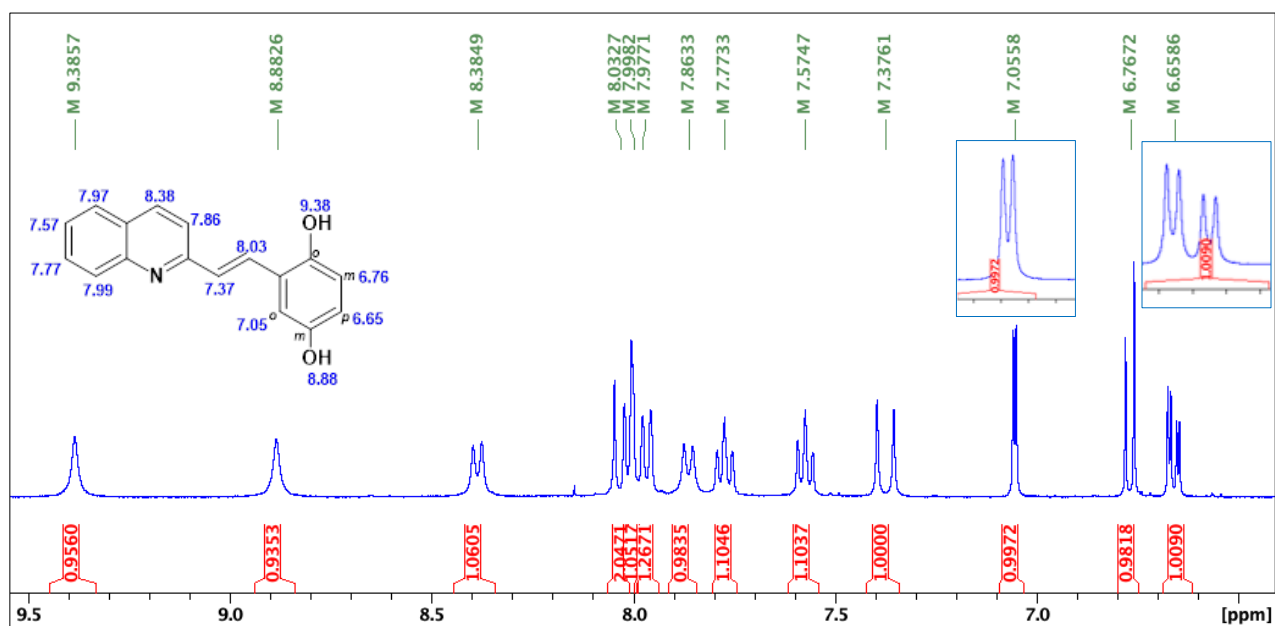


Figure 3.10: ¹H NMR spectrum of 74 in DMSO-*d*₆ at 600 MHz
(¹H NMR showing from 6.65 to 9.38 ppm and expanded signals 7.05 and 6.65 ppm).

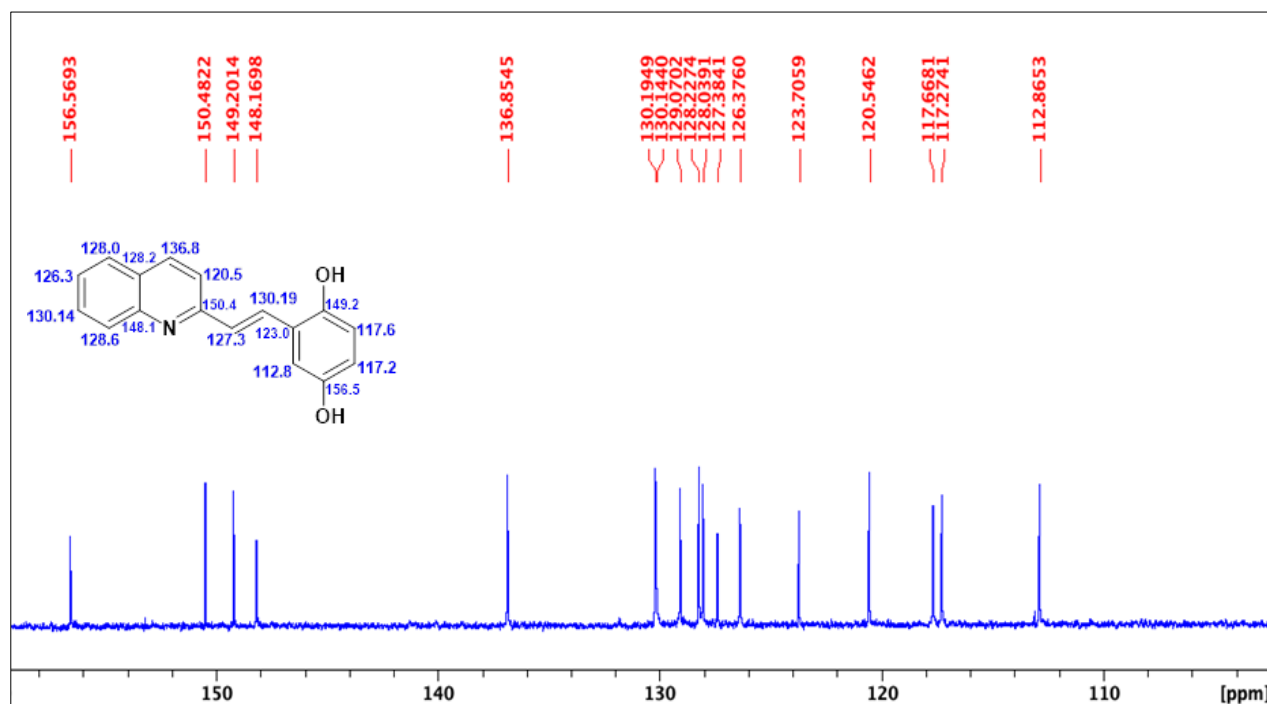


Figure 3.11: ¹³C NMR spectrum of 74 in DMSO-*d*₆ at 600 MHz
(¹³C NMR showing from 113 to 156 ppm).

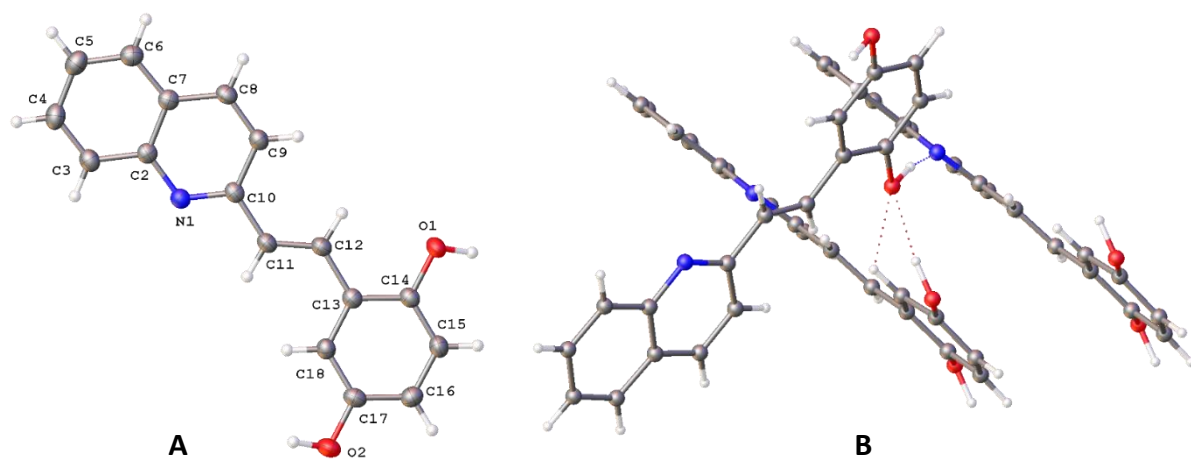


Figure 3.12: X-ray crystal structure of 74. (A) Molecular structure of 74. Displacement parameters shown at 50% probability; (B) Hydrogen bonding detail in 74 showing H-bonding (dotted lines) between each hydroxyl supported by weak CH...O interactions and an OH...N interaction to another molecule.

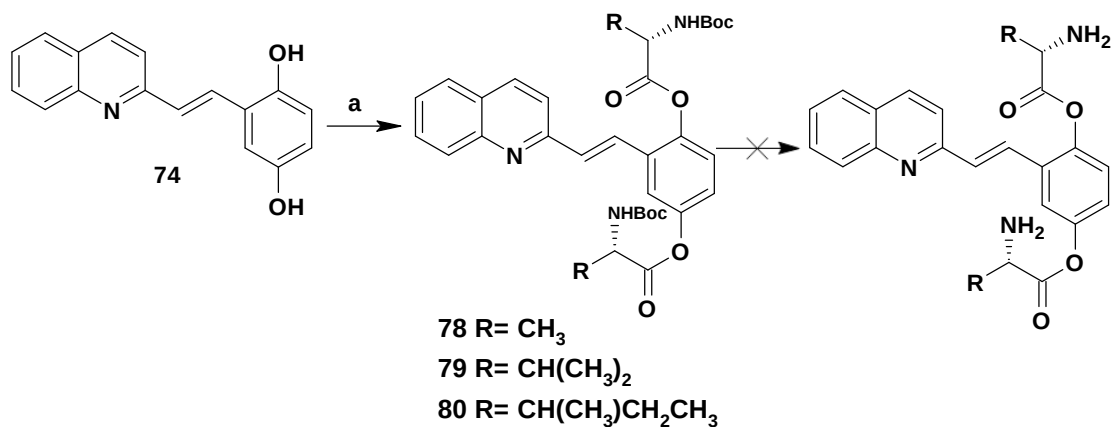
3.3.3 Attempted synthesis of amino acid ester prodrugs of **70** and **74**

Following the successful synthesis of pyrazinib amino acid prodrugs (**Chapter 2**), the aim was to synthesise amino acid prodrugs of **70** and **74** using the same coupling procedure. It is evident from Chapter 2 that the prodrug approach offers improved drug solubility and may also improve drug stability. As discussed earlier, compound **74** lacks good water solubility and exhibits chemical instability. Hence, the synthesis of amino acid prodrugs of **74** was investigated.

The HBTU coupling agent was used to couple the two hydroxyl groups of **74** with the carboxylic acid of N-Boc protected amino acids in the presence of DIPEA base (**Scheme 3.4**). The progress of the reaction was monitored by TLC. ¹H NMR analysis of the crude product indicated the coupling of the N-Boc protected amino acids to **74**. The crude products were purified by flash column chromatography to afford pure Boc protected amino acid analogues of **74** in a low yield ($\leq 10\%$). The low yield of Boc protected analogues of **74** could be attributed to challenges during both the reaction and purification stages. In the reaction stage, steric hindrance around the hydroxyl groups of compound **74** and the amino acid carboxylic acids may have limited the coupling efficiency leading to incomplete product formation. Additionally, compound **74** is chemically unstable and may have degraded under reaction conditions, further contributing to reduced yields. During purification stage, the separation of the product from impurities was challenging due to similar polarities, resulting in significant product loss during flash column chromatography. Together these factors likely account for overall low yield of the reaction.

This coupling reaction was attempted with three amino acids (**Table 3.2**). Spectroscopic characterisation was carried out on all Boc protected amino acid analogues of **74**. The ¹H and ¹³C NMR spectra of **80** are shown in Figures 3.13 and 3.14. The ¹H NMR shows the presence of protons attached to the methyl group of isoleucine signals at 1.02 and 0.92. Moreover, two signals due to the N-Boc group at 1.44 and 1.40 ppm, indicates the di-coupling of Boc isoleucine at the ring. The aromatic protons appear between 8.34 to 7.1 ppm. In the ¹³C NMR, the presence of two carbonyl signals at 171.9 and 171.4 ppm also indicates the di-coupling at positions 1 and 4 of the ring. Moreover, the carbon signal at 79 ppm is attributable to the Boc group while other carbon signals from 60 to 11 ppm correspond to isoleucine.

Scheme 3.4: Synthesis of Boc amino acid analogues of 74 by HBTU coupling method.



Reagents and conditions: (a): Boc amino acid, anhydrous acetonitrile, HBTU, DIPEA base, stirring at room temperature (RT) for 24 hours, 7.5 to 10%

Table 3.2: Boc protected amino acid analogues of 74 (78-80).

Compound number	Structure	Boc Amino acid	Yield (%)	IR ν_{max} cm ⁻¹ (ATR)
	R =			
78	CH ₃	Alanine	10	3366 (N-H), 1755 (C=O, ester), 1674 (C=O, Boc)
79	CH(CH ₃) ₂	Valine	9	3440 (N-H), 1759 (C=O, ester), 1680 (C=O, Boc)
80	CH(CH ₃)C ₂ H ₅	Isoleucine	7.5	3342 (N-H), 1762 (C=O, ester), 1701 (C=O, Boc)

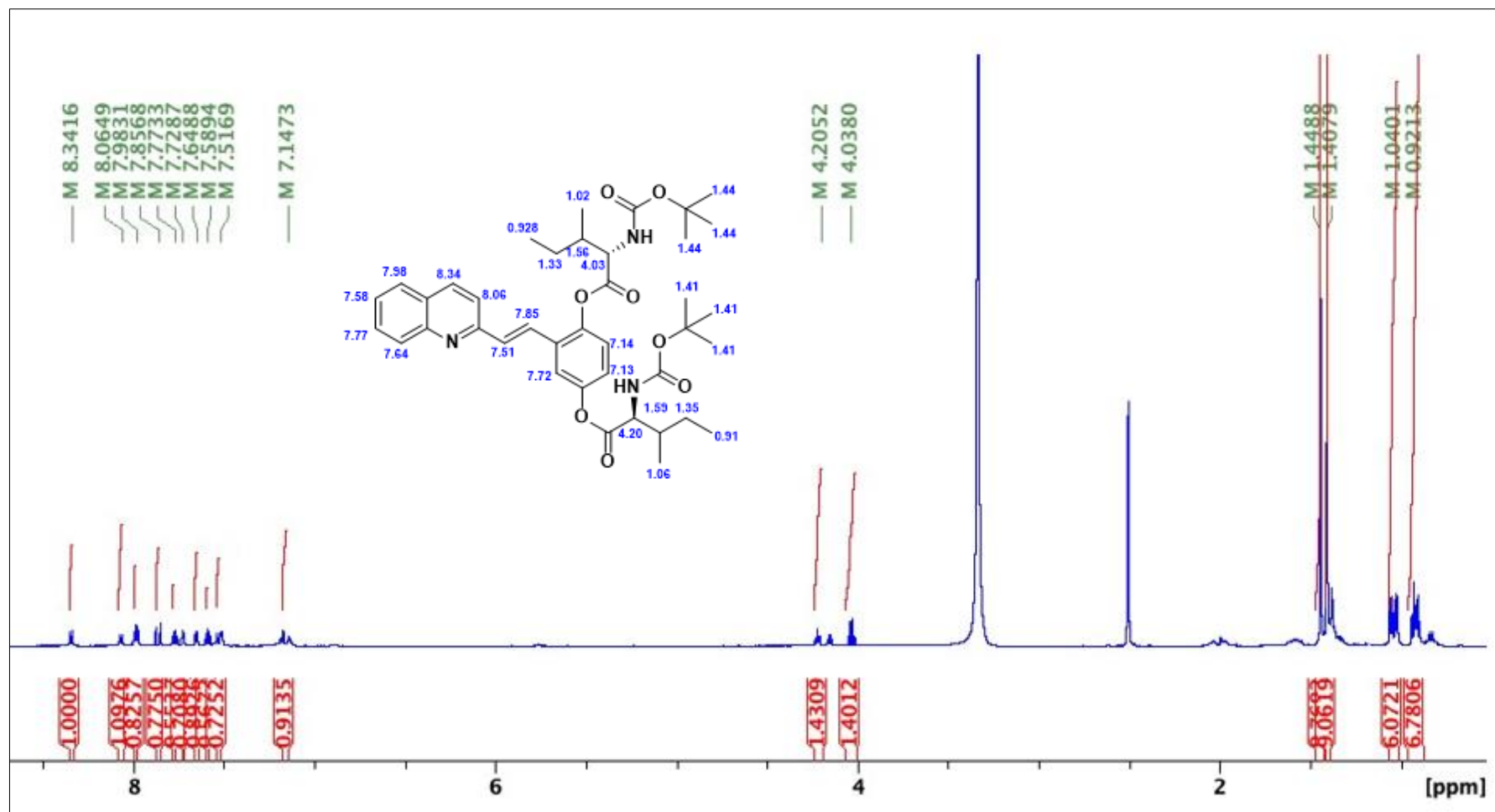


Figure 3.13: ^1H NMR of 80 in $\text{DMSO}-d_6$ at 600 MHz.
 (Note: DMSO signal: 2.50 ppm and water signal: 3.3 ppm)

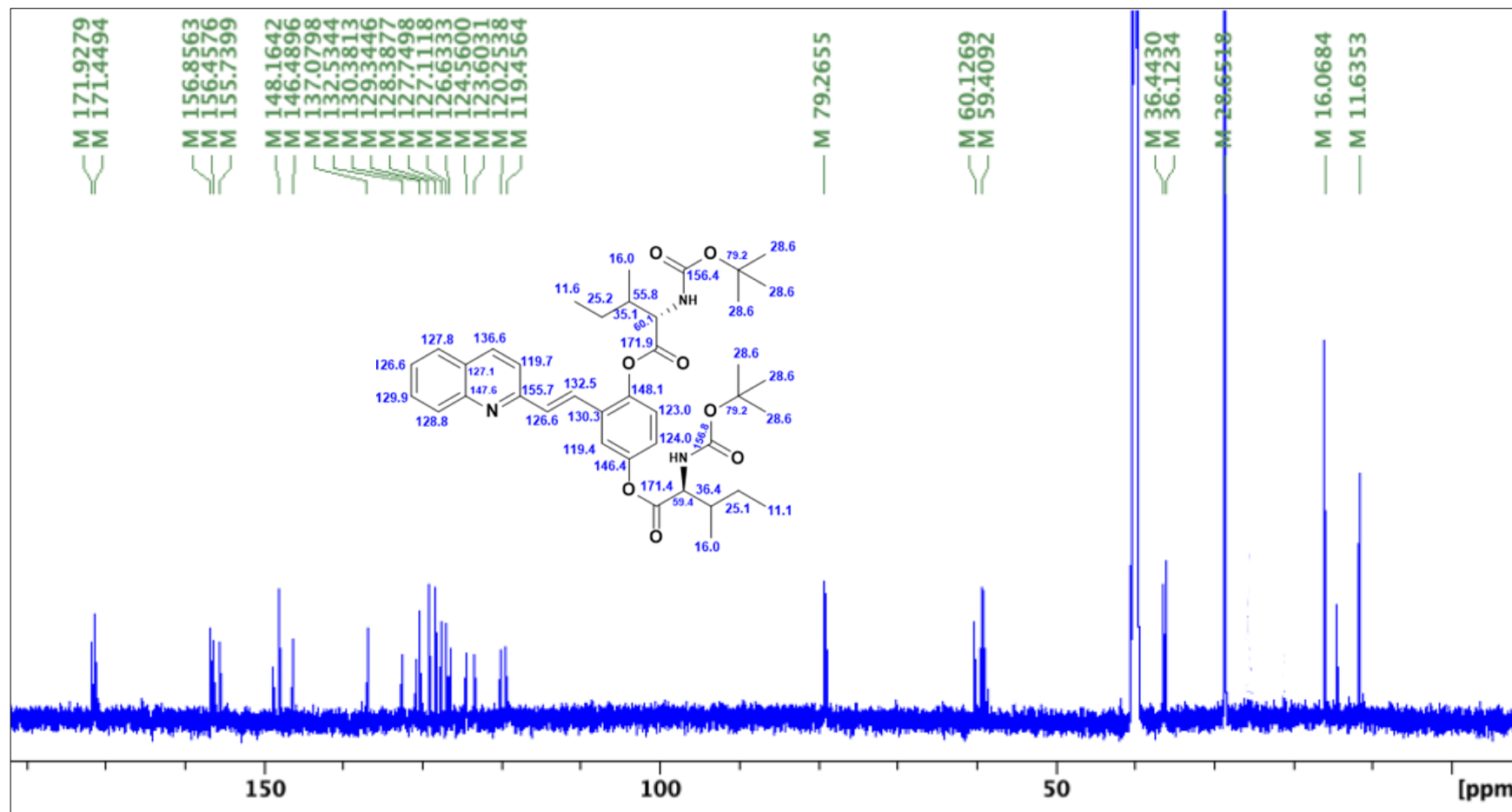


Figure 3.14: ^{13}C NMR spectrum of 80 in $\text{DMSO}-d_6$ at 600 MHz.

(Note: DMSO signal: 38.8 to 40.1ppm)

The next step of the synthesis involved deprotecting the Boc group attached to the amine group of the amino acid. The procedure outlined in Chapter 2 for deprotection of the Boc group using TFA was repeated. Although ^1H NMR indicated the deprotection of the Boc group, it also showed a mixture of **74** and the amino acid, with broad signals indicating the presence of hydroxy groups of **74** (Figure 3.15). Despite previously deprotecting a large number of Boc amino acid derivatives (Chapter 2), it was observed that amino acid analogues of **74** were short-lived, decomposing within a few hours to yield **74**. Repeated attempts to refine the reaction conditions, such as using lower amounts of TFA in DCM (1:3) at room temperature were unsuccessful. In all reactions, ^1H NMR consistently indicated the presence of both the amino acid and **74**. The ^1H NMR spectrum (Figure 3.15) demonstrates this mixture, showing broad signals at 9.8, 9.3, and 10.32 ppm in DMSO, two of which are characteristic of the hydroxy groups of **74** and the other could be the OH group of an amino acid.

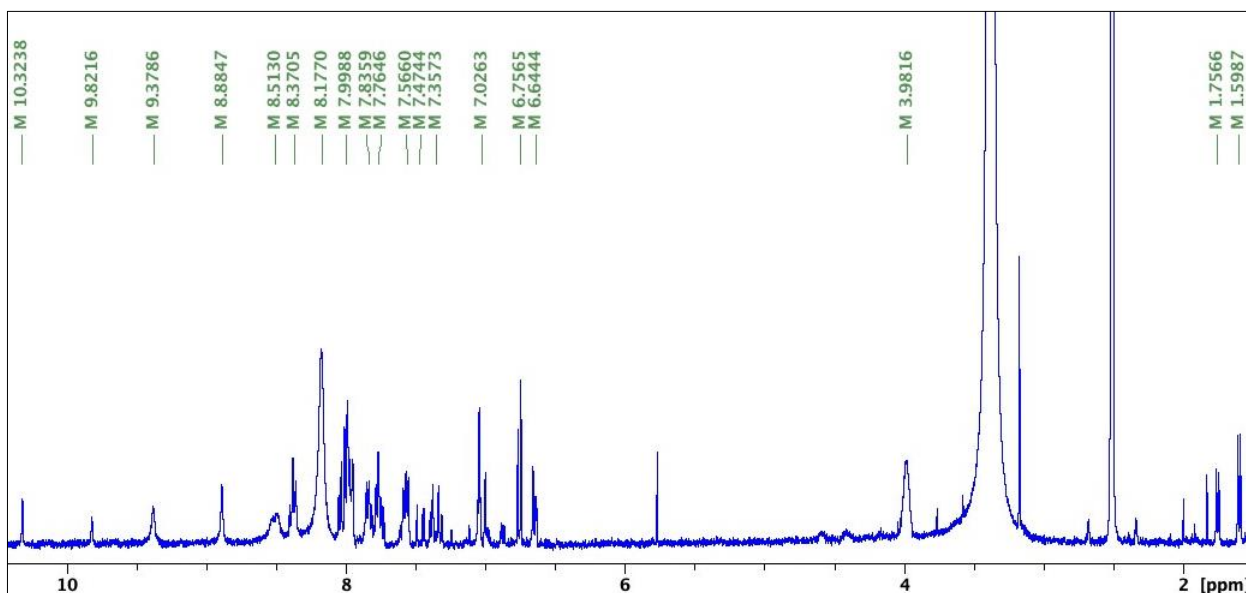


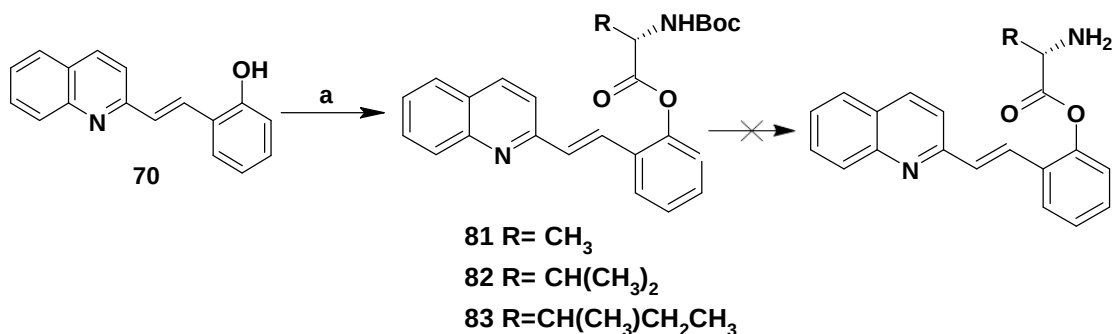
Figure 3.15: ^1H NMR of the reaction mixture of alanine analogue of **74** in $\text{DMSO}-d_6$ at 400 MHz post Boc deprotection step.

Additionally, attempts to synthesise amino acid prodrugs of **70** using the HBTU coupling method (Figures 3.16 and 3.17 and Table 3.3) also resulted in products (**81-83**) that converted to **70** upon deprotection (Figure 3.18). This instability of amino acid analogues of **70** was confirmed by ^1H NMR as the hydroxyl group of **70** was observed at 10.5 ppm (Figure 3.18).

The persistent instability of both amino acid analogues of **70** and **74**, as evidenced by ^1H NMR, underscores the inherent challenge in synthesizing stable prodrugs under these conditions. Despite various adjustments to the reaction protocols, the rapid decomposition of these analogues remains

a significant barrier, suggesting that alternative synthetic strategies or protective groups may be necessary to achieve the desired stability.

Scheme 3.5: Synthesis of Boc amino acid analogues of 70 by HBTU coupling method.



Reagents and conditions: (a): Boc amino acid, anhydrous acetonitrile, HBTU, DIPEA base, stirring at room temperature (RT), 8-12%

Table 3.3: Boc protected amino acid analogues of 70 (81-83).

Compound number	Structure	Boc Amino acid	Yield (%)	IR ν_{max} cm ⁻¹ (ATR)
	R =			
81	CH ₃	Alanine	10	1759 (C=O) 3440 (N-H), 1702 (C=O, Boc)
82	CH(CH ₃) ₂	Valine	12	1765 (C=O) 3339 (N-H), 1686 (C=O, Boc)

83	$\text{CH}(\text{CH}_3)\text{C}_2\text{H}_5$	Isoleucine	8	1746
				(C=O)
				3335 (N-
				H), 1678
				(C=O,
				Boc)

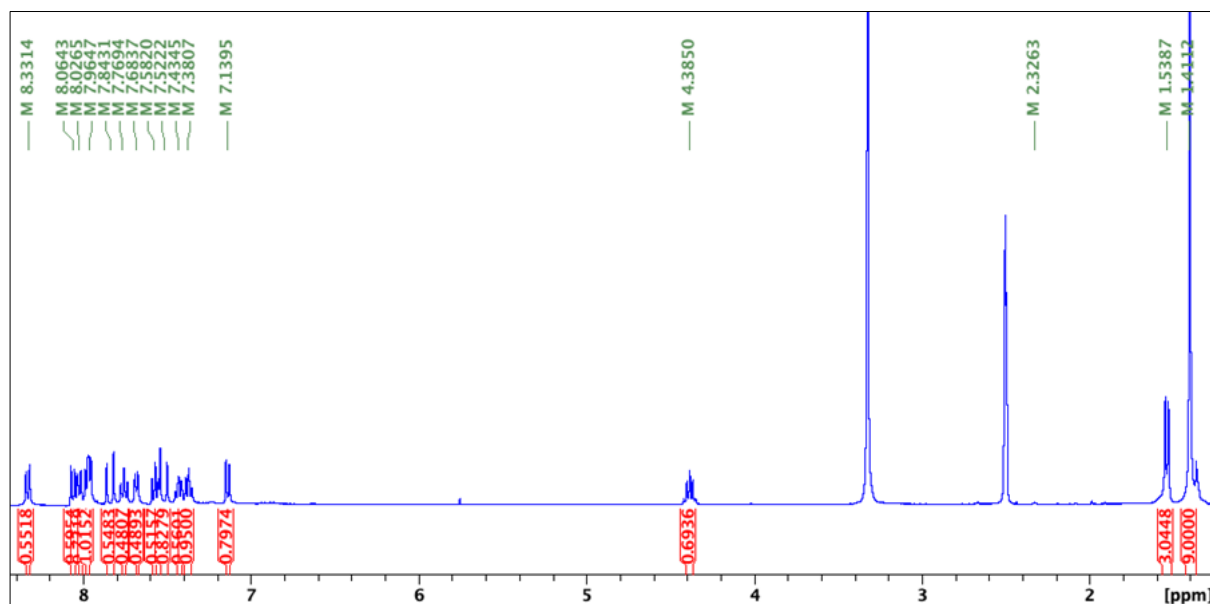


Figure 3.16: ^1H NMR of 81 in $\text{DMSO}-d_6$ at 600 MHz
(Note: DMSO signal: 2.50 ppm and water signal: 3.5 ppm)

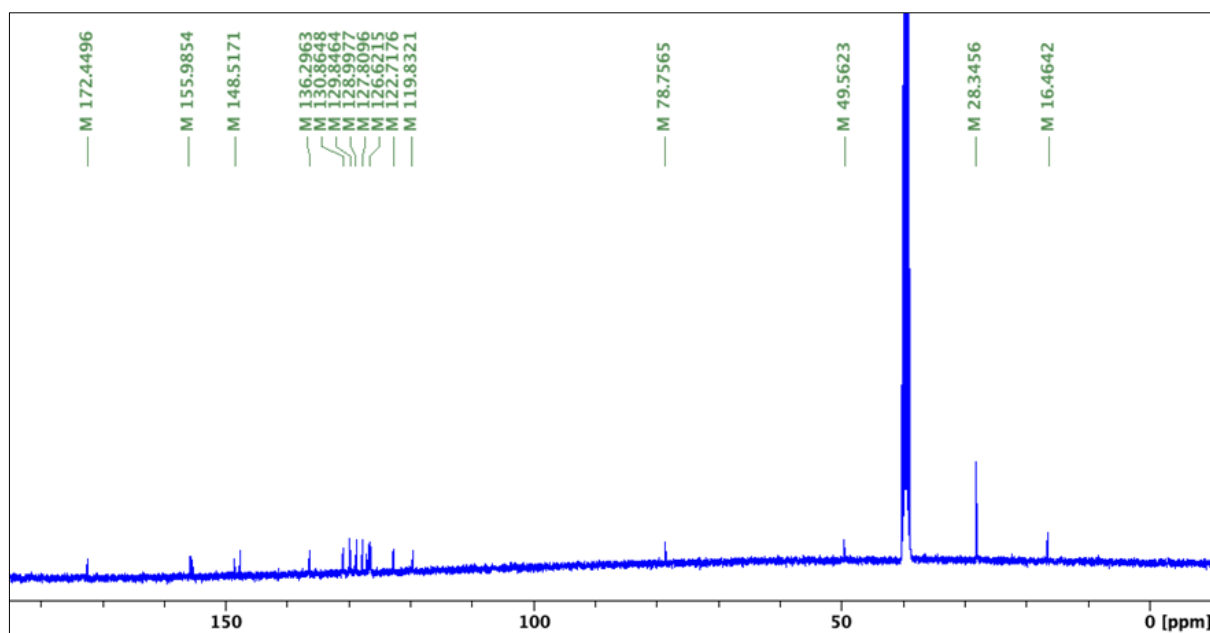


Figure 3.17: ^{13}C NMR spectrum of 81 in $\text{DMSO}-d_6$ at 600 MHz
(Note: DMSO signal: 38.8 to 40.1 ppm)

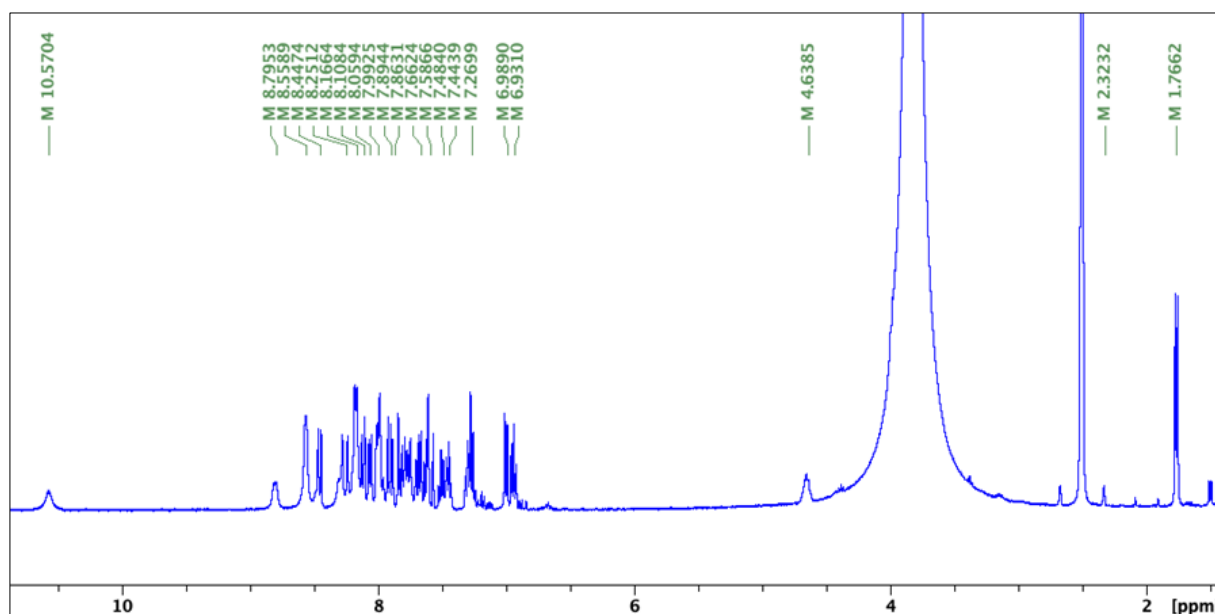
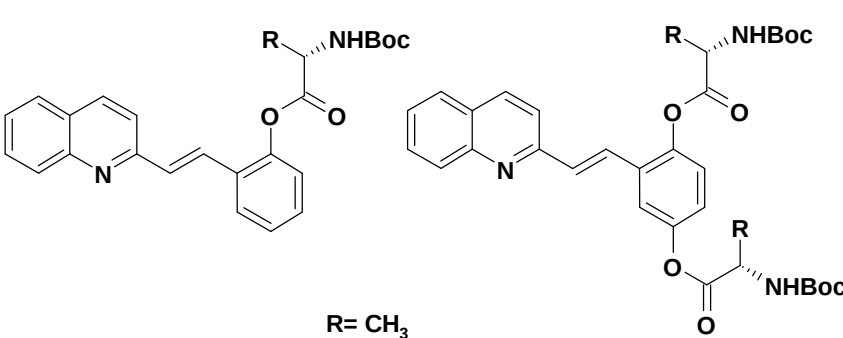


Figure 3.18: ^1H NMR of alanine analogue of **70** in $\text{DMSO-}d_6$ at 400 MHz post Boc deprotection step.

To overcome this problem, coupling of **70** and **74** by using other strategies such as coupling with an unprotected amino acid were attempted which also resulted in a failed reaction. Hence, we tried an alternative coupling approach using DCC with DMAP and $\text{HOBt.H}_2\text{O}$ which resulted in no coupling reaction. Strategies attempted to prepare amino acid prodrugs of **70** and **74** are shown in Table 3.4. Although HBTU coupling may have formed the amino acid prodrugs they readily converted to **70** and **74** after deprotection. In addition, HBTU coupling of **70** and **74** with Boc amino acids was highly limited by low yield. Ultimately, due to the persistent instability of amino acid prodrugs of **70** and **74**, further pursuit of these compounds was discontinued. This instability highlights the need for alternative prodrug strategies, such as carbamate linkages to resist hydrolysis or PEGylation to enhance solubility and reduce degradation.

Table 3.4: Attempted coupling reactions for the formation of amino acid prodrugs of **70 and **74**.**

 $R = \text{CH}_3$ $R = \text{CH}(\text{CH}_3)_2$ $R = \text{CH}(\text{CH}_3)\text{C}_2\text{H}_5$		
Attempted reactions	Reaction conditions	Reaction outcome
HBTU coupling	Boc amino acid (2.5 eq), anhydrous acetonitrile, HBTU (2.2 eq), DIPEA base (61 eq), stirring at RT for 24 hours	Boc amino acid coupled to 70 and 74 but after deprotection, readily hydrolysed
HBTU coupling	(L) amino acid (2.5 eq), anhydrous acetonitrile, HBTU (2.2 eq), DIPEA base (61 eq), stirring at RT for 24 hours	No reaction
DCC coupling	Boc amino acid (0.23 mmol), DCC (0.233 mmol), DMAP (0.23 mmol) in anhydrous acetonitrile (5 mL), stirring at RT for 24 hours	No reaction

3.4 Physicochemical analysis of **70** and its synthesised analogues: **76** and **77**

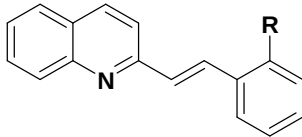
In Chapter 2, the aqueous solubility, chemical and metabolic stability of pyrazinib amino acid prodrugs were discussed and compared with the parent drug. In this Chapter, the aqueous solubility and stability including chemical and enzymatic stability of **76** and **77** will be discussed and compared with **70**.

3.4.1 Aqueous solubility studies

As discussed in Chapter 2, low aqueous solubility is a major problem encountered during the formulation of new chemical entities. For a drug to be absorbed, it must be present in the form of aqueous solution at the site of absorption.¹⁴⁰

The aqueous solubility of all three compounds (**70**, **76** and **77**) were determined by shake flask technique (discussed in Chapter 2). Both **70** and **76** are practically insoluble in water, with a solubility lower than the recommended minimum of 0.05 mg/mL for a potential drug candidate (**Table 3.5**). Compound **76** is an acetyl ester prodrug of **70**, which showed better solubility than **70** but its value was lower than 0.05 mg/mL, the recommended minimum solubility that is desirable for potential drug candidates.¹⁶⁵ In comparison, HCl salt of **76** (**77**) was soluble at 2.17 mg/mL in water, which is greater than the recommended minimum solubility of 0.05 mg/mL. The highest solubility was obtained for **77** which is reflective of the increase in hydrophilicity of the molecule (**Table 3.5**). As discussed earlier in Chapter 1, a salt form of drug is usually more water-soluble compared to the parent drug.¹⁴¹ Salt formations are performed on ionisable drugs, i.e. weakly acidic or basic drugs which may alter the physicochemical properties of a drug without modifying its chemical structure.¹⁴¹ Weakly basic drugs are moderately/poorly soluble in water. However, if the pH of the medium is reduced by the addition of acid, the solubility of the base increases. As the pH is reduced, the base is converted to a salt which is relatively more soluble in water.¹⁴² The addition of HCl formed a charged ion on the nitrogen of the quinoline ring, hence the solubility was improved, compared to **70** and **76** (**Table 3.5**). Compound **77** demonstrated a remarkable 6027-fold increase in aqueous solubility.

Table 3.5: Aqueous solubility values of 70 and its analogues: 76 and 77.

Compound number	Structure	Solubility (mg/mL)
		
70	R= OH	0.00036
76	R= OC(O)CH ₃	0.0034
77	R= OC(O)CH ₃ .HCl	2.17

3.4.2 *In vitro* stability studies

3.4.2.1 Chemical stability

The amino acid prodrugs of **70** and **74** were extremely limited by their instability after synthesis. Therefore, it was important to test the chemical stability of **70**, **76** and **77** before conducting biological studies. The chemical stability of **70**, **76** and **77** was evaluated by HPLC using Hypersil BDS C18 (dim 250 x 4.6 mm) column in phosphate buffer at pH 4, 7.4, and 9 over the period of 24 hours.

Figure 4.19 displays the chemical stability graphs of **70**, **76** and **77**. Compound **70** showed greater stability at earlier time points at pH 4 than the other pH values but dropped to 0% after 3 hours. At pH 7.4 and 9, it decreased significantly to 7.5% and 12.5% respectively, after 24 hours (**Figure 3.19 A**). Compound **76** demonstrated the highest overall stability, especially at pH 4, maintaining 70% stability after 24 hours. At pH 7.4 its stability decreased to 59% after 24 hours. However, at pH 9, its stability decreased more rapidly, reaching 0% after 24 hours (**Figure 3.19 B**). In contrast, **77** exhibited intermediate stability, performing better in acidic conditions with 36% remaining at pH 4 after 24 hours. At pH 7.4, its stability dropped to 7.5%, and at pH 9, it dropped to 0% after 24 hours, indicating its potential hydrolysis to **70** (**Figure 3.19 C**). Table 4.6 demonstrates the half-life of **70** and its analogues: **76** and **77** which was calculated that remained approximately at 50% at all pH values at any time point.

Overall, **76** is the most stable form, particularly in acidic and neutral environments, indicating the advantage of masking the phenolic group with an acetyl ester moiety. Compound **77** offers moderate stability, favouring acidic and neutral conditions over basic environment. Both **76** and **77** exhibited poor stability at pH 9, likely undergoing hydrolysis to form **70**. This instability is due to the ester bond in both compounds, which is particularly susceptible to cleavage in basic conditions. Based on the results obtained, **76** showed the superior stability at pH 4 and 7.4. A study of the effect

of esterase enzyme on **76** would be desirable for a comprehensive understanding of its activation to **70**. By studying esterase activity, the efficiency and rapidity of **76** conversion to **70** can be determined. Table 3.6 presents the half-life and kinetic reaction order for all three compounds. Empirical half-life was utilised as a practical approach when reaction order could not be determined due to non-linearity or insufficient degradation. For compounds that degraded < 50%, the reaction order was inferred by comparing the first and second half-lives. However, for compounds where degradation dropped < 25%, the reaction order could not be determined.

Table 3.6: Half-lives ($t_{1/2}$) and kinetic reaction order of **70 and its analogues: **76** and **77** in PBS (pH 4,7.4 and 9) analysed by HPLC. N=3.**

Compound number	pH 4	pH 7.4	pH 9
70	First $t_{1/2}$: 60 mins Second $t_{1/2}$: 80 mins Reaction order: second	First $t_{1/2}$: 50 mins Second $t_{1/2}$: 50 mins Reaction order: zero	First $t_{1/2}$: 60 mins Second $t_{1/2}$: 120 mins Reaction order: second
76	N/A	N/A	N/A
77	N/A	First $t_{1/2}$: 30 mins Second $t_{1/2}$: 80 mins Reaction order: second	N/A

N/A = Not available for the compounds whose stability is $\geq 50\%$ or $< 25\%$ over the period of 24 hrs.

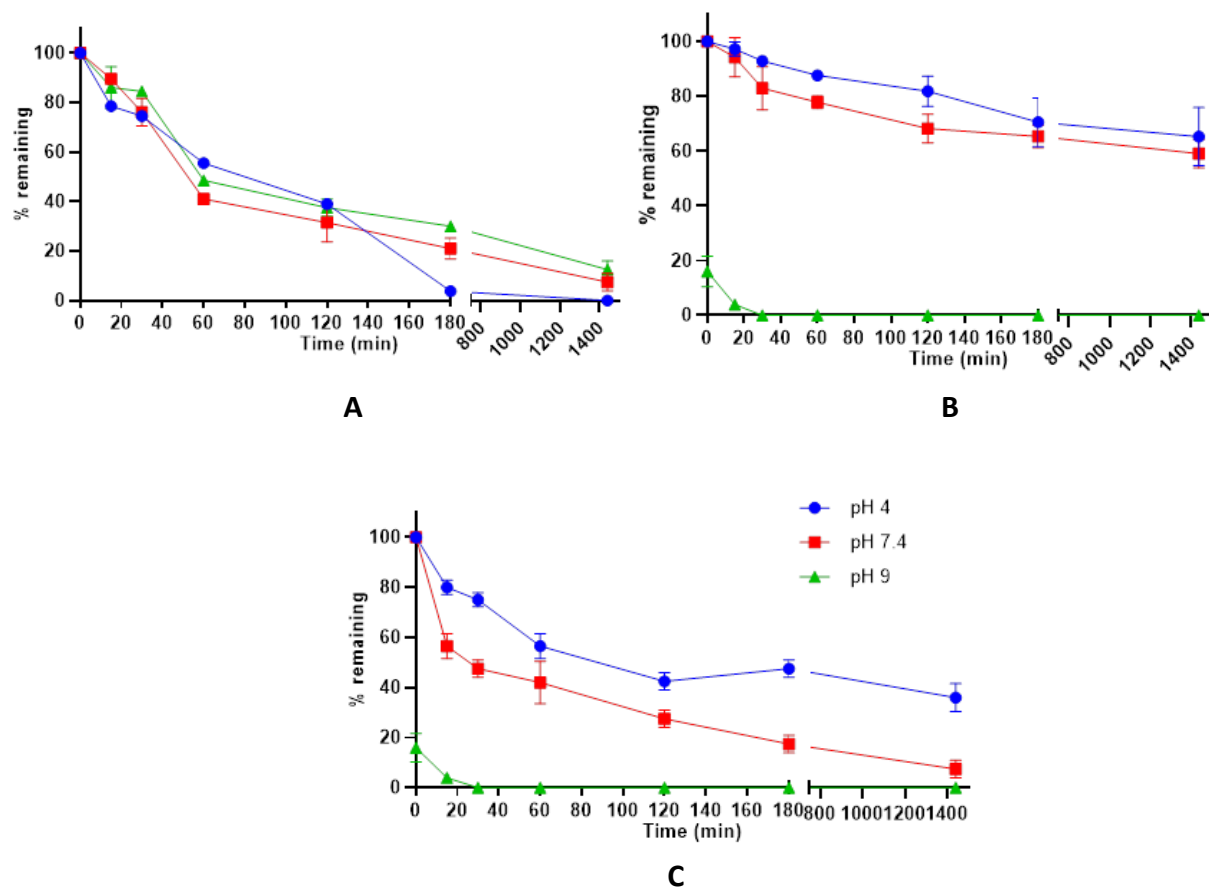


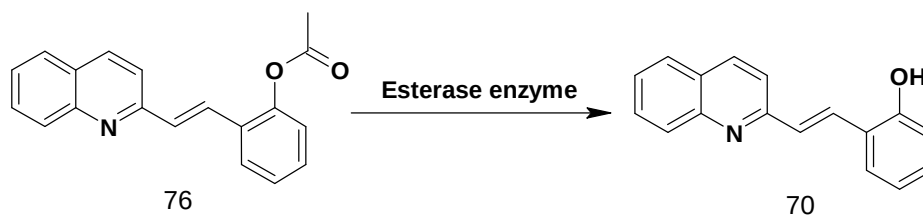
Figure 3.19: HPLC chemical stability study of 70 and its analogues 76 and 77 at three pH values; 4, 7.4 and 9. (A) Chemical stability profile of 70; (B) Chemical stability profile of 76; (C) Chemical stability profile of 77. N=3. Colour code: Blue= pH 4, Red= pH 7.4 and Green= pH 9

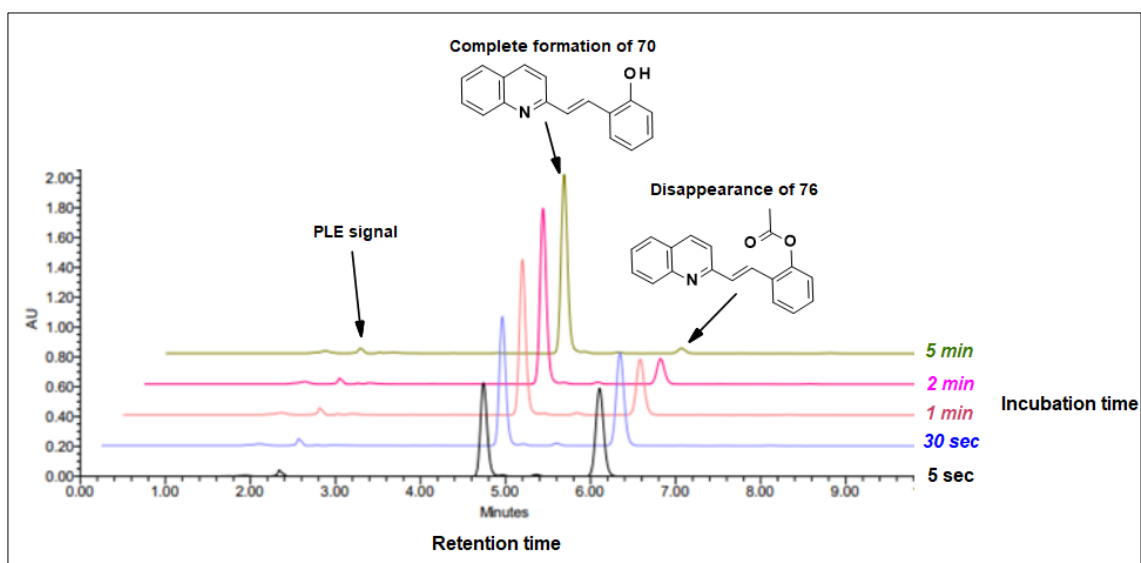
3.4.2.2 Enzymatic hydrolysis

As discussed in Chapters 1 and 2, a major step in prodrug design involves the conversion of prodrug to its active/parent drug in an efficient manner.⁶⁷ In this Chapter, an enzyme mediated hydrolysis of **76** was carried out by using a porcine liver esterase (PLE, Merck product no: E3019, specific activity 95.7 U/mg).

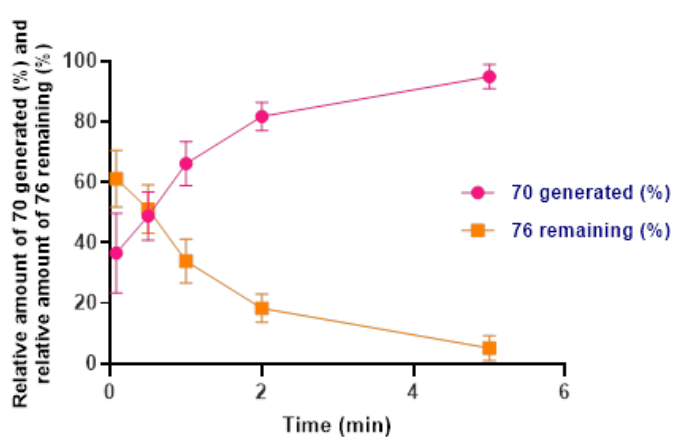
The reaction mixture containing **76** and PLE was incubated under physiological conditions (PBS, pH 7.4 at 37°C) for 5 minutes and the results were measured by RP-HPLC using a Hypersil BDS C18 (dim 250 x 4.6 mm) column (**Figure 3.20 A**). In the study, it was found that **76** readily converted to **70** within 2 to 5 minutes of incubation as there was a very small amount of **76** observed after 5 mins. In addition, a control sample of **76** was analysed and no conversion of **76** to **70** observed in the absence of PLE (**Figure 3.20 C**). As shown in Figure 3.20 B, approximately 18% of **76** remained and 81% of **70** was generated at 2 minutes. The data shows rapid conversion of **76** to **70** within 5 mins. Therefore, these findings confirm **76** as an ester prodrug due to its rapid conversion to its parent drug **70** in the presence of an esterase enzyme.

Scheme 3.6: Conversion of 76 to 70 in the presence of esterase enzyme at 37 °C in PBS (pH 7.4).

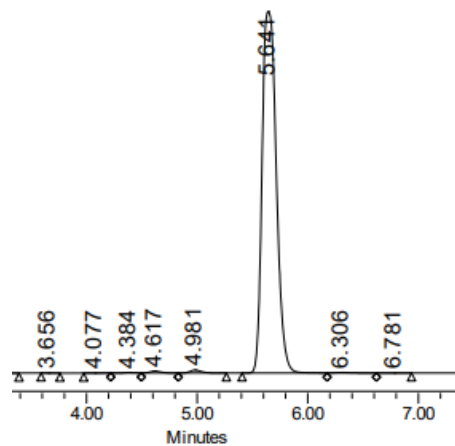




A



B



C

Figure 3.20: (A) Chromatograms of esterase mediated hydrolysis of 76 at retention time of 5.8 min and formation of 70 at retention time of 4.8 min; (B) Hydrolysis of 76 and formation of 70, calculated as % of peak area of compound 70 formed and compound 76 remaining; (C) Control sample of 76 without PLE enzyme, at a retention time of 5.64 mins.

3.5 Analysing the *in silico* physicochemical properties, pharmacokinetics and drug-likeness profile of **70** and its analogues: **76** and **77**

Previously in Chapter 2, the *in silico* physicochemical properties, pharmacokinetics and drug-likeness of pyrazinib and its prodrugs were discussed using the SwissADME web tool. In this section, we will discuss the SwissADME predicted physicochemical properties, pharmacokinetics profile and drug-likeness of **70** and its analogues: **76** and **77**.

3.5.1 Bioavailability radar of **70** and its analogues: **76** and **77**

As discussed in Chapter 2, a bioavailability radar is generated for all molecules which enables a first glance at the drug-likeness of a molecule. It takes into account 6 physicochemical properties including lipophilicity (LIPO), size (SIZE), polarity (POLAR), solubility (INSOLU), flexibility (FLEX) and saturation (INSATU) which create the most favourable physiochemical environment for oral bioavailability.¹¹³ The pink region in the radar represents an optimal range for oral bioavailability.¹¹³ For a small molecule to be considered a drug like molecule, it must fall entirely within that pink region.

Bioavailability radars generated for **70**, **76** and **77** in SwissADME are shown in Figure 3.21. The lipophilicity of all three compounds (**70**, **76** and **77**) falls on the border of the pink region, while some properties fall under the pink region, some away from the border and other outside the border. Based on bioavailability radars, all three compounds (**70**, **76** and **77**) are predicted not be orally bioavailable due to being too unsaturated which is caused by the presence of a *trans* double bond and aromatic systems. Moving further down on SwissADME, a list of physicochemical properties for each molecule was calculated, which be discussed in Section 3.5.2.

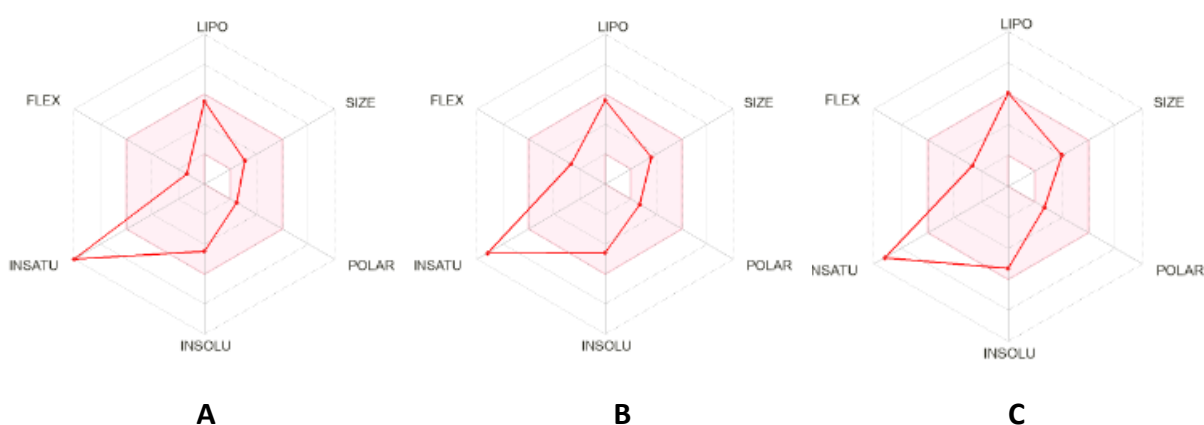


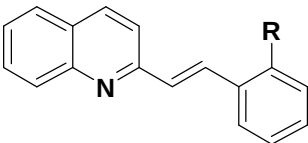
Figure 3.21: Bioavailability radars of (A) **70**; (B) **76**; and (C) **77**.

The pink area representing the range for each property: Size (MW 500-150 g/mol), Polarity (TPSA 120-30 Å²), solubility (Log S not higher than 6), Saturation (fraction of carbon not less than 0.25) and flexibility (no more than 9 rotatable bonds).

3.5.2 *In silico* physicochemical properties

In previous chapters, we discussed the physicochemical properties of compounds in detail using Lipinski's Ro5. SwissADME predicted physicochemical properties based on Lipinski's Ro5 for **70**, **76** and **77** are summarised in Table 3.7. Upon scrutiny of Table 3.7, all three compounds (**70**, **76**, and **77**) meet the criteria of Lipinski's Ro5 and are predicted to have a good oral bioavailability. Furthermore, molar refractivity and TPSA (discussed in Chapter 2) was also predicted. It can be seen that all three molecules have molar refractivity in the range of 130-40 m³/mol (**Table 3.6**). Moreover, all three Q compounds have TPSA in the range of 140-20 Å². As discussed in Chapter 2, if the number of rotatable bonds of any drug candidate exceeds 9 then it is predicted not to be orally bioavailable. In our case, all three molecules contain < 9 rotatable bonds (**Table 3.6**). Furthermore, the ideal unsaturation fraction ranges from 0.25 to 1, indicating that carbon atom fraction in *sp*³ hybridisation cannot be less than 0.25.¹⁴³ The unsaturation of all three molecules was indicated in the bioavailability radar (**Section 3.5.1**). Both **76** and **77** have a fraction Csp³ value of 0.05 compared to **70** with 0.00. Overall, all three Q molecules predicted to have good bioavailability based on Lipinski's Ro5, number of rotatable bonds, molar refractivity and TPSA, but highly limited by Csp³ fraction due to the presence of a double bond linking a quinoline ring and a substituted aromatic ring. Other than Lipinski's Ro5 SwissADME predicts the drug-likeness of a molecule using different rule-based filters: Veber, Ghose, Egan and Muegge along with a bioavailability score. For a molecule to be considered as a therapeutic candidate, it needs to have a bioavailability score of 0.10 or above. For **70** and its analogues (**76** and **77**), Swiss ADME predicted a bioavailability score of 0.55. This suggests that the parameter of drug-likeness is high, as all 3 molecules fit Lipinski's Ro5, Veber, Ghose, Egan and Muegge without any violations.

Table 3.7: *In silico* physicochemical properties of 70 and its analogues (76 and 77) using SwissADME.

 70 R= OH 76 R= OC(O)CH₃ 77 R= OC(O)CH₃·HCl								
Compound number	Chemical Formula	MW (g/mol)	HBA	HBD	LogP	MR (m ³ /mol)	TPSA (Å ²)	Fraction Csp3
70	C ₁₇ H ₁₃ NO	247.29	2	2	3.60	79.1	39.1	0.00
76	C ₁₉ H ₁₅ NO ₂	289.33	3	0	3.88	88.6	33.1	0.05
77	C ₁₉ H ₁₆ ClNO ₂	325.79	3	0	3.61	95.5	39.1	0.05

3.5.3 Water solubility and lipophilicity

As discussed in Chapter 2, SwissADME predicts the lipophilicity and water solubility by calculating LogP and Log S values. The LogP value indicates whether the compound is generally lipophilic. The LogP values given for **70**, **76**, and **77** are summarised in Table 3.7 are the consensus LogP.

The Log S (ESOL) values of **70**, **76**, and **77** are -4.47, -4.59, and -5.29, respectively. The water solubility of the compounds ranges from poorly soluble to highly soluble. Poor water-soluble values lie from -10 to -6, moderately soluble from -6 to -4, soluble from -4 to -2 and highly soluble are -2 to > 0.¹²² The solubility values reveal that none of the compounds are predicted to be soluble or highly soluble. Based on SwissADME predicted water solubility, all three molecules (**70**, **76**, and **77**) are moderately soluble. The *in silico* predicted solubility values were compared with experimentally determined values (Table 3.8). Both **70** and **76** are poorly water soluble according to experimental values, matching the *in silico* predicted Log S values (Table 3.8). However, for **77** there is a discrepancy between the predicted moderate solubility and experimental result showing it is water

soluble (2.17 mg/mL). This highlights the significance of validating *in silico* predictions with experimental data.

Other than water solubility, pharmacokinetic profiles of **70**, **76**, and **77** were also predicted (Section 3.5.4).

Table 3.8: *In silico* predicted water solubility values vs experimentally determined water solubility values of **70 and its analogues: **76** and **77**.**

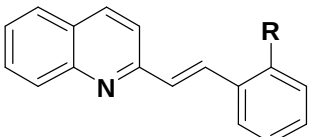
Compound number	In silico predicted water solubility (Log S)	Experimentally determined water solubility (mg/mL)
70	-4.47	0.00036
76	-4.59	0.0034
77	-5.29	2.17

3.5.4 Pharmacokinetic profile

As discussed in Chapter 2, the pharmacokinetic profile of a drug defines its ADME properties and SwissADME predicts various ADME parameters: gastrointestinal absorption, blood-brain barrier (BBB) access, interaction with CYP isoenzymes, and P-glycoprotein (P-gp). The results are indicated as 'yes' or 'no' depending on whether the molecule is predicted to be a substrate to P-gp or an inhibitor/non-inhibitor for CYP isoenzymes, respectively. P-gp and CYP isoenzymes (CYP3A4, CYP2D6, CYP2C9, CYP2C19 and CYP1A2) are discussed in Chapter 2.

Table 3.9 reveals the pharmacokinetic profile of **70**, **76** and **77**. All three molecules are predicted to have a high degree of gastrointestinal absorption and easily pass through the blood-brain barrier. Moreover, **70** and **76** are not predicted to be a P-gp substrates, which explains their good intestinal absorption and high bioavailability. On the other hand, **77** is predicted to be a P-gp substrate. Furthermore, **70** and **76** are predicted to be inhibitors of isoenzymes (Table 3.9) which may result in drug-drug interactions and could lead to toxicity. In contrast, **77** is not an inhibitor of any CYP isoenzymes. However, **76** is a prodrug of **70**, with **77** being a salt of **76**. Therefore, while the *in-silico* predictions suggest potential CYP (CYP1A2, CYP2C19, CYP2C9) inhibition by **76** and predict **77** to be a P-gp substrate, these effects are not expected occur in reality. This is because **76** and **77** should readily convert to **70** *in vivo* as shown in PLE esterase assay (Section 3.4.2.2). Once converted to **70**, the active drug exerts its own pharmacological effects.

Table 3.9: Pharmacokinetic profile of 70 and its analogues: 76 and 77 predicted by SwissADME web tool.

 70 R= OH 76 R= OC(O)CH₃ 77 R= OC(O)CH₃.HCl								
Compound number	GI absorption	Blood brain barrier	P-gp subst rate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
70	High	Yes	No	Yes	Yes	No	Yes	Yes
76	High	Yes	No	Yes	Yes	Yes	No	No
77	High	Yes	Yes	No	No	No	No	No

3.6 Computational analysis of 70 with CysLT receptor 1

3.6.1 3D and 2D interaction of 70 with CysLT receptor 1 proteins (6RZ4 and 6RZ5)

Compound **70** acts as an antagonist of CysLT₁ as it binds potently to CysLT₁. The 3D and 2D ligand-protein interaction maps of CysLT₁ antagonists pranlukast and zafirlukast are discussed in detail in Chapter 4. In this chapter, we performed docking analysis of **70** with the CysLT₁ PDB crystal structure and generated 3D and 2D interaction maps using the Flare software from Cresset.

The interactions of **70** with CysLT₁ were compared with the interactions formed by pranlukast and zafirlukast with CysLT₁. Docking was performed on Flare by drawing the ligand (**70**) and starting the docking calculations. Once docking was completed, a docking score was calculated and the docking pose with the lowest negative score was selected, as a very negative score corresponds to strong binding.

Figure 3.22 shows the 3D and 2D interactions of **70** with CysLT₁ protein 6RZ4. It can be seen that Ser A155 forms a strong hydrogen bond with the hydroxy group of **70** at a distance of 2.2 Å. On the other hand, residues including Phe 158, Val 186, Leu 257, His 190 and Leu 189 form hydrophobic interactions with the aromatic rings and *trans* double bond of **70**. However, no water molecule is observed to form an interaction with **70**. In contrast, pranlukast forms various interactions with

CysLT₁ protein 6RZ4 as discussed in Chapter 4. Hydrophobic interaction with Phe 158 and Leu 189 is common interactions between **70** and pranlukast formed with CysLT₁ protein 6RZ4.

The 3D and 2D interactions of **70** was also studied with CysLT₁ protein 6RZ5. As shown in Figure 3.23, Ser 155 forms a strong hydrogen bond with the hydroxy group of **70** at a distance of 2.5 Å. Other residues including Phe 158, Arg 253, Leu 257, His 190 and Tyr 108 form hydrophobic interactions with the aromatic rings and *trans* double bond of **70**. In contrast, as discussed in chapter 4, zafirlukast forms many interactions including hydrogen bond and hydrophobic interaction with protein 6RZ5. There is only one common interaction between **70** and zafirlukast, which is a hydrophobic interaction with Tyr 108. Compound **76** is expected to be rapidly hydrolysed to **70**, hence, docking was not performed for **76** or **77**.

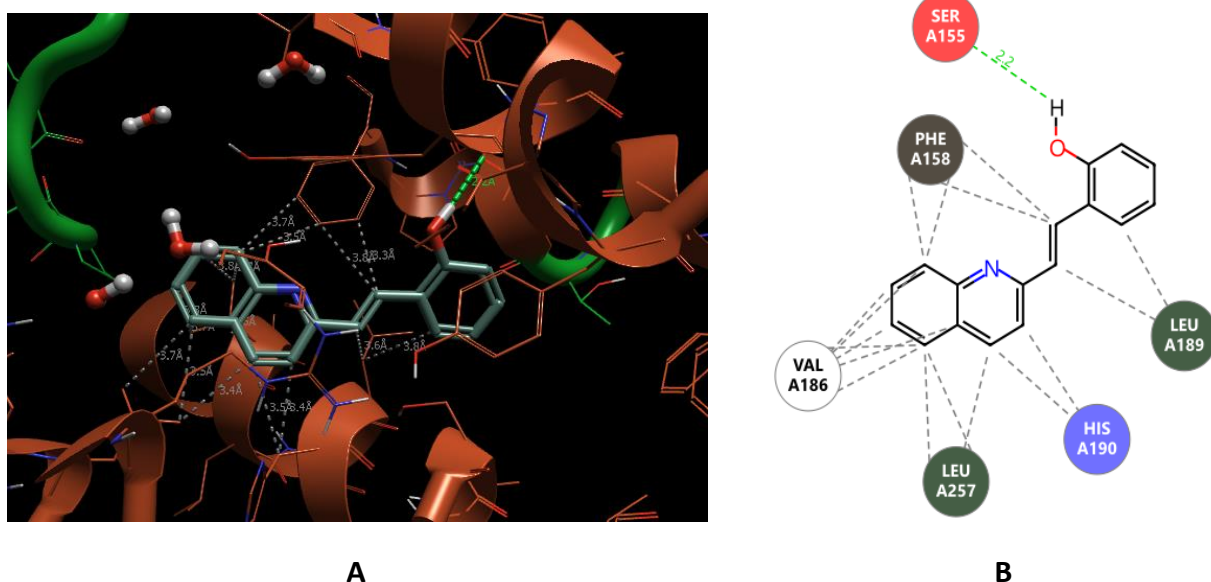
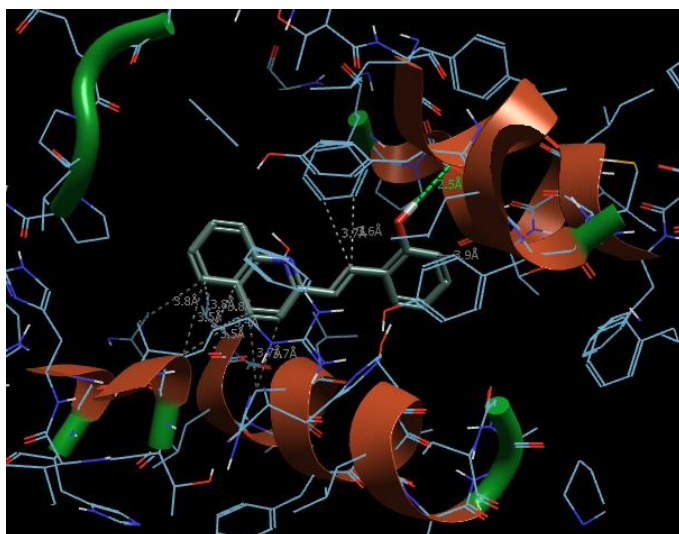
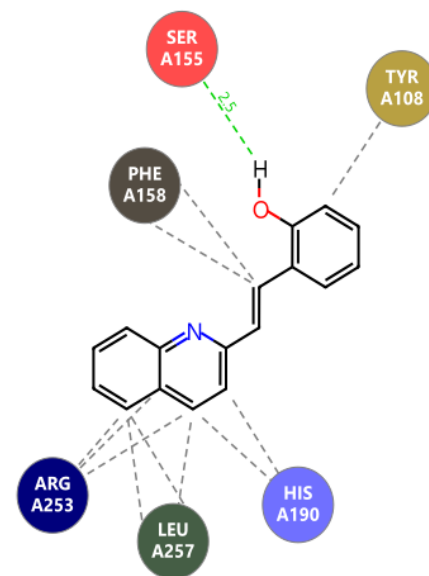


Figure 3.22: A) 3D ligand-protein interaction diagram of 70 with CysLT₁ (6RZ4); (B) 2D ligand-protein interaction diagram for 70 with CysLT₁ (6RZ4). Water molecules presented as capped stick in (A). Molecular interactions defined as: hydrogen bonding: maximum distance = 2.6 Å and hydrophobic interactions are at a distance of 3.3 to 4 Å. The dotted lines which represent strong hydrogen bonds are coloured in light green and hydrophobic contacts in grey.



A



B

Figure 3.23: (A) 3D ligand-protein interaction diagram of 70 with CysLT₁ (6RZ5); (B) 2D ligand-protein interaction diagram for 70 with CysLT₁ (6RZ5). Molecular interactions defined as: hydrogen bonding: maximum distance = 2.6 Å and hydrophobic interactions are at a distance of 3.3 to 4 Å. The dotted lines which represent strong hydrogen bonds are coloured in light green and hydrophobic contacts in grey.

3.7 Biological evaluation of **70** and its analogues: **76** and **77**

Previously, **70** was tested on human metastatic uveal melanoma (MUM) cell lines such as OMM2.5 cells, where it exhibited anti-cancer effects (**Section 3.1**). In this section anticancer effects of **76** and **77** were investigated using MUM cell lines. The *in vitro* cell viability and antiproliferative activity of **76** and **77** were investigated and compared with **70**. These experiments were performed by Dr. Valentina Tonelotto and Prof. Brendan Kennedy of the School of Biomolecular and Biomedical Science, UCD. In addition, **76** was assessed in the NCI-60 cell line screen, a panel of 60 human cancer cell lines used by NCI to evaluate anti-cancer activity. This screening was performed by M Kunkel (NCI).

3.7.1 Investigating the cytotoxicity and antiproliferative activity of **76** and **77**

Cell viability and antiproliferative activity of **70**, **76** and **77** were investigated using the MUM cell line; OMM 2.5. Cell viability was assessed using the MTT assay. MTT assay is a colorimetric assay widely used to measure metabolic activity: viability, proliferation and cytotoxicity.¹⁴⁴ MTT is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow, water-soluble tetrazolium dye. It is reduced to a formazan, an insoluble purple coloured product, by mitochondrial dehydrogenase enzyme in the metabolically active cells (**Figure 3.24**). Dose dependent graphs can be generated from the data obtained in this assay.¹⁴⁴ MTT is a simple and easily performed assay, provides quantitative results that can be easily analysed and can be applied to a wide range of cells.¹⁴⁴ However, some drugs potentially might interfere with MTT reduction which could affect the accuracy of results. For example, drugs that can alter mitochondrial activity, react with formazan and affect cell membrane integrity.

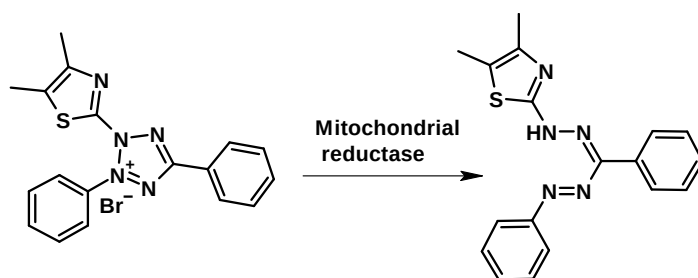


Figure 3.24: The reduction of MTT to formazan

In addition to the cell viability assay, the antiproliferative activity of **70** and **77** was evaluated using the clonogenic assay (carried out by Dr. Simone Marcone in School of Medicine, Trinity Translational Medical Institute, Trinity College Dublin). As discussed in Chapter 2, this *in vitro* cell survival assay measures the drug's ability to inhibit cell growth and colony formation.

3.7.1.1 Experimental details

3.7.1.1.1 Cell culture

OMM2.5 cells (kindly provided by Dr. Martine Jager, Leiden, The Netherlands) were cultured with complete media containing RPMI 1640 with GlutaMAX™ Supplement (Gibco; Waltham, MA, United States) + 10% fetal bovine serum (FBS; Gibco) + 2% penicillin-streptomycin (PEST), in T175 flasks. Culture flasks and plates were incubated at 37°C with 5% CO₂.

3.7.1.1.2 The MTT assay

The effect of **76** and **77** on OMM2.5 cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay as described previously.¹⁴⁵ Briefly, 5000 cells/well were seeded into 96-well plates and allowed to adhere overnight. Cells were treated in triplicates with either 0.5% DMSO (vehicle control) or 15% H₂O₂ or 2.5, 5, 10, 20 µM of **70**, **76**, and **77** prepared in complete media for 96 hours. Drug solution was removed, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye and serum-free media were added in a 1: 10 ratio, to each well and incubated in the dark for 2.5 hours at 37 °C. Following, 100% DMSO (1:1 ratio) was added to each well and absorbance measured at 570 nm using a SpectraMax® M2 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). This assay was used to measure the effect of three compounds on OMM2.5 cell viability by quantifying metabolic activity after 96 hours of treatment.

3.7.1.1.3 Clonogenic assay

OMM2.5 cells were seeded into 6-well plates at 2 x 10³ cells/ml (final volume 2 ml) and allowed to adhere overnight. The following day, cells were treated with either 0.5% DMSO (vehicle control) or 10, 20 µM **70** or **77** prepared in complete media. All drugs were dissolved in DMSO to prepare stock solutions. Cells were treated with 2 ml of desired drug solution per well in duplicate and incubated at 37°C with 5% CO₂ for 96 hours. Drug solutions were removed and fresh complete media was added to the plates and cells allowed to grow for an additional 8 days at 37°C with 5% CO₂. Clones were washed twice and fixed with 4% paraformaldehyde/formaldehyde for 10 mins at room temperature (RT). Colonies were stained with 0.5% crystal violet solution (Pro-Lab diagnostics PL700; Richmond Hill, ON, Canada) for 10 mins-2 hours at RT, shaker, washed and dried (once desired staining is achieved). Plates were imaged using the Gel Count™ system (Oxford Optronix; Oxford, UK) and analysed using the colony count (J Plugin in ImageJ v1.53e). The

clonogenic evaluated the ability of cells to survive treatment and retain their capacity to proliferate into colonies, indicating long-term survival and treatment impact.

3.7.1.1.4 Statistical analysis

Statistical analysis was performed using One-way ANOVA with Dunnett's Test for Multiple Comparisons in GraphPad Prism v7.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). IC₅₀ values were calculated using GraphPad Prism. Error bars represent mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (N = 3-5).

3.7.1.2 Cell viability assay of 70, 76 and 77 using MTT

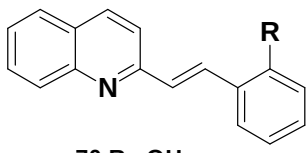
In this study, the cell viability of **70**, **76** and **77** were evaluated using MTT assay on OMM 2.5 cells over a 96-hour treatment. The results demonstrated a dose-dependent significant decrease in cell viability of all three compounds, with **70**, **76** and **77** solubilised in DMSO or **77** solubilised in water in comparison to 0.5% DMSO (**Figure 3.25 A-B**). The high concentrations resulted in significant reductions in viability, specifically, **70** reduced the viability to approximately 50% at 20 μ M, while **76** and **77** exhibited similar trends, reducing viability to around 50% and 40%, respectively, at the same concentration. However, **77** solubilised in water reduced viability up to 60%. The reduction in cell viability signifies that compounds are effective in inducing death of melanoma cells.

The dose-response curves further quantified these effects, which provides IC₅₀ values; a key indicator for a compound's potency. The IC₅₀ value represents the concentration of a drug required to inhibit 50% of the cell population.¹⁴⁶ The lower the IC₅₀ value, the higher the drug's potency.¹⁴⁶ The IC₅₀ potency classification is as follows: compounds with an IC₅₀ value of less than 1 μ M are considered highly potent, those with values between 1 and 10 μ M have moderate potency, and those with IC₅₀ values greater than 10 μ M are considered to have low potency.

The IC₅₀ values for **70** was determined to be 8.52 μ M, whereas, **76** and **77** (DMSO) showed IC₅₀ values of 8.85 μ M and 9.26 μ M, respectively (**Figure 3.25 C-E**). Interestingly, **77** solubilised in water showed an IC₅₀ value of 7.50 μ M (**Figure 3.25 F**). Moreover, the IC₅₀ values are used for ranking and categorised into classes as shown in Table 3.10.¹⁴⁷ Based on the findings of this study, all three compounds fall into moderate potency range (**Table 3.10**). These values suggest that these compounds are reasonably effective in inhibiting cell viability at micromolar concentration.

In summary, the results suggest that all three compounds (**70**, **76** and **77**) significantly reduced cell viability suggesting promising cytotoxic effects and potential drug candidates for further development as therapeutic agents for targeting metastatic uveal melanoma.

Table 3.10: IC₅₀ range and IC₅₀ values for 70 and its analogues: 76 and 77 on OMM 2.5 cell lines after 96 hours treatment.

 70 R= OH 76 R= OC(O)CH₃ 77 R= OC(O)CH₃.HCl		
Compound number	IC ₅₀ values (μM)	Compound's potency ¹⁴⁷
70	8.52 in DMSO	Moderate
76	8.85 in DMSO	Moderate
77	9.22 in DMSO	Moderate
	7.50 in water	Moderate

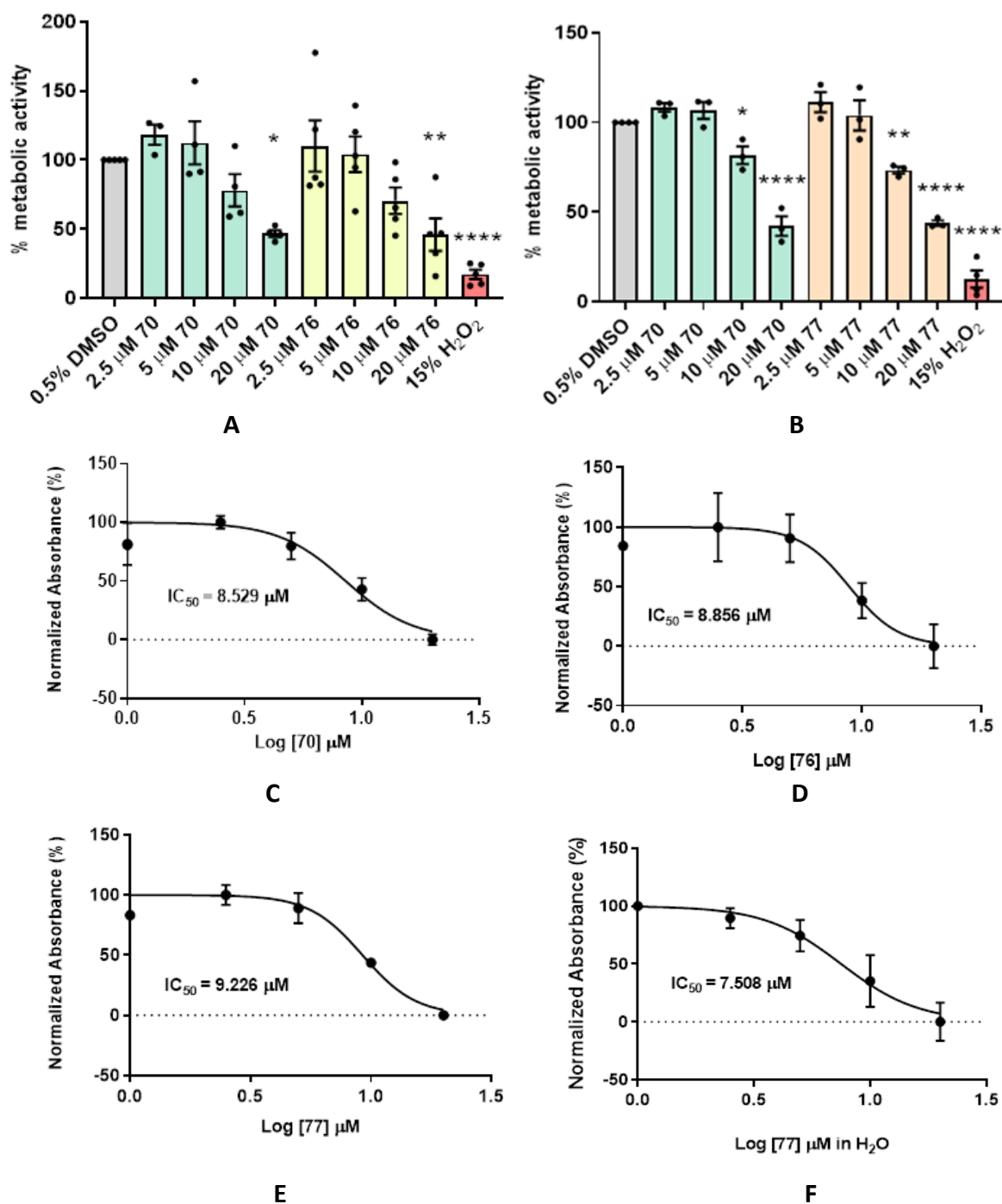


Figure 3.25: 70 and its analogues: 76 and 77 reduces the viability of OMM2.5 cells. (A-B) Dose dependent graphs of 70, 76 and 77 in DMSO and water at 2.5, 5, 10, 15 and 20 μ M concentrations; (C-F) IC_{50} values of 70, 76 and 77 in (E) DMSO and (F) water, respectively. The values represent the mean $\pm$ SEM (N = 3-5). Error bars represent mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, ** $p < 0.0001$.**

3.7.1.3 Antiproliferative activity of 70 and 77 on the OMM 2.5 cell line using clonogenic assay

The antiproliferative activity of **77** was evaluated and compared to **70** using the clonogenic assay. Compound **77** was dissolved in DMSO as its aqueous solubility was unknown at the time of the experiment. OMM 2.5 cells were treated with 10 and 20 μM concentration of each compound for 96 hours, with 0.5% v/v DMSO serving as a vehicle control. The concentrations for the clonogenic assays were selected based on the parallel design of MTT and clonogenic experiments. The minimum concentrations reducing cell viability in the MTT assay were used for the clonogenic assays without calculating the IC_{50} . The results demonstrate that both **70** and **77** reduced the cell survival of treated cells. Treatment with **77** (10 μM) yielded a similar reduction with greater viability as shown in Figure 3.26. At 20 μM , **70** further decreased the survival fraction to 10-15%, whereas, **77** almost completely eradicated the survival colonies, indicating as a stronger antiproliferative effect (**Figure 3.26**). The statistical analysis confirmed that these reductions were highly significant compared to the vehicle control.

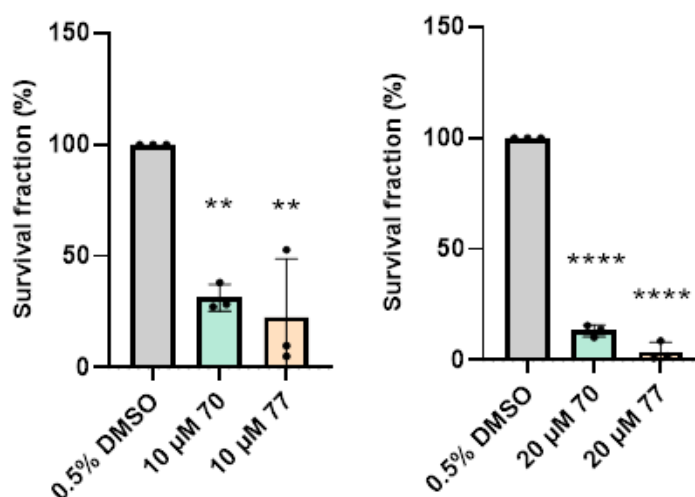


Figure 3.26: Antiproliferative activity of 70 and 77 in OMM 2.5 cell lines at 10 and 20 μM concentrations by clonogenic assay. Error bars represent mean $\pm$ SEM, * p < 0.05, ** p < 0.01, **** p < 0.0001. (N = 3-5).

3.7.2 Investigating the anticancer activity of **76** in the NCI-60 Human Tumour Cell line screen

NCI and NCI-60 cell lines have been discussed in chapter 2. In this work, **76** was submitted to the NCI and progressed to 5 dose testing stage. Compounds that are submitted to NCI, undergo a 1 dose screen at 1×10^{-5} M concentration in the full NCI-60 panel.¹⁴⁸ Compounds that significantly demonstrate growth inhibition in the 1 dose screen can progress further to 5 dose assays. The 5-dose assay calculates three parameters for each compound:

- 1- GI_{50} : the concentration resulting in a 50% growth inhibition of cells as compared to vehicle control treated cells.
- 2- TGI: the total growth concentration
- 3- LC_{50} (cytotoxicity): the concentration required to induce 50% lethality of cells i.e.

Compound **76** was selected for evaluation in the NCI-60 5-dose cell line screen. Since both **76** and **77** (HCl salt of **76**) convert to the parent compound **70** *in vivo*, the choice of **76** over **77** does not affect the outcome of the study, as their activity ultimately depends on the release of **70**. The evaluation assessed GI_{50} , TGI and LC_{50} in the NCI panel of 60 cell lines, using the sulphorhodamine B (SRB) protein assay available from the NCI DTP database.¹⁴⁸ Compound **76** showed good potency in all the cells lines tested. Its best potency was in CCRF-CEM leukaemia cells ($GI_{50} = 0.22 \mu M$) and UO-31 renal cancer cells ($GI_{50} = 0.62 \mu M$). The dose-response graphs generated for leukaemia and renal cancer cell lines are shown in Figure 3.27. Dose-response graphs for the remaining cancer cell lines are available in the appendices section. The NCI-60 panel includes a fixed set of 60 cancer cell lines representing various cancer types, such as melanoma, breast cancer, and leukaemia. However, it does not include uveal melanoma cell lines. Since, there is particular interest in UM, further studies were conducted separately UM cell lines to explore the anticancer effects of compound **76** (Section 3.7.1).

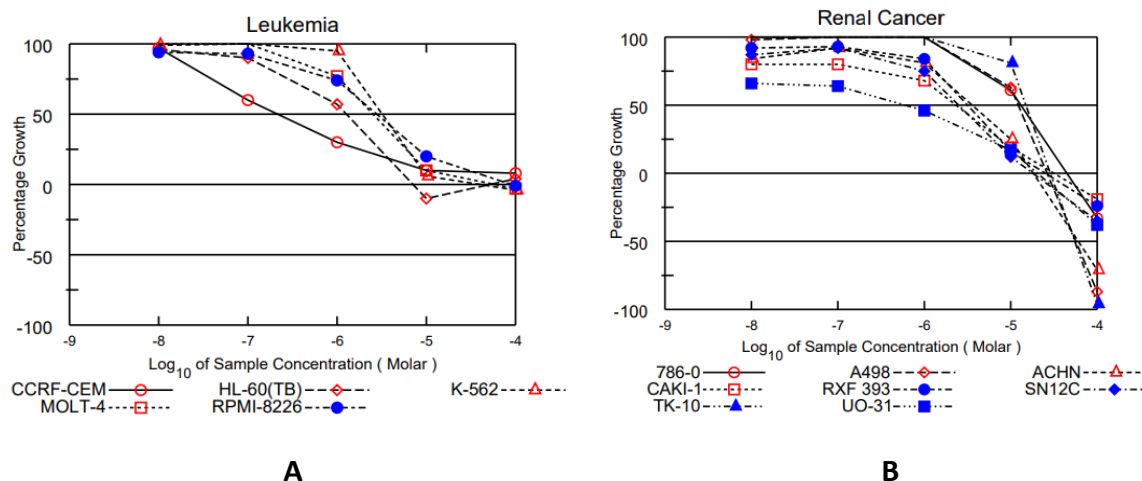


Figure 3.27: Dose response curves generated by NCI-60 screening of **76 for (A) 5 leukaemia cancer cell lines; (B) 8 renal cancer cell lines.**

The NCI has developed a COMPARE algorithm that can examine the dose-response patterns of anti-cancer agents. By utilizing these algorithms, researchers can possibly discover the probable mechanism of action of a test compound and whether its response pattern ascertain or it is distinct or similar to any of the standard prototype compounds in the NCI database.¹⁴⁹ Furthermore, once the various cellular molecular targets in the 60 cell lines have been characterised, researchers may have the ability to select compounds that are most likely to interact with a specific target.¹⁴⁹ The NCI-60 COMPARE analysis provided an extensive evaluation of the growth response of 60 different human cancer cell lines to our investigational quininib analogue, **76**. COMPARE analysis of **76** was carried out by Dr. Valentina Tonelotto. Using the COMPARE algorithm, the entire database of tested compounds is ranked by the similarity of the GI_{50} response patterns of the cell lines to **76**. This analysis includes data for the targeted investigational oncology agents (IOA) set, which comprises 180 clinically approved agents and 820 investigational agents. The heat map generated through this analysis visually represents the Pearson correlation patterns of the GI_{50} values. The hierarchical clustering of the NCI-60 growth response patterns using the COMPARE algorithm reveals significant insights into the activity profile of **76**. The top 25 compounds with similar response patterns to **76** are detailed in the table below (**Table 3.10**), highlighting their correlation coefficients and known mechanisms of action.

In summary, the evaluation of **76** across the NCI-60 cell line panel demonstrates its potent antiproliferative and cytotoxic effects, with particularly strong activity observed in CCRF-CEM leukaemia cells and UO-31 renal cancer cells. The dose-response data and GI50 values affirm **76's** efficacy. Furthermore, the COMPARE analysis further elucidates the compound's activity profile by comparing its response patterns with those of other agents, revealing significant correlations with known oncology drugs and investigational agents. This comprehensive evaluation highlights the potential of **76** as a promising candidate in cancer treatment, providing a foundation for further development and investigation into its therapeutic mechanisms.

Table 3.11: The top 24 compounds with similar response patterns to 76, correlation coefficients and known mechanisms of action.

Compound	Correlation	Mechanism of action/Target
SN-011	0.58	STING (stimulator of interferon genes)
AMPC	0.57	TFF3 (Trefoil factor 3)
Quercetin	0.53	Known as an antioxidant with various biological activities
Resatorvid	0.51	TLR4 (Toll-like receptor 4)
PNU-74654	0.49	Inhibits beta-catenin, involved in Wnt signaling pathway
CB-103	0.48	Notch signaling pathway
Cerulenin	0.48	FASN (fatty acid synthase)
Vismodegib	0.48	SMO (smoothened, hedgehog pathway)
LY-3410738	0.47	IDH2 (isocitrate dehydrogenase 2)
PI-103	0.47	PI3K (phosphoinositide 3-kinase)
PT-2385	0.46	HIF-2 alpha (hypoxia-inducible factor 2 alpha)
Aminolevulinic Acid	0.46	Used as a photosensitizer in photodynamic therapy
CC-90009	0.46	CRBN (cereblon)
Chlorambucil	0.46	Acts as a DNA alkylator or alkylating agent.
Arsenic Trioxide	0.46	Induces apoptosis in cancer cells
Chelerythrine	0.45	PKC (protein kinase C)
AZD-3458	0.45	PI3K
ST-3595	0.44	HDAC (histone deacetylase)
Tenalisib	0.44	PI3K

CA3	0.43	YAP-TEAD (Yes-associated protein-TEA domain family member)
Melphalan Hydrochloride	0.43	DNA alkylator or alkylating agent
C-75	0.43	FASN
Pyridoclax	0.43	Mcl-1 (myeloid cell leukaemia 1)
BAY-1816032	0.43	BUB1 (Budding uninhibited by benzimidazoles 1)

3.8 Chapter summary

This chapter focuses on enhancing the physicochemical properties of quinoline containing **70** and its analogue **74** which are poorly water-soluble and chemically unstable drugs with demonstrated anticancer activity *in vivo* and *in vitro* in colon cancer and uveal melanoma cell lines. A hydrochloride salt of **74**, known as **72** was previously formulated, showing good aqueous solubility and potent anticancer effects *in vivo* and *in vitro* in colon cancer cell lines. Compound **72** was tested for chemical stability at three pH values (4, 7.4 and 9) and exhibited poor chemical stability at pH 7.4 and 9. To address these challenges, the initial plan was to synthesise amino acid prodrugs of **74**.

Compound **70** and **74** were synthesised using the Perkin condensation method. The synthesis of amino acid prodrugs of **74** was attempted by HBTU coupling method. Unfortunately, the amino acid prodrug formation of **74** was unsuccessful due to the poor chemical stability of the resulting compounds. Given that **70** has only one hydroxyl group it was hypothesised that this might simplify the coupling process, but amino acid prodrugs of **70** also demonstrated limited chemical stability.

Subsequently, the synthesis of acetyl ester prodrug of **70**, referred to as **76** was explored. Although **76** exhibited poor water solubility, a hydrochloride salt **77** was formulated to improve this issue. Compound **77** demonstrated very good water solubility, compared to both **70** and **76**. Chemical stability assessment at pH 4, 7.4 and 9 revealed that **70** exhibited poor stability, **76** showed the best stability across all pH values and **77** had moderate stability particularly at pH 4 compared to 7.4 and 9. The anticancer effects of **70**, **76** and **77** were evaluated in MUM cell lines, and all three compounds exhibited potent cell viability effects indicating their potential as therapeutic agents despite their physicochemical challenges. Moreover, compound **76** was assessed by the NCI in the NCI-60 panel and successfully progressed to 5-dose testing stage. Since both **76** and **77** convert to the parent compound **70** *in vivo*, the choice of **76** does not impact the study outcome.

This study presents a comprehensive analysis of the synthesis and physicochemical evaluation of analogues of **70** and **74**. While amino acid prodrug formation was challenging, the acetyl ester prodrug and its hydrochloride salt (**76** and **77**) demonstrated significant improvements in solubility and stability. Future work should focus on optimising prodrug strategies and exploring alternative to amino acids e.g. glucose or phosphate esters. Extended *in vivo* studies are necessary to assess the pharmacokinetics and therapeutic efficacy of **76** and **77** in relevant cancer models. *In vivo* toxicity of **70** and its analogues **76** and **77** will be discussed in Chapter 5.

Chapter 4: Identification of novel cysteinyl leukotriene receptor 1 (CysLT₁) antagonists by ligand- based virtual screening

4.1: Introduction

4.1.1 G-protein coupled receptors: Cysteinyl leukotriene receptors

Cellular receptors are composed of proteins that coordinate the functions of different cells in the biological system.¹⁵⁰ Cellular receptors can be broadly categorised into internal receptors, ion channel receptors, enzyme-linked receptors and G protein coupled receptors (GPCRs).¹⁵⁰ GPCRs are the main focus of this study and are a diverse group of human membrane proteins that mediate cellular signals through ligand binding which activates G-proteins.¹⁵¹ However, abnormal GPCR functions are related to human diseases including cancer, asthma and cardiac disease which affect the patient survival rates. Therefore, more than 30% of drugs approved by the FDA target GPCRs and in 2018 the annual sales of these drugs were more than \$180 billion.¹⁵² Cysteinyl leukotriene receptors type 1 and 2 (CysLT₁ and CysLT₂) belong to the family of GPCRs. CysLT₁ and CysLT₂ receptors are potent lipid mediators, activated by endogenous leukotrienes including leukotriene C₄ (LTC₄), leukotriene D₄ (LTD₄) and leukotriene E₄ (LTE₄) which are derived from arachidonic acid.¹⁵³ CysLT₁ receptor is most highly expressed in lung smooth muscle cells, intestinal macrophages and the spleen.¹⁵⁴ In contrast, CysLT₂ receptor is most highly expressed in the heart, adrenal medulla, placenta and peripheral blood leukocytes.¹⁵⁵

4.1.1.1 Cysteinyl leukotriene receptor 1 antagonists

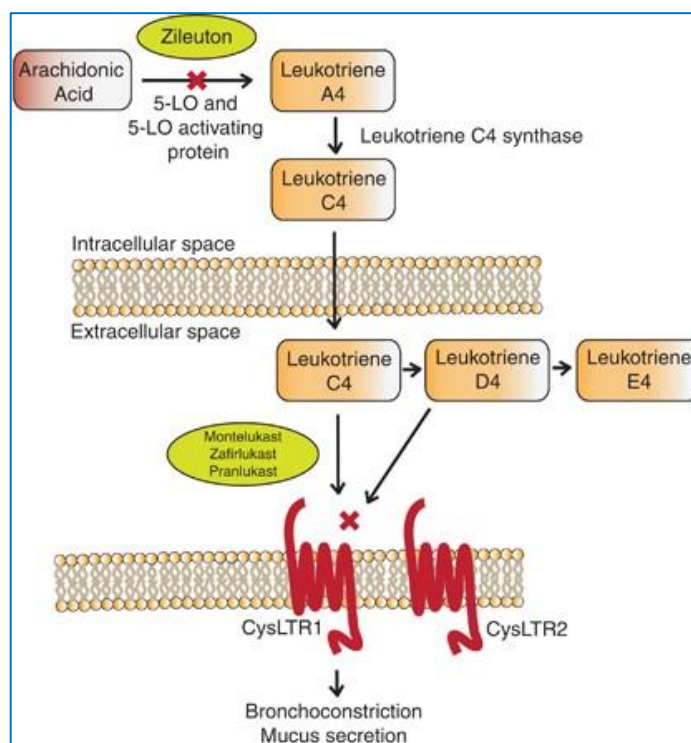
Selective antagonists of CysLT₁ such as montelukast, pranlukast and zafirlukast are clinically approved drugs to treat asthma by blocking the actions of endogenous leukotrienes (LTC₄, LTD₄ and LTE₄) on the CysLT₁ receptor (**Figure 4.1 A**). These drugs competitively bind to CysLT₁ receptor, preventing cysteinyl leukotrienes from activating it (**Figure 4.1 A**). This blockade inhibits bronchoconstriction and decreases mucus secretion. Furthermore, these antagonists prevent recruitment and activation of inflammatory cells, thus reducing inflammation in the airways. These drugs are effective for targeting CysLT₁. It is important to note that there is no known clinically approved antagonist for CysLT₂ at present.¹⁵⁴ Chemical structures of pranlukast and zafirlukast are shown in Section 4.3.1.

To gain knowledge of the structure-based mechanism of CysLT₁ receptors, Luginina *et al.* determined the crystal structure of the CysLT₁ receptor in complexes with zafirlukast and pranlukast at 2.5 Å and 2.7 Å resolution respectively.¹⁵⁶ According to Luginina *et al.* the structure-based mechanism of the CysLT₁ receptor revealed a unique ligand-binding mode and signalling mechanism. Moreover, Luginina *et al.* revealed that zafirlukast and pranlukast are very chemically distinct ligands and show variations in contact within the binding pocket despite having overall similar binding positions.¹⁵⁶ Additionally, MK-571 is acknowledged as a potent and selective pre-

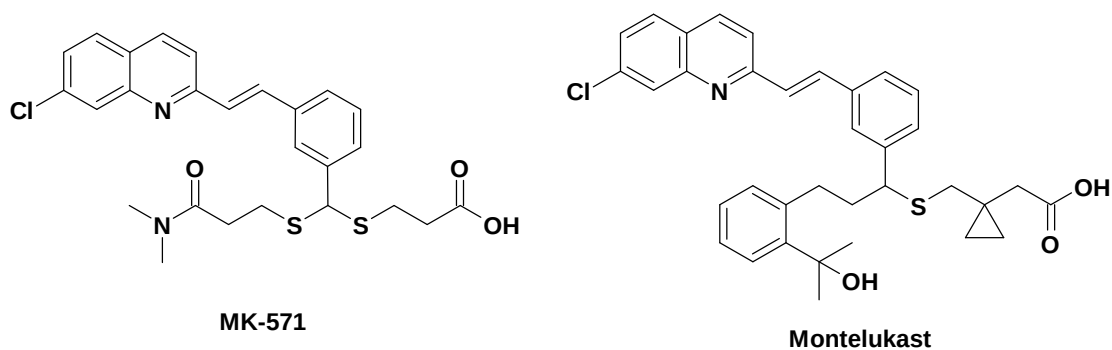
clinical antagonist of the CysLT₁ receptor, functioning in the same manner as zafirlukast, montelukast and pranlukast.¹⁵⁷ Furthermore, the quinoline-containing MK-571 is commonly used as an inhibitor of multidrug resistance protein-1 (MRP-1).¹⁵⁷ The chemical structures of MK-571 and montelukast are shown in Figure 4.1 B.

CysLT promote cancer progression by enhancing cell proliferation, survival, and metastasis through inflammatory signalling pathways, contributing to a supportive tumour microenvironment. The CysLT pathway is involved in multiple signalling pathways and serves various functions across different tissues.¹⁵⁸ The CysLT contribute to cell survival and proliferation through the CysLT₁ receptor.¹⁵⁸ Numerous *in vitro* studies have indicated that CysLT₁ antagonists reduce cell viability and induce cell death in several types of cancer cells, including colorectal, urological, breast, lung, and neurological malignancies.¹⁵⁸ For example, montelukast have been shown to inhibit tumour growth in a xenograft model of human HCT-116 colon cancer.¹⁵⁹ Additionally, angiogenesis is a hallmark of cancer (discussed in detail in Chapter 5), and CysLT₁ signalling stimulates the production of the pro-angiogenic mediator vascular endothelial growth factor (VEGF) *in vitro*. In contrast, CysLT₁ antagonists have been shown to modulate vascular permeability and reduce VEGF levels in murine models of allergic asthma as well as in asthmatic patients.¹⁶⁰

Transitioning from these established therapeutic agents, we now turn our attention to the methodologies employed in the identification of new hit compounds. Techniques such as virtual screening and computational modelling play crucial roles in discovering novel CysLT₁ receptor antagonists. These strategies enable the identification and optimization of promising candidates, paving the way for the development of next-generation therapeutics.



A



B

Figure 4.1: (A): Illustration of mechanism of action of selective CysLT₁ receptor antagonists; (B) Chemical structure of MK-571 and montelukast.

Image A taken from Tse, S. M *et al.*¹⁶¹

4.1.2 Strategies for identification of hits

A hit is a chemical compound typically identified through computational screening and shows activity against a specific target. Hit identification is a crucial step in the drug discovery process, as it helps researchers to identify compounds that have the potential to bind to a specific drug target of interest and modulate its activity in a desired way. The primary goal of hit identification is to discover validated hit series with the highest likelihood of being developed into effective drugs.¹⁶²

Various well-established strategies are employed to identify hits, including high-throughput screening (HTS), fragment-based drug discovery, virtual screening, and nuclear magnetic resonance (NMR) screening.¹⁶³ Furthermore, depending on the characteristics of the project and available resources, a combination of different strategies can be applied.¹⁶³ However, the focus of this study is on virtual screening (VS), a powerful method that leverages computational techniques to predict how well a compound can bind to a target, thereby accelerating the identification of promising drug candidates.

4.1.3.1 Virtual screening (VS)

To discover an innovative hit is an expensive and prolonged process. It is estimated that a typical drug discovery cycle can take up to 10 to 17 years, from lead identification to clinical trials with a cost of 2 billion USD.¹⁶⁴ In the early 1990's combinational chemistry and HTS offered great promise for accelerating the drug discovery process by stimulating the synthesis and screening of large libraries of compounds in a short period of time. However, many identified hits in this process failed during lead optimisation.¹⁶⁵ Therefore, it was necessary to find an alternative approach that could save a significant amount of resources. Identifying hits through VS represented as a major step forward in the initial stages of drug discovery. The first VS was reported in 1997.¹⁶⁵ VS is an efficient computer-assisted drug design technique that evaluates large libraries of small molecules to find new drug candidates that are most likely to bind to a drug target.¹⁶⁴ Ready-made libraries are available that are designed for particular drug targets, such as GPCRs, ion channels and nuclear receptors.¹⁶⁴ Chemical databases containing compounds (**Table 4.1**) are designed using drug-like properties based on Lipinski's Ro5 or other criteria such as Veber's rules and Ghose's rules. These guidelines ensure that the compounds have properties that make them more likely to be effective, bioavailable drugs. VS is complementary to HTS, mainly in terms of its high accuracy in finding the most appropriate novel hit through the filtration of large libraries of compounds and its reduced cost in the modern drug discovery process.¹⁶⁶ VS utilises two main approaches to identify novel hits: ligand based virtual screening (LBVS) and structure based virtual screening (SBVS).¹⁶⁴

Table 4.1. Common chemical databases and compound collections available from vendors or institutes.¹⁶⁵

Chemical databases	Type	Number of compounds	Website/reference
ZINC	Public	> 750 million	https://zinc.docking.org/
PubChem	Public	118 million	https://pubchem.ncbi.nlm.nih.gov/
Chem Spider	Public	129 million	https://www.chemspider.com/
ChEMBL	Public	>1 million	https://www.ebi.ac.uk/chembl/
Asinex	Commercial	550,000	https://www.asinex.com/screening-libraries-(all-libraries)
Enamine	Commercial	> 6.75 billion	https://enamine.net/compound-collections/real-compounds/real-database
Drug Bank	Public	500,000	https://go.drugbank.com/
ChemDiv	Commercial	1.6 million	https://www.chemdiv.com/catalog/screening-compounds/

4.1.3.1.1 Ligand-based virtual screening (LBVS)

LBVS is a technique widely employed in drug discovery. It is used when the structure of the target protein is unknown, but the structure of an active reference compound or ligand is known and used as a template to search for new compounds that share similar properties and may have similar biological activity as the reference compound.¹⁶⁷ Hence, the aim of LBVS is to identify compounds with similar physicochemical properties, chemical functionality and shape parameters to the reference compound which increases the chance of finding biologically potent compounds. LBVS is performed by comparing molecular descriptors of known/reference compounds with the molecular descriptors of databases.¹⁶⁷ Furthermore, one of the most common classification techniques is the number of dimensions of the represented descriptors including 1D, 2D and 3D. 1D involves pharmacokinetic properties such as molecular weight, the number of hydrogen bond acceptors and donors and physicochemical properties such as LogP value and solubility (Log S). 2D involves molecular topology such as number of aromatic rings in the molecule, connectivity index and carbo index which calculate molecular similarity.¹⁶⁴ 3D provides information about the molecular properties of the molecule in terms of the spatial distribution of atoms and chemical groups.

4.1.3.1.2 Structure based virtual screening (SBVS)

SBVS, also known as receptor-based virtual screening (RBVS), aims to predict the best orientation and position of the ligand within the binding site of the target molecule so that both molecules form a stable complex.¹⁶⁸ This technique is commonly used in drug discovery to identify and optimise small molecules that bind to target proteins, enzymes, and other biomolecules. It gained widespread use in the 1990s due to advancements in techniques, increased computational power, and greater access to structural data of target molecules.¹⁶⁴ One of the main advantages of the SBVS technique includes cost-effectiveness and its capacity to significantly reduce the time required to screen large libraries of small molecules. However, SBVS has limitations, primarily due to the complexity of ligand binding interactions, which can pose challenges in accurately predicting the correct binding pose and classifying the compounds.¹⁶⁷ Despite these challenges, SBVS remains a valuable tool in the drug discovery process, providing insights that guide the development of potential therapeutics.

4.2 Specific aims of chapter 4

1. To prepare CysLT₁ receptor proteins (6RZ4 and 6RZ5) using Flare software.
2. To perform water analysis on protein 6RZ4 using Flare.
3. To perform LBVS for identification of potential hits for the further development of CysLT₁ selective antagonists by using Cresset's advanced LBVS platform Blaze.
4. To investigate the *in vitro* antagonist effect of the selected hits on human CysLT₁ receptor.

4.3 Materials and methods

All the computational and VS studies were performed utilising Cresset's computer aided drug design (CADD) software, Flare 4.0 version and Blaze platform. Flare is a software for structure-based drug design and Blaze is an advanced LBVS software. The workflow is shown in Figure 4.2.

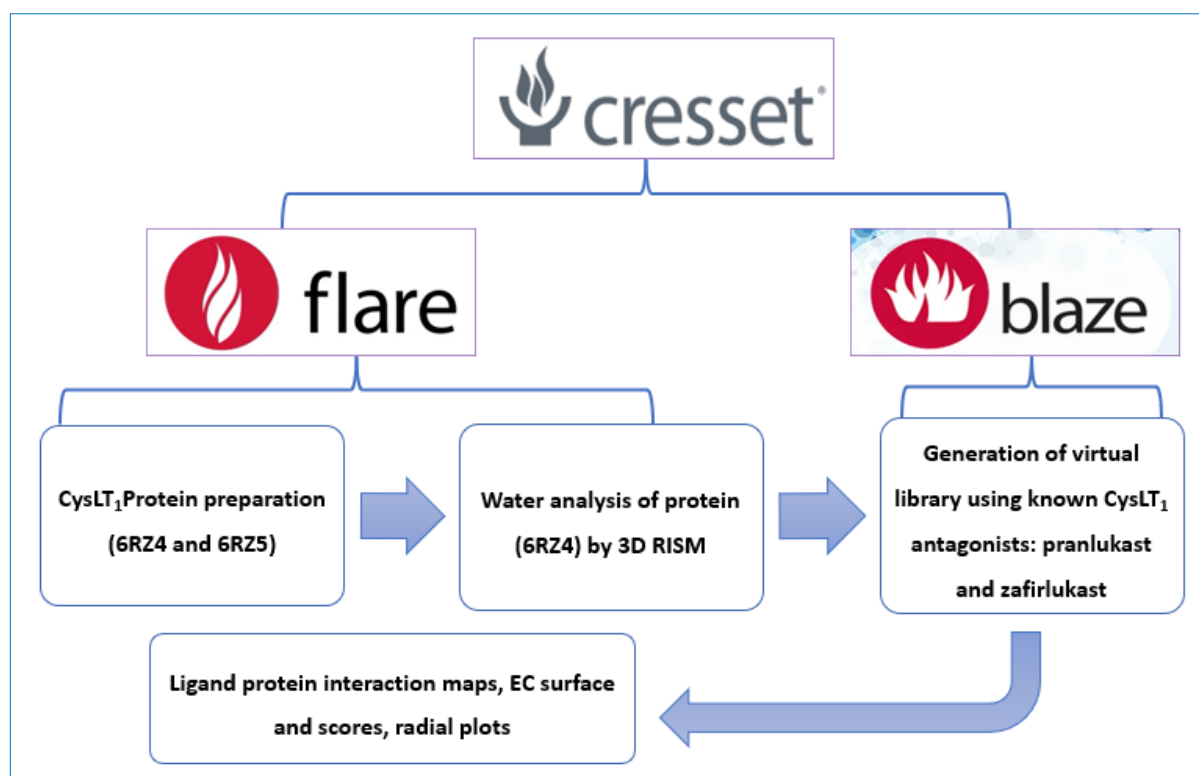


Figure 4.2 Workflow of Cresset's software used in this study; EC= electrostatic complementarity, RISM= reference interference site model.

4.3.1 Protein preparation

The aim of this work was to identify novel antagonists of CysLT₁ by LBVS. Prior to carrying out LBVS, it was essential to prepare the proteins of interest. Initially, 3D crystal structures of CysLT₁ receptor proteins were downloaded from the RCSB protein data bank (PDB) by using the PDB codes 6RZ4 and 6RZ5 in complex with ligand molecules, pranlukast and zafirlukast, at resolutions of 2.5 and 2.7 Å, respectively (**Figures 4.3** and **4.4**).¹³⁶ Proteins were prepared to ensure that they were in correct structural state for analysis. This involves checking for alternate positions of amino acid side chains, and selecting the best conformation based on how well the side chain fits (its occupancy) or how it interacts with its surroundings. Using Flare's automated protein preparation tool, missing side chains and small gaps in the structure (up to two amino acids) were filled in, and the ends of the protein were capped to make them chemically complete. It also removes water outside the active site.

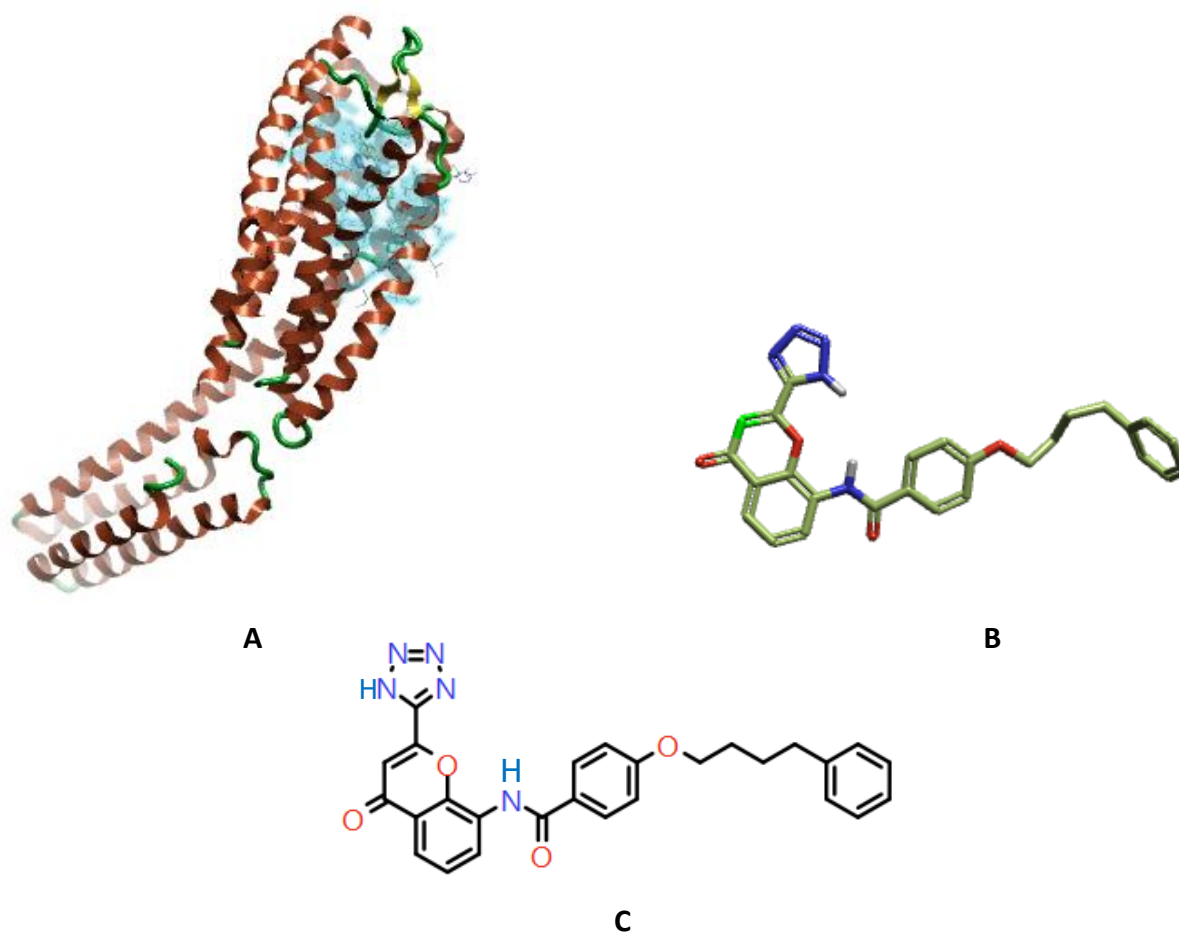


Figure 4.3: (A) Schematic cartoon model of crystal structure of protein 6RZ4, resolution of 2.5 Å.¹³⁶ Active site highlighted in blue; (B) 3D structure of pranlukast is shown, carbon atoms are coloured in green and yellow, nitrogen atoms are coloured in blue, oxygen atoms are coloured in red and hydrogen atoms are coloured in grey where shown; (C) 2D structure of pranlukast.

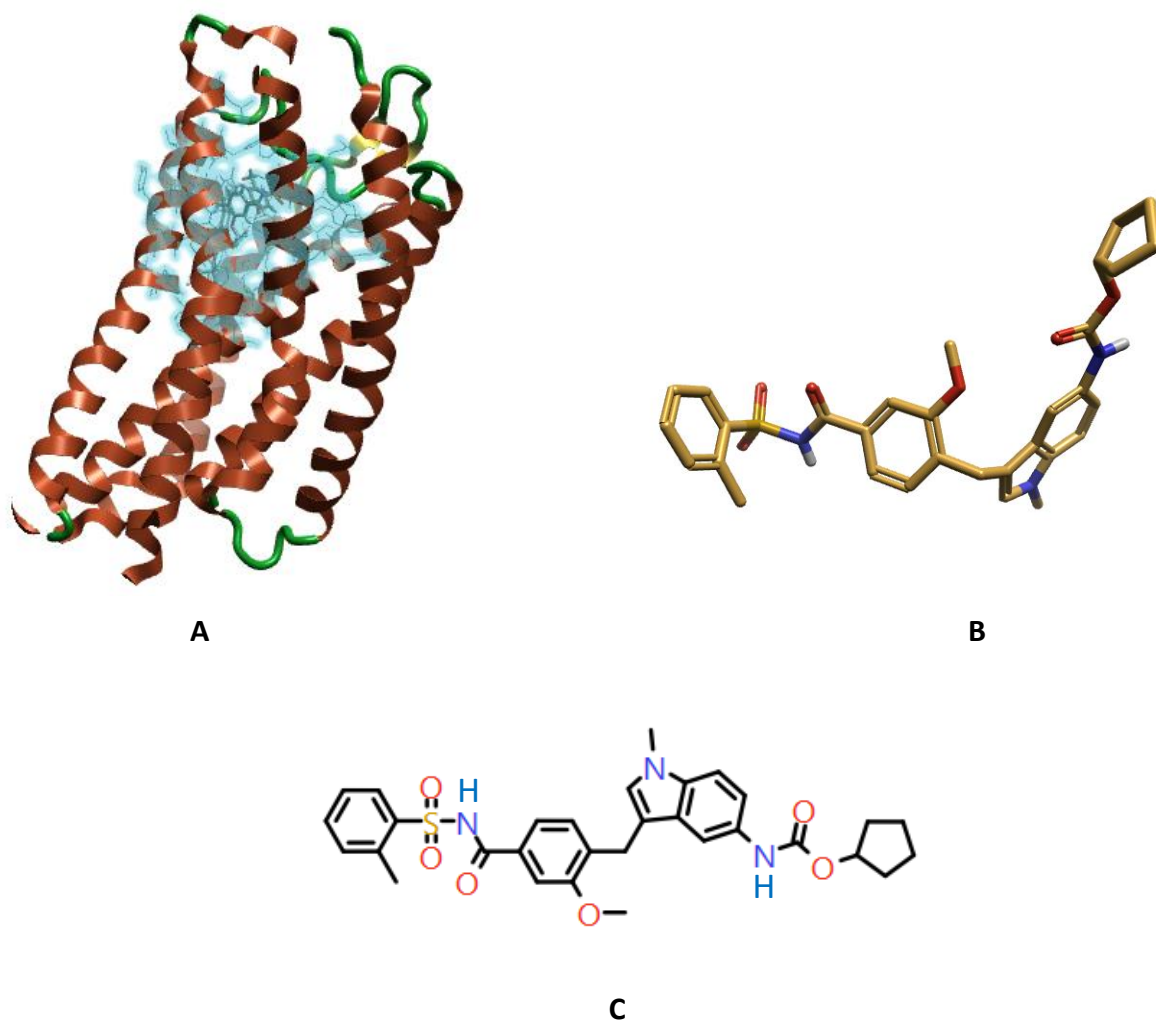


Figure 4.4: (A) Schematic cartoon model of crystal structure of protein 6RZ5, resolution of 2.7 Å.136 Active site highlighted in blue; (B) 3D structure of zafirlukast is shown, carbon atoms are coloured in green, nitrogen atoms are coloured in blue, oxygen atoms are coloured in red, sulphur atoms are coloured in yellow and hydrogen atoms are coloured in grey where shown; (C) 2D structure of zafirlukast is shown.

4.3.2 Water analysis on pranlukast bound to CysLT₁ protein (6RZ4) using 3D RISM

Flare software was utilised to perform water analysis of pranlukast bound to protein 6RZ4. A 3D RISM (reference interference site model) approach was used. Cresset's force field, XED method, was selected. Then, the ligand and protein were selected and 3D-RISM calculations were started which generated the iso-surfaces calculations which revealed the molecular insights into water analysis. Water analysis could not be performed for protein 6RZ5, due to the absence of water molecules around zafirlukast.

4.3.3 LBVS workflow to identify novel hits for CysLT₁ protein (6RZ4 and 6RZ5)

Pranlukast and zafirlukast were used as reference ligands for the LBVS workflow. To initiate the Blaze search, a test run was prepared using the Asinex database. Then, the template generated from the test run was used to identify hits similar to pranlukast and zafirlukast using all databases in Blaze. Similarity was assessed using 50% 3D electrostatic potential and 50% shape. The generated field was then used to virtually screen for additional hits with similar field points and biological activities. For VS the top 2,000 compounds were selected and filtered in Flare based on Blaze scores (0 to +1) and docking scores (-12 to -15) to generate a set of 90 hits (47 hits for protein 6RZ4 and 43 hits for protein 6RZ5). The 90 selected hits were manually filtered based on the calculated Blaze score to generate a set of 4 compounds (2 hits for 6RZ4 and 2 hits for 6RZ5) that were commercially available and purchased for biological testing.

4.3.4 Generation of 3D and 2D ligand protein interaction maps

The Flare software was used to generate 3D and 2D maps of ligand-protein interactions. These maps illustrate intermolecular interactions through dotted lines, representing contacts, on the diagrams. In the 2D map, each residue that interacts with the ligand is depicted by a coloured circle. The circle includes the residue's 3-letter code, chain number, and colour, following the RasMol 'shapely' colour scheme.¹⁶⁹ The 3D and 2D maps of pranlukast, zafirlukast and selected hit molecules are discussed in Section 4.4.

4.3.5 *In vitro* antagonist activity of CysLT₁ protein (6RZ4 and 6RZ5) hit compounds using human CysLT₁ receptor cellular based assay

This experiment was performed by Eurofins (commercial service) according to their standard protocols. The procedure outlined is taken from Eurofins. CHO-K1 cells were suspended in DMEM buffer (supplied by Invitrogen) and plated at a density of 30,000 cells/well in microplates. Fluorescent probe Fluo4 Direct (Invitrogen), mixed with probenidol in HBSS buffer (supplied by Invitrogen) supplemented with 20 mM Hepes (pH 7.4), was added to each well and incubated with the cells for 60 minutes at 37°C, followed by 15 minutes at 22°C. The microplate reader (FlipR,

Molecular Devices) then added the test compound or HBSS buffer, followed 5 minutes later by 0.1 nM LTD₄ or HBSS buffer (basal control). Fluorescence intensity, which correlates with cytosolic Ca²⁺ concentration was measured to assess changes. Results were expressed as percent inhibition compared to the control response to 0.1 nM LTD₄. MK 571 was used as a standard reference antagonist, tested at various concentrations to generate a concentration-response curve and determine its IC₅₀ value.

4.4 Results and discussion

4.4.1 The importance of protein preparation

The increasing availability of protein crystal structures continues to have a huge effect on drug discovery, improving the ability to recognise key protein ligand interactions. The ability to generate bespoke crystallographic data can highlight additional chemistry opportunities that can propel a project forward in new directions. However, like all data, the information must be processed and analysed to ensure the reliability of the structure and that the maximum information is obtained. This process is covered by the general method of protein preparation. The purpose of protein preparation is to generate one or more protein models that represent the bioactive conformation(s) of the protein when ligands are bound and also examine the stability of protein structure. This can be a single representation, but it is more likely to be an ensemble of closely related structures showing the concerted movements of protein and ligand upon binding. To release the full potential of crystallography, careful structural preparation is vital. A badly prepared protein structure can lead to misinterpretation of results and can retard project development. Badly prepared structures can lead to confusing interaction analysis, incorrect assignment of binding mode and in general can greatly reduce the value of crystallographic input into a project. There are multiple tools which automate the process of protein preparation, such as Flare. In general, protein preparation is an essential step prior to carrying out any computational studies, in order to add hydrogen atoms, optimize hydrogen bonds, remove atomic clashes, examine the protein for gaps, assign optimal protonation states and compare two or more-protein structures.

4.4.2 Comparison of protein residue sequence and structure of CysLT₁ proteins (6RZ4 and 6RZ5) by alignment and superposition

Before importing the prepared proteins into Blaze, it was important to compare the residue sequence of CysLT₁ proteins and their structure by alignment and superposition. Prior to carrying out residue sequence and structural comparison of proteins, missing sidechains were added, extra chains were removed and gaps between the amino acid sequence in the protein structures were filled. Once the proteins were prepared then all other chains such as surfactants were removed.

Furthermore, the proteins were checked for the presence of BRIL inserts (a two-point insertion connected through N- and C-terminal to form continuous extension of two anti-parallel α helices of the membrane protein) and BRIL inserts were removed from protein 6RZ4, by splitting the amino acid chain from the end where sequence number changes to 1000's (**Figure 4.5**). There was no BRIL inserts present in protein 6RZ5. After removing BRIL (**Figure 4.5 B**), the gaps between the amino acids sequence of protein 6RZ4 were filled. Hence, the proteins were ready for alignment and superposition.

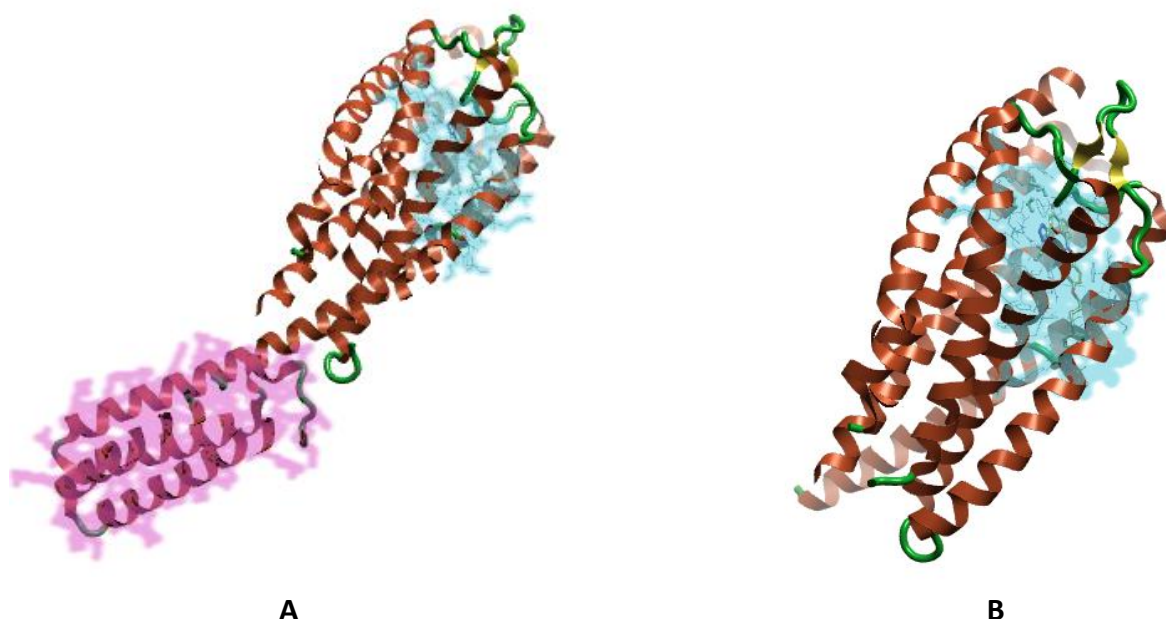


Figure 4.5: (A) Schematic cartoon model of protein 6RZ4 fused with BRIL highlighted in purple, secondary structure colour scheme for protein and active site highlighted in blue; (B) Schematic cartoon model of protein 6RZ4 without BRIL.

The protein sequence similarity of CysLT₁ proteins 6RZ4 and 6RZ5 were compared by alignment and the structural similarity was compared by superposition. Protein sequence alignment arranges the amino acid sequences of two or more proteins to find regions of identity or similarity across the entire sequence. The sequence for the imported proteins and their relative alignment is shown in the alignment table (**Figure 4.6**). Furthermore, a sequence similarity table showing the similarity matrix percentage calculated during protein alignment which means that the amino acid residue sequence of both proteins is 98.75% similar.

6RZ4	LSSHKKKAIGHIMVVTA AFLVSEFMPYHIQRTIHLHFLHNETKPCDSVLRMQKSUVVITLSLAASNCCFDPLLYFFSG
6RZ5	LSSHKKKAIGHIMVVTA AFLVSEFMPYHIQRTIHLHFLHNETKPCDSVLRMQKSUVVITLSLAASNCCFDPLLYFFSG

Figure 4.6: Protein sequence alignment table of CysLT₁ proteins 6RZ4 and 6RZ5. Aligned residues are highlighted on a bright green background: shows regions that are conserved i.e., perfect alignment.

Once the protein sequence alignment was completed then aligned proteins can be superposed. Structural superposition requires the structure of two proteins in order to compare by overlaying two protein structures in 3D space. The superposition tab in Flare used least squares fit of equivalent C. α carbon atoms to superpose the proteins and calculate an RMSD between the structures. Root square standard deviation (RMSD) is one of the simplest measures to score the similarity of two protein structures.¹⁷⁰ RMSD calculates the squared difference between equivalent atoms in two structures defined as,

$$\text{Equation 4.1: } RMSD = \sqrt{\frac{\sum d_i^2}{n}}$$

Where d_i stands for the distance between each of the n pairs of equivalent atoms in two optimally superposed structures.¹⁷⁰ In practice, a single representative atom per residue is chosen such as C-alpha or C-beta. A RMSD value ≤ 2 indicates structural similarity; as the value of RMSD increases, the structural similarity decreases.¹⁷⁰ In this study, the RMSD calculated between protein 6RZ4 and 6RZ5 using Flare was 1.66 Å. Proteins 6RZ4 and 6RZ5 were superposed and the ligands associated with them were also superposed within the active site. (Figure 4.7 A and B).

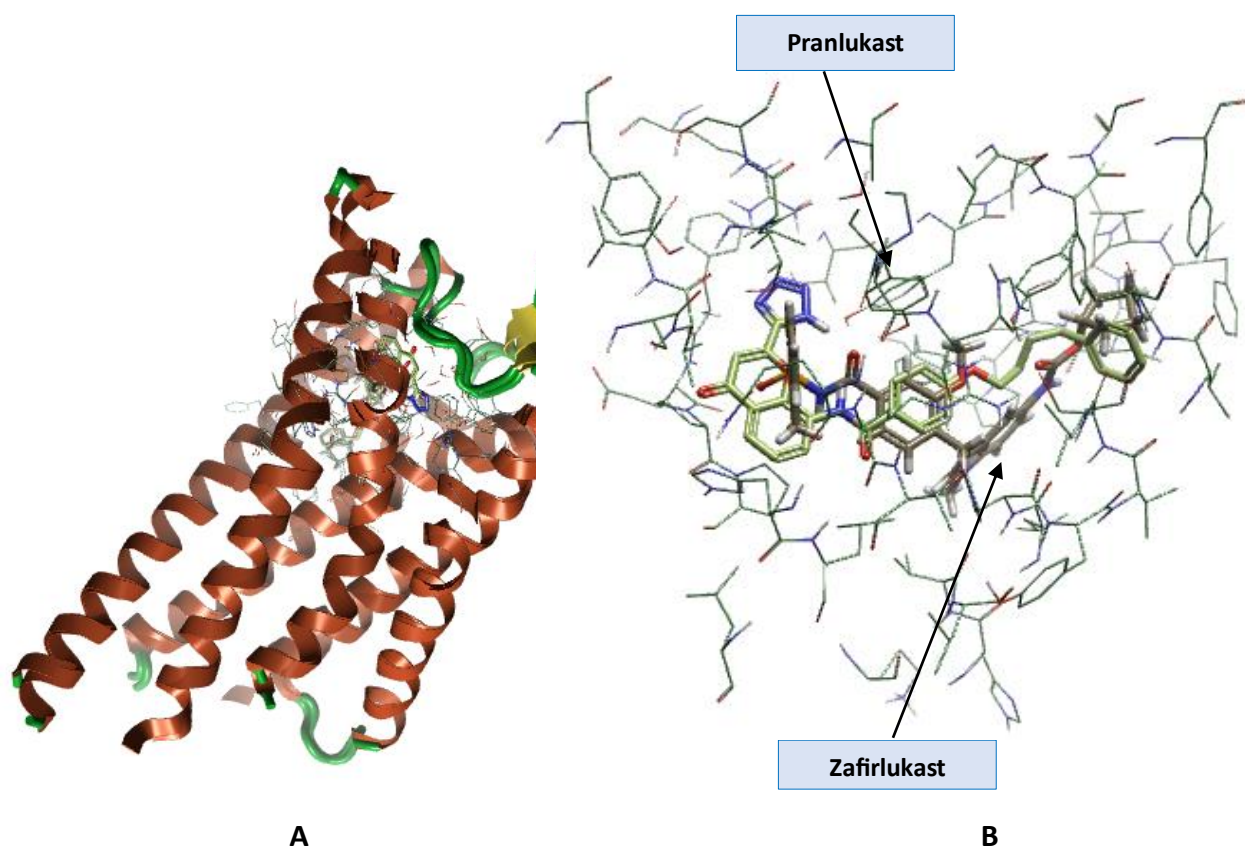


Figure 4.7: (A) Cartoon model of structural superposing of 6RZ4 and 6RZ5; (B) Superposed pranlukast and zafirlukast within the active site, carbon atoms are coloured in green for pranlukast and grey for zafirlukast, nitrogen atoms are coloured in blue, oxygen atoms are coloured in red, sulphur atoms are coloured in yellow and hydrogen atoms are coloured in grey.

4.4.3 Water analysis of protein 6RZ4 by 3D-RISM approach

The crystal structure of protein 6RZ4 has shown the presence of seven water molecules in the active site (**Figure 4.8**). To understand and exploit the stability of crystallographic water molecules present in the active site of 6RZ4, a 3D-RISM approach was utilised in Flare to compute and highlight these water molecules. There was no crystallographic water molecules present in the active site of protein 6RZ5; therefore, no water analysis was required for 6RZ5.

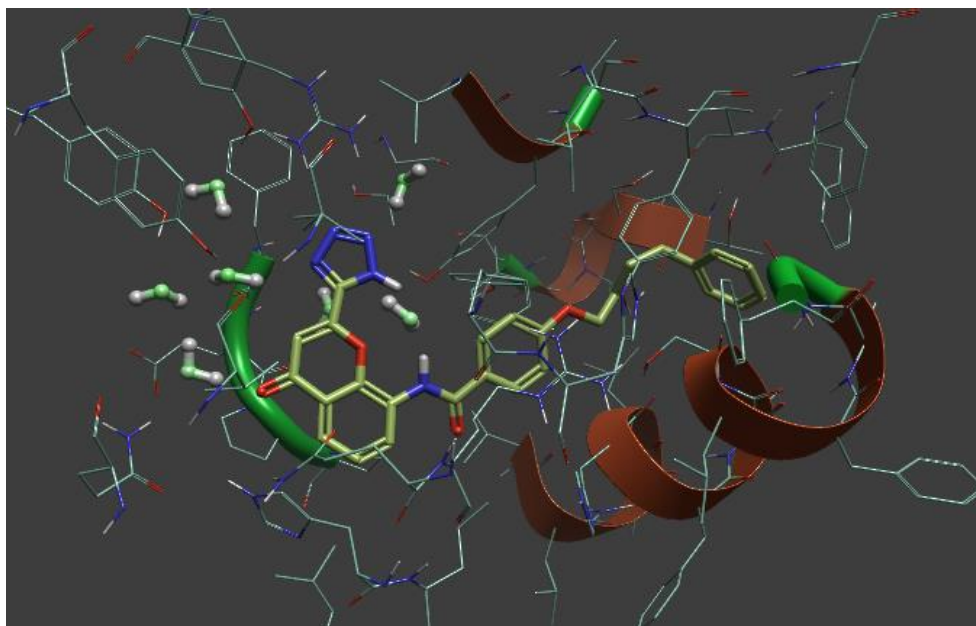
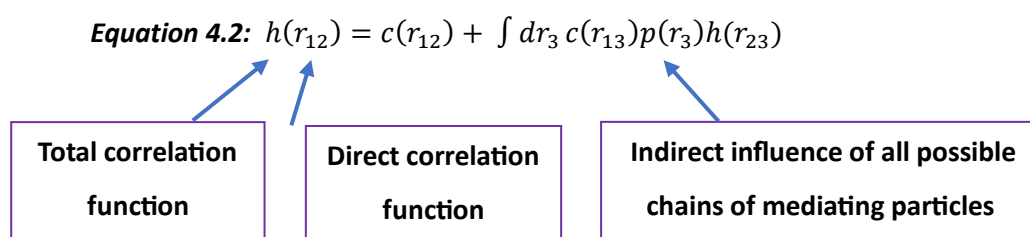


Figure 4.8: Schematic cartoon model of pranlukast bound to protein 6RZ4 surrounded by water molecules are shown as capped stick, oxygen atoms are coloured in green and hydrogen atoms are coloured in grey.

RISM is a modern approach to solvation based on the molecular Ornstein-Zernike equation (**Equation 4.2**). 3D-RISM has seen increasing use as a method to investigate the location and stability of water molecules in a protein. Conceptually, 3D-RISM is equivalent to running an infinite-time molecular dynamics simulation on the solvent (keeping the solute fixed), and then extracting the density of solvent particles. The molecular Ornstein-Zernike equation presents the total correlation function $h(r_{12})$ between two particles as contributions from a direct correlation $c(r_{12})$ and from indirect contributions propagating through all possible chains of mediating particles. This equation cannot be solved directly, but an approximate solution can be obtained by guessing a suitable closure relation derived from statistical mechanics, and then iterating until self-consistency is reached.



The output of a 3D-RISM calculation consists of free energy (ΔG) iso-surfaces, which represent the stability of a water molecule in the active site. These iso-surfaces map regions coloured by negative and positive ΔG values, as shown in Figure 4.9. Water molecules associated with negative ΔG values (colour coded with green) are characterized as 'happy' (favourable) water. In other words, these water molecules are predicted to be more stable in the active site of the protein. On the other hand, water molecules associated with positive ΔG values (colour coded with red) represent 'unhappy' (unfavourable) water molecules, which are less stable. The 3D-RISM calculation on 6RZ4 clearly shows the labelled ΔG values calculated for the seven water molecules present in the active site (**Figure 4.9**). In this case, all seven water molecules in the active site have negative ΔG values, characterizing them as 'happy' water molecules. This indicates that all water molecules in the active site are predicted to be stable based on the 3D-RISM calculation.



Figure 4.9: 3D-RISM model of stable water molecules in the active site of 6RZ4 indicated by negative (green) ΔG iso-surfaces.

4.4.4 Summarising ligand-protein interaction maps of pranlukast and zafirlukast with CysLT₁ proteins (6RZ4 and 6RZ5)

Ligand-protein interaction maps were analysed using Flare to visualise the 3D interactions and to generate 2D interaction maps. Luginina *et.al* described the key ligand-protein interactions for pranlukast and zafirlukast (**Figure 4.10**) which can be compared with those analysed in Flare. The purpose of summarising these interactions is to compare and contrast them with the top four hit molecules identified by the LBVS platform Blaze (**Section 4.4.7**). Luginina *et.al*. indicated that the extracellular (EC) part of the CysLT₁ binding pocket is lined with polar and charged residues that interact with the ligands.¹⁷¹ Important residues include R79 (Arg 79), which forms a salt bridge to the tetrazole moiety of pranlukast with a distance of 2.6 Å.¹⁷¹ In contrast zafirlukast does not directly contact with R79 (Arg 79).¹⁵¹ Y104 (Tyr 104) made extensive polar interactions with the tetrazole group and benzopyran moieties of pranlukast, as well as with the sulphonamide of zafirlukast.¹⁵¹ V277 (Val 277) formed hydrophobic interactions with both ligands.¹⁷¹

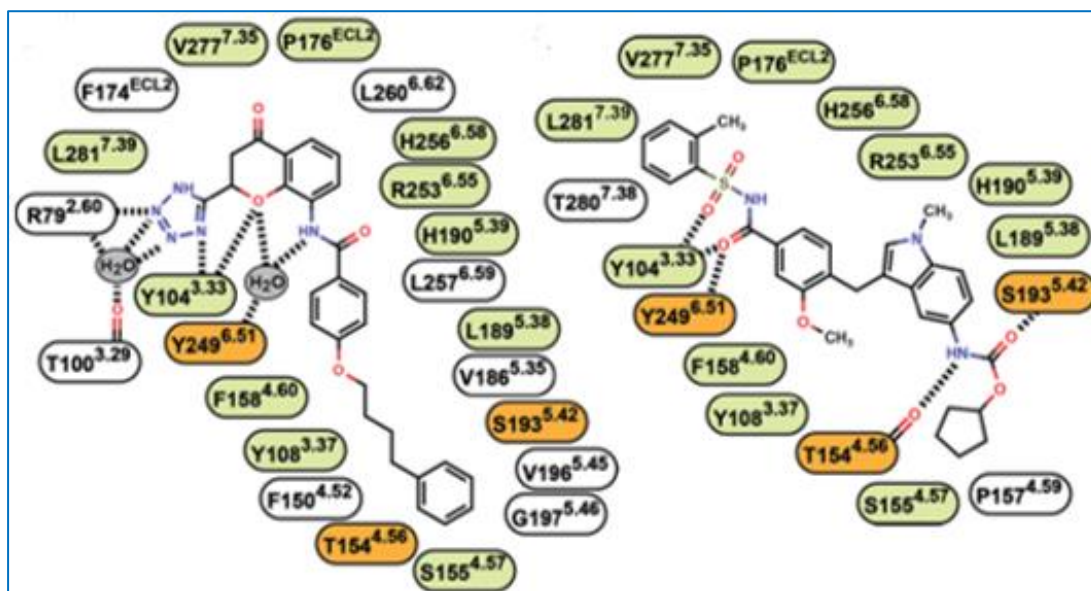


Figure 4.10: 2D ligand-protein interaction maps for pranlukast and zafirlukast. Residues engaged in the same type of interactions with both ligands are coloured in light green. Orange colour represents the same residue but a different type of interaction. White colour represents the interactions that are not common between the two ligands.

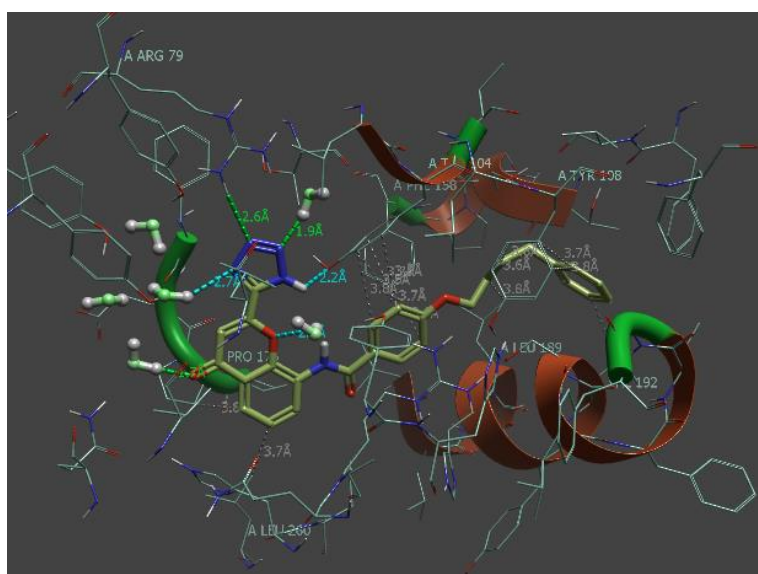
Image taken from Luginina. *et.al*.¹⁷¹

In our analysis using Flare, pranlukast forms hydrogen bonds and hydrophobic interactions with the amino acid residues of CysLT₁ protein (6RZ4) as shown in Figure 4.11 and table 3.2. Arg A79 forms a strong hydrogen bond with the tetrazole ring of the ligand at a distance of 2.6 Å. Tyr A104 forms a weak hydrogen bond with the tetrazole ring at a distance of 2.2 Å. HOH A2106 (water molecule) forms a strong hydrogen bond with the tetrazole ring of the ligand molecule at a distance of 1.9 Å. HOH A2102 (water molecule) forms a strong hydrogen bond with carbonyl group at a distance of 1.7 Å. HOH 2111 (water molecule) forms a weak hydrogen bond with the oxygen of the ligand at a distance of 2.4 Å. Other residues in the interaction map form hydrophobic interactions with pranlukast at distances ranging from 3.5 to 3.9 Å (**Figure 4.11 A and B**).

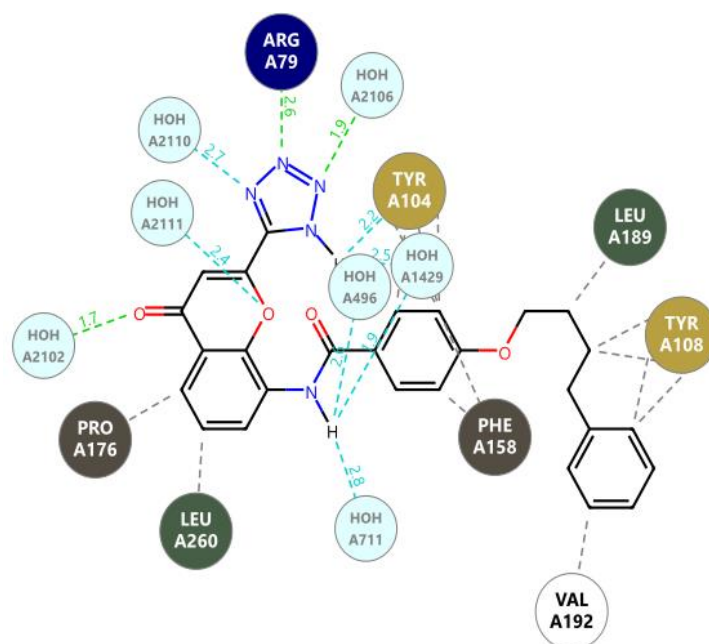
Figure 4.12 outlines the 3D and 2D interaction maps of zafirlukast with CysLT₁ protein (6RZ5). It forms hydrogen bonds and hydrophobic contacts (**Table 4.2**). Tyr B249, forms a strong hydrogen bond with the carbonyl group of the amide moiety of zafirlukast at a distance of 1.8 Å. Tyr B104 has more than one interaction with zafirlukast; the hydrogen of the hydroxy group of the tyrosine amino acid forms a strong hydrogen bond with the carbonyl oxygen of the ligand's amide bond at a distance of 1.8 Å. The same hydrogen of the tyrosine amino acid forms a weak hydrogen bond with the sulphonamide group at a distance of 2.7 Å. Ser B193, a serine amino acid, forms a strong hydrogen bond with the carbonyl oxygen group of the ligand's amide bond at a distance of 2.2 Å. Thr B154, a threonine amino acid, forms a strong hydrogen bond with the secondary amide group at a distance of 1.82 Å. Other residues in the interaction map form hydrophobic interactions with zafirlukast with distances ranging from 3.4 to 3.9 Å, (**Figure 4.12 A and B**).

Common interactions between Flare generated and Luginina. *et.al.* ligand-protein interaction maps are discussed. Both pranlukast and zafirlukast interact with residue Tyr 104 which Luginina. *et.al.* indicated this as an important interaction due to its polar interaction with the tetrazole ring of pranlukast and the sulphonamide group of pranlukast and zafirlukast, respectively. However, in the Flare generated interaction maps, Tyr 104 forms a weak hydrogen bond with both the tetrazole ring and the sulphonamide group. Moreover, Val B277 forms a hydrophobic interaction with zafirlukast which is mentioned by Luginina. *et.al.* From Table 4.2, it is clear that pranlukast is stable in the active site and has a total of 15 interactions with the residues and water molecules present in the active site, out of which 6 are hydrophobic. However, the active site itself outlines a hydrophilic cavity due to the presence of water molecules that interact with the ligand. In contrast, zafirlukast has a total of 11 interactions with the residues of CysLT₁ protein (6RZ5) out of which 7 are hydrophobic interactions. Additionally, pranlukast's interaction with residue A79 at 2.6 Å is a common interaction identified in both the Flare-generated and Luginina. *et. al.*'s studies. The protein structures (6RZ4 and 6RZ5) appear suitable for VS based on the detailed interaction maps provided. The

differentiation in interaction profiles between pranlukast and zafirlukast can be leveraged to refine screening strategies and predict potential new ligands more effectively.

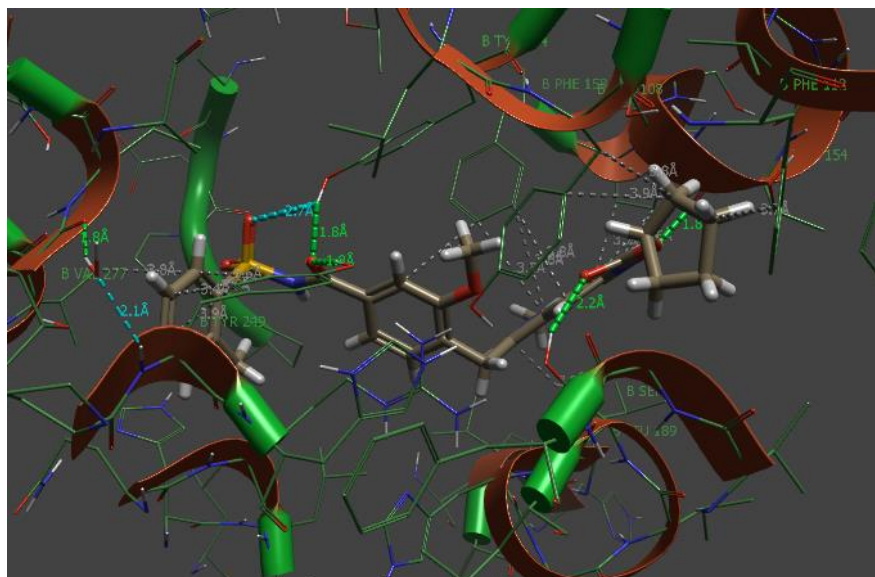


A

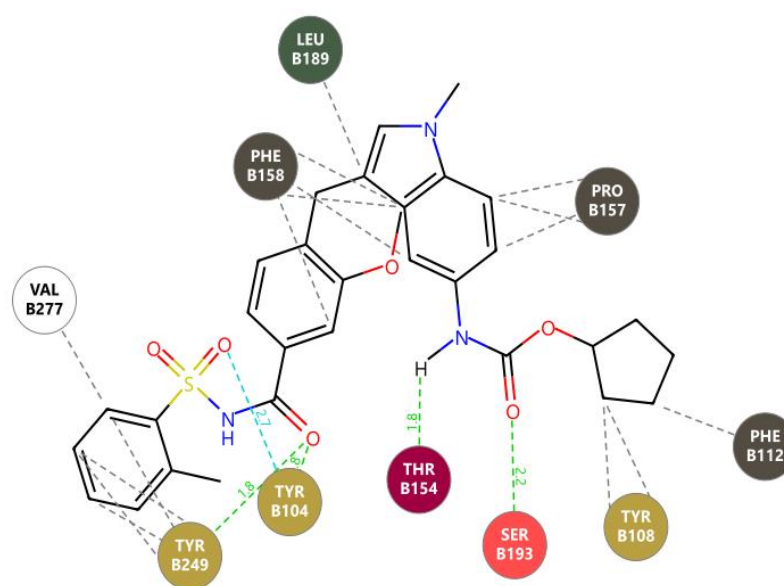


B

Figure 4.11: (A) 3D ligand-protein interaction diagram for pranlukast with CysLT₁ (6RZ4); (B) 2D ligand-protein interaction diagram for pranlukast with CysLT₁ (6RZ4). Water molecules presented as capped stick in (A). Each residue, water molecule or cofactor interacting with the ligand in 2D map is represented by a coloured circle reporting the residue's 3-letters code, chain number and colour according to the RasMol 'shapely' colour scheme. Molecular interactions defined as: strong/average hydrogen bonding: maximum distance = 2.6 Å, weak hydrogen bonding: maximum distance = 3.0 Å and hydrophobic maximum distance: 3.3 to 4 Å. The dotted lines which represent strong hydrogen bonds are coloured in light green, weak H-bonds in cyan and hydrophobic contacts in grey.



A



B

Figure 4.12: (A) 3D ligand-protein interaction diagram for zafirlukast with CysLT₁ (6RZ5) (B) 2D ligand-protein interaction diagram for zafirlukast with CysLT₁ (6RZ5). Each residue, water molecule or cofactor interacting with the ligand in 2D map is represented by a coloured circle reporting the residue's 3-letters code, chain number and colour according to the RasMol 'shapely' colour scheme. Molecular interactions defined as: strong/average hydrogen bonding: maximum distance = 2.6 Å, weak hydrogen bonding: maximum distance = 3.0 Å and hydrophobic maximum distance: 3.3 to 4 Å. The dotted lines which represent strong hydrogen bonds are coloured in light green, weak H-bonds in cyan and hydrophobic contacts in grey.

Table 4.2: Ligand-protein interactions of pranlukast and zafirlukast with 6RZ4 and 6RZ5 generated in Flare.

Ligand	Residue involved with hydrogen bonding	Distance (Å)	Water molecules in the active site involved with hydrogen bonding	Distance (Å)	Residue involved with hydrophobic interaction	Distance (Å)	Total number of interactions
Pranlukast	Arg A79	2.6	HOH A2106	1.9	Pro A176		15
	Tyr A104	2.2	HOH A2110	2.7	Leu A260	N/A	
			HOH A2102	1.7	Phe A158		
			HOH A2111	2.4	Val A192		
			HOH A496	2.5	Leu A189		
			HOH A1429	2.5	Tyr A104		
			HOH A711	2.8			
Zafirlukast	Tyr B104 (x2)	1.8 & 2.7			Val B277		11
	Tyr B249	1.8	N/A	N/A	Tyr B249	N/A	
	Thr B154	1.8			Tyr B108		
	Ser B193	2.2			Phe B112		
					Pro B157		
					Leu B189		
					Phe B158		

4.4.5 Displaying Electrostatic Complementarity (EC) surfaces and radial plots of pranlukast and zafirlukast.

4.4.5.1 EC surfaces of pranlukast and zafirlukast

Electrostatic interactions between small molecules and their respective receptors are a key contributor to the enthalpic component of the binding free energy ΔG . Assessing the electrostatic match between ligands and binding pockets provides important insights into why ligands bind and what could be changed to improve binding, and informs the design of polar/enthalpic binders, which typically have better selectivity and pharmacokinetic parameters than entropic binders.¹⁷² Electrostatic complementarity (EC) surface colouring and scores within Flare are an extension of the protein interaction potential functions. EC maps provide a visual interaction of how a ligand matches electrostatically with the corresponding protein. A quantitative EC score provides information on how well the electrostatics of a ligand match that of the protein active site and can accordingly prove invaluable for understanding ligand binding, SAR and the design of new molecules that target the protein. Figure 4.13 displays the EC surface maps of pranlukast and zafirlukast. The EC colouring shows the minimum and maximum EC, thus, areas coloured in green on the EC maps of each ligand in Figure 4.13 shows the perfect complementarity and areas coloured in red show electrostatic clash i.e., poor electrostatic complementarity. The EC scores will be discussed in Section 4.4.5.2 with the radial plot properties of ligands.

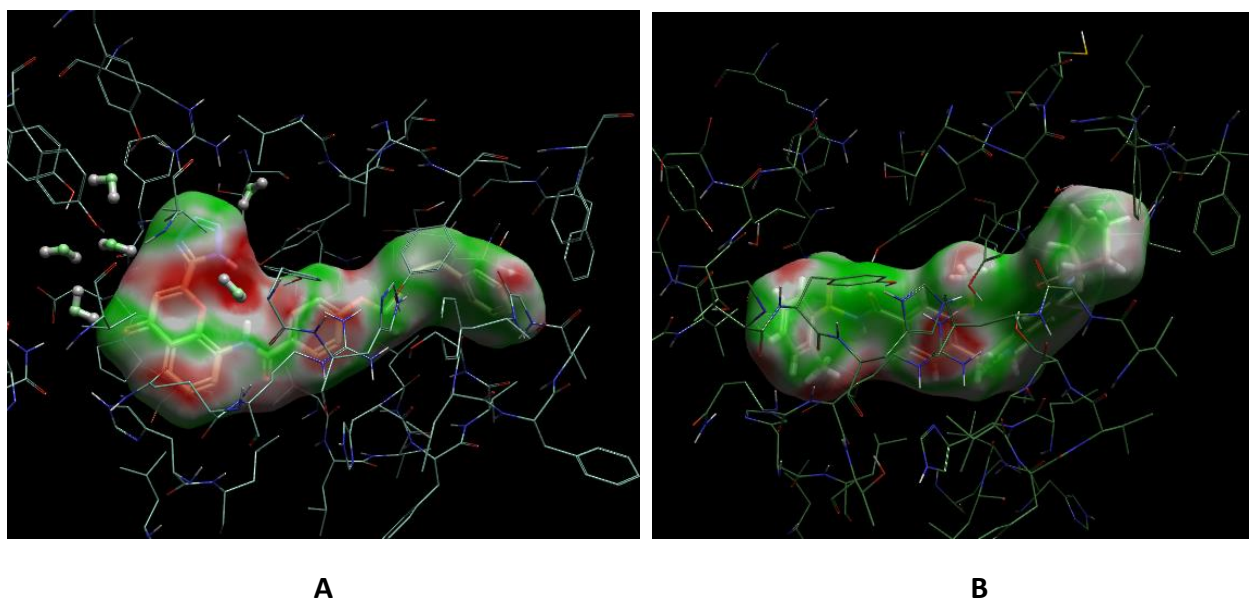


Figure 4.13: (A) EC surface shown on pranlukast; (B) EC surface shown on zafirlukast. Key: Poor electrostatic complementarity (red) and perfect electrostatic complementarity (green)

4.4.5.2 Radial plots of ligand molecules

Radial plots are an intuitive visual tool to summarize multiple numerical data into a single core, calculating a multi-parameter score to help select the molecules with the best fit to the ideal property profile.²⁴ An example of a radial plot window is shown in Figure 4.14. Radial windows and properties of pranlukast and zafirlukast are presented in the ligands table of Flare software (**Figure 4.14**). Radial plot properties summarise numerical data for a molecule which may include EC score, docking scores, Blaze scores, and physicochemical properties such as molecular weight (MW), topological surface area (TPSA) and lipophilicity (SLogP). In addition, data can be selected or deselected for inclusion in the radial plot. The radial plot is based on the idea that molecular properties which are 'perfect' should be displayed at the centre of the radial plot as shown in Figure 4.14. Thus, a molecule with near-perfect properties should have a radial plot with a small, encapsulated area. Conversely, poor properties would be plotted at the edge of the radial plot such that a molecule with sub-ideal properties would have a large, enclosed area.

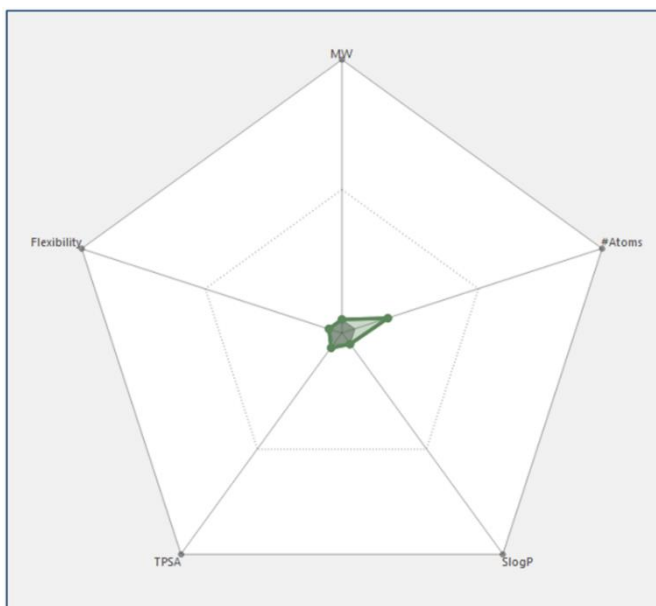


Figure 4.14: Example of a radial plot window for a single molecule.

Image is taken from Cresset group's website.¹⁷³

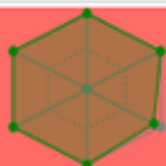
The radial plot window and properties of ligand molecules pranlukast and zafirlukast are displayed in Figure 4.15. In Flare, the radial plot properties are combined into a single radial plot score (RPS) [0 (unacceptable) to 1 (acceptable)] that represents the fit of a compound to the ideal properties profile. The RPS value for pranlukast was 0.267 and zafirlukast was 0.016 (**Figure 4.15**). In this case, there were no Blaze and docking scores available for the ligand molecules. An EC score was computed for both ligands, with pranlukast corresponding to 6RZ4 (EC score = 0.218) and zafirlukast corresponding to 6RZ5 (EC score = -0.135). The computed EC score is the normalized surface integral

of the complementarity score that effectively displays the average value of that score over the surface of the ligand. The EC scores range from 1 (perfect complementarity) to -1 (perfect clash). The EC score of pranlukast is 0.218 which means pranlukast has good complementarity with the protein, but it's not a perfect fit. In contrast, the EC score of zafirlukast is -0.135 which indicates some clash or poor complementarity towards the associated protein. Other than EC score, physicochemical properties were also calculated such as MW, TPSA, SLogP, no of rotatable bonds and Lipinski's Ro5. As discussed in Chapter 2, Lipinski's Ro5 assesses the drug likeness of a chemical compound that would make it likely orally active drug. It is based on specific physicochemical properties such as MW less than 500, LogP value not greater than 5, hydrogen bond acceptors no more than 10 and no less than 5 hydrogen bond donors. To be orally active a drug should have no more than 1 violation.¹⁷⁴

Figure 4.15 shows the property columns coloured based on how each value fits the profile set in the Radial Plot Properties window. For instance, if the calculated score is lower than the accepted range of each property the column colour will get darker and if the score is outside the range, then the column will appear as 'red' colour. If the calculated score is closer to the accepted range, then the column will appear in lighter shade and if the score is within the range than the column will appear in 'green'. It can be seen that pranlukast has some reasonable physicochemical properties in terms of TPSA, MW and SLogP as they are coloured in 'lighter red shade' in comparison to zafirlukast. For instance, pranlukast has 0 violations of Ro5. On the other hand, zafirlukast has two violations of Ro5 rule. In summary, both ligands have a majority of their properties coloured in darker shades (unfavourable) but they will be further compared with selected hits of 6RZ4 and 6RZ5 proteins (**Figures 4.24 and 4.25**).

Radial Plot	MW	EC	#Atoms	SlogP	TPSA	Rof5	#RB
0.267 	480.5	0.218	36	4.4	114.1	0	9

A

Radial Plot	MW	EC	#Atoms	SlogP	TPSA	Rof5	#RB
0.016 	574.7	-0.135	41	5.7	116.1	2	10

B

Figure 4.15: Radial plot and molecular properties of (A) Pranlukast; (B) Zafirlukast. Radial plot windows in the ligand table are coloured according to radial plot score (RPS). Radial plot window with an RPS 0 (unacceptable) are coloured in red and 1 (acceptable) are coloured in green.

4.4.6 Performing LBVS using Blaze to identify potential hit molecules targeting CysLT₁ receptor protein

In our pursuit to identify novel hits for the CysLT₁ receptor, Cresset's Blaze platform was employed. Blaze is Cresset's LBVS platform which uses the shape and 3D electrostatic character of known ligands to rapidly search large chemical collections for molecules with similar properties. It is a VS system that contains the infrastructure to manage compound collections and the associated conformation populations. It automatically records additions and removals from any collection and handles duplication across collections. The major advantage of Blaze includes the VS of 30 million compounds in just a few hours, enabling huge time and cost savings. It also reduces the need to purchase libraries of chemical compounds. Users can simply hand over the best quality leads from VS to the lab, making early-stage drug development more cost-effective, and reducing the environmental impact caused by wasted chemicals. Other VS platforms are available in the market such as Lead Builder, but it identifies hits in a database of 1.5 million compounds only in comparison to Blaze.

To perform Blaze LBVS, five steps are involved. The first step is the selection of reference ligands that are known to interact with the target of interest and have the desired activity or potency. In this study the reference ligands for Blaze LBVS are pranlukast and zafirlukast.

The second step involves the calculation of molecular descriptors, wherein, various molecular descriptors are calculated for the reference ligands, such as 2D or 3D field point patterns. In this study both ligands have pre-computed conformations and the field points of both ligand molecules are added to produce the Blaze pharmacophore seed (**Figure 4.16**). Field points are condensed representations of electrostatic, hydrophobic and shape properties. They are placed in the areas of local extrema in these potential energy functions. The size of the field points corresponds to the strength of the field at that point in space. The presence of the field point suggests that this is the area of the ligand which forms the favourable interaction with the protein receptor.

The third step is the generation of a virtual library using Blaze. The virtual library of compounds is generated by screening a large database of compounds for those that have similar molecular descriptors to the reference ligands. In this case, a Blaze test run was conducted prior to a full Blaze screen by using the Asinex library. The Asinex library is reasonably small but diverse and could provide good indicative examples of the type of results likely to be returned from a full Blaze screen, and run in approximately 2 to 4 hours. The results of the Blaze test run showed that none of the hits matched the field points of the reference ligands around the tetrazole ring of pranlukast and the sulfonamide group of zafirlukast. As a result, modifications were applied by adding field

constraint values, which force the alignment algorithm to prioritise matching the field points in specific places around the ligands. In this case the field constraint values were added around the tetrazole ring of pranlukast and the sulfonamide group of zafirlukast (**Figure 4.16**). Once the constraint values were added, a full Blaze search was conducted using all available databases.

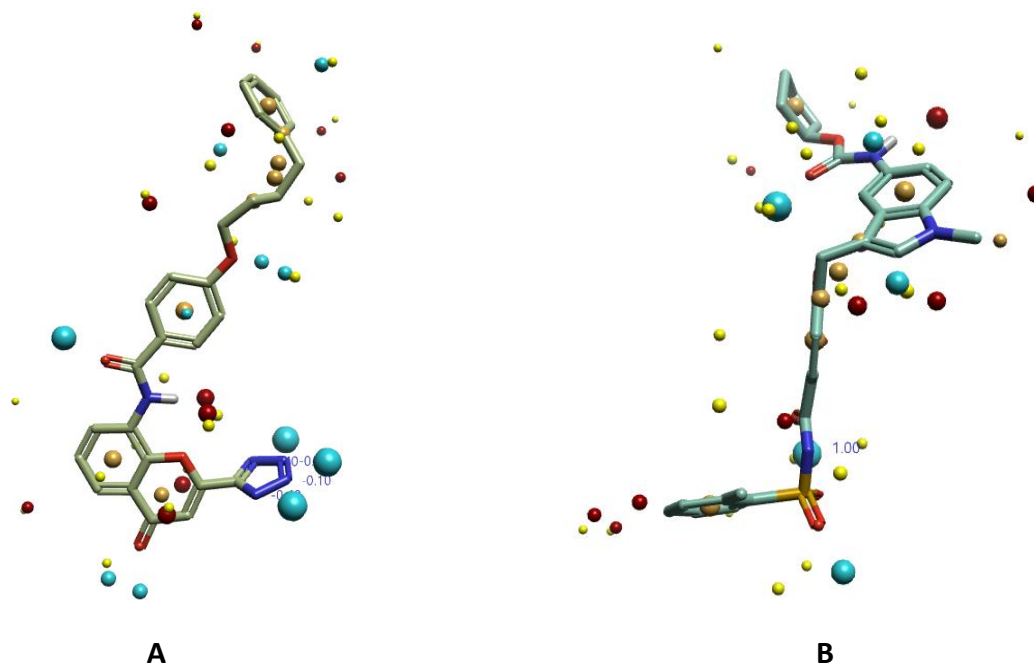


Figure 4.16: (A) 3D structure of pranlukast with its associated field points and calculated field constraint values around the tetrazole ring; (B) 3D structure of zafirlukast with its associated field points and calculated field constraint values around the sulphonamide group. Field points in blue correspond to an area where electrostatics are negative; red corresponds to areas where electrostatics are positive; yellow corresponds to van der Waals surface area and orange points are for hydrophobicity.

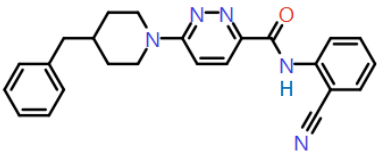
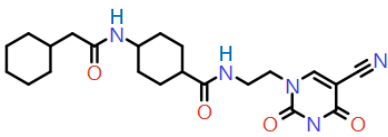
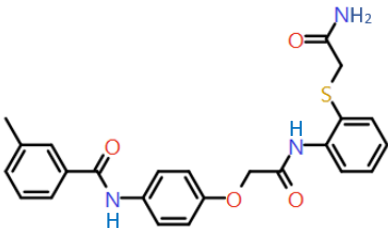
Once the full Blaze search was done, the fourth step is the filtration of hits using various criteria, such as Blaze score and docking score, to narrow down the number of compounds to a manageable size. Blaze and docking scores are scoring function values, an essential component in molecular docking that can be used to identify the most stable binding ligand to a protein receptor from among a large data set. The aim was to filter the compounds based on Blaze and docking scores. The higher the score value, the more similar the potential hit molecule is to the reference ligand. Thus, 2,000 hits whose 3D field points are matched with the field points of the reference ligands were obtained. The Blaze file containing the 2,000 compounds was downloaded in Flare and filtered using Blaze score and docking score to further narrow the selection of compounds down to 90 hits (47 hits for 6RZ4 and 43 for 6RZ5). The list of 90 hits was further narrowed down to 4 hits (2 hits for 6RZ4 and 2 hits for 6RZ5). These 4 hits were manually selected based on their high Blaze score and

commercial availability. Table 3.3 displays the top 8 potential hits (**84-99**) for each protein, along with their corresponding Blaze scores and chemical structure.

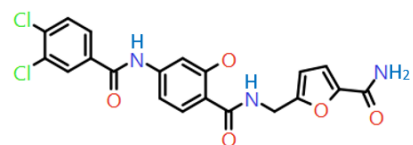
To summarize, **84** and **85** had the highest Blaze scores for 6RZ4. However, **84** for 6RZ4 could not be purchased due to shipping issues. Therefore, **86** was selected alongside **85** for further progress (Table 4.3). For 6RZ5, **92** and **93** had the highest Blaze scores among the hit list, but **93** was not commercially available and hence could not be used. **94**, **95**, and **96** also had availability issues, so we selected **97** for purchase, hence **92** and **97** were selected for further progression (Table 4.3).

The fifth step involves the validation of hits in which the selected compounds are validated through experimental testing, such as *in vitro* and *in vivo* assays, to determine their actual activity and potential as drug candidates (explained in Section 4.4.9). Interestingly, none of the CysLT₁ (6RZ4) hits (**84-91**) contain the tetrazole ring, one of the major structural features in pranlukast. In contrast, the CysLT₁ (6RZ5) hits (**92-99**) contain a sulfonamide group (similar to zafirlukast).

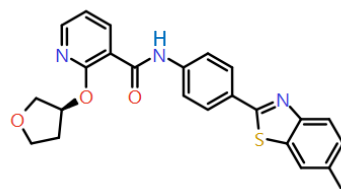
Table 4.3: Top 8 hit molecules for CysLT₁ protein (6RZ4 and 6RZ5) along with their Blaze score and chemical structure.

CysLT ₁ Protein	Compound number	Blaze score	2D chemical structure
6RZ4	84	0.6728	
	85	0.6423	
	86	0.6403	

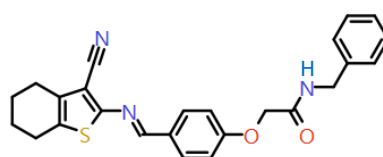
87 0.6382



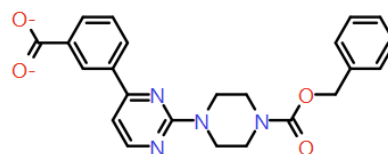
88 0.6353



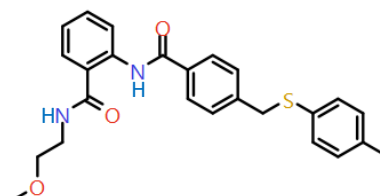
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90 0.6297

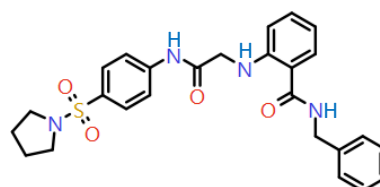


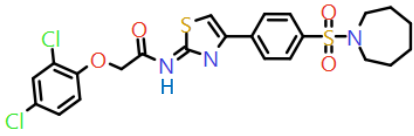
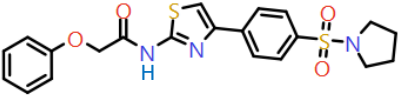
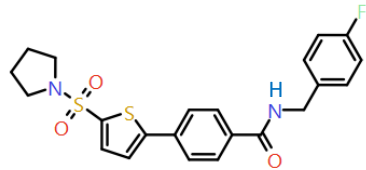
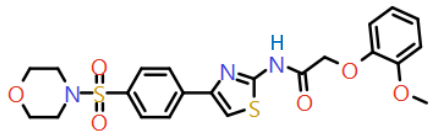
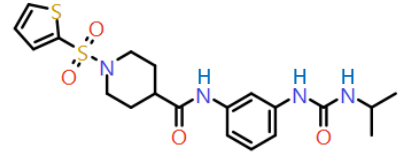
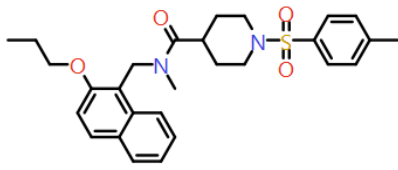
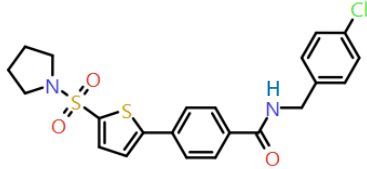
91 0.6276



6RZ5

92 0.6608



93	0.6577	
94	0.6457	
95	0.6395	
96	0.6380	
97	0.6360	
98	0.6349	
99	0.6330	

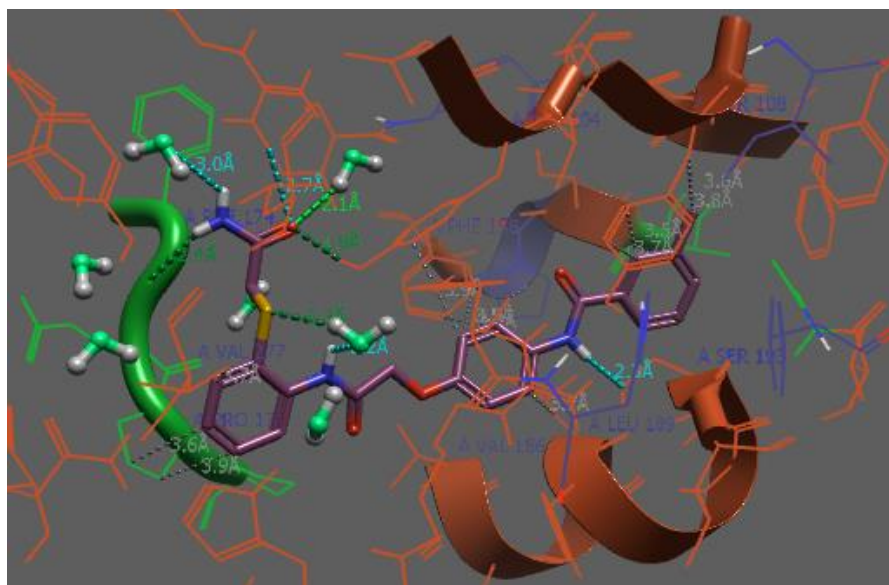
4.4.7 Summarising ligand-protein interaction maps of selected hit molecules with CysLT₁ receptor

Ligand protein interactions of the reference ligands, pranlukast and zafirlukast with CysLT₁ in co-crystals 6RZ4 and 6RZ5 were explained in detail in Section 4.4.4. Now, the ligand-protein interactions of the 4 selected hits (**85**, **86**, **92** and **97**) with CysLT₁ will be discussed.

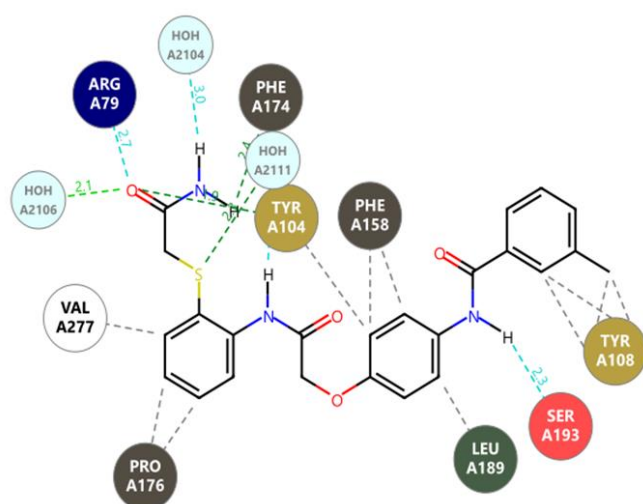
4.4.7.1 Ligand protein interaction maps of **85** and **86** with protein 6RZ4

The 3D and 2D interaction maps in Figure 4.17 outline the interaction of the **85** with the CysLT₁ receptor protein from X-ray co-crystal 6RZ4 and water molecules present in the active site. **85** has network of interactions such as hydrogen bonds and hydrophobic contacts with one water molecule out of seven water molecules present in the active site and amino acid residues of CysLT₁ protein (6RZ4) as shown in Table 4.4. The hydrogen of water molecule HA 2106 present in the active site forms an average hydrogen bond with the nitrogen atom of the cyano group of **85** with a distance of 2.1 Å. Tyr A104 forms a weak hydrogen bond with nitrogen atom of nitrile group with a distance of 1.8 Å. Phe A174 forms an average hydrogen bond with the hydrogen atom of **85** with a distance of 2.1 Å. Interactions with this residue were not seen for pranlukast in our Flare model. Notably, an interaction with Arg 79 is missing.

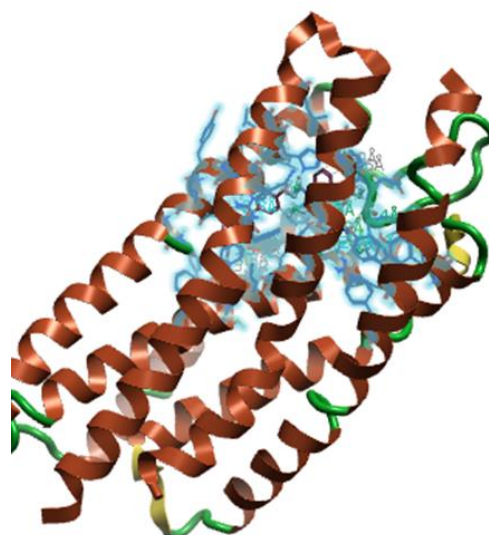
Compound **86** has a lower number of interactions. Other residues form hydrophobic interactions with the molecule as shown in Table 4.3. The 3D and 2D interaction maps in Figure 4.18 outline the interaction of **86** with the CysLT₁ receptor protein from X-ray co-crystal 6RZ4 and water molecules present within the active site. Compound **86** has network of interactions such as hydrogen bonds, hydrophobic contacts and aromatic-aromatic interactions with three of seven water molecules present in the active site and amino acid residues of CysLT₁ protein (6RZ4) (**Table 4.3**). The hydrogen of HOH A2106 forms a strong hydrogen bond with the oxygen atom of the carbonyl of amide bond of **86** with a distance of 2.1 Å. The hydrogen atom of water molecule HOH A2111, forms an average hydrogen bond with the sulfur atom of **86** with a distance of 2.3 Å. HOH A2104, the oxygen atom of water molecule forms a weak hydrogen bond with the hydrogen atom of the **86** with a distance of 3.0 Å. The hydrogen atom of the amine group of the arginine amino acid Arg 79 residue forms a weak hydrogen bond with oxygen group of the carbonyl group of the molecule at a distance of 2.7 Å. The oxygen atom of Ser A193 forms a weak hydrogen bond with the hydrogen atom of the amide group at a distance of 2.3 Å. In summary, **86** forms a diverse network of interactions including hydrogen bonds, hydrophobic contacts, and aromatic-aromatic interactions, with key water molecules and amino acid residues within the active site of the CysLT₁ receptor protein, highlighting its potential role in stabilising the protein-ligand complex.



A



B



C

Figure 4.18: (A) 3D ligand-protein interaction diagram of 86 with CysLT₁ (6RZ4); (B) 2D ligand-protein interaction diagram of 86 with CysLT₁ (6RZ4); (C) Schematic cartoon model of 86 bound to CysLT₁ protein (6RZ4), active site highlighted in light blue. Water molecules presented as ball and stick in (A). Each residue, water molecule or cofactor interacting with the ligand in 2D map is represented by a coloured circle reporting the residue's 3-letters code, chain number and colour according to the RasMol 'shapely' colour scheme. Molecular interactions defined as: hydrogen bonding: maximum distance = 2.6 Å, weak hydrogen bonding: maximum distance = 3.0 Å and hydrophobic: 3.3 to 4 Å. The dotted lines which represent strong hydrogen bonds are coloured in light green, weak H-bonds in cyan and hydrophobic contacts in grey.

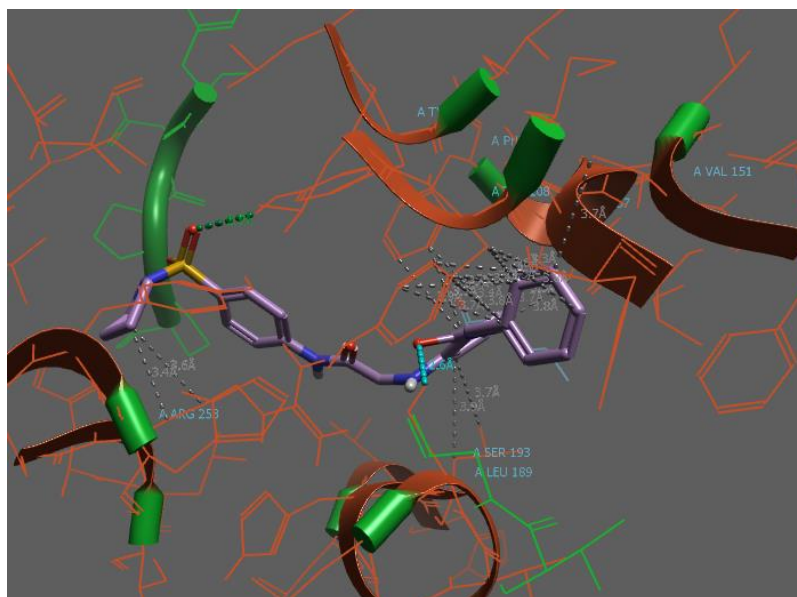
Table 4.4 shows the interactions between hit molecules, **85** and **86** and the CysLT₁ protein (6RZ4) as well as associated water molecules. Compound **85** has a total of 7 interactions with residues of CysLT₁ protein (6RZ4) and associated water. In contrast, **86** has a total of 13 interactions with the residues of the CysLT₁ protein (6RZ4) and associated water. Both hit molecules have common interactions with Tyr A104, Phe A174, and a water molecule (A2106). Additionally, both hit molecules exhibit similar interactions to pranlukast. Only one water molecule of seven, (A2106) interacts with **85**, while three water molecules (A2106, A2111, and A2104) interact with **86**. Despite the absence of a tetrazole ring in both hit molecules, they still form hydrogen bonds. HOH A2106 forms an average hydrogen bond with the nitrile group in **85** whereas in pranlukast, A2106 forms a strong hydrogen bond with the nitrogen of the tetrazole ring. In contrast, A2106 also forms a strong hydrogen bond with the oxygen atom of the amide bond in **86**. On the other hand, tetrazoles are relatively strong NH acids but also exhibit weak base properties. Despite being weak bases, tetrazoles effectively interact with both hydrogen bond donors and acceptors, and water molecules can serve as both. Therefore, the tetrazole moiety in pranlukast forms a stronger hydrogen bond with the water molecule in the active site compared to **86**.

Table 4.4: Ligand-protein interaction of pranlukast, 85 and 86 with CysLT₁ protein (6RZ4).

Interactions	Pranlukast	Distance Å	85	Distance Å	86	Distance Å
Residue	Arg A79	2.6	Phe A174	2.1	Arg A79	2.7
involved	Tyr A104	2.2	Tyr A104	1.8	Tyr A104	2.2
with hydrogen					Phe A174	2.4
bonding					Ser A193	2.3
Water	HOH A2106	1.9	HOH	2.1	HOH A2106	2.1
molecules	HOH A2110	2.7	A2106		HOH A2111	2.2
involved with	HOH A2102	1.7			HOH A2104	3.0
hydrogen	HOH A2111	2.4				
bonding	HOH A496	2.5				
	HOH A429	2.5				
	HOH A711	2.8				
Residue	Tyr A104	N/A	Tyr A104	N/A	Tyr A104	N/A
involved with	Phe A158		Tyr A108		Tyr A108	
hydrophobic	Pro A176		Phe A158		Phe A158	
interaction	Leu A189		Val A186		Pro A176	
	Val A192				Leu A189	
	Leu A260				Val A277	
Total number of interactions	15		7		13	

4.4.7.2 Ligand protein interaction maps of **92** and **97** with protein 6RZ5

Figure 4.19 and 4.20 displays the 3D and 2D interaction maps of **92** and **97** with CysLT₁ receptor protein (6RZ5). Compound **92** has a network of interactions such as hydrogen bonds and hydrophobic contacts with important amino acid residues of CysLT₁ protein (6RZ5) as shown in Table 4.5. Tyr B104, forms an average hydrogen bond with the oxygen of the sulfonamide group of the **92** at a distance of 2.5 Å. The hydroxy group of Ser B193 forms a weak hydrogen bond with the amide carbonyl group of **92** at a distance of 2.6 Å. Other residues form hydrophobic interactions with **92** (Table 4.5).



A

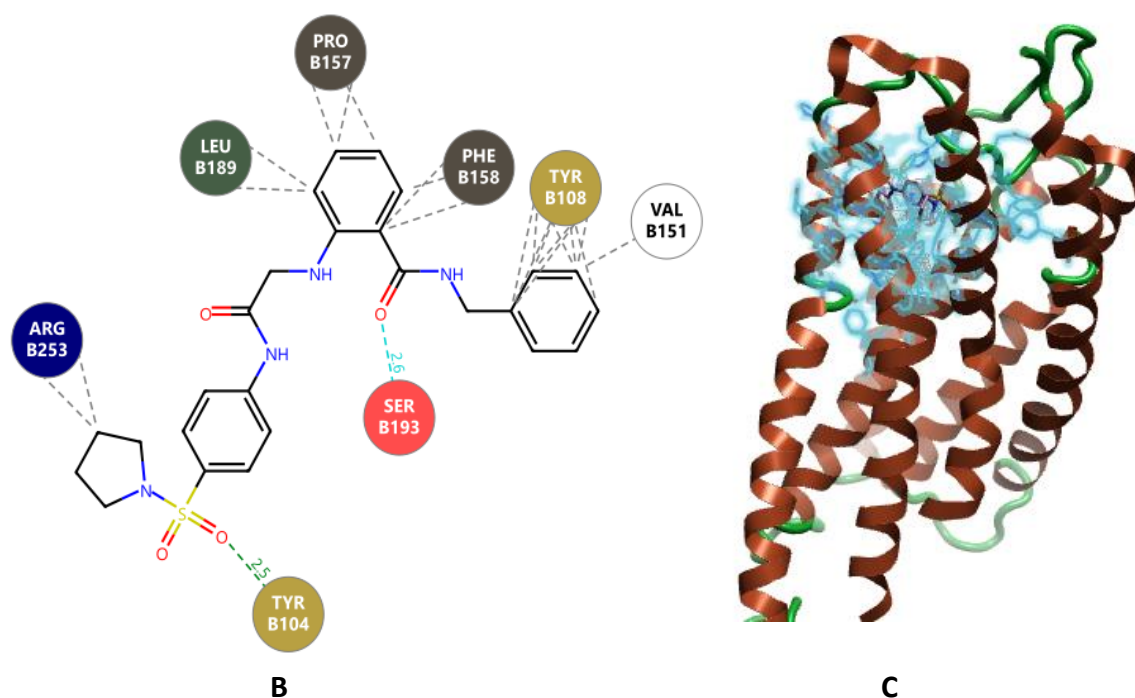
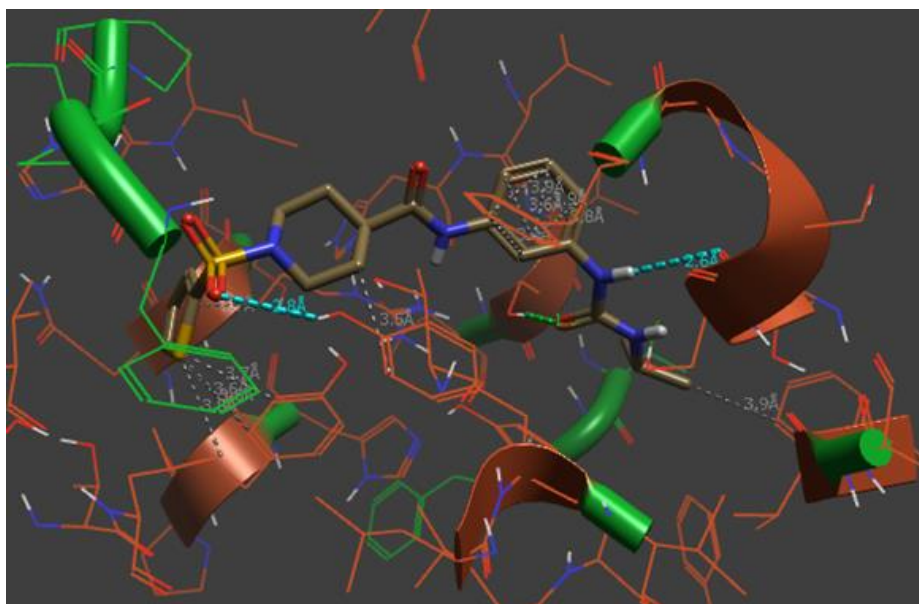
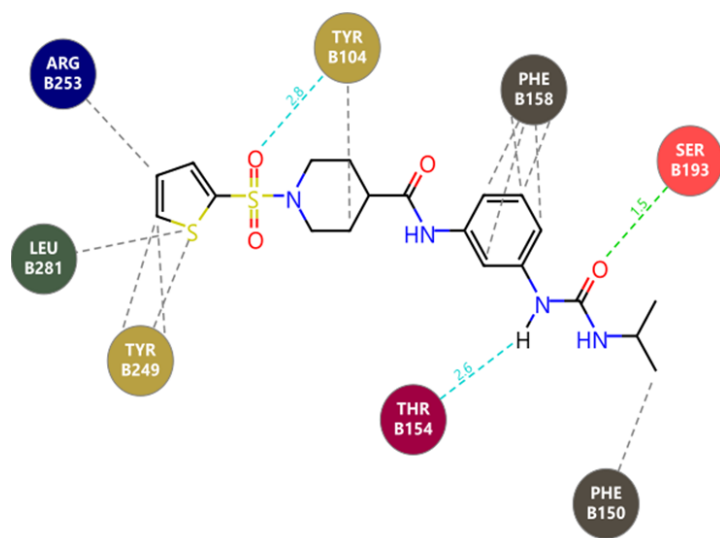


Figure 4.19: (A) 3D ligand-protein interaction diagram of 92 with CysLT₁ (6RZ5); (B) 2D ligand-protein interaction diagram of 92 with CysLT₁ (6RZ5); (C) Schematic cartoon model of 92 bound to CysLT₁ protein (6RZ5), active site highlighted in light blue. Each residue interacting with the ligand in 2D map is represented by a coloured circle reporting the residue's 3-letters code, chain number and colour according to the RasMol 'shapely' colour scheme. Molecular interactions defined as: strong/average hydrogen bonding: maximum distance = 2.6 Å, weak hydrogen bonding: maximum distance = 3.0 Å and hydrophobic atoms are up to 3.3 to 4 Å. The dotted lines which represent strong hydrogen bonds are coloured in light green, weak H-bonds in cyan and hydrophobic contacts in grey.

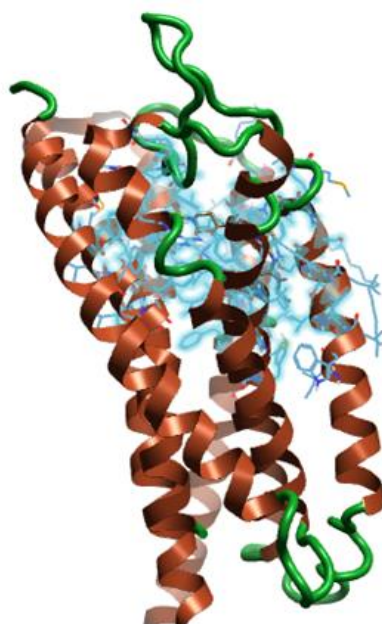
Compound **97** has a network of interactions including hydrogen bonds and hydrophobic interactions with 6RZ5 as shown in Table 4.5. The hydrogen atom of Ser B193 forms a strong hydrogen bond with the amide carbonyl group of **97** at a distance of 1.5 Å. The hydroxy group of Tyr B104 forms a weak hydrogen bond with the oxygen of the sulphonamide group of **97** at a distance of 2.8 Å. Other residues form hydrophobic interactions with **97** as shown in Table 4.5. Moreover, Leu B281 and Tyr 249 form hydrophobic interactions with the thiophene sulfur atom of **97**. Other residues form hydrophobic interactions with carbon atoms only. Some hydrophobic interactions are common with zafirlukast (Phe158).



A



B



C

Figure 4.20: (A) 3D ligand-protein interaction diagram of 97 with CysLT₁ (6RZ5); (B) 2D ligand-protein interaction diagram of 97 with CysLT₁ (6RZ5); (C) Schematic cartoon model of 97 bound to CysLT₁ protein (6RZ5), active site highlighted in light blue. Each residue, water molecule or cofactor interacting with the ligand in 2D map is represented by a coloured circle reporting the residue's 3-letters code, chain number and colour according to the RasMol 'shapely' colour scheme. Molecular interactions are defined as., hydrogen bonding: maximum distance = 2.6 Å, weak hydrogen bonding: maximum distance = 3.0 Å, and Hydrophobic atoms are C or S which are bonded only to C, S or H are up to 3.3 to 4 Å. The dotted lines which represent strong hydrogen bonds are coloured in light green, weak H-bonds in cyan and hydrophobic contacts in grey.

Compound **92** has 8 and **97** has 9 interactions with the residues of CysLT₁ protein (6RZ5) as shown in Table 4.5. Tyr B104 forms a hydrogen bond with a sulphonamide group present in both hits (**92** and **97**). Tyr B104 has weaker hydrogen bonding with both **92** and **97** than with zafirlukast. In comparison, Tyr B104 has two interactions with zafirlukast; the hydroxy group forms a strong hydrogen bond with amide the carbonyl group at a distance of 1.8 Å and a weak hydrogen bond with the sulfonamide group at a distance of 2.7 Å. Additionally, **97** contains a thiophene sulfur which forms a hydrophobic interaction with Leu B281. Leu B281 does not have any interaction with **92**.

Table 4.5: Ligand-protein interactions of zafirlukast, 92 and 97 with CysLT₁ protein (6RZ5).

Interactions	Zafirlukast	Distance Å	92	Distance Å	97	Distance Å
Residue involved with hydrogen bonding	Tyr B104	1.8 & 2.7	Tyr B104	2.5	Tyr B104	2.8
	Thr B154	1.8	Ser B193	2.6	Thr B154	2.6
	Ser B193	2.2			Ser B193	1.5
	Tyr B249	1.8				
Residue involved with hydrophobic interaction	Tyr B108	N/A	Arg A253	N/A	Leu B281	N/A
	Phe B112		Leu B189		Tyr B249	
	Pro B157		Pro B157		Arg B253	
	Phe B158		Phe B158		Phe B158	
	Leu B189		Tyr B108		Phe B150	
	Val B277		Val B151		Tyr B104	
	Tyr B249					
Total number of interactions	11		8		9	

4.4.8 Discussion of EC surfaces, Blaze scores, EC scores and radial plots of 85, 86, 92 and 97

4.4.8.1 EC surfaces of hit molecules

As discussed previously, EC maps highlight which parts of the ligand-protein interface are electrostatically suboptimal. The EC score provides information on how well the electrostatics of a ligand match that of the protein active site. Figure 4.21 displays the EC surface maps of hit molecules **85** and **86** for CysLT₁ protein (6RZ4). The perfect and clashing EC regions are coloured in green and red respectively. There is an electrostatic clash (red colour) between the pyrimidine ring of **85** and HOH A2111. In contrast, there is an electrostatic clash between HOH A2111 and the amine group of **86** as well as between benzene ring of **86** and the nitrogen of ARG A253 residue (**Figure 4.21 B**).

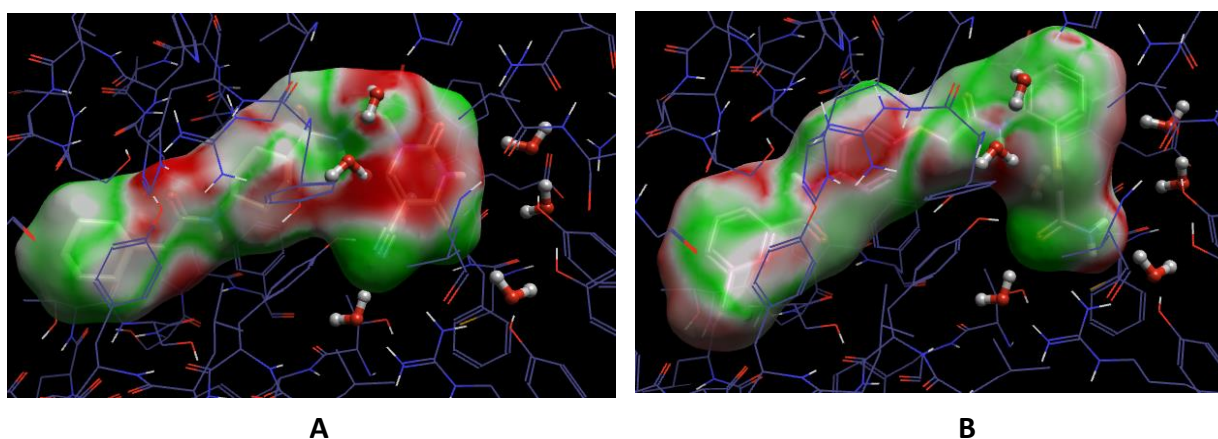


Figure 4.21: (A) EC surface for 85 with CysLT₁ (6RZ4); (B) EC surface for 86 with CysLT₁ (6RZ4). Poor electrostatic complementarity (red) and perfect electrostatic complementarity (green). Water molecules presented as ball and stick.

Figure 4.22 displays the EC surface maps of **92** and **97** with CysLT₁ (6RZ5). Both molecules **92** and **97** displays electrostatic clash (red) in key functional regions such as sulfonamide group, benzene ring and the secondary amide group. Both hits show electrostatic clash around the sulfonamide group. Overall, **97** show fewer overall electrostatic clashes than **92**, suggesting a more favourable electrostatic interaction profile. The reduced clashes in **97** could lead to improved binding affinity and stability within the CysLT₁ receptor protein 6RZ5. The EC scores will be further discussed with the Blaze scores and radial plot properties of all 4 hit molecules.

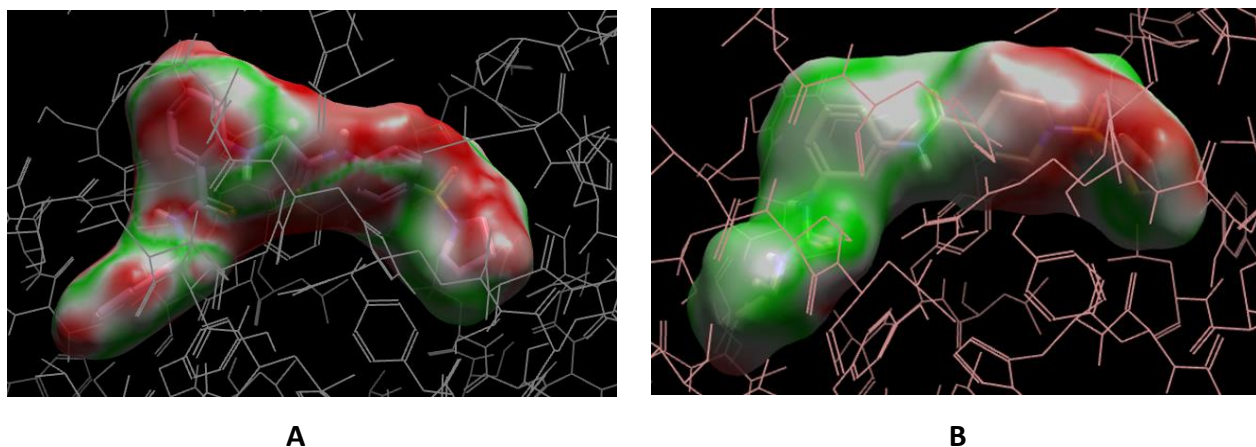


Figure 4.22: (A) EC surface for 92 with CysLT₁ (6RZ5); (B) EC surface for 97 with CysLT₁ (6RZ5). Poor electrostatic complementarity (red) and perfect electrostatic complementarity (green).

4.4.8.2 Blaze, EC scores and radial plots of hit molecules

As explained earlier in Section 4.4.5.2, radial plot properties represent numerical data for a molecule including calculated EC score, docking score, Blaze score and physicochemical properties such as molecular weight (MW), TPSA, lipophilicity (SLogP). The Blaze score indicates the similarity of the hit molecule to the reference ligand, the docking score indicates the binding affinity of docked hit molecule with proteins and the EC score is a computed normalized surface integral of the complementarity score that effectively displays the average value of the score over the ligand's surface. Blaze scores range from 0 to +1, docking scores, range from -12 to -15 and EC scores from 1 (perfect EC) to -1 (poor EC).

Figure 4.23 displays the Blaze, docking and EC scores, the radial plot window and physicochemical properties of CysLT₁ (6RZ4) hits **85** and **86** coloured according to the score values and how each value fits the profile set in the radial plot properties window. The Blaze scores of **85** and **86** are 0.642 and 0.640 respectively. The docking scores of **85** and **86** are -12.04 and -12.54 which lie within the range of -12 to -15 (a more negative docking score indicates a stronger protein-ligand complex). The EC scores of both molecules are 0.087 and 0.108. However, the EC value of pranlukast is 0.218 which is comparatively higher than the EC scores of **85** and **86**. The physicochemical properties of both hit molecules such MW, LogP, TPSA and Ro5 are acceptable as they are inside the radial plot window. Both hits have MW less than 480 and log P values less than 5. Pranlukast has a MW of 480 and a log P of 4.4 and showed no violations of Ro5. Compound **85** and **86** have no violations of Ro5 and could be further analysed for their biological activity. Moreover, the radial plot score (RPS) values of **85** and **86** are 0.404 and 0.410 which are comparatively better than pranlukast's RPS value of 0.267. In

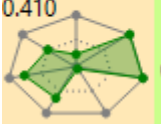
summary, selected CysLT₁ (6RZ4) hits **85** and **86** have more drug-like physicochemical properties as compared to pranlukast.

Radial Plot	MW	EC	#Atoms	SlogP	TPSA	Ro5
0.267 	480.5	0.218	36	4.4	114.1	0

A

Radial Plot	BLAZE_SCORE ▾	Docking Score	EC	MW	SlogP	TPSA	Ro5
0.404 	0.6423	-12.045	0.087	429.5	1.6	131.4	0

B

Radial Plot	BLAZE_SCORE ▾	Docking Score	EC	MW	SlogP	TPSA	Ro5
0.410 	0.6403	-12.545	0.108	449.5	3.8	110.5	0

C

Figure 4.23: (A) Table of Blaze scores, docking scores, EC scores and radial plot window of pranlukast; (B) CysLT₁ (6RZ4) hit 85; (C) CysLT₁ (6RZ4) hit 86. Radial plot window with an overall RPS 0 (unacceptable) are coloured in red, 0.5 (acceptable) are coloured in yellow, and 1 (acceptable) are coloured in green.

Note: Blaze and docking scores are only available for hit molecules.

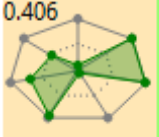
Figure 4.24 displays the Blaze, docking and EC scores, the radial plot window and physicochemical properties of CysLT₁ (6RZ5) hits **92** and **97** coloured according to the score values and how each value fits the profile set in the Radial Plot Properties window. The RPS values of **92** and **97** are 0.406 and 0.543 which are comparatively higher than zafirlukast's RPS value of 0.016.

The Blaze scores of **92** and **97** are 0.660 and 0.636 respectively. Both **92** and **97** have good docking scores (-12.02 and -12.05). The, EC scores of **92** and **97** are -0.041 and 0.089 respectively. Compound **92** has lower EC score and **97** has a higher EC score as compared to zafirlukast's EC score. The physicochemical properties of **92** are inside the radial plot window (LogP, TPSA and Ro5). Compound **92** has a MW 492 and a reasonable LogP value of 3.5 and does not violate Ro5. Compound **97** has a MW of 450.5 and LogP value of 3.3 leading to 0 violations of Ro5. In comparison,

zafirlukast has a MW 757 and a LogP of 5.7 and showed 2 violations of Ro5. Both hits of 6RZ5 do not violate the Ro5 and can be further progressed to *in vitro* and *in vivo* studies.

Radial Plot	MW	EC	#Atoms	SlogP	TPSA	Rof5
0.016 	574.7	-0.135	41	5.7	116.1	2

A

Radial Plot	BLAZE_SCORE ▾	Docking Score	EC	MW	SlogP	TPSA	Rof5
0.406 	0.6608	-12.024	-0.041	492.6	3.5	107.6	0

B

Radial Plot	BLAZE_SCORE	Docking Score	EC	MW	SlogP	TPSA	Rof5
0.543 	0.636	-12.053	0.089	450.6	3.3	107.6	0

C

Figure 4.24: (A) Table of Blaze scores, docking scores, EC scores and radial plot window of zafirlukast; (B) CysLT₁ (6RZ5) hit 92; (C) CysLT₁ (6RZ5) hit 97. Radial plot window with an overall RPS 0 (unacceptable) are coloured in red, 0.5 (acceptable) are coloured in yellow, and 1 (acceptable) are coloured in green.

Note: Blaze and docking scores are only available for hit molecules.

4.4.9 Evaluating the *in vitro* antagonist activity of CysLT₁ protein (6RZ4 and 6RZ5) hit compounds using human CysLT₁ receptor cellular based assay

Following LBVS to identify potential antagonists of the CysLT₁ receptor, an *in vitro* cellular-based assay was performed to evaluate the antagonist activity of selected 4 hit compounds **85**, **86**, **92** and **97**. The purpose of this assay was to evaluate the antagonist activity of the four compounds at the human CysLT₁ receptor expressed in transfected CHO cells (CHO-K1), determined by measuring their effect on LTD₄ agonist (0.1 nM) induced cytosolic calcium ion mobilization using a fluorometric detection method. Previous studies have shown that LTD₄ produces a potent agonist effect at the CysLT₁ receptor.¹⁷⁵ This experiment was performed as a commercial service by Eurofins in accordance with their validation standard operating procedure.

All four hit compounds (**85**, **86**, **92** and **97**) were tested at 20 and 50 µM, as these were the fixed concentrations provided by Eurofins for this assay. For the antagonist effect, results are expressed as % inhibition of the control reference agonist response for each selected hit molecule at both tested concentrations (**Figure 4.25**). HBSS buffer was used as a basal control, while the standard reference antagonist MK 571 with an IC₅₀ value of 1 nM was used as a positive control. Inhibition between 25 and 50% indicates weak to moderate effects, inhibition lower than 25% is not considered significant, and inhibition higher than 50% is considered to represent significant effects of the test compounds. 50% is the most common cut-off value for further investigation.

In this case, the hit molecules **85** and **86** showed inhibition lower than 25% at both tested concentrations, thus the values are not considered significant (**Table 4.6**). On the other hand, the hit molecule **92** showed moderate effects as its % inhibition is very close to the cut-off value of 50% at both concentrations (**Table 4.6**). Compound **97** showed the strongest antagonist effect as its % inhibition is higher than 50% (cut-off value) at both concentrations (**Table 4.6**). A positive control tested in this experiment was MK 571, with an IC₅₀ value of 1 nM. These findings collectively demonstrate that **97** effectively inhibited the LTD₄-induced calcium ion mobilization in CHO cells expressing the CysLT₁ receptor. Therefore, it suggests that **97** serves as antagonist of CysLT₁ receptor. The rank order of potency observed was: **97** > **92** > **85** > **86**. In summary, compounds **92** and **97** demonstrated the antagonist effect and will be utilised for further *in vivo* zebrafish studies.

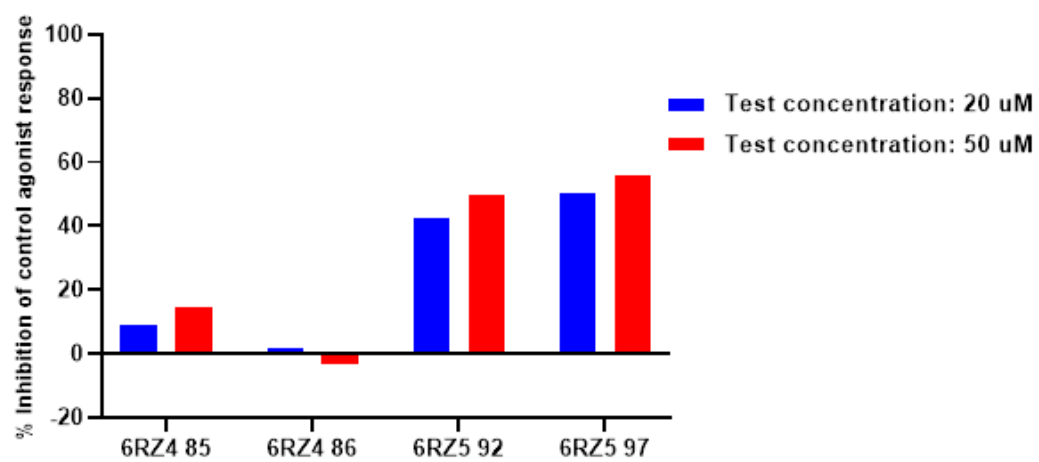
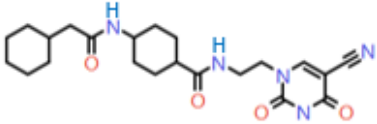
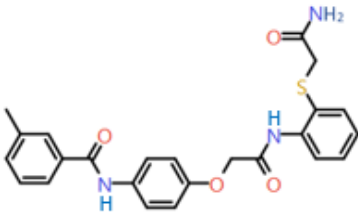
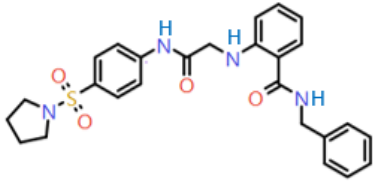
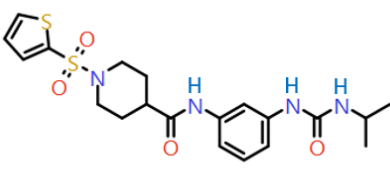


Figure 4.25: Antagonist effect on CysLT₁ receptor protein by 85, 86, 92, and 97 in CHO-K1 cells determined by measuring their effect on LTD₄ agonist (0.1 nM) induced cytosolic calcium ion mobilisation using a fluorometric detection method.

Table 4.6: Table of tested concentrations and % inhibition of control agonist response of selected hit molecules of CysLT₁ receptor protein (6RZ4 and 6RZ5).

CysLT ₁ Protein	Compound number	Structure of hits	Concentration (μ M)	% Inhibition of control agonist response
6RZ4	85		20	8.9
			50	14.7
	86		20	1.8
			50	-3.4
6RZ5	92 [#]		20	42.5
			50	49.6
	97 [#]		20	50.5
			50	56.0

Note: #: compounds 92 and 97 will be further tested in an in vivo zebrafish model.

4.5 Chapter summary

This chapter has primarily focused on the identification of CysLT₁ receptor antagonists by LBVS using Cresset's lead finder platform, Blaze. Crystal structures of CysLT₁ (6RZ4) with pranlukast and CysLT₁ (6RZ5) with zafirlukast at 2.7 and 2.5 Å resolution, respectively were used.¹³⁶ Pranlukast and zafirlukast were used as a reference template to generate a set of 47 and 43 hit molecules for CysLT₁ protein (6RZ4 and 6RZ5). From these, 4 hit molecules were selected for receptor-binding studies, and the inhibition levels for **92** and **97** were ≥ 50% of the control reference agonist response. In the *in vitro* receptor binding assay, compound **97** demonstrated the strongest antagonist effect, making it the best performer among the hit molecules.

In addition, Cresset's Flare software, was utilised to visualise the target structures and potential ligands within the binding site. The set of 4 selected hits identified in this work (**85**, **86**, **92** and **97**) is an interesting addition to this study. Future work could involve running molecular dynamics (MD) simulations to analyse the stability of the selected hit molecules with the target protein, free energy perturbation and SAR studies to improve potency.

In vivo toxicity and anti-angiogenic activity in a zebrafish model of **92** and **97** will be discussed in Chapter 5.

Chapter 5: Evaluation of *in vivo* toxicity and anti-angiogenic activity of small molecules using zebrafish model

5.1 Introduction

5.1.1 Zebrafish model for human disease studies

Zebrafish (*Danio rerio*) were introduced for genetic studies initially in the early 1980s.¹⁷⁶ The zebrafish model has emerged as a highly valuable model organism in disease and drug discovery research. Studies have proved genetic similarity between zebrafish and humans, with 70% of human genes having at least one zebrafish orthologue.¹⁷⁶ Moreover, over 80% of human disease-related genes have a zebrafish counterpart.¹⁷⁷ The genetic congruence between zebrafish and humans facilitates the modelling of human disease with high levels of accuracy.¹⁷⁸ While mouse models are continuing to be frequently used in medical research, zebrafish offer distinct advantages, including transparent embryos for real-time observation, rapid development, high fecundity, and cost-effectiveness.¹⁷⁹ All of these characteristics and evidence have increased the use of zebrafish as a model for comprehending human genetic disorders, such as cancer, and have highlighted their potential for *in vivo* screening of new therapies.¹⁷⁹ In cancer research, zebrafish models are used to study angiogenesis and metastasis across various types of cancers such as melanoma, pancreatic cancer and leukaemia.¹⁸⁰ The ability to visualize tumour growth, angiogenesis, and metastasis in live zebrafish is a significant advantage.¹⁸⁰ Additionally, high-throughput drug screening in zebrafish identifies compounds with potential anti-cancer properties.^{180, 181} Over the past decade, the zebrafish model has solidified its position as a robust platform with extensive applications in systems pharmacology and toxicology.¹⁸¹

In addition to their extensive application in cancer research, zebrafish can also be used for studying a spectrum of human diseases including cardiovascular, neurological and metabolic disorders.¹⁸² In summary, the zebrafish model is an indispensable tool in medical research due to its genetic similarity to humans and their rapid organ development.¹⁸² The zebrafish model provides profound insights into the mechanisms of various diseases and facilitate high-throughput drug screening.¹⁸³ As a result, it significantly contributes to the discovery and development of new treatments for a wide range of human diseases.¹⁸³

5.1.1.1 Zebrafish embryonic development

The transparent nature of zebrafish embryos and larvae permits direct observation of internal processes such as organ development and function, which is particularly advantageous for real-time studies of developmental biology and disease progression.¹⁸⁴ Zebrafish also exhibit rapid development with major organs forming within 24 hours post-fertilisation and embryos hatching by 2-3 days, allowing for rapid data generation.¹⁸⁵ Their embryonic development is characterised by well-defined stages, including cleavage, gastrulation, segmentation, and organogenesis, all of which

can be easily monitored and studied due to the transparency and external development of the embryos.¹⁸⁵ Figure 5.1 illustrates the embryonic developmental stages. After fertilisation, the blastomere undergoes cleavage and gastrulation. Somites, also known as segmented tissue units, are embryonic structures in zebrafish that give rise to vertebrae, skeletal muscles and dermis during early development. They begin forming between 10.33 hours post fertilisation (hpf) and continue through 24 hpf, indicating segmentation. At the 13-somite stage (24 hpf), the circulatory system becomes visible with a heartbeat, and organogenesis continues with the development of the neuromeres, otoliths, liver anlage, and intestinal anlage.¹⁸⁵ Pectoral fins form at 48 hpf, and the embryo becomes responsive to touch. Hatching typically occurs between 48 hpf and 72 hpf, marking the completion of early organogenesis.¹⁸⁵ At this stage, the free-swimming embryo possesses a protruding mouth, a functional pronephros, and an intestinal tract. Intravenous delivery can be performed starting from 24 hours to 48 hours.¹⁸⁵ The well characterised stages of zebrafish embryonic development features make zebrafish ideal for evaluating the *in vivo* toxicity of drugs, enabling real-time assessment of toxic effects of drugs on organ development and function.¹⁸⁶

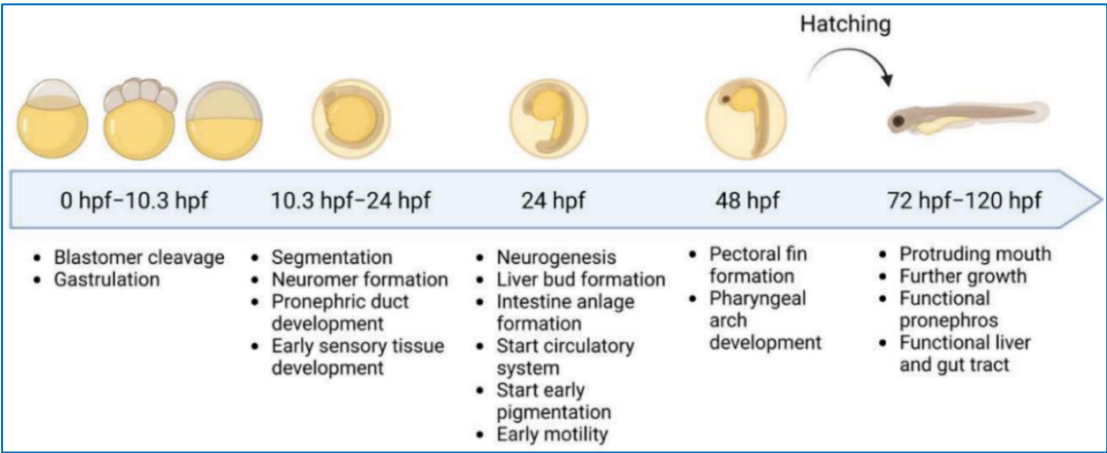


Figure 5.1: Schematic representation of early developmental stages of zebrafish embryo.

The diagram represents the zygote period when the embryo is at single cell stage runs from 0.75 to 2 hpf, then blastomer period runs from 2.2 to 5.3 hpf and gastrula period runs from 5.2 to 10 hpf. In the schematic diagram it shows that the segmentation period starts from bud stage (10 hpf) to somite stage (14 hpf) and segmentation period continues to 24 hpf.

Image is taken from Bondue *et al.*¹⁸⁵

5.1.2 Zebrafish model for *in vivo* toxicity screening of drugs

Toxicity testing is crucial in the evaluation of newly developed drugs prior to human use. It serves not only to determine the safety of a test substance but also to thoroughly characterise its potential toxic effects.¹⁸⁷ The primary principle of toxicity testing is to assess the effects of test substance on

laboratory animals, with the aim of understanding their potential toxic effects on humans.¹⁸⁷ Additionally, these tests involve exposing laboratory animals to high doses to evaluate potential hazards to humans who may be exposed at much lower doses.¹⁸⁷ In general, toxicity testing begins *in vitro* using various cell lines at different concentrations of drug substance. The substance is then tested in several animal models (rodents, dogs, rabbits) but these animal models required extensive data compilation for each substance from individual laboratories.¹⁸⁸ These tests are time consuming, costly and labour intensive. This traditional approach concentrated on observing the phenotypic effects of the chemical compounds in cells or animal model.¹⁸⁹ Hence, a more appealing method called the 3R concept (reduction, replacement and refinement) aims to minimise animal use.¹⁸⁹

Fish have traditionally been used in toxicity testing of individual substances and effluents. Nowadays, zebrafish are commonly utilized to test for developmental toxicity as well as general toxicity.¹⁸¹ Zebrafish reduce reliance on higher vertebrae (mice and rats) in early drug developmental stages, aligning with the principle of 3Rs. The zebrafish model has emerged as a suitable model for *in vivo* toxicity screening of drugs for potential use to treat human disease.¹⁸¹ The zebrafish model has been approved by the FDA for toxicity and safety evaluation for newly develop drugs.¹⁹⁰ The use of zebrafish embryos is increasingly favoured for examining the toxicity of drug candidates replacing the use of adult fish due to their transparent nature during early developmental stages which provides direct observation of organ development and toxic effects without invasive procedures.¹⁹⁰ Additionally, zebrafish embryos are cost effective, short-lived, have low variability, and are very useful in imaging studies.¹⁹⁰ Figure 5.2 shows a morphology of a normal zebrafish embryo at 48 hpf. At this stage major sensory organs (eyes) and craniofacial features are fully developed. Blood flow and un-looped heart region and various components of brain and spine can be distinguished (Figure 5.2).

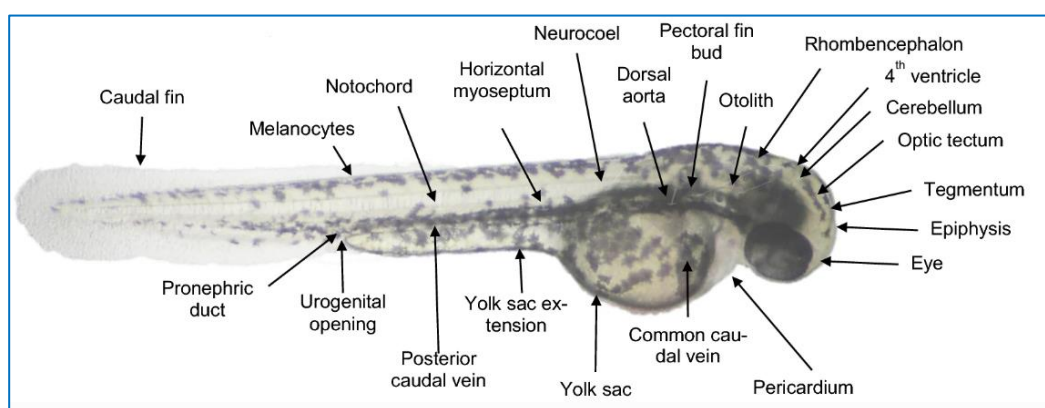


Figure 5.2: Diagram of a morphology of a normal 48 hpf zebrafish embryo

Image taken from Hellfeld *et al.*¹⁹¹

In vivo toxicity can be assessed by an acute toxicity experiment.¹⁸⁷ Acute toxicity is a short-term evaluation of a potential drug/chemical substance and provides a guideline to determine the appropriate dosage for long-term studies and the basis for designing other testing programs.¹⁸⁷ The embryos are treated with chemical substances at different concentrations, the data containing the adverse effect is obtained to generate a dose-response graph and LC₅₀ value (lethal concentration at which 50% of the embryos do not survive) is obtained which is a measure of compound's toxicity.¹⁹² A zebrafish embryo is considered dead when extensive full-body necrosis or early embryonic degeneration are observed.¹⁹²

In addition to lethal endpoints, acute toxicity can also reveal teratological effects/phenotypic defects which are abnormalities in growth and development of embryos.¹⁹³ The transparency of embryos facilitates the detection of teratological effects which include head and tail malformations (curved, and short), pericardial oedema, yolk sac oedema, and growth delays.¹⁹³ By integrating teratology into acute toxicity, researchers can gain a comprehensive understanding of lethal and non-lethal toxicities of chemical substances on embryonic development.¹⁹³

5.1.3 Angiogenesis and cancer

Angiogenesis is a physiological process through which new blood vessels form from pre-existing vessels, a crucial mechanism of growth, development and wound healing.¹⁹⁴ This process is tightly regulated by pro-angiogenic and anti-angiogenic factors.¹⁹⁵ These factors are molecules that either promote or inhibit the formation of new blood vessels.¹⁹⁵ However, in cancer, this balance is disrupted.¹⁹⁵ Tumour cells manipulate angiogenesis to their advantage by producing and releasing pro-angiogenic factors including VEGF briefly discussed in Chapter 3, fibroblast growth factors (FGFs) and others into the surrounding microenvironment (**Figure 5.3**).¹⁹⁶

VEGF is a signal protein produced by cells that stimulates the formation of blood vessels.¹⁹⁷ It binds to specific receptors on the surface of endothelial cells, which are the cells lining the inside of blood vessels.¹⁹⁷ This binding activates various signalling pathways that promote the proliferation, migration and survival of these cells, leading the formation of new blood vessels.¹⁹⁷ These new vessels supply the tumour with oxygen and nutrients. Among activated endothelial cells, some differentiate into tip cells. Tip cells lead the sprouting process (finger-like projections) that guide the new vessel's direction.¹⁹⁷ Furthermore, FGFs belong to the family of growth factors and promote growth and differentiation of endothelial cells. FGFs bind to FGF receptors on the cell surface, triggering signalling cascades that result in cell proliferation, migration and the formation of new blood vessels.¹⁹⁸

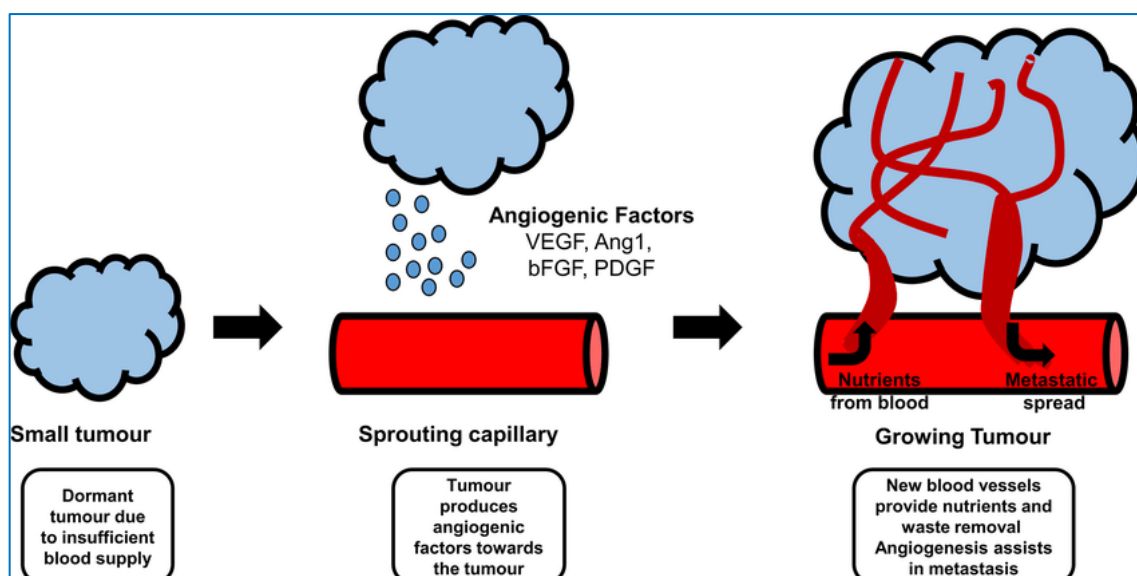


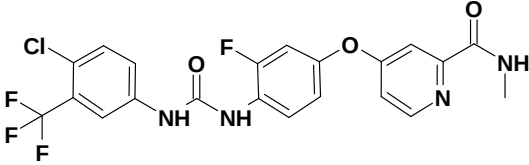
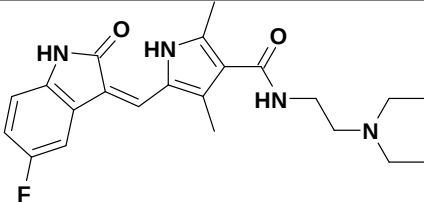
Figure 5.3: Schematic diagram of tumour angiogenesis process.

The process of angiogenesis in tumour growth involves three main stages: (1) a small tumour remains dormant due to insufficient blood supply, (2) the tumour releases angiogenic factors such as VEGF, FGF, Ang1, and PDGF, which stimulate new blood vessel formation (sprouting capillaries) and (3) these new blood vessels support tumour growth by providing nutrients and removing waste, while also facilitating metastatic spread to other tissue.

Image taken from Olsen *et al.*¹⁹⁹

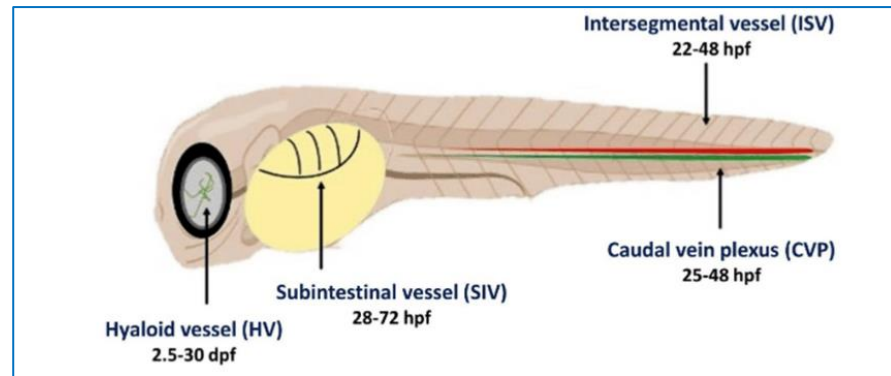
In summary, dysregulated angiogenesis is a hallmark of cancer that drives tumour growth, progression and metastasis.²⁰⁰ Clinically, targeting angiogenesis is well established as a promising strategy in cancer therapy. Angiogenic inhibitors aim to disrupt the formation and function of tumour blood vessels, thus starving the tumour of its blood supply and impeding its growth.²⁰⁰ Table 5.1 shows examples of FDA approved drugs that inhibit angiogenesis by targeting VEGF. These anti-angiogenesis strategies are used together with other anticancer chemotherapeutics.²⁰⁰ For instance, bevacizumab specifically targets VEGF and has been successfully used in combination with chemotherapy to improve outcomes for the cancer patient.²⁰⁰ However, it was observed that the side effects of bevacizumab increased when it was used in the treatment of colorectal cancer in combination with IFL (a chemotherapeutic regimen consisting irinotecan, fluorouracil and leucovorin) leading to bleeding complications.²⁰⁰ Furthermore, it was observed that the combination therapy of bevacizumab with paclitaxel improved the outcomes for advanced non-small lung cancer patients but severe pulmonary haemorrhage was observed.²⁰⁰ In the pursuit of understanding the intricate process of angiogenesis in cancer, the utilization of innovative model systems such as zebrafish has been invaluable.²⁰¹

Table 5.1: Examples of FDA approved anti-angiogenic agents.

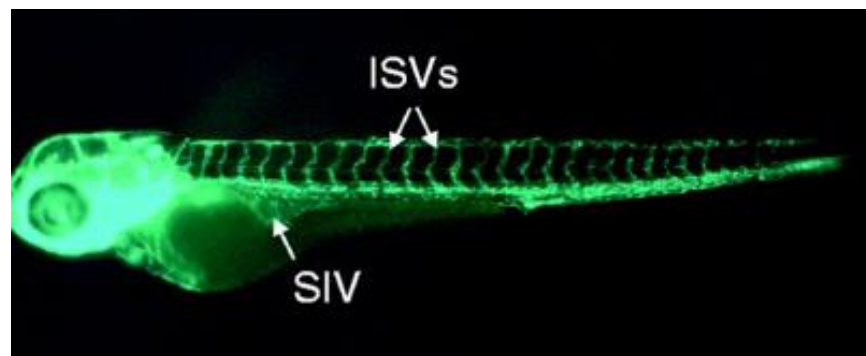
Compound number	Angiogenesis Inhibitor generic name	Chemical structure	FDA-approved indication
100	Bevacizumab	N/A: Monoclonal antibody	Colorectal and non-small lung cancer
101	Regorafenib		Colorectal and gastrointestinal cancer
102	Sunitinib		Gastrointestinal cancer

5.1.3.1 Zebrafish angiogenesis screens

As discussed earlier in this chapter zebrafish have emerged as a valuable model organism in biomedical and drug development research. The transparent nature of zebrafish allows direct observation of blood vessel development using advanced imaging techniques.²⁰¹ This combined with their rapid development and genetic similarity of their angiogenic pathway to those in humans, makes an ideal model for studying the dynamics of their blood vessel formation.²⁰¹ In angiogenesis screens zebrafish are typically exposed to chemical libraries to observe the resultant effects on vascular development. Vasculogenesis in the zebrafish begins at 12 hpf.²⁰² By 24 hpf angiogenic intersegmental blood vessels (ISVs) begin to form and are fully developed at 48 hpf.²⁰² Sub-intestinal vessels (SIVs) start developing in the gut at 28 hpf and reach full maturity at 72 hpf facilitating the evaluation of anti-angiogenic agents.²⁰² Figure 5.4 (A) displays an image of the development stage and periods of angiogenic blood vessel within the zebrafish. Moreover, during an angiogenic screen, transgenic zebrafish lines (*Tg (fli1: EGFP)*) are utilised which express enhanced green fluorescent proteins (EGFP) in its vasculature, thus enabling real time analysis and quantification of angiogenesis (**Figure 5.4 B**).²⁰³ These screens facilitate the identification of compounds that can either promote or inhibit angiogenesis, offering insights into potential treatments for cancer.²⁰³



A



B

Figure 5.4: Angiogenic blood vessels formation within the zebrafish embryos. (A) Development stage and periods for the formation of angiogenic blood vessels; (B) Fluorescent microscopic image of vasculature of 48 hpf zebrafish embryo displaying ISV (blood vessels located between the somites) and SIV (a major vein located beneath the intestines).

Images A and B are taken from Tang *et al.*¹⁹¹

5.2 Specific aims of chapter 5

1. To evaluate the *in vivo* toxicity and safety profile of pyrazinib (**54**) and its isoleucine prodrug (**69**), CysLT₁ targeting analogues **70**, **76** and 6RZ5 hit molecules (**92** and **97**). Structures of all the compounds are shown in Figure 5.5.
2. To evaluate the anti-angiogenic activity of compounds **54**, **69**, **70**, **76**, **92** and **97** in blood vessels (SIV and ISV) of zebrafish embryos.

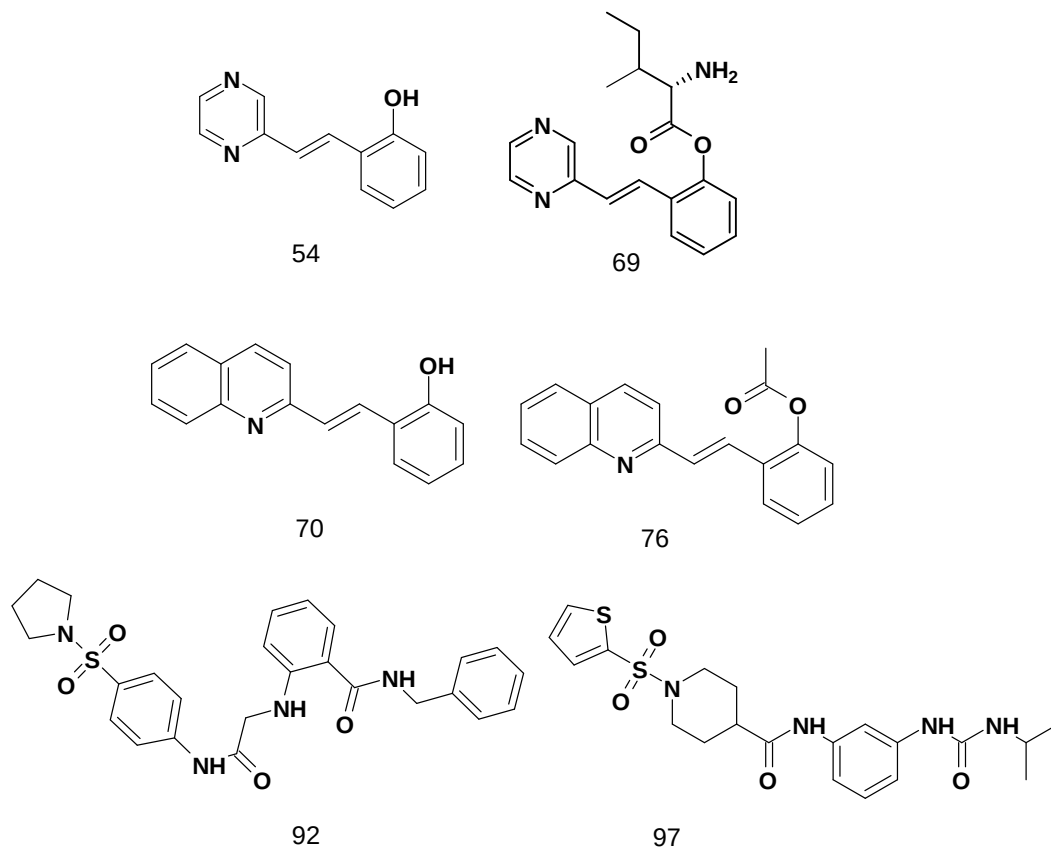


Figure 5.5: Chemical structure of compounds **54**, **69**, **70**, **76**, **92** and **97** evaluated for *in vivo* toxicity and anti-angiogenic activity in zebrafish model.

5.3 Materials and methods

All experiments were performed in accordance with Bioreperia's standard operating procedures (SOPs) and guidelines.

5.3.1 Zebrafish maintenance and eggs collection

Adult zebrafish (*Danio rerio*) were used for breeding and as a source of fertilized eggs. Adult zebrafish were maintained in an aquarium containing RO (reverse osmosis) water, with red ocean sea salt added to achieve a conductivity of ~800-1500 mS. Breeding groups of female: male (4:2) adult fish were placed in the aquarium the evening before the collection of eggs. The following morning, eggs were collected from the breeding tank and placed in a petri dish with the help of a sieve containing E3 medium [embryo growth medium (0.29 M NaCl, 0.01 M KCl, 0.01 M CaCl₂·2H₂O, 0.04 M MgCl₂·6H₂O in 2L water)]. Then, developed fertilised eggs were selected under microscope for the experimental studies.

5.3.2 Stock preparation of small molecules

Compounds **54**, **69**, **70**, **76**, **92**, **97** and montelukast were dissolved in 100% DMSO to make a 300 mM stock solution for experimental use and stored in -20 °C. All other reagents such as E3 and PTU medium were prepared as described in Sections 5.3.1 and 5.3.4.

5.3.3 *In vivo* toxicity assay

The *in vivo* toxicity was assessed at a range of concentrations. Each drug was tested individually in five concentrations selected from (312.5, 62.5, 12.5, 2.5, 0.5 and 0.1 µM) prepared from its stock solution and diluted in E3 medium. Zebrafish embryos 48 hours post fertilisation (hpf) (*Tg (fli1: EGFP)*) in E3 medium were distributed into a 96 well plate, one embryo in each well. For each drug, 100-120 embryos were used, i.e., 20 embryos per concentration. The E3 medium was removed from the wells and embryos were treated with 100 µL of DMSO (vehicle control: 0.1% v/v in E3 medium), **54**, **69**, **70**, **76**, **92**, **97** and montelukast. The embryos were incubated at 28°C for 72 hours and examined for lethal and non-lethal toxicities under a microscope (Nikon SMZ 1500) at timepoints of 24, 48 and 72 hours (**Figure 5.6**). Lethal toxicity refers to the ability of a drug to cause the death of zebrafish embryos, whereas non-lethal toxicity that can cause varying degrees of phenotypic defect within zebrafish embryos without resulting in death. Percentage alive (%) was calculated as follows:

$$\frac{\text{Total number of fish alive}}{\text{Total number of fish exposed}} \times 100$$

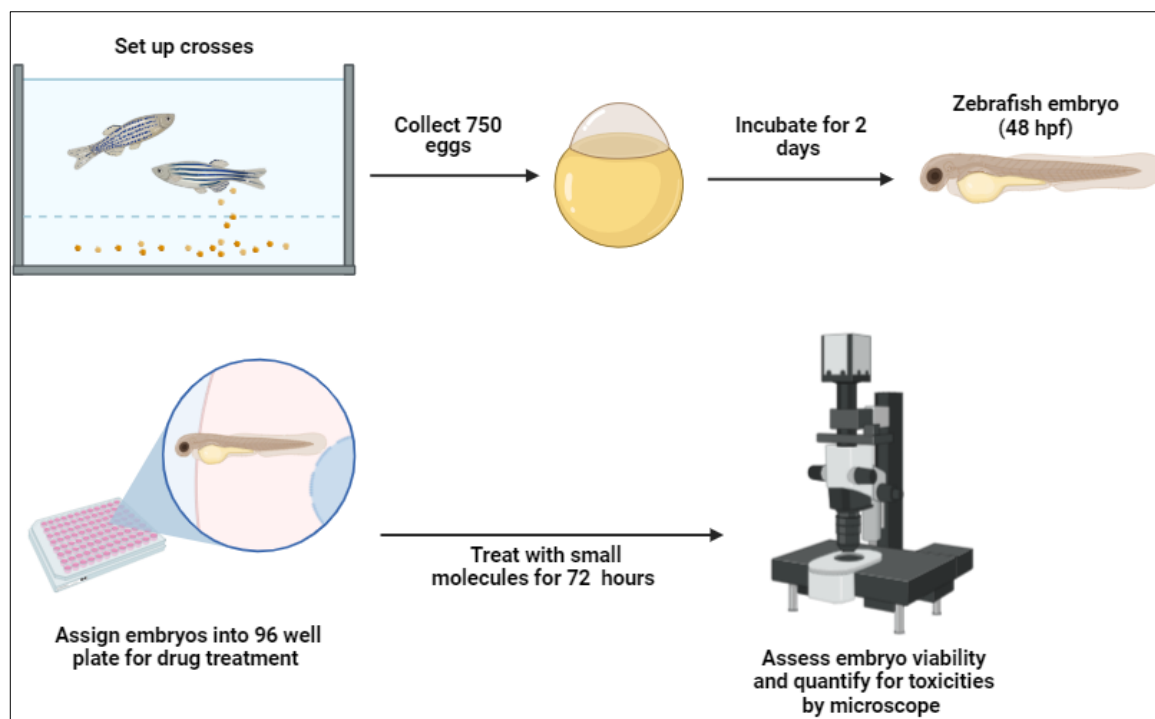


Figure 5.6: Schematic diagram of in vivo toxicity assay in *Tg (fli1: EGFP)* zebrafish embryos. Eggs were collected following mating and placed in an incubator at 28 °C. 48 hpf embryos were plated in 96 well plate, treated with small molecules (54, 69, 70, 76, 92, 97, and montelukast) at concentrations from 312.5 to 0.1 μ M for 72 hours and examined for lethal and non-lethal toxicities under microscope at timepoints of 24, 48 and 72 hours.

5.3.4 Sub-intestinal vessels (SIV) assay

Zebrafish embryos [*Tg (fli1: EGFP)*] were collected and kept at 28°C in PTU medium (0.03% PTU in 16.5 mL E3 medium). When the zebrafish embryos reached 48 hpf, they were distributed into 96 well plate using 10 embryos per drug. The PTU medium (100 μ L) was removed from each well and embryos were treated with DMSO (0.1% v/v in PTU medium), **54** (12.5 μ M), **69** (12.5 μ M), **92** (312.5 μ M), **97** (62.5 μ M) and montelukast (12.5 μ M), respectively. Embryos were incubated at 28°C for 24 hours. Following treatment, the embryos were anesthetised and imaged at 72 hpf under fluorescence microscope (Nikon SMZ 1500) (**Figure 5.7**).

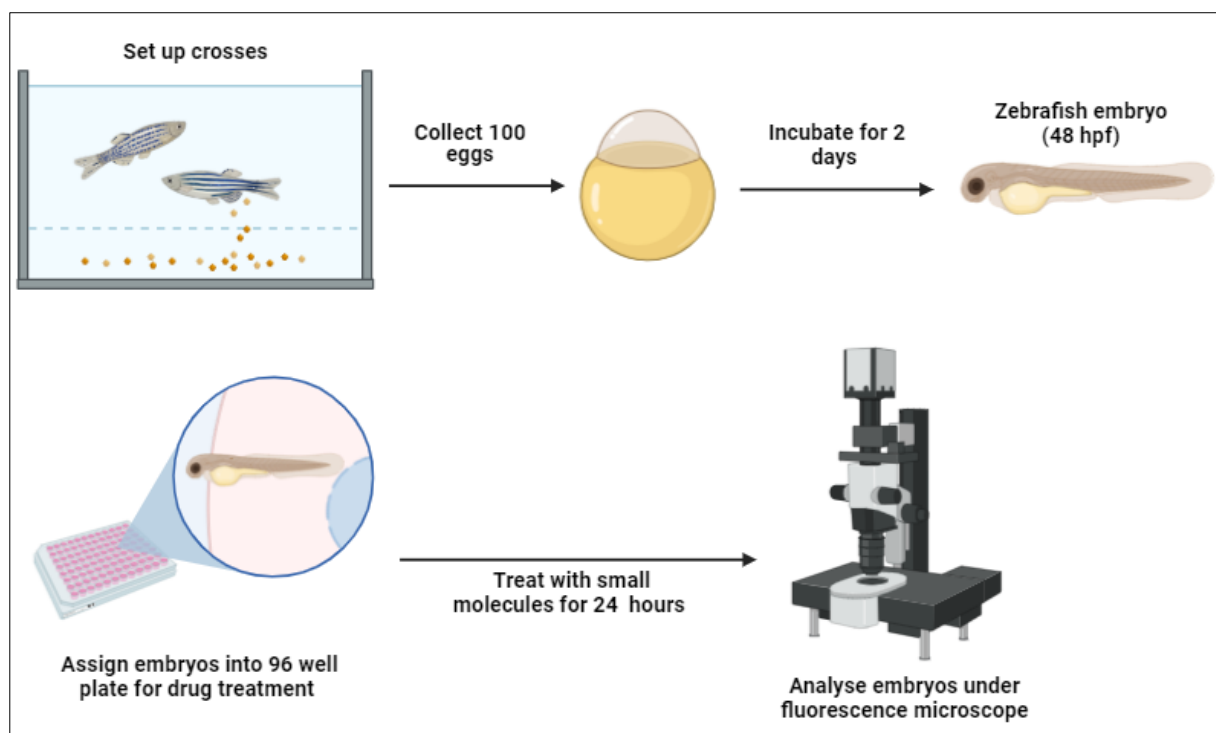


Figure 5.7: Schematic diagram of anti-angiogenic assay in SIVs using *Tg (fli1: EGFP)* zebrafish embryos. Eggs were collected following mating and placed in an incubator at 28 °C. 48 hpf were plated in 96 well plate, treated with small molecules (54 at 12.5 μ M, 69 at 12.5 μ M, 92 at 312.5 μ M, 97 at 62.5 μ M and montelukast at 12.5 μ M) for 24 hours and analysed under fluorescent microscope.

5.3.5 Quantification of SIVs

The anti-angiogenic activity of the drugs was determined on the basis of, firstly, the number of ectopic sprouts protruding out of the SIV basket structure and, secondly, the average area of a SIV basket. Zebrafish (72 hpf) were examined under fluorescence microscope (Nikon SMZ 1500) for the presence of ectopic sprouting and area of treated SIVs. The average number of SIV sprouts and average area of SIVs of zebrafish embryos was quantified using ImageJ software (Java based image processing programme). Both the number of ectopic sprouts and the area of the SIV baskets in drug-treated groups were compared to the DMSO-treated control group.

5.3.6 Intersegmental vessels (ISV) assay

4 hpf [*Tg (fli1: EGFP)*] zebrafish embryos were plated 1 per well in a 96 well cell plate, using 10 embryos per drug in PTU medium (0.03% PTU in 16.5mL E3 medium). The PTU medium (100 μ L) was removed and embryos were treated with 100 μ L of DMSO (0.1% v/v in PTU medium), **54** (12.5 μ M), **69** (12.5 μ M), **70** (12.5 μ M), **76** (12.5 μ M) **92** (312.5 μ M), **97** (62.5 μ M) and montelukast (12.5 μ M), respectively. Drug treated embryos were incubated at 28°C for 20 hours. After treatment, the embryos were anaesthetised and imaged at 24 hpf under fluorescence microscope (Nikon SMZ 1500) as shown in Figure 5.8.

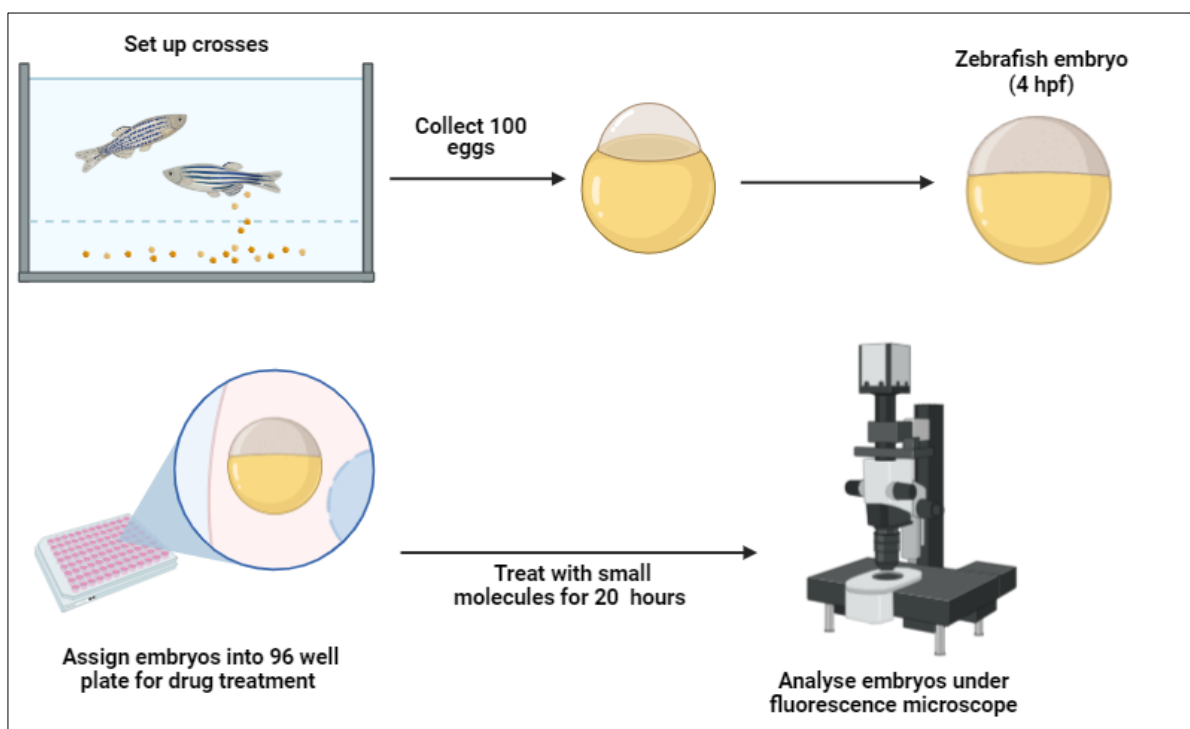


Figure 5.8: Schematic diagram of anti-angiogenic assay in ISVs using *Tg (fli1: EGFP)* zebrafish embryos. Eggs were collected following mating and placed at room temperature. 4 hpf were plated in 96 well plate, treated with small molecules (**54** at 12.5 μ M, **69** at 12.5 μ M, **70** at 12.5 μ M, **76** at 12.5 μ M **92** at 312.5 μ M, **97** at 62.5 μ M, and montelukast at 12.5 μ M) for 24 hours and analysed under fluorescent microscope.

5.3.7 Quantification of ISVs

To assess the anti-angiogenic effect of the tested compounds, the length of intersegmental vessels (ISVs) in zebrafish embryos was quantified. Zebrafish embryos (4 hfp) were treated with different compounds (**54**, **69**, **70**, **76**, **92**, **97** and montelukast). Zebrafish (24 hpf) were examined under fluorescence microscope (Nikon SMZ 1500). ISV length was measured from the point where the vessel branches off from the dorsal aorta (DA) to the dorsal longitudinal anastomotic vessel (DLAV) (**Figure 5.9**). Quantification was performed using ImageJ software (Java based image processing programme). For each embryo, at least 10 ISVs were measured, and the average ISV length was calculated. This procedure was repeated for each experimental group (N= 10 embryos per drug). The mean ISV length for each treatment group was compared to the DMSO control group to assess the extent of angiogenesis inhibition.

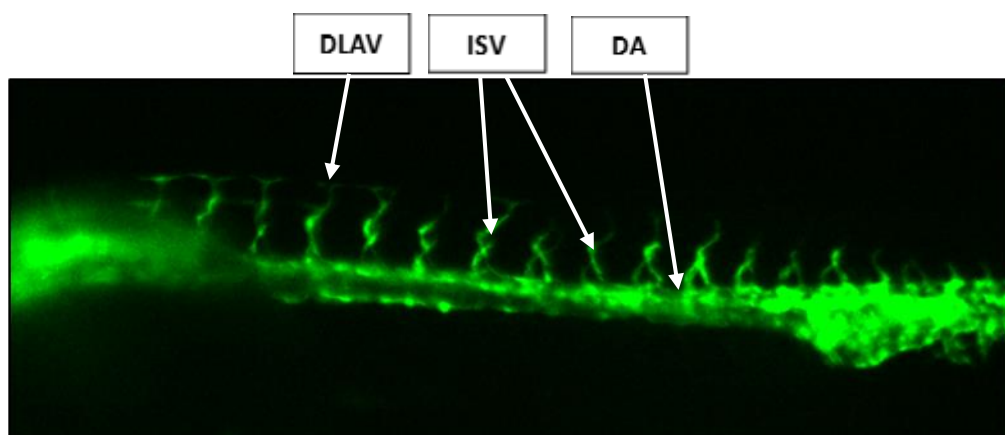


Figure 5.9: A normal intersegmental vasculature of a 24 hpf zebrafish embryo highlighting major structures such as dorsal longitudinal anastomotic vessel (DLAV), intersegmental vessel (ISV) and dorsal aorta (DA).

5.3.8 Imaging

For all assays, a Nikon SMZ 1500 microscope and a digital camera was used for analysis and taking images. Following treatment, embryos were treated with 1 drop of tricaine (anaesthesia) until they were unresponsive to touch. Prior to imaging, embryos were positioned in the centre of a well and images were taken at a magnification of 3x for *in vivo* toxicity assay, 8x for SIV angiogenic assay and 10x for ISV angiogenic assay.

5.3.9 Statistical analysis

Significance between control group and treatment groups were evaluated by one-way analysis of variance (ANOVA) in GraphPad Prism v8.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Error bars represent mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ns (non-significant). (N=10-20).

5.4 Results and discussion

To demonstrate the application of zebrafish angiogenesis and toxicity assays, the effects of various of small molecules studied in this thesis including Q analogues (**70** and **76**), hit molecules from LBVS (**92** and **97**), pyrazinib (**54**) and its prodrug (**69**) were investigated on zebrafish embryos. Initially, the *in vivo* toxicity of these compounds was evaluated to establish safe concentration ranges suitable for subsequent anti-angiogenic evaluations.

5.4.1 Evaluation of *in vivo* lethal and non-lethal toxicity of small molecules in zebrafish embryos.

5.4.1.1 Lethal toxicity effects in zebrafish embryos

It is often difficult to translate toxic effects from cell culture to *in vivo* toxicology. The zebrafish model fills a niche between *in vitro* and *in vivo* models. General toxicity determines the lethal and non-lethal toxicities from microscopic examination of the embryos over three days. To evaluate the lethal toxic effects of **54**, **69**, **70**, **76**, **92**, **97** and montelukast, zebrafish embryos (48 hpf) were screened at various concentrations to determine the lethal concentration (LC₅₀) at which 50% of the embryos do not survive.

The exposure of zebrafish embryos to different concentrations of drugs revealed that toxicity is both time and concentration dependent. In order to determine LC₅₀, a dose response curve of each drug was generated based on timepoints of 24, 48 and 72 hours as shown in figure 5.9. DMSO (0.1% v/v) used as a vehicle control exhibited no lethal effects across all three timepoints, indicating low toxicity.

Compound **69** (0.1 -312.5 μ M) showed lethal effects at highest dose (312.5 μ M) while its parent drug, **54** (0.1-312.5 μ M) resulted in 100% mortality at higher doses (62.5 and 312.5 μ M) within 24 hours of exposure. The LC₅₀ value was calculated as an average of all three time points for both compounds from dose response curves (**Figure 5.10 A and B**). The LC₅₀ revealed that **54** has a lower LC₅₀ (32 μ M) compared to its prodrug, **69** (158 μ M) indicating higher toxicity for **54** (**Table 5.2**).

Q analogues, **76** (0.1 – 312.5 μ M) and its parent drug **70** (0.1 – 312.5 μ M) also exhibited lethal effects at the highest dose (312.5 μ M), causing 100% embryo mortality within 24 hours (**Figure 5.10 E and F**). The LC₅₀ values of **76** (157 μ M) and **70** (142 μ M) were comparable, with **76** being slightly less toxic than **70** (**Table 5.2**).

The *in vivo* toxicity of selected hit compounds **92** and **97** was evaluated alongside montelukast, a clinically approved CysLT₁ antagonist (discussed in Chapter 4). Compounds **92** (0.5 – 312.5 μ M), **97** (0.5 – 312.5 μ M) and montelukast (0.02 – 12.5 μ M) exhibited no lethal toxic effects, with over 80%

embryo survival at highest concentrations tested across at all time points (**Figure 5.10 C, D and G**). Consequently, their LC₅₀ values were not calculated (**Table 5.2**).

A drug is considered non-toxic, if more than 50% of embryos remain alive. This *in vivo* toxicity data suggests that **92**, **97** and montelukast exhibit no significant toxicity associated at all tested concentrations. In contrast, compounds **69**, **70** and **76** demonstrated lethal toxic effects at highest concentration (312.5 µM), while compound **54** showed lethality at both 62.5 and 312.5 µM.

This study also monitored non-lethal effects within the zebrafish embryos upon drug exposure such as developmental abnormalities, changes in physical behaviour or physiological alterations of zebrafish embryos (discussed in Section 5.4.1.2).

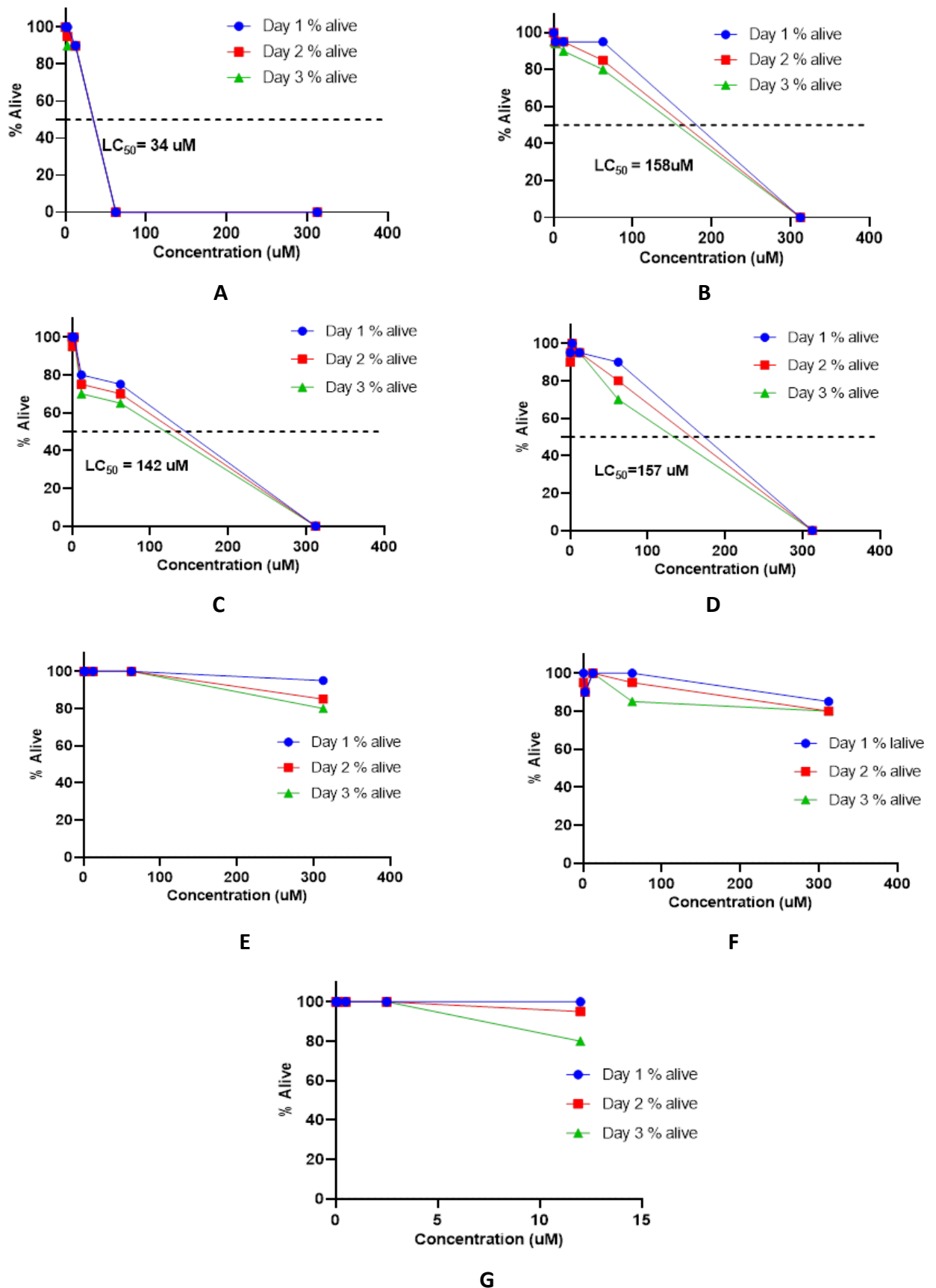


Figure 5.10: Dose response curves of zebrafish embryos (48 hpf) exposed to (A) 54; (B) 69; (C) 70; (D) 76; (E) 92; (F) 97 and (G) Montelukast based on 24-, 48- and 72-hours exposure timepoints (N=20).

Table 5.2: LC₅₀ data from the in vivo toxicity assay using zebrafish embryos based on 24, 48 and 72-hours exposure of small molecules.

Compound number	Test compound	Exposure time (hours)	LC ₅₀ (μM) values
54	Pyrazinib	24	34
		48	34
		72	34
69	Pyrazinib isoleucine	24	161
		48	158
		72	155
70	-	24	145
		48	142
		72	140
76	-	24	161
		48	155
		72	155
92	-	24	Nd
		48	Nd
		72	Nd
97	-	24	Nd
		48	Nd
		72	Nd
-	Montelukast	24	Nd
		48	Nd
		72	Nd

Note: Nd = not detectable, > 50% embryos were alive.

5.4.1.2 Non-lethal toxicity effects in zebrafish embryos

During *in vivo* toxicity studies, zebrafish embryos were evaluated for non-lethal toxicities (NLTs) leading to phenotypic defects such as pericardial oedema, yolk sac oedema, chorion defects, tail deformations (upcurved and short tail fish), and pigmentation loss after drug exposure and microscope examination of embryos over three days; 24, 48, and 72 hours. Figure 5.10 illustrates the percentage of embryos with NLTs across different concentrations and time points, and Figure 5.11 illustrates healthy embryos and examples of phenotypic defects from different tested groups. Control groups treated with DMSO (0.1% v/v) exhibited no spontaneous defects, indicating healthy embryo development (**Figure 5.12 A**).

Compound **69** showed consistent pericardial oedema across all concentrations and time points, sporadic yolk sac oedema at higher concentrations, chorion and tail deformations at various concentrations, and no pigmentation loss, with an overall NLT incidence of 5-15%. Moderate (2.5 μ M) and higher concentrations (12.5 μ M and 62.5 μ M) indicated cumulative toxicity with increasing NLTs, respectively (**Figure 5.11 B**). In contrast, **54** exhibited frequent pericardial oedemas across all concentrations, yolk sac oedema at higher concentrations and later time points, chorion defects, consistent tail deformations, and no pigmentation loss, with an NLT incidence of 5-15%. Lower concentrations (0.1 μ M, 0.5 μ M, and 2.5 μ M) maintained stable NLTs (5%), while the highest concentration (12.5 μ M) showed a sharp increase in NLTs (15%) by Day 3, indicating significant cumulative non-lethal toxicity (**Figure 5.11 A**).

Compound **76** demonstrated varied pericardial oedema, more frequent at higher concentrations, common yolk sac oedema, chorion defects, tail deformations, and rare pigmentation loss, with an NLT incidence of 5-15% (**Figure 5.11 F**). Compound **70** showed pericardial oedema at higher concentrations and early time points, yolk sac oedema at most concentrations, common chorion defects, frequent tail deformations at higher concentrations, and some pigmentation loss, with an overall NLT incidence of 5-15% (**Figure 5.11 E**). Furthermore, CysLT₁ hit antagonists, **92**, **97** and montelukast exhibited occasional tail deformations at higher concentrations and some pigmentation loss, with an NLT incidence of 0-5%, indicating minimal non-lethal toxicities (**Figure 5.11 C, D and G**).

In the study assessing the toxicity of various compounds using zebrafish embryos, pericardial oedema yolk sac oedema, chorion defects, tail deformations, and pigmentation loss emerged as significant indicators of non-lethal toxicities (NLTs) induced by each tested compound. **69** and **54** showed high incidences of multiple defects, particularly pericardial oedema and tail deformations. They demonstrated dose-dependent toxicity. Compound **76** and **70** also induced moderate incidences, frequently causing pericardial and yolk sac oedema. **92**, **97**, and montelukast displayed

minimal defects with NLT incidences ranging from negligible to low percentages, suggesting low toxicity at the tested concentrations.

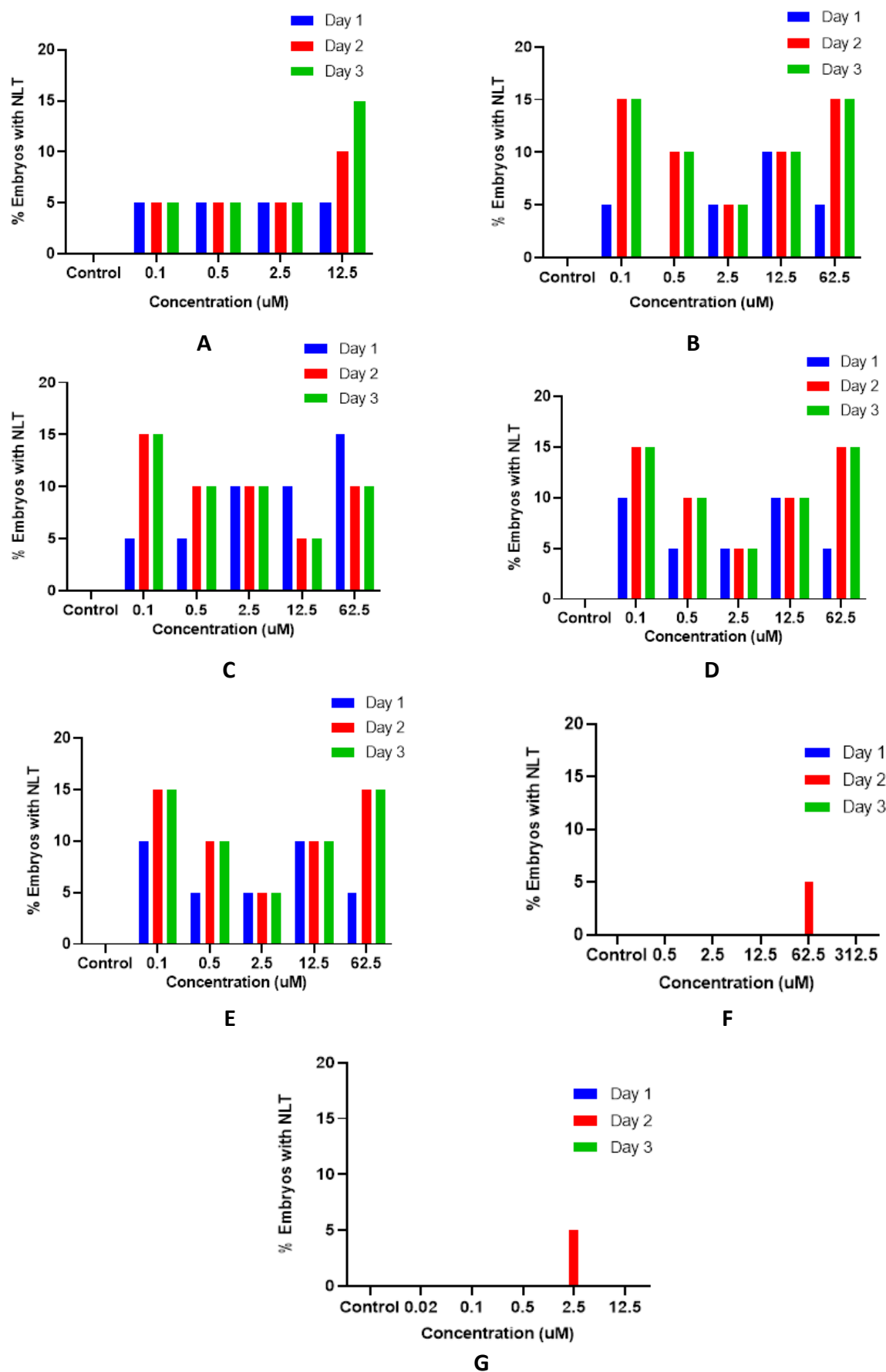


Figure 5.11: Non-lethal toxicities within zebrafish embryos caused by (A) 54; (B) 69; (C) 70; (D) 76; (E) 92; (F) 97 and (G) montelukast at timepoints of 24, 48 and 72 hours. N=20.

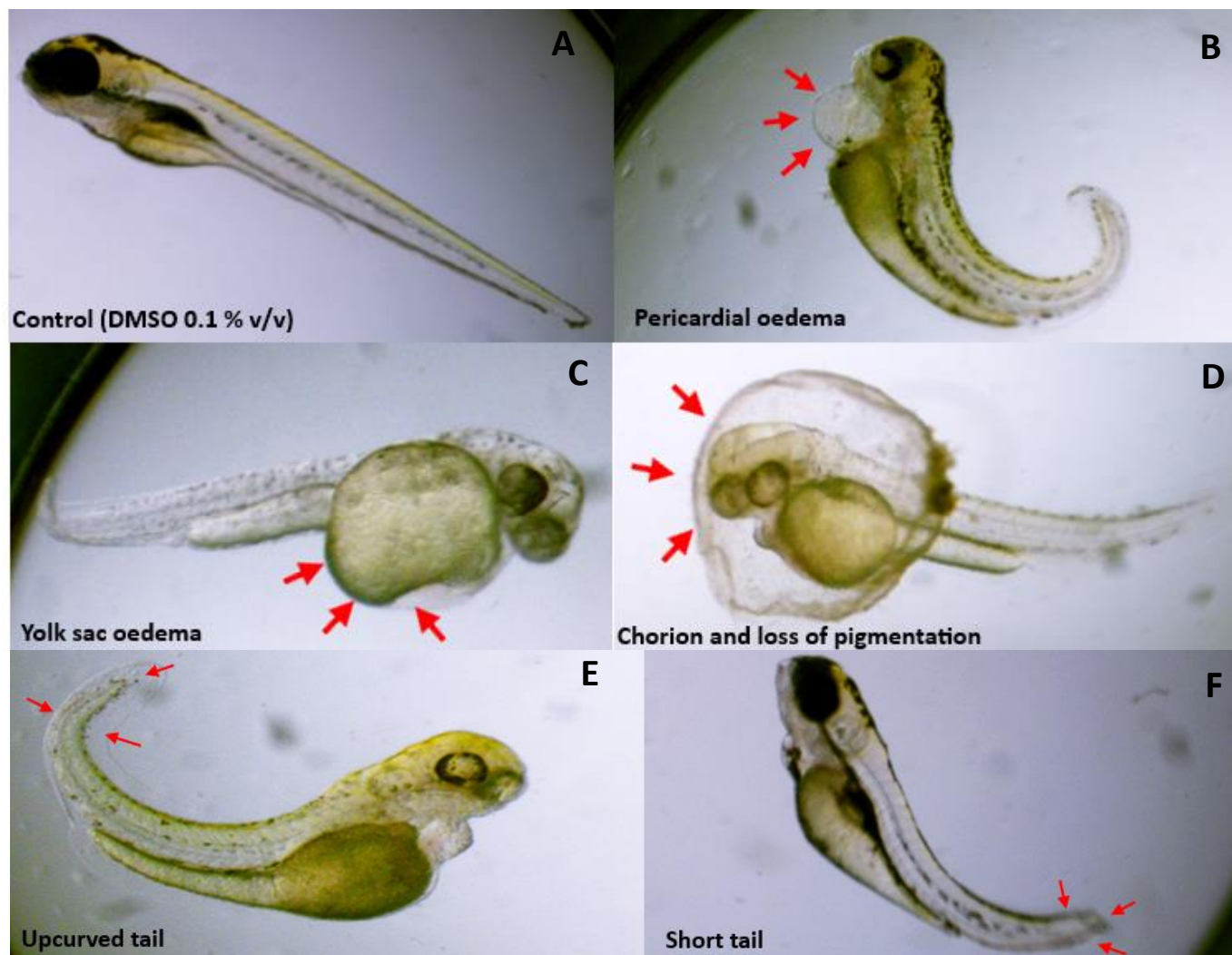


Figure 5.12: Microscopic images of (A) Healthy zebrafish embryo in DMSO 0.1% v/v; and example of phenotypic defects observed within zebrafish upon treatment with; (B) 54 at 12.5 μM ; (C) 69 at 62.5 μM ; (D) 76 at 2.5 μM ; Tail deformations (E-F) Tail deformations: E; 70 at 0.5 μM (upcurved tail) and F; 92 at 312.5 μM (short tail).

5.4.2 Evaluating the anti-angiogenic activity of small molecules in angiogenic vessels of zebrafish embryos.

To assess the anti-angiogenic activity of a series of test compounds including: **54**, **69**, **70**, **76**, **92**, **97** and montelukast, an evaluation was conducted using vascularized regions of zebrafish embryos: the sub-intestinal vessels (SIV) and intersegmental vessels (ISV). Initially, a safe concentration was determined for each compound through *in vivo* toxicity assay (**Section 5.4.1**). These concentrations were then applied in the subsequent anti-angiogenesis studies to ensure that observed effects on vascular development were attributable to the compounds' actions rather than to potential toxic side effects.

5.4.2.1 Sub-intestinal vessels (SIVs)

The anti-angiogenic activity of **54**, **69**, **92**, **97**, and montelukast were evaluated in the SIV region. Zebrafish embryos at 48 hpf were treated with the following concentrations: **54** at 12.5 μ M, **69** at 12.5 μ M, **92** at 312.5 μ M, **97** at 62.5 μ M, and montelukast at 12.5 μ M for a duration of 24 hours. At 72 hpf, the embryos were examined for the presence of ectopic sprouting from the sub-intestinal vessels (SIVs) using criteria such as the number of sprouts protruding from the basket structure and the area of a normal SIV basket (**Figure 5.14**). SIV is a major vein located beneath the intestine, is highly vascularised and typically characterised by a smooth, basket-like formation comprising 6 to 7 vessel compartments (**Figure 5.13 A**).

Interestingly, the administration of **69** and **92** led to the emergence of ectopic sprouts, suggesting a pro-angiogenic effect, as evidenced by the increase in new vessel growth without a substantial reduction in the overall SIV basket area (**Figures 5.13 and 5.14**). This indicates that **69** and **92** may enhance angiogenic activity by stimulating endothelial cell proliferation and migration, potentially through the upregulation of angiogenic growth factors or signalling pathways.

In marked contrast, treatments with **54**, **97** and montelukast did not induce ectopic sprouting from the SIV basket and resulted in a significant reduction in the overall area of the SIV basket as compared to vehicle control (**Figure 5.13 and 5.14**). These compounds appear to exert anti-angiogenic effects and may inhibit key processes involved in vessel formation, such as endothelial cell activation and migration. The reduction in SIV basket area, coupled with the absence of ectopic sprouting, suggests that **54**, **97** and montelukast may interfere with angiogenic signalling pathways or growth factors. A previous study by Eskicioglu *et al.* suggested that montelukast may have anti-angiogenic properties.²⁰⁴ They evaluated montelukast in an *in vivo* rat model of ovarian hyperstimulation syndrome (OHSS), demonstrating that montelukast limited the VEGF expression

which is associated with negative effects on angiogenesis.²⁰⁴ However, these effects were not statistically significant and further studies are required to confirm its efficacy.²⁰⁴

Together, these findings suggest that **69** and **92** may promote angiogenesis in zebrafish embryos, as evidenced by the significant increase in the number of ectopic sprouts from the SIV basket without a substantial reduction in the SIV basket area. On the other hand, **54**, **97** and montelukast may exhibit anti-angiogenic effects in zebrafish embryos.

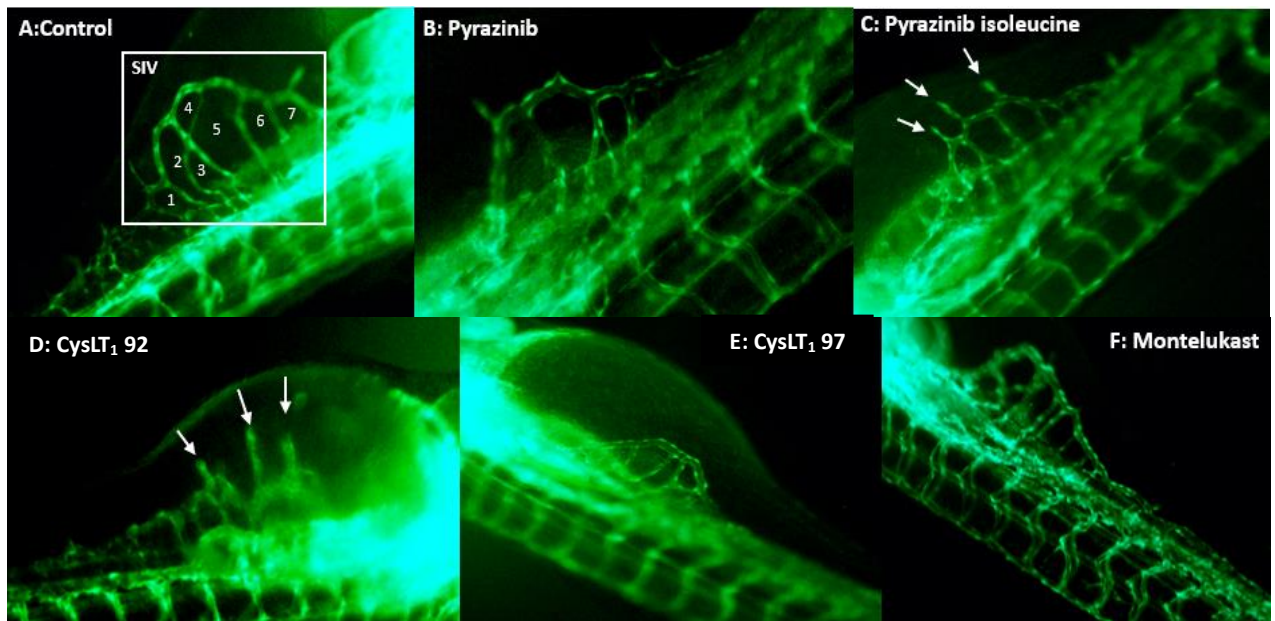


Figure 5.13: (A-F) Live fluorescence images of SIV baskets of zebrafish embryos (72 hpf) treated for 24 hours with (A) DMSO 0.1% v/v; (B) 54 (pyrazinib) at 12.5 μ M; (C) 69 (pyrazinib isoleucine) at 12.5 μ M; (D) 92 at 312.5 μ M; (E) 97 at 62.5 μ M; (F) Montelukast at 12.5 μ M.

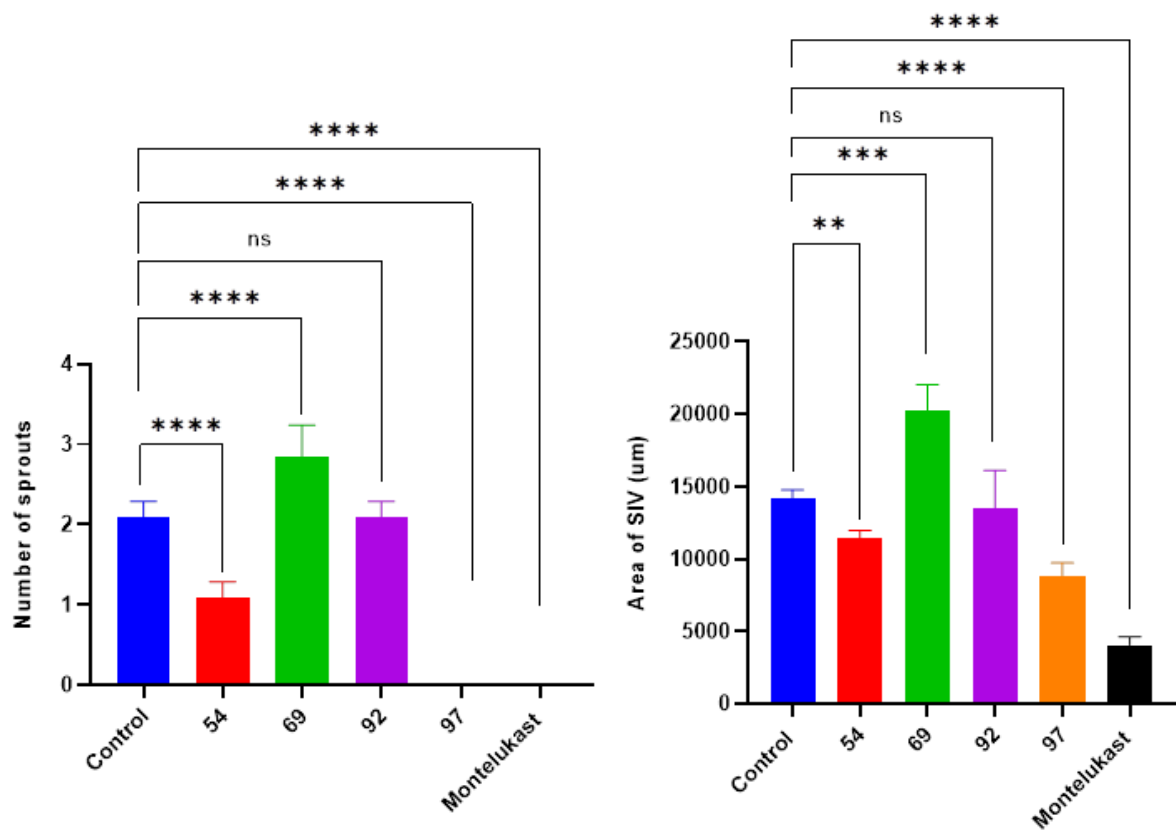


Figure 5.14: Quantification of the average number of SIV sprouts and average area of SIV of zebrafish embryos. N=10. One-way analysis of variance (ANOVA) was performed for statistical analysis. Error bars represent mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ or ns (non-significant).

5.4.2.2 Intersegmental vessels (ISVs)

ISVs in zebrafish embryos serve as an established model for studying angiogenesis due to their predictable and well-characterised development. The ISVs are particularly sensitive to pro- and anti-angiogenic factors, making them an ideal system for evaluating the anti-angiogenic effect of therapeutic agents.

In this study, the anti-angiogenic activity of several small molecules was assessed by measuring the length of ISVs in zebrafish embryos tested with different compounds. The results demonstrated a significant reduction in ISV length across all drug-treated groups compared to the DMSO control, which represents normal ISV development (**Figure 5.15**). Compounds **54**, **69**, **70**, **76**, **92**, **97** and montelukast all exhibited significant anti-angiogenic activity, as indicated by their ability to significantly reduce ISV length. Among these, **70** showed the most pronounced effect, suggesting it possesses potent anti-angiogenic properties (**Figure 5.15 and 5.16**).

The comparison of **54** with **69** reveals a notable difference in anti-angiogenic activity. Compound **54** is a potent inhibitor of angiogenesis, significantly reducing the length of ISV and demonstrating a strong suppressive effect on vessel formation. However, **69** which is a prodrug of **54** demonstrated a lesser reduction in ISV length (**Figure 5.15 and 5.16**). This suggests that the conversions of **69** to the active drug (**54**) could be slow or incomplete in this model, thereby reducing its overall effectiveness compared to the parent compound hence, leading to a reduced anti-angiogenic effect.

Comparing **70** with **76**, both compounds exhibit considerable anti-angiogenic activity, but **70** has pronounced reduction in ISV length (**Figure 5.15 and 5.16**). Findings from previous study by Murphy *et al.* demonstrated that **70** exerts a strong anti-angiogenic effect in zebrafish ISV. This difference suggests that **70** may have a stronger or more direct mechanism of action in inhibiting angiogenesis. However, it remains unknown whether **76** was converted to **70** in this model.

The comparison of **92**, **97**, and montelukast with DMSO revealed statistically significant inhibition of ISV length (**Figure 5.15 and 5.16**). However, no direct statistical comparison was made between **92**, **97** and montelukast. While these analogues show significant inhibition, further analysis is required to determine if their effects are stronger than montelukast. This suggests that these analogues could enhance anti-angiogenic properties, possibly by improving their affinity for angiogenesis-related targets or by affecting additional pathways involved in vascular development.

Interestingly, compounds **69** and **92** context dependent effects on angiogenesis in zebrafish promoting sprouting in SIV, a vein network while delaying vessel formation in ISV which are arteries. This contrasting activity potentially could arise from differences in the vascular microenvironment, signalling pathways, and structural features of veins versus arteries. SIV contains more active angiogenesis compared to ISV, thus favoured pro-angiogenic effects.

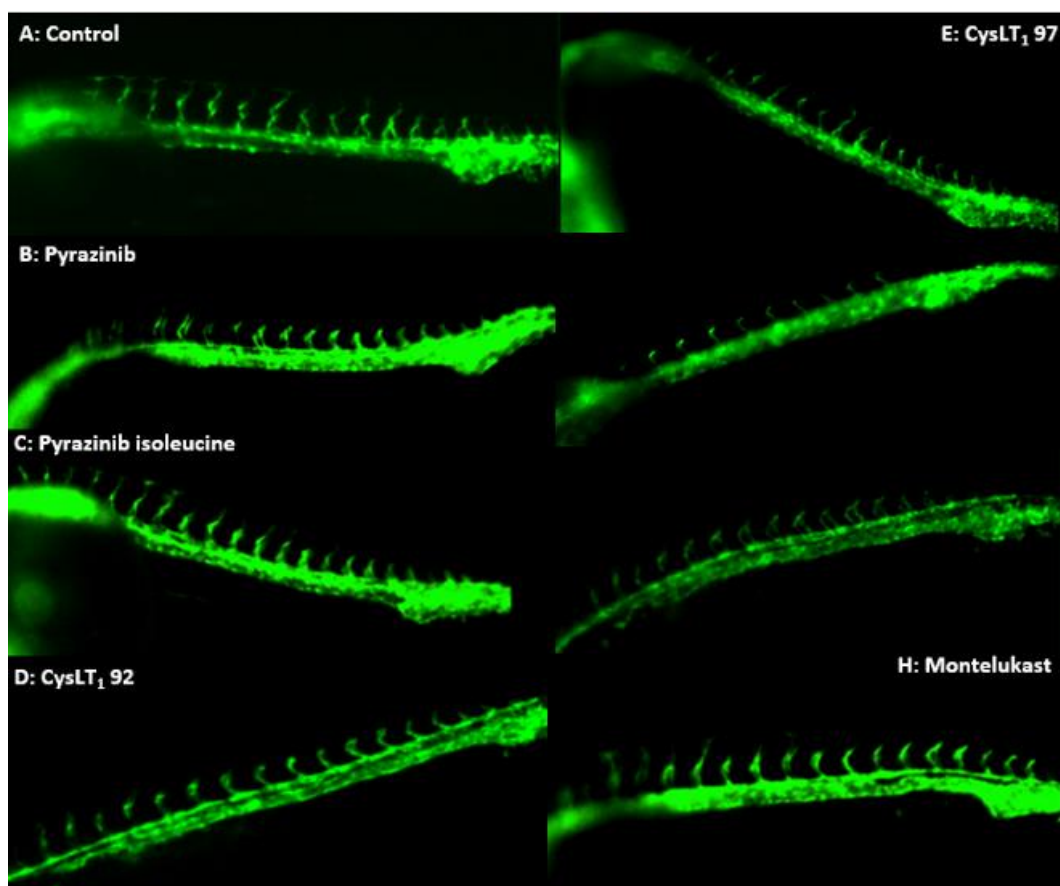


Figure 5.15: Live fluorescence images of ISVs of zebrafish embryos (24 hpf) treated for 20 hours with (A) DMSO 0.1% v/v; (B) 54 (pyrazinib) at 12.5 μ M; (C) 69 (pyrazinib isoleucine) at 12.5 μ M (D) 92 at 312.5 μ M; (E) 97 at 62.5 μ M; (F) 70 at 12.5 μ M; (G) 76 at 12.5 μ M and (H) Montelukast at 12.5 μ M.

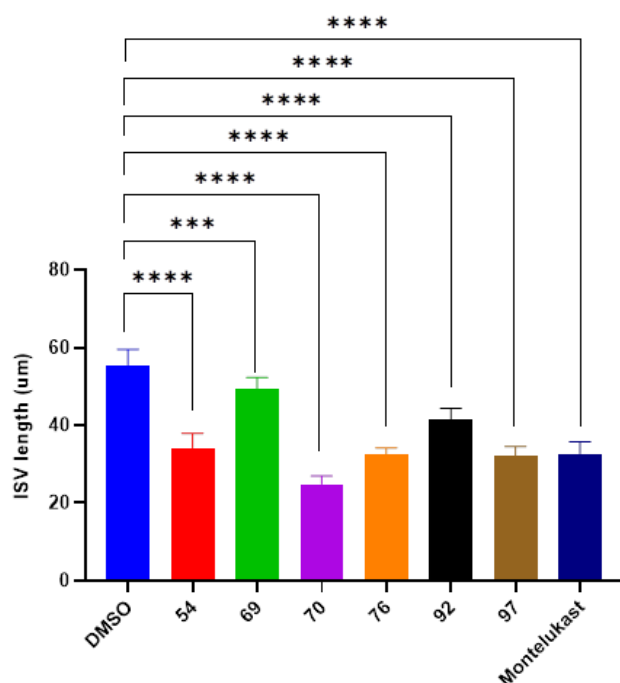


Figure 5.16: Quantification of the average length of ISV of zebrafish embryos treated with DMSO 0.1% v/v, 54 at 12.5 μ M, 69 at 12.5 μ M, 70 at 12.5 μ M, 76 at 12.5 μ M 92 at 312.5 μ M, 97 at 62.5 μ M, and montelukast at 12.5 μ M. N=10. One-way analysis of variance (ANOVA) was performed for statistical analysis. Error bars represent mean $\pm$ SEM, *** $p < 0.001$, **** $p < 0.0001$. N=10.

5.5 Chapter summary

This chapter focuses on evaluating the toxicity and anti-angiogenic effects of various small molecules using zebrafish models. These small molecules include quininib analogues targeting CysLT₁, hit compounds from virtual screening of CysLT₁, pyrazinib, and its amino acid prodrug. Zebrafish models are ideal for this study because their genetic similarity to humans and transparent embryos make it easy to study disease mechanisms and drug toxicity.

This chapter described experiments in which zebrafish embryos were exposed to different concentrations of compounds to determine lethal and non-lethal toxicities. Pyrazinib (**54**), pyrazinib isoleucine (**69**), Q1 (**70**), and Q28 (**76**) exhibited lethality at higher concentrations, while CysLT₁ hits (**92** and **97**), and montelukast demonstrated no significant toxic effects at concentrations from 312.5 to 0.1 μ M. Non-lethal toxicities, such as phenotypic defects and physiological changes, were also observed for all the compounds, including pericardial and yolk sac oedema, tail deformations, and pigmentation loss.

The anti-angiogenic activity of these small molecules was evaluated in zebrafish vasculature focusing specifically on sub-intestinal (SIV) and intersegmental (ISV) blood vessels. In the SIV, **92** and **69** promoted ectopic sprouting, indicating pro-angiogenic activity, while **54**, **97**, and montelukast showed anti-angiogenic effects by reducing the overall SIV basket area. However, in the ISV analysis, all drug treated groups showed a significant reduction in length, indicating a delay in ISV formation. **70** exhibited the strongest inhibition, while **54** was more effective than its prodrug **69**. Similarly, **70** showed stronger effects than its acetyl ester prodrug **76**. While montelukast and CysLT₁ **92** and **97** demonstrated significant inhibition. Based on these results, compound **97** is the more promising among the set of CysLT₁ hits. Compound **97** and **76** will be chosen for further progression along the development pipeline.

Chapter 6: Conclusion & Future work

6.1: Concluding remarks

Gastrointestinal cancers, particularly oesophageal adenocarcinoma (OAC) and uveal melanoma (UM) represent distinct yet aggressive malignancies with poor survival rates and limited treatment options. OAC especially is difficult to treat with a 5-year survival rate of less than 20%. Current therapies including neoadjuvant chemotherapy and chemoradiation, are only effective in a subset of patients, with the lack of biomarkers and approved radiosensitisers compounding the difficulty in treatment. UM though rare, has a high metastatic potential and similarly lacks effective systemic treatment. These two cancer types highlight the pressing need for novel therapeutic approaches that can overcome the limitations of current treatment strategies.

This thesis aimed to address these challenges by focusing on the development of new analogues of pyrazinib and quininib, two promising anti-cancer agents. Pyrazinib was found to be a potential radiosensitiser while, quininib targets the Cysteinyl leukotriene receptor 1 (CysLT₁), an important mediator in UM progression. The main challenge for both compounds was their suboptimal physicochemical properties including water solubility. This thesis aimed to overcome this limitation by synthesising their water-soluble analogues and evaluating their biological activity. This thesis also aimed to do virtual screening to identify CysLT₁ potential antagonists.

Chapter 1 introduced the significant burden of gastrointestinal and ocular cancers, setting the context for the research. It provided a comprehensive overview of the current treatment strategies for OAC and UM, highlighting the limitations in existing therapies. In particular, the poor solubility and bioavailability of several potent anti-cancer agents limit their clinical application. The chapter also presented the importance of improving the physicochemical properties of drugs, particularly through prodrug design and other strategies to improve water solubility.

Chapter 2 focused on pyrazinib (**54**), a radiosensitiser that was discovered through *in vivo* screening in zebrafish and tested in OAC models. Although pyrazinib showed promising radio-sensitising activity, its poor water solubility hindered further development. This chapter discussed different synthetic approaches, such as Perkin condensation and metal-catalysed reactions, to optimise the synthesis of pyrazinib. Pyrazinib amino acid prodrugs were synthesised to improve the poor water solubility of pyrazinib. Notably, the pyrazinib-isoleucine prodrug (**69**) significantly increased its aqueous solubility. However, despite the successful solubility enhancement, the prodrugs exhibited reduced radio-sensitising activity, indicating the need for further optimisation to maintain therapeutic effectiveness.

Chapter 3 addressed the water solubility and stability limitations of Quininib and its structural analogues. Quininib (Q1, **70**) is another potent anti-cancer small molecule, identified as an inhibitor of the CysLT₁ receptor, suffers from similar solubility challenges as pyrazinib. Chapter 3 focused on the synthesis of Q1 analogues: Q28 (**76**) and Q29 (**77**) to improve both water solubility and chemical stability. These analogues were chemically characterised, and their water solubility was evaluated. Compound **77** exhibited significantly improved solubility, while **76** demonstrated better chemical stability across different pH conditions. The biological evaluation in metastatic uveal melanoma (MUM) cell lines showed that these analogues retained potent anti-cancer activity. Furthermore, the analogues of **70** demonstrated anti-angiogenic properties, crucial for preventing cancer metastasis. Additionally, amino acid prodrugs of **70** was attempted but it was unsuccessful due to difficulties in coupling the amino acids to the hydroxyl group of **70**. This chapter demonstrated that structural modifications to **70** could enhance its physicochemical properties without compromising its biological activity.

Chapter 4 switched the focus to identifying hit antagonists targeting the CysLT₁ receptor, a key player in cancer cell survival and metastasis. A ligand-based virtual screening (LBVS) approach was utilised to identify novel hits, showing strong binding affinity to the CysLT₁ receptor. The chapter detailed the computational methods, including protein-ligand docking and interaction mapping, that led to the discovery of several promising CysLT₁ antagonists. These antagonists were evaluated for their biological activity *in vitro*, demonstrating potential as new therapeutic agents for cancer.

Chapter 5 focused on the *in vivo* evaluation using zebrafish embryos. Zebrafish owing to their transparency and rapid organ development served as an efficient model for evaluating the toxicity and anti-angiogenic effects of the small molecules studied in this thesis. Pyrazinib, CysLT₁ hits and Q1 analogues were tested, and all tested drugs demonstrated acceptable safety profiles in the zebrafish model. Importantly, small molecules including compound **97** and **76** significantly inhibited angiogenesis, a key process in cancer progression. The results of this chapter confirmed the potential of compounds **97** and **76** for further preclinical testing and showcased the utility of zebrafish models in drug screening for cancer therapies.

Overall, this thesis successfully addresses critical challenges in drug development of OAC and UM by improving the physicochemical properties of pyrazinib and quinib. By using synthetic approaches and prodrug design, novel analogues with enhanced solubility and stability were designed and synthesised. However, the radiosensitising activity of pyrazinib was compromised in prodrug form, hence these findings pave the way for further optimisation. Quininib analogues, particularly **76** and **77**, showed promise as anti-UM and anti-angiogenic agents, with significant

improvements in water solubility and chemical stability. The use of zebrafish model was a valuable asset providing rapid and efficient *in vivo* toxicity and efficacy testing. These findings provide a strong foundation for the continued development of pyrazinib and quininiib analogues with the potential for advancing them to clinical testing. A conceptual diagram presented in Figure 6.1 illustrates the key elements of the thesis.

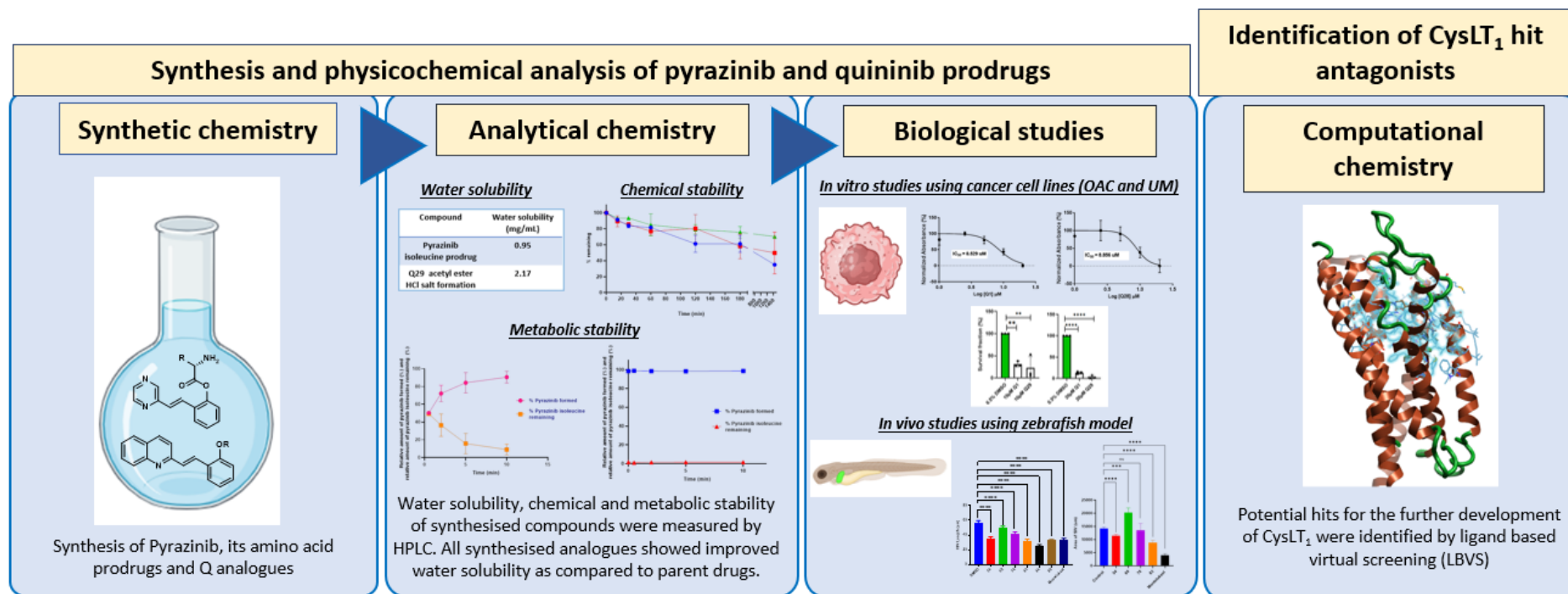


Figure 6.1: Concept diagram of main findings from the thesis. This diagram illustrates the synthesis and physicochemical analysis of pyrazinib and quinib prodrugs, whose properties were evaluated using HPLC, including water solubility, chemical stability, and metabolic stability. The subsequent steps involved *in vitro* biological studies using OAC and UM cell lines, as well as *in vivo* evaluations in zebrafish models. Additionally, the identification of potential CysLT₁ antagonists was achieved through ligand-based virtual screening (LBVS), highlighting new therapeutic candidates for further development.

6.2: Future directions

The research conducted in this PhD thesis has led to the identification of several promising directions for future studies, which are detailed below.

Chapter 2- Pyrazinib and its amino acid prodrugs

1- Optimisation of MAOS assisted Heck reaction for Pyrazinib synthesis

- **Objective:** To further optimise the MAOS assisted Heck reaction for the synthesis of pyrazinib, aiming to increase the yield from the current 36% to > 90%. This will involve investigating and refining key reaction parameters, including catalyst concentration, choice of base and reaction temperature.
- **Optimization of Catalyst Concentration:** Investigate the effect of varying the concentration of palladium (II) acetate to balance between reaction efficiency and by-product formation. Perform a systematic study using catalyst concentrations ranging from 0.5 mol% to 5 mol% and analyse their impact on reaction yield and purity.
- **Exploration of alternative bases:** Test alternative bases such potassium hydroxide, potassium carbonate, sodium acetate and cesium carbonate. Evaluate their impact on reaction yield, rate and formation of by-products.
- **Fine tuning of reaction temperature:** Optimise reaction temperature by testing range of temperatures (140°C, 150°C, 170°C) to identify the optimal temperature for maximum yield and minimum by-product formation.
- **Scale up:** After optimisation it is essential to confirm the improved conditions are scalable. Perform the reaction at a larger scale under the optimised conditions and assess reproducibility. Address any challenges related to scalability, such as heat transfer or mixing.

2- Further enhancing the water-solubility of pyrazinib amino acid prodrugs

- **Objective:** To further enhance the water solubility of pyrazinib amino acid prodrugs by introducing amino acid moieties at the amine side of the molecule.
- **Methodology:** Employ standard peptide coupling method such as HBTU to attach amino acid chains to the amine group on the amino acid side of pyrazinib.
- **Water solubility:** Quantify for solubility improvement using HPLC analysis and compare it with the original prodrug and parent drug, pyrazinib.

3- *In vivo* zebrafish xenograft model

- **Objective:** Assess the anticancer efficacy of pyrazinib and its prodrugs and their impact on tumour invasion.
- **Methodology:** Utilise zebrafish xenograft model, injecting labelled tumour cells such as OE33 cells and track tumour growth and migration using fluorescence microscopy.

Chapter 3- Quininib analogues

1- Alternative prodrug strategies

- **Objective:** To explore alternative prodrug strategies, such as carbamate linkage or PEGylation can improve water solubility while protecting the prodrugs of **70** and **74** from premature degradation.
- **Methodology:** Synthesise carbamate-based prodrugs of **70** and **74** by reacting with various carbamoyl chloride derivatives. Synthesise PEG-conjugated derivatives of **70** and **74** using various PEG chain lengths (e.g., PEG-200 or PEG-400).
- **Physicochemical properties:** Measure water solubility and conduct chemical stability at acidic, neutral and basic pH of these prodrugs to determine degradation kinetics and pathway.

2- Drug metabolism studies

- **Objective:** Analyse liver metabolism pathways for **70** and **74** to identify potential metabolites and their activity.
- **Methodology:** Use liver microsome assays followed by LC-MS/MS to identify and quantify metabolites. Characterise all major metabolites and evaluate their therapeutic or adverse effects using functional assays.

Chapter 4- CysLT₁ hit antagonists

1- Structure activity relationship (SAR) studies

- **Objective:** Refine SAR for promising hits like compound **97** to improve activity and pharmacokinetic properties.
- **Methodology:** Synthesise analogues with variations in hydrophilic and hydrophobic substitutions. For example, inclusion of bulky hydrophobic moiety (cyclopentane, cyclohexane or phenyl) at carbamide functional group. Additionally, incorporate extra carbonyl group to form stronger hydrogen bonds with the receptor. Evaluate these

analogues for binding affinity with CysLT₁ receptor. Define key structural features contributing to activity and solubility.

2- Validation of anticancer potential

- **Objective:** Evaluate the anticancer activity of newly synthesised analogues of compound **97** using *in vitro* and *in vivo* models.
- **Methodology:** Conduct proliferation assays (MTT and clonogenic assays) on lung and colon cancer cell lines. Test *in vivo* toxicity and anticancer activity of the most promising novel compounds in zebrafish embryo model.

Chapter 7: Experimental details

Materials and Methods

All reagents were commercially available and were used without further purification, unless otherwise stated. All anhydrous reactions were performed under an atmosphere of nitrogen. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance Spectrometer operating at 600 and 400 MHz using an internal standard of tetramethylsilane (TMS) and deuterodimethylsulfoxide ($\text{DMSO-}d_6$) as a solvent by Dr. John O'Brien in the School of Chemistry, Trinity College Dublin. All chemical shift (δ) values are given in ppm and coupling constant (J) in Hz. The following abbreviations for multiplicities are used: Doublet (d), triplet (t), singlet (s), broad singlet (br s), doublet of doublet (dd), triplet of doublet (td), multiplet (m), and septet (sept). FTIR analysis were carried out as ATR by using a Perkin Elmer FTIR spectrometer version 10.6.0 with neat samples and frequency of bands were observed in wavenumbers (cm^{-1}). X-ray crystallography data was collected by Dr Brendan Twamley of the School of Chemistry, Trinity College, Dublin. The melting point analysis was carried out using Stuart melting point |SMP 201| apparatus and microwave assisted synthesis was carried out using CEM Discover 2.0 microwave synthesiser.

Thin Layer Chromatography analysis (TLC) was performed on Merck Silica gel 60 TLC aluminium sheets with fluorescent indicator visualising with UV light at 254 nm and 365 nm. Purification of compounds was performed by flash column chromatography using Silica gel from Merck (pore size 60 Å, product no: 231-545-4). All products isolated were homogenous on TLC.

Analytical HPLC studies

All HPLC assays to described in this thesis were performed on the Waters Alliance E2695 HPLC instrument. Isocratic elution was applied with a mobile phase of acetonitrile (70%) and water (30%). Compounds were detected using 2998 photodiode array detector (PDA), flow rate was 1 mL/min and injection volume was 10 μL . Total run time was 10 minutes for all the compounds.

Analytical HPLC stability studies were performed using a Hypersil BDS C18 (dim 250 x 4.6 mm) column (cat no: 10344413, Thermo Fisher Scientific). Stock solutions were prepared by dissolving 2.5 mg of compound into 5 mL of mobile phase. Phosphate buffers at pH values of 4, 7.4 and 9 were prepared in accordance with the British Pharmacopeia 2023. 60 μL of stock solution was diluted with 2 mL appropriate buffer, shaken and injected immediately. Samples were withdrawn and analysed at $t = 0.5$ min, 15 min, 30 min, 60 min, 120 min, 180 min and 1440 min.

Aqueous solubility was tested using XDB-C18 Solv Save (dim 150 x 3.0 mm) column (cat no: 49597, Apex scientific). An amount of approximately 2 mg of each drug accurately weighed out and suspended in a corresponding amount of distilled water in a vial (Wheaton sample vials with rubber lined caps, Sigma-Aldrich Z 256 188-1PAK). This suspension was placed on a mechanical shaking

plate (Mini gyro-rocker, SSM3, Stuart) and agitated at 400 shakes/min for 24 hours. After 24 hours, the suspensions were further filtered, diluted with mobile phase, and the filtrate was assayed by HPLC using the same conditions as described above.

Metabolic stability studies were performed using a porcine liver esterase enzyme and human blood plasma. Stock solutions were prepared by dissolving 3 mg of compound into 1 mL of mobile phase. 1 mL of stock solution was diluted with 1 mL of appropriate enzyme/ plasma solution, vortexed and then incubated in a water bath at 37 °C for 10 minutes. Samples were withdrawn at t= 0.5 min, 2 min, 5 min and 10 min, quenched with ice-cold stop solution [ACN: MeOH (1:1)]. The samples were filtered and analysed by HPLC.

7.1 General methods for the synthesis of pyrazinib

7.1.1 General method 1: Conventional Perkin condensation

7.1.1.1 Synthesis of (*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl acetate (55)

2-Hydroxybenzaldehyde (4.88 g, 4.17 mL, 40 mmol) was dissolved in acetic anhydride (20 mL), followed by the addition of 2-methylpyrazine (3.76 g, 3.65 mL, 40 mmol). The resulting mixture was stirred at reflux temperature (180°C) for 5-6 days. Then, the solvent was evaporated *in vacuo* under reduced pressure to afford a brown/yellow crystalline solid in a 15% yield (1.4 g). The product was carried forward for hydrolysis without further purification.

IR (ATR; ν_{max}/cm^{-1}) 1758 cm^{-1} (C=O)

^1H NMR (600 MHz DMSO- d_6) δ 8.84 (d, 1H, J = 1.3 Hz, ArH, pyrazine ring), 8.65 (d, 1H, J = 1.4 Hz, ArH, pyrazine ring), 8.53 (d, 1H, J = 2.4 Hz, ArH, pyrazine ring), 7.92 (dd, 1H, J = 1.5 and 8 Hz, ArH), 7.74 (d, 1H, J = 16 Hz), 7.44 (t, 1H, J = 7 Hz, ArH), 7.41 (d, 1H, J = 16 Hz), 7.36 (t, 1H, J = 7 Hz, ArH), 7.20 (dd, 1H, J = Hz ArH), 2.38 (s, 3H, CH₃ acetate group)

^{13}C NMR (600 MHz DMSO- d_6) δ 169.7, 150.7, 149.0, 145.0, 144.6, 143.9, 130.3, 128.9, 127.4, 127.3, 127.1, 126.8, 123.7, 21.2

7.1.1.2 Hydrolysis of (*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl acetate to obtain (*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenol (54)

Pyrazinib acetate (1.4 g, 5.83 mmol) was dissolved in ethyl acetate/ethanol (1:1 mixture, 40 mL). The resulting mixture was stirred at 100°C, followed by the addition of saturated NaOH aqueous solution (2 mL). The reaction was monitored by TLC (R_f = 0.34 cm, mobile phase: n-hexane: ethyl acetate (1:1)). The solvent was then removed *in vacuo* and the crude mixture was purified by using

flash column chromatography over silica gel (hexane: ethyl acetate, gradient 1:1 to 1:2) to afford a bright yellow solid in a 12% yield (0.85 g).

Melting point: 207 °C

IR (ATR; ν_{max}/cm^{-1}) 3301 cm^{-1} (Broad OH)

^1H NMR (600 MHz DMSO- d_6) δ 10.01 (br s, 1H, OH), 8.73 (d, 1H, J = 1.4 Hz, ArH, pyrazine ring), 8.60 (d, 1H, J = 1.5 Hz, ArH, pyrazine ring), 8.48 (d, 1H, J = 2.5 Hz, ArH, pyrazine ring), 8.03 (d, 1H, J = 16 Hz), 7.64 (dd, 1H, J = Hz, ArH), 7.37 (d, 1H, J = 16 Hz), 7.18 (t, 1H, J = 7 Hz, ArH), 6.92 (d, 1H, J = Hz ArH), 6.86 (t, 1H, J = 7 Hz, ArH)

^{13}C NMR (600 MHz DMSO- d_6) δ 156.3, 151.7, 144.9, 144.2, 143.1, 130.4, 130.3, 127.9, 124.0, 123.1, 119.8, 116.5

HRMS (ESI): $\text{C}_{12}\text{H}_{10}\text{N}_2\text{O}$ requires 198.1065; found $[\text{M}+\text{H}]^+$ 199.1140

Purity (HPLC): 97%

7.1.2 General method 2: Zinc catalysed reaction using microwave technology for the synthesis of pyrazinib (54)

2-Hydroxybenzaldehyde (1.22 g, 1.04 mL, 10 mmol) and 2-methylpyrazine (0.94 g, 0.91 mL, 10 mmol) were added into a microwave vial (10 mL) followed by the addition of zinc chloride (0.061 g, 0.45 mmol). The vial was placed into the microwave and heated at 140°C for 5 hours. The crude reaction mixture appeared as a brown mixture which was purified by the flash column chromatography over silica gel (n-hexane: ethyl acetate isocratic 60:40) to afford a bright yellow solid (0.5% yield, 0.01g).

7.1.3 General method 3: Cobalt catalysed reaction for the synthesis of pyrazinib (54)

2-Hydroxybenzaldehyde (1.22 g, 1.04 mL, 10 mmol) and 2-methylpyrazine (0.94 g, 0.91 mL, 10 mmol) were dissolved in water (10 mL) followed by the addition of catalyst, cobalt chloride (0.026 g, 10 mmol %). The reaction mixture was stirred vigorously at reflux temperature (120°C) for 24 hours. After 24 hours, the reaction mixture was cooled to room temperature and dissolved in water (50 mL). Then, the aqueous layer was extracted with ethyl acetate (100 mL x 3) and washed with brine (30 mL). The organic layer was dried by filtration through anhydrous sodium sulfate and concentrated *in vacuo*. The crude appeared as a brown oil which was purified by flash column chromatography over silica gel (n-hexane: ethyl acetate isocratic 60:40) to afford a bright yellow solid (1% yield, 0.02g).

7.1.4 General method 4: Calcium catalysed reaction for the synthesis of pyrazinib (54)

2-Hydroxybenzaldehyde (1.22 g, 1.04 mL, 10 mmol) and 2-methylpyrazine (0.94 g, 0.91 mL, 10 mmol) were dissolved in water (10 mL) followed by the addition of calcium triflate (0.169 g, 5 mol%) and Bu_4NPF_6 (0.077 g, 2 mol%). The reaction mixture was stirred at 130°C for 24 hours. After 24 hours, the reaction mixture was cooled to room temperature. The crude product was further purified by flash column chromatography over silica gel (n-hexane: ethyl acetate isocratic 60:40) to afford bright yellow solid (4% yield, 0.08g).

7.1.5 General method 5: Heck reaction using microwave technology for the synthesis of pyrazinib (54)

Palladium (II)acetate (15 mg, 0.066 mmol) and triethylamine (0.2 mL, 1.43 mmol) were added into a microwave vial (10 mL) followed by the addition of 2-bromophenol (0.2444 g, 0.137 mL, 1.3 mmol) and 2-vinylpyrazine (0.275 g, 0.275 mL, 2.6 mmol). Then, the vial was sealed and placed in the microwave, where the solution was heated at 160°C, for specified amount of time. After heating, the crude mixture was purified by flash column chromatography over silica gel (n-hexane: ethyl acetate isocratic 60:40) to afford a bright yellow solid (36% yield, 0.112g).

7.2 Formation of pyrazinib amino acid prodrugs (56-62)

To a solution of pyrazinib (**54**) (0.3g, 1.5 mmol) and N-Boc protected amino acid (2.5 eq) in dry acetonitrile (30 mL). Then, HBTU (2 eq) and DIPEA base (61 eq) was added dropwise under nitrogen. The resulting mixture was stirred at room temperature under a nitrogen atmosphere for 24 hours. The solvent was removed under reduced pressure and the residue was extracted with DCM (50 mL x 3). The combined organic extracts were washed with saturated aqueous solution of potassium hydrogen sulfate (30 mL), saturated aqueous sodium bicarbonate (30 mL) and brine (30 mL). The organic layer containing the product was dried with anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The crude product appeared as brown/yellow oil, purified by flash column chromatography over silica gel (hexane: ethyl acetate, isocratic 70:30).

(E)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl)-L-glycinate (56) was prepared from (E)-2-(2-(Pyrazin-2-yl)vinyl)phenol and Boc protected L-glycine as a pale-yellow sticky solid in 48% yield (0.168 g).

IR (ATR; $V_{\text{max}}/\text{cm}^{-1}$): 3200 cm^{-1} (N-H), 1755 cm^{-1} (C=O), 1709 cm^{-1} (Boc, C=O)

^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 8.93 (d, 1H, J = 1.3 Hz, ArH), 8.63 (d, 1H, J = 1.4 Hz, ArH), 8.51 (d, 1H, J = 2.3 Hz ArH), 7.97 (dd, 1H, J = 1.3 and 8.0 Hz, ArH), 7.75 (d, 1H, J = 16 Hz), 7.45 (t, 1H, J = 7 Hz, ArH),

7.40 (d, 1H, $J = 16$ Hz), 7.35 (t, 1H, $J = 6.9$ Hz, ArH), 7.18 (dd, 1H, $J = 1.3$ and 8 Hz, ArH), 4.2 (s, 2H, CH₂), 1.38 (s, 9H, Boc, CH₃ ×3).

¹³C NMR (600 MHz, DMSO-*d*₆) δ 172.8, 156.2, 151.0, 149.1, 145.1, 144.9, 144.1, 144.0, 143.7, 130.4, 129.1, 127.76, 127.4, 127, 123.3, 78.6, 49.9, 28.5

HRMS (ESI): C₁₉H₂₁N₃O₄ requires 355.1534; found [M+H]⁺ 356.1611

(*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl)-L-alaninate (57) was prepared from (*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenol and Boc protected *L*-alanine as a pale-yellow sticky solid in 52% yield (0.18 g).

IR (ATR; V_{max}/cm^{-1}): 3311 cm⁻¹(N-H), 1765 cm⁻¹ (C=O), 1701 cm⁻¹ (Boc C=O)

¹H NMR (600 MHz, DMSO-*d*₆) δ 8.97 (d, 1 H, $J = 1.4$ Hz, ArH), 8.62 (d, 1H, $J = 7.8$ Hz, ArH), 8.52 (d, 1H, $J = 7.8$ Hz, ArH), 7.94 (dd, 1H, $J = 1.2$ and 8.1 Hz, ArH), 7.76 (d, 1H, $J = 16$ Hz), 7.44 (t, 1H, $J = 7.4$ Hz, ArH), 7.40 (d, 1H, $J = 16$ Hz), 7.36 (t, 1H, $J = 7.3$ Hz, ArH), 7.13 (dd, 1H, $J = 1.5$ and 7.3 Hz, ArH), 4.36 (q, 1H, CH), 1.48 (d, 3H, $J = 7.2$ Hz, CH₃), 1.38 (s, 9H, Boc, CH₃ ×3).

¹³C NMR (600 MHz, DMSO-*d*₆) δ 172.8, 156.2, 151.0, 149.1, 145.1, 144.9, 144.1, 144.0, 143.7, 130.4, 129.1, 127.76, 127.4, 127, 123.3, 79.1, 50.2, 28.5, 21.2, 17.1

HRMS (ESI): C₂₀H₂₃N₃O₄ requires 369.1776; found [M+H]⁺ 370.1856

(*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl)-L-valinate (58) was prepared from (*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenol and N-Boc protected *L*-valine as a yellow sticky solid in 55% yield (0.190 g).

Melting point: 179 °C

IR (ATR; V_{max}/cm^{-1}) 3333 cm⁻¹ (N-H), 1754 cm⁻¹ 1686 cm⁻¹ (Boc C=O)

¹H NMR (400 MHz DMSO-*d*₆) δ 8.97 (d, $J = 1.3$ Hz, 1H, ArH), 8.61 (d, 1H, $J = 7.8$ Hz, ArH), 8.51 (d, 1H, $J = 7.8$ Hz, ArH), 7.94 (dd, 1H, $J = 1.4$ and 8.1 Hz, ArH), 7.81 (d, 1H, $J = 16.10$ Hz, ArH), 7.43 (t, 1H, $J = 7.3$ Hz, ArH), 7.40 (d, 1H, $J = 16.0$ Hz, ArH), 7.36 (t, 1H, $J = 7.4$ Hz, ArH), 7.11 (dd, 1H, $J = 7.3$ Hz, ArH), 4.15 (t, 1H, CH), 2.26 (m, 1H, CH), 1.38 (s, 9H, CH₃ ×3), 1.05 (dd, 6H, $J = 9.0$ Hz, CH₃ ×2).

¹³C NMR (400 MHz DMSO-*d*₆) δ 171.6, 156.5, 151.0, 148.9, 144.8, 144.0, 143.7, 130.4, 129.1, 127.7, 127.6, 127.4, 127.0, 123.3, 79.0, 60.4, 29.9, 28.5, 19.6, 19.1

HRMS (ESI): C₂₂H₂₇N₃O₄ requires 397.0919; found [M+H]⁺ 398.1000

(E)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl)-L-leucinate (59) was prepared from (E)-2-(2-(Pyrazin-2-yl)vinyl)phenol and Boc protected L-leucine as a pale-yellow sticky solid in 57% yield (0.195 g).

IR (ATR; V_{max}/cm^{-1}): 3298 cm^{-1} (N-H), 1764 cm^{-1} (C=O), 1702 cm^{-1} (Boc C=O)

^1H NMR (600 MHz, DMSO- d_6) δ 8.92 (d, 1H, ArH), 8.60 (d, 1H, $J = 7.6$ Hz, ArH), 8.52 (d, 1H, $J = 7.7$ Hz, ArH), 7.93 (dd, 1H, $J = 1.3$ and 8.1 Hz, ArH), 7.77 (d, 1H, $J = 16.2$ Hz, ArH), 7.43 (t, 1H, $J = 7.4$ Hz, ArH), 7.41 (d, 1H, $J = 16.0$ Hz, ArH), 7.36 (t, 1H, $J = 7.3$ Hz, ArH), 7.11 (dd, 1H, $J = 1.4$ and 7.1 Hz, ArH), 4.28 (q, 1H, CH), 1.79 (m, 1H, CH), 1.71 (m, 1H, CH), 1.38 (s, 9H, $CH_3 \times 3$), 0.93 (dd, 6H, $J = 1.5$ and 6.7 Hz, $CH_3 \times 2$)

^{13}C NMR (600 MHz, DMSO- d_6) δ 172.1, 156.1, 155.9, 150.5, 148.5, 144.4, 143.7, 143.4, 129.9, 128.6, 127.1, 127.0, 126.9, 126.6, 122.8, 78.6, 59.7, 37.2, 28.9, 24.3, 22.6, 21.3

HRMS (ESI): $C_{23}H_{30}N_3O_4$ requires 411.1626; found $[M+H]^+$ 412.1705

(E)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl)-L-serinate (60) was prepared from (E)-2-(2-(Pyrazin-2-yl)vinyl)phenol and Boc protected L-serine as a pale-yellow sticky solid in 54% yield (0.130g).

IR (ATR; V_{max}/cm^{-1}): 2975 cm^{-1} (N-H), 1769 cm^{-1} (C=O), 1709 cm^{-1} (Boc, C=O)

^1H NMR (600 MHz, DMSO- d_6) δ 8.96 (d, $J = 1.4$ Hz, 1H, ArH), 8.60 (d, 1H, $J = 7.5$ Hz, ArH), 8.52 (d, 1H, $J = 2.64$ Hz, ArH), 7.95 (dd, 1H, $J = 1.6$ and 8.11 Hz, ArH), 7.78 (d, 1H, $J = 16.35$ Hz), 7.58 (t, 1H, $J = 7.3$ Hz, ArH), 7.44 (d, 1H, $J = 7.86$ Hz, ArH), 7.39 (d, 1H, $J = 16.1$ Hz), 7.37 (t, 1H, $J = 7.1$ Hz, ArH), 7.10 (dd, 1H, $J = 1.5$ and 8.11 Hz, ArH), 4.45 (t, 1H, CH), 3.80 (m, 1H, CH), 3.70 (m, 1H, CH_2), 1.38 (s, 9H, $3 \times CH_3$), 1.33 (s, 9H, $3 \times CH_3$)

^{13}C NMR (600 MHz, DMSO- d_6) δ 170.6, 156.0, 151.0, 149.0, 144.8, 144.2, 143.7, 130.4, 129.0, 127.7, 127.6, 127.5, 126.9, 123.7, 79.2, 73.6, 61.6, 55.3, 28.6, 27.5

HRMS (ESI): $C_{25}H_{31}N_3O_7$ requires 485.1175; found $[M+H]^+$ 486.1255

(E)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(S)-2-((tert-butoxycarbonyl)amino)-2-(4-hydroxyphenyl)acetate (61) was prepared from (E)-2-(2-(Pyrazin-2-yl)vinyl)phenol and Boc protected L-tyrosine as a pale-yellow sticky solid in 60% yield (0.2 g).

IR (ATR; V_{max}/cm^{-1}): 3335 cm^{-1} (N-H), 1763 cm^{-1} (C=O), 1705 cm^{-1} (Boc, C=O)

^1H NMR (600 MHz, DMSO- d_6) δ 9.04 (d, 1H, $J = 1.3$ Hz, ArH), 8.62 (d, 1H, $J = 7.4$ Hz, ArH), 8.53 (d, 1H, $J = 2.64$ Hz, ArH), 7.93 (dd, 1H, $J = 1.6$ and 8.11 Hz, ArH), 7.71 (d, 1H, $J = 16.3$ Hz), 7.45 (t, $J = 7.3$

Hz, 1H, ArH), 7.40 (d, 1H, $J = 16.1$ Hz), 7.36 (t, 1H, $J = 7.0$ Hz, ArH), 6.91 (d, 1H, $J = 7.86$ Hz, ArH), 6.84 (d, 1H, $J = 7.3$ Hz, ArH), 4.54 (t, 1H, CH), 2.00 (m, 1H, CH₂), 1.33 (s, 9H, 3 × CH₃), 1.27 (s, 9H, 3 × CH₃)
¹³C NMR (600 MHz, DMSO-*d*₆) δ 172.8, 156.2, 151.0, 149.1, 145.1, 144.9, 144.1, 144.0, 143.7, 130.4, 129.1, 127.76, 127.4, 127, 123.3, 79, 50, 28.5, 21.2, 17.1

HRMS (ESI): C₃₁H₃₅N₃O₇ requires 561.1499; found [M+H]⁺ 562.1580

2-((*E*)-2-(Pyrazin-2-yl)vinyl)phenyl(2*S*)-2-((tert-butoxycarbonyl)amino)-3-methylpentanoate (62)

was prepared from (*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenol and Boc protected *L*-isoleucine as a pale-yellow sticky solid in 60% yield (0.2 g).

IR (ATR; V_{max} /cm⁻¹): 3275 cm⁻¹ (N-H), 1761 cm⁻¹ (C=O), 1703 cm⁻¹ (Boc, C=O)

¹H NMR (600 MHz, DMSO-*d*₆) δ 8.98 (d, 1H, $J = 1.4$ Hz, ArH), 8.62 (d, 1H, $J = 2.5$ Hz, ArH), 8.51 (d, $J = 2.5$ Hz, 1H, ArH), 7.95 (dd, $J = 1.6$ and 8.19 Hz, 1H, ArH), 7.70 (d, $J = 16.5$ Hz, 1H), 7.52 (t, $J = 7.69$ Hz, 1H, ArH), 7.41 (d, $J = 16.2$ Hz, 1H), 7.37 (t, 1H, $J = 7$ Hz, ArH), 6.93 (dd, $J = 8.19$ Hz, 1H, ArH), 4.3 (m, 1H, CH), 2.00 (m, 2H, CH₂), 1.8 (m, 1H, CH), 1.08 (d, $J = 7.4$ Hz, 3H, CH₃), 0.97 (d, $J = 7.3$ Hz, 3H, CH₃)
¹³C NMR (600 MHz, DMSO-*d*₆) δ 171.6, 167.4, 151.0, 149.0, 144.9, 144.1, 143.8, 130.4, 129.2, 127.76, 127.71, 127.5, 127.0, 123.3, 79.1, 59.4, 36.2, 28.6, 25.5, 15.9, 11.6.

HRMS (ESI): C₂₃H₃₀N₃O₄ requires 411.1626; found [M+H]⁺ 412.1705

7.3 Removal of Boc protecting group from 56-62 to yield 63-69

7.3.1 General method 1: Removal of Boc protecting group using oxalyl chloride reaction

To a stirring solution of relative pyrazinib Boc amino acid analogue **56-62** (0.5 mmol) in methanol (3 mL), oxalyl chloride (1.5 mmol, 3 eq) was added. The reaction was monitored by TLC (mobile phase: hexane: ethyl acetate 1:1). Upon complete conversion of pyrazinib Boc amino acid analogue, the solvent was evaporated *in vacuo*. Then, the crude mixture was extracted with DCM (5 mL) and washed with deionized water (5 mL) twice. The organic layer was dried over with anhydrous Na₂SO₄ and the solvent was removed *in vacuo*.

7.3.2 General method 2: TFA: DCM reaction

The relative pyrazinib Boc amino acid analogue **56-62** (0.5 mmol) was dissolved in DCM (3 mL) and cooled to 0°C. Then, trifluoroacetic acid (3 mL) was added. The mixture was stirred at room temperature until complete on TLC (mobile phase: hexane: ethyl acetate 1:1), typically within 10 minutes. The solvent was reduced *in vacuo*. The crude mixture was dissolved in water (5 mL) and washed with DCM (5 mL x 3). The aqueous layer containing the product was concentrated *in vacuo* and the remaining water was removed in a freeze dryer to afford the product.

(E)-2-(2-(Pyrazin-2-yl)vinyl)phenyl-L-glycinate (63) was prepared from (E)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl) glycinate (**56**) as a pale-yellow gel in 46% yield (0.069 g).

IR (ATR; V_{max}/cm^{-1}) 3102 cm^{-1} (NH₂), 1751 cm^{-1} (C=O)

¹H NMR (600 MHz DMSO-*d*₆) δ 8.85 (d, 1H, *J* = 1.2 Hz, ArH), 8.64 (d, 1H, *J* = 2.5 Hz, ArH), 8.55 (d, 1H, *J* = 2.5 Hz ArH), 7.94 (dd, 1H, *J* = 1.3 and 7.9 Hz, ArH), 7.81 (d, 1H, *J* = 16 Hz), 7.48 (t, 1H, *J* = 7 Hz, ArH), 7.46 (d, 1H, *J* = 16 Hz), 7.38 (t, 1H, *J* = 6.8 Hz, ArH), 7.25 (dd, 1H, *J* = 1.3 and 8 Hz, ArH), 3.8 (s, 2H, CH₂)

¹³C NMR (600 MHz DMSO-*d*₆) δ 169.0, 150.7, 148.3, 145.2, 144.5, 144.0, 130.6, 128.9, 127.4, 127.2, 126.9, 123.8, 51.2

HRMS (ESI): C₁₇H₁₉N₃O₂ requires 255.3795; found [M+H]⁺ 256.3873

Purity (HPLC): 75%

(E)-2-(2-(Pyrazin-2-yl)vinyl)phenyl-L-alaninate (64) was prepared from (E)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl)-L-alaninate (**57**) as a yellow gel in 62% yield (0.09 g).

IR (ATR; V_{max}/cm^{-1}) 3048 cm^{-1} (NH₂), 1754 cm^{-1} (C=O)

¹H NMR (600 MHz DMSO-*d*₆) δ 8.86 (d, 1H, *J* = 2.2 Hz ArH), 8.64 (d, 1H, *J* = 2.4 Hz ArH), 8.56 (d, 1H, *J* = 2.5 Hz ArH), 8.00 (dd, 1H, *J* = 1.3 and 7.8 Hz ArH), 7.75 (d, 1H, *J* = 16.3 Hz), 7.47 (t, 1H, *J* = 7 Hz ArH), 7.43 (d, 1H, *J* = 16.0 Hz), 7.41 (t, 1H, *J* = 7 Hz, ArH), 7.24 (dd, 1H, *J* = 1.3 and 8 Hz, ArH), 4.6 (q, 1H, CH), 1.68 (d, 3H, *J* = 7.4 Hz, CH₃)

¹³C NMR (600 MHz DMSO-*d*₆) δ 169.7, 158.3, 150.7, 148.1, 145.1, 144.5, 144.0, 130.6, 129.1, 128.0, 127.7, 127.5, 127.1, 123.2, 48.7, 16.4

HRMS (ESI): C₁₄H₁₃N₃O₂ requires 269.1159; found [M+H]⁺ 270.1237

Purity (HPLC): 99.6%

(*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl-*L*-valinate (65) was prepared from (*E*)-2-(2-(pyrazin-2-yl)vinyl)phenyl (tert-butoxycarbonyl)-*L*-valinate (**58**) as a yellow solid in 92% yield (0.151 g).

Melting point: 180 °C

IR (ATR; *V*_{max}/cm⁻¹) 3100 cm⁻¹ (NH₂), 1769 cm⁻¹ (C=O)

¹H NMR (600 MHz DMSO-*d*₆) δ 8.85 (d, 1H, *J* = 1.4 Hz ArH), 8.64 (d, 1H, *J* = 2.5 Hz ArH), 8.55 (d, 1H, *J* = Hz ArH), 7.99 (dd, 1H, *J* = 1.4 and 7.2 Hz ArH), 7.80 (d, 1H, *J* = 16.3 Hz), 7.47 (t, 1H, *J* = 7 Hz ArH), 7.44 (d, 1H, *J* = 15.9 Hz), 7.43 (t, 1H, *J* = 7 Hz, ArH), 7.24 (dd, *J* = 1.4 and 7 Hz, 1H, ArH), 4.41 (t, 1H, CH), 1.25 (m, 1H, *J* = 2.1 Hz, CH₂), 1.14 (dd, 6H, *J* = 1.5 and 7 Hz CH₃×2)

¹³C NMR (600 MHz DMSO-*d*₆) δ 168, 158.4, 150.6, 148.1, 145.0, 144.5, 144.0, 128.9, 127.9, 127.6, 127.5, 127.0, 123.2, 58.0, 30.0, 18.5, 18.4

HRMS (ESI): C₁₇H₁₉N₃O₂ requires 297.1472; found [M+H]⁺ 298.1550

Purity (HPLC): 98.56%

(*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl-*L*-leucinate (66) was prepared from (*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl)-*L*-leucinate (**59**) as yellow gel in 75% yield (0.128 g).

IR (ATR; *V*_{max}/cm⁻¹) 3351 cm⁻¹ (NH₂), 1768 cm⁻¹ (C=O)

¹H NMR (600 MHz DMSO-*d*₆) δ 8.83 (d, 1H, *J* = 1.3 Hz, ArH), 8.73 (d, 1H, *J* = 2.3 Hz, ArH), 8.63 (d, 1H, *J* = 2.6 Hz, ArH), 7.99 (dd, 1H, *J* = 1.4 and 7.68 Hz, ArH), 7.76 (d, 1H, *J* = 15.8 Hz), 7.51 (t, 1H, *J* = 2.4 Hz, ArH), 7.49 (d, 1H, *J* = 15.9 Hz), 7.39 (t, 1H, *J* = 7.7 Hz, CH), 7.21 (dd, 1H, *J* = 1.9 and 7.6 Hz, ArH), 3.15 (q, 1H, CH), 1.79 (m, 1H, CH₂), 1.71 (m, 1H, CH₂), 0.91 (dd, *J* = 1.4 and 6.7 Hz, 6H, CH₃ ×2).

¹³C NMR (600 MHz DMSO-*d*₆) δ 169.4, 150.5, 148.1, 144.9, 144.6, 144.2, 130.7, 130.6, 128.9, 127.9, 127.7, 127.4, 126.8, 51.3, 40.2, 22.6, 22.3.

HRMS (ESI): C₁₈H₂₁N₃O₂ requires 311.1627; found [M+H]⁺ 312.1706

Purity (HPLC): 94.2%

(*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl-*L*-serinate (67) was prepared from (*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl(tert-butoxycarbonyl)-*L*-serinate (**60**) as a yellow gel in 24% yield (0.03 g).

IR (ATR; *V*_{max}/cm⁻¹) 3442 cm⁻¹ (NH₂ or OH overlapping signal), 1765 cm⁻¹ (C=O)

¹H NMR (600 MHz DMSO-*d*₆) δ 8.85 (d, 1H, *J* = 1.2 Hz ArH), 8.64 (d, 1H, *J* = 4 Hz ArH), 8.55 (d, 1H, *J* = 2.4 Hz ArH), 7.99 (dd, 1H, *J* = 1.3 and 7.6 Hz ArH), 7.81 (d, 1H, *J* = 16 Hz), 7.48 (t, 1H, *J* = 7 Hz ArH),

7.46 (d, 1H, J = 16 Hz), 7.43 (t, 1H, J = 7 Hz, ArH), 7.25 (dd, 1H, J = 1.3 and 8 Hz, ArH), 4.7 (t, 1H, CH), 3.62 (m, 2H, CH₂)

¹³C NMR (600 MHz DMSO-*d*₆) δ 168, 150.7, 148.3, 145.2, 144.5, 144.0, 130.6, 128.9, 128.1, 127.6, 127.5, 127.1, 123.4, 65.1, 58.2

HRMS (ESI): C₁₅H₁₅N₃O₂ requires 285.2998; found [M+H]⁺ 286.3076

Purity (HPLC): 94.3%

(*E*)-2-(2-(Pyrazin-2-yl)vinyl)phenyl (S)-2-amino-2-(4-hydroxyphenyl)acetate (68) was prepared from (*E*)-2-(2-(pyrazin-2-yl)vinyl)phenyl (S)-2-((tert-butoxycarbonyl)amino)-2-(4-hydroxyphenyl)acetate (**61**) as a brown/yellow gel in 75% yield (0.129 g).

IR (ATR; V_{max}/cm^{-1}) 3171 cm⁻¹ (NH₂), 1741 cm⁻¹ (C=O)

¹H NMR (600 MHz DMSO-*d*₆) δ 8.85 (d, 1H, J = 1.2 Hz ArH), 8.64 (d, 1H, J = 2.5 Hz, ArH), 8.55 (d, 1H, J = 2.5 Hz ArH), 7.99 (dd, 1H, J = 1.3 and 7.9 Hz, ArH), 7.81 (d, 1H, J = 16 Hz), 7.48 (t, 1H, J = 7 Hz ArH), 7.46 (d, 1H, J = 16 Hz), 7.43 (t, 1H, J = 7 Hz, ArH), 7.25 (dd, 1H, J = 8 Hz, ArH), 6.71 (d, 1H, J = 8.5 Hz, ArH), 4.70 (t, 1H, CH), 4.07 (d, 2H, CH₂)

¹³C NMR (600 MHz DMSO-*d*₆) δ 170.9, 157.6, 150.7, 148.0, 145.2, 144.5, 143.7, 131.0, 130.5, 128.9, 128.1, 127.6, 127.1, 123.4, 115.8, 53.9, 35.5

HRMS (ESI): C₂₁H₁₉N₃O₃ requires 361.4073; found [M+H]⁺ 362.4079

Purity (HPLC): 90%

2-((*E*)-2-(Pyrazin-2-yl)vinyl)phenyl (2S)-2-amino-3-methylpentanoate (69) was prepared from 2-((*E*)-2-(pyrazin-2-yl)vinyl)phenyl (2S)-2-((tert-butoxycarbonyl)amino)-3-methylpentanoate (**62**) as a yellow solid in 78% yield (0.133 g).

Melting point: 183 °C

IR (ATR; V_{max}/cm^{-1}) 3359 cm⁻¹ (NH₂), 1766 cm⁻¹ (C=O)

¹H NMR (600 MHz, DMSO-*d*₆) δ 8.84 (d, 1H, J = 1.4 Hz, ArH), 8.64 (d, 1H, J = 2.4 Hz ArH), 8.55 (d, J = 2.4 Hz, 1H, ArH), 7.98 (dd, J = 1.5 and 8.19 Hz, 1H, ArH), 7.80 (d, J = 16.5 Hz, 1H), 7.52 (d, J = 7.69 Hz, 1H, ArH), 7.48 (d, J = 16.2 Hz, 1H), 7.44 (t, 1H, ArH), 7.23 (dd, J = 8.19 Hz, 1H, ArH), 4.50 (m, 1H, CH), 2.15 (m, 2H, CH₂), 1.9 (m, 1H, CH), 1.11 (d, J = 7.2 Hz, 3H, CH₃), 0.99 (d, J = 7.1 Hz, 3H, CH₃)

¹³C NMR (600 MHz, DMSO-*d*₆) δ 167.8, 150.1, 147.5, 144.5, 144.1, 143.7, 130.1, 128.4, 127.4, 127.2, 127.0, 126.4, 122.6, 56.7, 36.1, 25.0, 14.3, 11.5

HRMS (ESI): C₁₈H₂₁N₃O₂ requires 311.1627; found [M+H]⁺ 312.1706

Purity (HPLC): 98.7%

7.4 General method for the formation of 70 and its analogues

7.4.1 Conventional Perkin condensation

The appropriate hydroxybenzaldehyde (10 mmol) was dissolved in acetic anhydride (30 mL), followed by the addition of 2-methylquinoline (1.43 g, 1.35 mL, 10 mmol). The resulting mixture was stirred at reflux temperature (140°C) for 24 hours. Then, the solvent was evaporated *in vacuo* and the crude mixture was purified by flash column chromatography over silica gel (hexane: ethyl acetate, gradient 60:40 to 50:50).

(*E*)-2-(2-(Quinoline-2-yl)vinyl)phenyl acetate (76) was synthesised from 2-hydroxybenzaldehyde and 2-methylquinoline as a yellow solid in 93% yield (2.76 g).

Melting point: 160°C

IR (ATR; $V_{\max}/\text{cm}^{-1}$): 1749 cm^{-1} (C=O)

^1H NMR (600 MHz DMSO- d_6) δ 8.37 (d, 1H, J = 9.1 Hz, ArH), 8.02 (d, 1H, J = 8.1 Hz, ArH), 7.97 (d, 1H, J = 8.5 Hz, ArH), 7.91 (d, 1H, J = 8.5 Hz, ArH), 7.80 (d, 1H, J = 16 Hz), 7.77 (d, 1H, J = 8 Hz, ArH), 7.58 (t, 1H, J = 8 Hz, ArH), 7.52 (d, 1H, J = 16 Hz), 7.42 (dd, 1H, J = 7.5 and 1.5 Hz, ArH), 7.36 (t, 1H, J = 8.6 Hz, ArH), 7.21 (dd, 1H, J = 2.2 and 8.8 Hz, ArH), 2.42 (s, 3H, acetate).

^{13}C NMR (600 MHz DMSO- d_6) δ 169.3, 159.2, 155.3, 148.8, 146.4, 136.5, 132.1, 130.4, 130.3, 129.3, 128.7, 128.3, 127, 126.3, 124.7, 123, 122.6, 120.8 120.1, 21.3

HRMS (ESI): $\text{C}_{19}\text{H}_{15}\text{NO}_2$ requires 289.1105; found $[\text{M}+\text{H}]^+$ 290.1184

Purity (HPLC): 98.1%

(*E*)-2-(2-(Quinoline-2-yl)vinyl)-1,4-phenylene diacetate (75) was synthesised from 2,5-dihydroxybenzaldehyde and 2-methylquinoline as a dark brown solid in 93% yield (2.00 g).

IR (ATR; $V_{\max}/\text{cm}^{-1}$) 1758 cm^{-1} (C=O)

^1H NMR (600 MHz DMSO- d_6) δ 8.38 (d, 1H, J = 9.1 Hz), 8.13 (d, 1H, J = 8.1 Hz), 7.97 (d, 1H, J = 8.5 Hz), 7.89 (d, 1H, J = 8.5 Hz), 7.77 (d, 1H, J = 16 Hz), 7.59 (dd, 1H, J = 8 Hz, 1.2 Hz), 7.54 (d, 1H, J = 16 Hz), 7.27 (d, 1H, J = 8.6 Hz), 7.18 (dd, 1H, J = 8.8 Hz, 2.2 Hz), 2.45 (s, 3H, acetate), 2.32 (s, 3H, acetate).

^{13}C NMR (600 MHz, DMSO- d_6) δ 169.9, 169.8, 159.2, 155.3, 148.8, 146.4, 136.5, 132.1, 130.4, 130.3, 129.3, 128.7, 128.3, 127.0, 126.3, 124.7, 123, 122.6, 120.8 120.1, 21.3, 21.2

7.4.1.1 Hydrolysis of acetate or diacetate group of compounds 75 and 76

The appropriate acetate analogue was dissolved in ethyl acetate/ethanol (1:1 mixture, 30 mL). The resulting mixture was stirred at reflux temperature (100°C) followed by the addition of saturated NaOH aqueous solution (10 mL) and the reaction was monitored by TLC. Then, the solvent was evaporated in *vacuo* under reduced pressure. The crude mixture was purified over silica gel by flash column chromatography (hexane: ethyl acetate, gradient 1:1 to 1:2).

(E)-2-(2-(Quinolin-2-yl)vinyl)phenol (70) was obtained from (E)-2-(2-(quinoline-2-yl)vinyl)phenyl acetate (**76**) as a bright yellow solid in 95% yield (1.6 g).

Melting point: 216 °C

IR (ATR; V_{max}/cm^{-1}) 3008 cm^{-1} (Broad, OH)

^1H NMR (600 MHz, DMSO- d_6) δ 10.01 (br s, 1H, OH), 8.31, (d, 1H, J = 8.7 Hz, ArH), 8.05 (d, 1H, J = 16 Hz), 7.98 (d, 1H, J = 9.3 Hz, ArH), 7.92 (dd, 1H, J = 1.3 and 8.6 Hz, ArH), 7.73 (d, 1H, J = 8.3 Hz, 1.3), 7.53 (dd 1, J = 1.2 and 7.9 Hz, ArH), 7.66 (dd, 1H, J = 1.3 and 7.9 Hz, ArH), 7.46 (d, 1H, J = 16 Hz), 7.15 (d, 1H, J = 2.8 Hz, ArH), 6.94 (d, 1H, J = 8.65 Hz, ArH), 6.81 (t, 1H, J = 7.5 Hz, ArH)

^{13}C NMR (600 MHz, DMSO- d_6) δ 156.2 155.3, 149.2, 148.1, 136.8, 130.19, 130.14 129.1, 128.2, 128.0, 127.3, 126.3, 123.7, 120.5, 117.6, 117.2, 112.8

HRMS (ESI): $C_{17}H_{13}NO$ requires 247.1046; found $[M+H]^+$ 248.1123

Purity (HPLC): 92%

(E)-2-(2-(Quinolin-2-yl)vinyl)benzene-1,4-diol (74) was obtained from (E)-2-(2-(quinoline-2-yl)vinyl)-1,4-phenylene diacetate (**75**) as a dark brown solid in 85% yield (1.2 g).

Melting point: 200 °C

IR (ATR; V_{max}/cm^{-1}) 3054 cm^{-1} (Broad, OH)

^1H NMR (600 MHz, DMSO- d_6) δ 9.38 (br s 1H, OH), 8.88 (br s, 1H, OH), 8.38, (d, 1H, J = 8.3 Hz, ArH), 8.03 (d, 1H, J = 16 Hz), 7.99 (d, 1H, J = 8.3 Hz, ArH), 7.97 (dd, 1H, J = 1.4 and 8.6 Hz, ArH), 7.86 (d, 1H, J = 8.6 Hz, ArH), 7.77 (dd 1, J = 1.4 and 7.9 Hz, ArH), 7.57 (dd, 1H, J = 1.0 and 7.9 Hz, ArH), 7.37 (d, 1H, J = 16 Hz), 7.05 (d, 1H, J = 2.8 Hz), 6.75 (d, 1H, J = 8.6 Hz, ArH), 6.65 (dd, 1H, J = 3.0 and 9.0 Hz, ArH)

^{13}C NMR (600 MHz, DMSO- d_6) δ 156.5, 150.4, 149.2, 148.1, 136.8, 130.19, 130.14, 129.0, 128.2, 128.0, 127.3, 126.3, 126.3, 123.7, 120.5, 117.6, 117.2, 112.8

HRMS (ESI): $C_{17}H_{13}NO_2$ requires 263.1056; $[M+H]^+$ found 264.1130

Purity (HPLC): 90%

Appendices

Appendix 1: X-ray crystallography data related to chapter 4

Compound 70 (Q1)

A specimen of $C_{17}H_{13}NO$, approximate dimensions 0.096 mm x 0.145 mm x 0.366 mm, was used for the X-ray crystallographic analysis. The X-ray intensity data were measured ($\lambda = 0.71073 \text{ \AA}$) on a Bruker D8 Quest ECO with an Oxford Cryostream Cryosystem low temperature device using a MiTeGen micromount. See Table 1 for collection parameters and exposure time.

A total of 2316 frames were collected. The total exposure time was 10.19 hours. The frames were integrated with the Bruker SAINT software package using a narrow-frame algorithm. The integration of the data using a monoclinic unit cell yielded a total of 25481 reflections to a maximum θ angle of 28.27° ($0.75 \text{ \AA}$ resolution), of which 3009 were independent (average redundancy 8.468, completeness = 99.8%, $R_{\text{int}} = 2.63\%$, $R_{\text{sig}} = 1.80\%$) and 2543 (84.51%) were greater than $2\sigma(F^2)$. The final cell constants of $a = 12.7251(2) \text{ \AA}$, $b = 6.66010(10) \text{ \AA}$, $c = 14.7632(3) \text{ \AA}$, $\beta = 103.3500(5)^\circ$, volume = $1217.38(4) \text{ \AA}^3$, are based upon the refinement of the XYZ-centroids of 9978 reflections above $20 \sigma(I)$ with $5.672^\circ < 2\theta < 56.48^\circ$. Data were corrected for absorption effects using the multi-scan method (SADABS). The ratio of minimum to maximum apparent transmission was 0.958. The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.9700 and 0.9920.

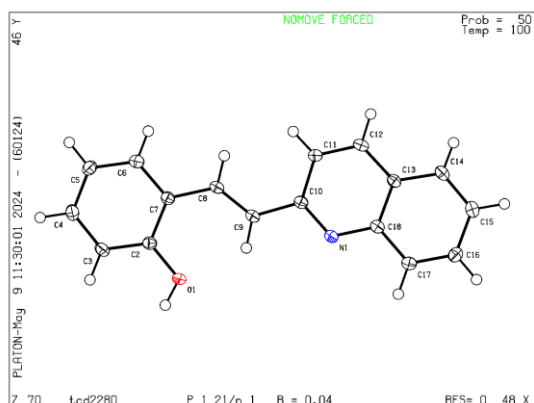
The structure was solved with the SHELXT structure solution program using Intrinsic Phasing and refined with the SHELXL refinement program using Least Squares minimisation within the OLEX2 Software Package, using the space group $P2_1/n$, with $Z = 4$ for the formula unit, $C_{17}H_{13}NO$. The final anisotropic full-matrix least-squares refinement on F^2 with 176 variables converged at $R1 = 4.02\%$, for the observed data and $wR2 = 11.75\%$ for all data. The goodness-of-fit was 1.061. The largest peak in the final difference electron density synthesis was $0.359 \text{ e}^-/\text{\AA}^3$ and the largest hole was $-0.191 \text{ e}^-/\text{\AA}^3$ with an RMS deviation of $0.048 \text{ e}^-/\text{\AA}^3$. On the basis of the final model, the calculated density was 1.349 g/cm^3 and $F(000)$, 520 e^- .

Refinement Note: Donor OH hydrogen refined with restraints (DFIX).

Table 1: Data collection details for 70.

Axis	dx/mm	2 θ /°	ω /°	ϕ /°	χ /°	Width/°	Frames	Time/s	Wavelength/Å	Voltage/kV	Current/mA	Temperature/K
Omega	61.247	19.02	198.15	102.00	54.76	0.50	386	15.00	0.71073	50	20.0	100
Omega	61.247	19.02	198.15	153.00	54.76	0.50	386	15.00	0.71073	50	20.0	100
Omega	61.247	19.07	198.13	317.84	54.76	0.50	386	15.00	0.71073	50	20.0	100
Omega	61.247	19.02	198.15	204.00	54.76	0.50	386	15.00	0.71073	50	20.0	100
Omega	61.247	23.34	202.40	36.00	54.76	0.50	386	20.00	0.71073	50	20.0	100
Omega	61.247	19.02	198.15	51.00	54.76	0.50	386	15.00	0.71073	50	20.0	100

Structure Tables



A clear light yellow, plate-shaped crystal of tcd2280 was mounted on a MiTeGen micromount with NVH immersion oil. Data were collected from a shock-cooled single crystal at 100(2) K on a Bruker D8 Quest ECO three-circle diffractometer with a sealed X-ray tube using a graphite monochromator and a Bruker PHOTON III C7 detector. The diffractometer was equipped with an Oxford Cryostream 800 low temperature device and used Mo K_{α} radiation ($\lambda = 0.71073 \text{ \AA}$). All data were integrated with SAINT and a multi-scan absorption correction using SADABS was applied.^[1,2] The structure was solved by dual methods using XT and refined by full-matrix least-squares methods against F^2 by XL using Olex2.^[3-5] All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms were refined with isotropic displacement parameters. Some were refined freely and some on calculated positions using a riding model with their U_{iso} values constrained to 1.5 times the U_{eq} of their pivot atoms for terminal sp^3 carbon atoms and 1.2 times for all other carbon atoms. Crystallographic data for the structures reported here have been deposited with the Cambridge Crystallographic Data Centre.^[6] CCDC 2222222 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures. This report was generated using FinalCif.^[7]

Table 1. Crystal data and structure refinement for tcd2280

CCDC number	
Empirical formula	$\text{C}_{17}\text{H}_{13}\text{NO}$
Formula weight	247.28
Temperature [K]	100(2)
Crystal system	monoclinic
Space group (number)	$P2_1/n$ (14)
a [Å]	12.7251(2)
b [Å]	6.66010(10)
c [Å]	14.7632(3)
α [°]	90
β [°]	103.3500(5)
γ [°]	90
Volume [Å ³]	1217.38(4)
Z	4
ρ_{calc} [gcm ⁻³]	1.349
μ [mm ⁻¹]	0.084
$F(000)$	520
Crystal size [mm ³]	0.366×0.145×0.096
Crystal colour	clear light yellow
Crystal shape	plate
Radiation	Mo K_{α} ($\lambda=0.71073 \text{ \AA}$)
2θ range [°]	5.67 to 56.55 (0.75 Å)
Index ranges	$-16 \leq h \leq 16$ $-8 \leq k \leq 8$ $-19 \leq l \leq 19$
Reflections collected	25481
Independent reflections	3009 $R_{\text{int}} = 0.0263$ $R_{\text{sigma}} = 0.0180$

Completeness to $\theta = 25.242^\circ$	99.9 %
Data / Restraints / Parameters	3009/1/176
Absorption correction $T_{\min}/T_{\max}$ (method)	0.7144/0.7457 (multi-scan)
Goodness-of-fit on F^2	1.061

Final R indexes [$\geq 2\sigma(I)$]	$R_1 = 0.0402$ $wR_2 = 0.1108$
Final R indexes [all data]	$R_1 = 0.0481$ $wR_2 = 0.1175$
Largest peak/hole [$\text{e}\text{\AA}^{-3}$]	0.36/−0.19

Refinement details for 70.

Donor OH hydrogen refined with restraints (DFIX).

Table 2. Atomic coordinates and U_{eq} [$\text{\AA}^2$] for tcd2280

Atom	<i>x</i>	<i>y</i>	<i>z</i>	U_{eq}
O1	0.66584(7)	0.24477(12)	0.21405(5)	0.0208(2)
H1	0.6853(13)	0.224(2)	0.1626(8)	0.041(4)
N1	0.76117(7)	0.71968(13)	0.44534(6)	0.0149(2)
C2	0.60684(8)	0.09225(16)	0.23668(7)	0.0156(2)
C3	0.57303(9)	-0.06864(16)	0.17601(7)	0.0181(2)
H3	0.592648	-0.072134	0.117723	0.022
C4	0.51147(9)	-0.22274(17)	0.19960(8)	0.0194(2)
H4	0.489258	-0.330847	0.157560	0.023
C5	0.48191(9)	-0.21997(16)	0.28479(8)	0.0192(2)
H5	0.438866	-0.324569	0.300941	0.023
C6	0.51630(9)	-0.06231(16)	0.34551(7)	0.0175(2)
H6	0.497037	-0.061918	0.403960	0.021
C7	0.57861(8)	0.09714(15)	0.32397(7)	0.0149(2)
C8	0.61013(8)	0.25592(16)	0.39314(7)	0.0155(2)
H8	0.588163	0.237612	0.449867	0.019
C9	0.66609(8)	0.42439(16)	0.38710(7)	0.0161(2)
H9	0.689697	0.445815	0.331342	0.019
C10	0.69347(8)	0.57743(15)	0.45978(7)	0.0147(2)
C11	0.64856(8)	0.57991(16)	0.53991(7)	0.0161(2)
H11	0.601847	0.474975	0.549710	0.019
C12	0.67297(8)	0.73343(16)	0.60227(7)	0.0167(2)
H12	0.642700	0.736321	0.655365	0.020
C13	0.74352(8)	0.88878(15)	0.58791(7)	0.0153(2)
C14	0.77236(9)	1.05459(16)	0.64898(7)	0.0183(2)
H14	0.742801	1.066395	0.702245	0.022
C15	0.84261(9)	1.19790(16)	0.63165(7)	0.0192(2)
H15	0.862055	1.307785	0.673174	0.023
C16	0.88604(9)	1.18225(17)	0.55213(7)	0.0186(2)
H16	0.935078	1.281574	0.540866	0.022
C17	0.85839(9)	1.02548(16)	0.49086(7)	0.0172(2)

H17	0.887313	1.018208	0.437107	0.021
C18	0.78687(8)	0.87469(15)	0.50768(7)	0.0146(2)

U_{eq} is defined as 1/3 of the trace of the orthogonalized U_{ij} tensor.

Table 3. Anisotropic displacement parameters [$\text{\AA}^2$] for tcd2280. The anisotropic displacement factor exponent takes the form: $-2\pi^2 [h^2(a^*)^2 U_{11} + k^2(b^*)^2 U_{22} + \dots + 2hka^*b^* U_{12}]$

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
O1	0.0292(4)	0.0199(4)	0.0172(4)	−0.0035(3)	0.0136(3)	−0.0061(3)
N1	0.0165(4)	0.0157(4)	0.0129(4)	0.0000(3)	0.0044(3)	0.0017(3)
C2	0.0146(5)	0.0165(5)	0.0163(5)	0.0003(4)	0.0049(4)	0.0012(4)
C3	0.0184(5)	0.0216(5)	0.0152(5)	−0.0033(4)	0.0057(4)	0.0010(4)
C4	0.0176(5)	0.0193(5)	0.0208(5)	−0.0055(4)	0.0036(4)	−0.0006(4)
C5	0.0183(5)	0.0171(5)	0.0232(5)	0.0000(4)	0.0065(4)	−0.0023(4)
C6	0.0184(5)	0.0186(5)	0.0163(5)	0.0011(4)	0.0059(4)	0.0012(4)
C7	0.0147(5)	0.0154(5)	0.0149(5)	0.0001(4)	0.0042(4)	0.0021(4)
C8	0.0171(5)	0.0173(5)	0.0131(4)	0.0001(4)	0.0054(4)	0.0023(4)
C9	0.0178(5)	0.0181(5)	0.0133(5)	−0.0010(4)	0.0057(4)	0.0013(4)
C10	0.0147(5)	0.0159(5)	0.0137(5)	0.0009(4)	0.0038(4)	0.0024(4)
C11	0.0167(5)	0.0177(5)	0.0152(5)	0.0011(4)	0.0061(4)	−0.0003(4)
C12	0.0170(5)	0.0211(5)	0.0131(5)	0.0010(4)	0.0059(4)	0.0026(4)
C13	0.0147(5)	0.0177(5)	0.0136(5)	−0.0006(4)	0.0034(4)	0.0025(4)
C14	0.0187(5)	0.0213(5)	0.0150(5)	−0.0034(4)	0.0043(4)	0.0018(4)
C15	0.0203(5)	0.0182(5)	0.0181(5)	−0.0032(4)	0.0020(4)	0.0013(4)
C16	0.0182(5)	0.0176(5)	0.0192(5)	0.0017(4)	0.0029(4)	−0.0016(4)
C17	0.0181(5)	0.0191(5)	0.0149(5)	0.0014(4)	0.0049(4)	0.0008(4)
C18	0.0140(5)	0.0162(5)	0.0132(4)	0.0008(4)	0.0025(4)	0.0026(4)

Table 4. Bond lengths and angles for 70.

Atom–Atom	Length [$\text{\AA}$]
O1–H1	0.862(9)
O1–C2	1.3502(13)
N1–C10	1.3304(13)
N1–C18	1.3714(13)

C2–C3	1.3992(15)
C2–C7	1.4158(14)
C3–H3	0.9500
C3–C4	1.3836(15)
C4–H4	0.9500

C4–C5	1.3936(15)
C5–H5	0.9500
C5–C6	1.3843(15)
C6–H6	0.9500
C6–C7	1.4049(14)
C7–C8	1.4604(14)
C8–H8	0.9500
C8–C9	1.3428(15)
C9–H9	0.9500
C9–C10	1.4625(14)
C10–C11	1.4277(13)
C11–H11	0.9500
C11–C12	1.3629(14)
C12–H12	0.9500
C12–C13	1.4175(15)
C13–C14	1.4193(14)
C13–C18	1.4205(14)
C14–H14	0.9500
C14–C15	1.3715(15)
C15–H15	0.9500
C15–C16	1.4123(15)
C16–H16	0.9500
C16–C17	1.3730(15)
C17–H17	0.9500
C17–C18	1.4152(15)
Atom–Atom–Atom	Angle [°]
C2–O1–H1	112.8(11)
C10–N1–C18	119.43(8)
O1–C2–C3	121.61(9)
O1–C2–C7	118.72(9)
C3–C2–C7	119.68(10)
C2–C3–H3	119.5

C4–C3–C2	121.01(10)
C4–C3–H3	119.5
C3–C4–H4	119.9
C3–C4–C5	120.25(10)
C5–C4–H4	119.9
C4–C5–H5	120.5
C6–C5–C4	118.92(10)
C6–C5–H5	120.5
C5–C6–H6	118.7
C5–C6–C7	122.50(10)
C7–C6–H6	118.7
C2–C7–C8	124.51(9)
C6–C7–C2	117.62(9)
C6–C7–C8	117.86(9)
C7–C8–H8	115.7
C9–C8–C7	128.53(9)
C9–C8–H8	115.7
C8–C9–H9	117.5
C8–C9–C10	125.01(9)
C10–C9–H9	117.5
N1–C10–C9	115.60(9)
N1–C10–C11	121.50(9)
C11–C10–C9	122.87(9)
C10–C11–H11	120.1
C12–C11–C10	119.74(10)
C12–C11–H11	120.1
C11–C12–H12	120.0
C11–C12–C13	120.00(9)
C13–C12–H12	120.0
C12–C13–C14	123.55(9)
C12–C13–C18	117.24(9)
C14–C13–C18	119.20(10)
C13–C14–H14	119.8

C15–C14–C13	120.47(10)
C15–C14–H14	119.8
C14–C15–H15	120.0
C14–C15–C16	120.05(10)
C16–C15–H15	120.0
C15–C16–H16	119.5
C17–C16–C15	120.92(10)

C17–C16–H16	119.5
C16–C17–H17	120.0
C16–C17–C18	120.10(10)
C18–C17–H17	120.0
N1–C18–C13	122.07(9)
N1–C18–C17	118.67(9)
C17–C18–C13	119.26(9)

Table 5. Torsion angles for tcd2280

Atom–Atom–Atom–Atom	Torsion Angle [°]
O1–C2–C3–C4	–179.19(9)
O1–C2–C7–C6	179.39(9)
O1–C2–C7–C8	–0.86(15)
N1–C10–C11–C12	1.64(16)
C2–C3–C4–C5	0.04(17)
C2–C7–C8–C9	2.50(18)
C3–C2–C7–C6	–0.37(15)
C3–C2–C7–C8	179.38(10)
C3–C4–C5–C6	–0.82(16)
C4–C5–C6–C7	1.02(17)
C5–C6–C7–C2	–0.43(16)
C5–C6–C7–C8	179.81(10)
C6–C7–C8–C9	–177.75(10)
C7–C2–C3–C4	0.56(16)
C7–C8–C9–C10	179.38(10)
C8–C9–C10–N1	171.57(10)
C8–C9–C10–C11	–10.38(17)
C9–C10–C11–C12	–176.30(10)
C10–N1–C18–C13	0.09(15)
C10–N1–C18–C17	–179.54(9)
C10–C11–C12–C13	–0.61(16)
C11–C12–C13–C14	179.48(10)

C11–C12–C13–C18	–0.58(15)
C12–C13–C14–C15	178.88(10)
C12–C13–C18–N1	0.88(15)
C12–C13–C18–C17	–179.49(9)
C13–C14–C15–C16	0.60(16)
C14–C13–C18–N1	–179.18(9)
C14–C13–C18–C17	0.45(15)
C14–C15–C16–C17	0.48(16)
C15–C16–C17–C18	–1.08(16)
C16–C17–C18–N1	–179.76(9)
C16–C17–C18–C13	0.60(15)
C18–N1–C10–C9	176.72(9)
C18–N1–C10–C11	–1.36(15)
C18–C13–C14–C15	–1.05(15)

Table 6. Hydrogen bonds for 70.

D—H...A [Å]	d(D—H) [Å]	d(H...A) [Å]	d(D...A) [Å]	<(DHA) [°]
O1—H1...N1 ^{#1}	0.862(9)	1.873(9)	2.7270(11)	170.8(16)
C9—H9...O1	0.95	2.15	2.8204(12)	126

Symmetry transformations used to generate equivalent atoms:

#1: 1.5-X, -0.5+Y, 0.5-Z;

Compound 74 (Q7)

A specimen of $C_{17}H_{13}NO_2$, approximate dimensions 0.120 mm x 0.140 mm x 0.260 mm, was used for the X-ray crystallographic analysis. The X-ray intensity data were measured ($\lambda = 1.54178 \text{ \AA}$) on a Bruker Apex Kappa Duo with an Oxford Cobra Cryosystem low temperature device using a MiTeGen micromount. See Table 1 for collection parameters and exposure time.

A total of 1738 frames were collected. The total exposure time was 21.68 hours. The integration of the data using a monoclinic unit cell yielded a total of 14737 reflections to a maximum θ angle of 70.41° ($0.82 \text{ \AA}$ resolution), of which 2386 were independent (average redundancy 6.176, completeness = 98.1%, $R_{\text{int}} = 7.68\%$, $R_{\text{sig}} = 5.24\%$) and 1644 (68.90%) were greater than $2\sigma(F^2)$. The final cell constants of $a = 4.9249(2) \text{ \AA}$, $b = 21.0805(9) \text{ \AA}$, $c = 12.4976(6) \text{ \AA}$, $\beta = 99.911(3)^\circ$, volume = $1278.13(10) \text{ \AA}^3$, are based upon the refinement of the XYZ-centroids of 2231 reflections above $20 \sigma(I)$ with $8.317^\circ < 2\theta < 138.6^\circ$. Data were corrected for absorption effects using the Multi-Scan method (SADABS). The ratio of minimum to maximum apparent transmission was 0.782. The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.8340 and 0.9180.

The structure was solved with the SHELXT structure solution program using Intrinsic Phasing and refined with the SHELXL refinement package using Least Squares minimisation with Olex2, using the space group $P2_1/n$, with $Z = 4$ for the formula unit, $C_{17}H_{13}NO_2$. The final anisotropic full-matrix least-squares refinement on F^2 with 189 variables converged at $R1 = 6.11\%$, for the observed data and $wR2 = 17.87\%$ for all data. The goodness-of-fit was 1.080. The largest peak in the final difference electron density synthesis was $0.237 \text{ e}^-/\text{\AA}^3$ and the largest hole was $-0.313 \text{ e}^-/\text{\AA}^3$ with an RMS deviation of $0.065 \text{ e}^-/\text{\AA}^3$. On the basis of the final model, the calculated density was 1.368 g/cm^3 and $F(000)$, 552 e^- .

Table 1: Data collection details for 74.

Axis	dx/mm	2 θ /°	ω /°	ϕ /°	χ /°	Width/°	Frames	Time/s	Wavelength/Å	Voltage/kV	Current/mA	Temperature/K
Omega	50.000	108.90	96.00	168.00	-54.74	2.00	67	55.00	1.54184	50	0.6	100
Omega	50.000	-49.30	190.66	64.00	54.74	2.00	67	30.00	1.54184	50	0.6	100
Omega	50.000	108.90	343.40	264.00	64.50	2.00	69	55.00	1.54184	50	0.6	100
Omega	50.000	-49.30	299.02	160.00	-64.50	2.00	58	30.00	1.54184	50	0.6	100
Phi	50.000	109.30	4.12	4.00	23.00	2.00	92	55.00	1.54184	50	0.6	100
Omega	50.000	108.90	343.40	72.00	64.50	2.00	69	55.00	1.54184	50	0.6	100
Omega	50.000	94.14	353.81	9.30	81.44	2.00	36	55.00	1.54184	50	0.6	100
Omega	50.000	-49.30	190.65	32.00	54.74	2.00	67	30.00	1.54184	50	0.6	100
Omega	50.000	108.90	96.00	24.00	-54.74	2.00	67	55.00	1.54184	50	0.6	100
Omega	50.000	-11.30	228.66	153.00	54.74	2.00	67	30.00	1.54184	50	0.6	100
Omega	50.000	108.90	343.40	24.00	64.50	2.00	69	55.00	1.54184	50	0.6	100
Omega	50.000	108.90	96.00	72.00	-54.74	2.00	67	55.00	1.54184	50	0.6	100
Omega	50.000	-49.30	190.66	360.00	54.74	2.00	67	30.00	1.54184	50	0.6	100
Omega	50.000	-49.30	299.02	64.00	-64.50	2.00	58	30.00	1.54184	50	0.6	100
Omega	50.000	108.90	96.00	48.00	-54.74	2.00	67	55.00	1.54184	50	0.6	100
Omega	50.000	108.90	343.40	312.00	64.50	2.00	69	55.00	1.54184	50	0.6	100
Omega	50.000	108.90	96.00	192.00	-54.74	2.00	67	55.00	1.54184	50	0.6	100
Omega	50.000	-49.30	299.02	256.00	-64.50	2.00	58	30.00	1.54184	50	0.6	100
Omega	50.000	-49.30	190.65	192.00	54.74	2.00	67	30.00	1.54184	50	0.6	100

Omega	50.000	108.90	96.00	72.00	-54.74	2.00	67	55.00	1.54184	50	0.6	100
Omega	50.000	-11.30	337.02	204.00	-64.50	2.00	39	30.00	1.54184	50	0.6	100
Omega	50.000	108.90	343.40	192.00	64.50	2.00	69	55.00	1.54184	50	0.6	100
Omega	50.000	-49.30	299.02	96.00	-64.50	2.00	58	30.00	1.54184	50	0.6	100
Omega	50.000	108.90	343.40	72.00	64.50	2.00	69	55.00	1.54184	50	0.6	100
Phi	50.000	-47.74	343.92	340.00	23.00	2.00	96	30.00	1.54184	50	0.6	100
Phi	50.000	109.30	4.12	4.00	23.00	2.00	92	55.00	1.54184	50	0.6	100

Table 2. Crystal data and structure refinement for 74.

Identification code	tcd1648b
Empirical formula	C ₁₇ H ₁₃ NO ₂
Formula weight	263.28
Temperature	100(2) K
Wavelength	1.54178 Å
Crystal system	Monoclinic
Space group	P2 ₁ /n
Unit cell dimensions	a = 4.9249(2) Å β = 90°. b = 21.0805(9) Å γ = 99.911(3)°. c = 12.4976(6) Å α = 90°.
Volume	1278.13(10) Å ³
Z	4
Density (calculated)	1.368 Mg/m ³
Absorption coefficient	0.725 mm ⁻¹
F(000)	552
Crystal size	0.26 x 0.14 x 0.12 mm ³
Theta range for data collection	4.158 to 70.409°.
Index ranges	-5 ≤ h ≤ 5, -25 ≤ k ≤ 25, -15 ≤ l ≤ 14
Reflections collected	14737
Independent reflections	2386 [R(int) = 0.0768]
Completeness to theta = 67.679°	99.7 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7533 and 0.5891
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	2386 / 2 / 189
Goodness-of-fit on F ²	1.080

Final R indices [$I > 2\sigma(I)$]	R1 = 0.0611, wR2 = 0.1622
R indices (all data)	R1 = 0.0898, wR2 = 0.1787
Largest diff. peak and hole	0.237 and -0.313 e.Å ⁻³

Table 3. Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$)**For 90. $U(\text{eq})$ is defined as one third of the trace of the orthogonalized U^{ij} tensor.**

	x	y	z	$U(\text{eq})$
O(1)	7742(4)	2144(1)	4201(2)	32(1)
N(1)	2370(4)	3744(1)	7585(2)	25(1)
O(2)	13522(5)	1598(1)	8291(2)	39(1)
C(2)	414(5)	4209(1)	7549(2)	24(1)
C(3)	-313(6)	4422(1)	8537(2)	28(1)
C(4)	-2266(6)	4878(1)	8537(2)	33(1)
C(5)	-3601(6)	5151(1)	7553(2)	34(1)
C(6)	-2914(6)	4956(1)	6592(2)	31(1)
C(7)	-920(5)	4480(1)	6562(2)	26(1)
C(8)	-163(6)	4246(1)	5587(2)	26(1)
C(9)	1820(5)	3795(1)	5640(2)	26(1)
C(10)	3106(5)	3550(1)	6663(2)	24(1)
C(11)	5283(5)	3072(1)	6772(2)	25(1)
C(12)	6161(5)	2761(1)	5971(2)	26(1)
C(13)	8373(5)	2289(1)	6119(2)	24(1)
C(14)	9105(6)	1977(1)	5219(2)	25(1)
C(15)	11184(6)	1526(1)	5363(2)	29(1)
C(16)	12649(6)	1394(1)	6389(2)	29(1)
C(17)	11989(6)	1716(1)	7282(2)	28(1)
C(18)	9839(5)	2143(1)	7141(2)	26(1)

Table 4. Bond lengths [Å] and angles [°] for TCD1648b.

O(1)-H(1)	0.990(10)	C(12)-C(13)	1.463(4)
O(1)-C(14)	1.378(3)	C(13)-C(14)	1.402(4)
N(1)-C(2)	1.371(3)	C(13)-C(18)	1.389(4)
N(1)-C(10)	1.331(3)	C(14)-C(15)	1.386(4)
O(2)-H(2)	0.988(10)	C(15)-H(15)	0.9500
O(2)-C(17)	1.377(3)	C(15)-C(16)	1.387(4)
C(2)-C(3)	1.416(3)	C(16)-H(16)	0.9500
C(2)-C(7)	1.414(4)	C(16)-C(17)	1.392(4)
C(3)-H(3)	0.9500	C(17)-C(18)	1.378(4)
C(3)-C(4)	1.361(4)	C(18)-H(18)	0.9500
C(4)-H(4)	0.9500		
C(4)-C(5)	1.413(4)	C(14)-O(1)-H(1)	106(3)
C(5)-H(5)	0.9500	C(10)-N(1)-C(2)	119.0(2)
C(5)-C(6)	1.366(4)	C(17)-O(2)-H(2)	108(3)
C(6)-H(6)	0.9500	N(1)-C(2)-C(3)	118.7(2)
C(6)-C(7)	1.409(4)	N(1)-C(2)-C(7)	122.4(2)
C(7)-C(8)	1.422(4)	C(7)-C(2)-C(3)	119.0(2)
C(8)-H(8)	0.9500	C(2)-C(3)-H(3)	119.7
C(8)-C(9)	1.357(4)	C(4)-C(3)-C(2)	120.5(3)
C(9)-H(9)	0.9500	C(4)-C(3)-H(3)	119.7
C(9)-C(10)	1.422(4)	C(3)-C(4)-H(4)	119.6
C(10)-C(11)	1.460(4)	C(3)-C(4)-C(5)	120.7(3)
C(11)-H(11)	0.9500	C(5)-C(4)-H(4)	119.6
C(11)-C(12)	1.331(4)	C(4)-C(5)-H(5)	120.2
C(12)-H(12)	0.9500	C(6)-C(5)-C(4)	119.6(3)

C(6)-C(5)-H(5)	120.2	C(11)-C(12)-C(13)	124.9(2)
C(5)-C(6)-H(6)	119.4	C(13)-C(12)-H(12)	117.6
C(5)-C(6)-C(7)	121.2(3)	C(14)-C(13)-C(12)	120.4(2)
C(7)-C(6)-H(6)	119.4	C(18)-C(13)-C(12)	121.7(2)
C(2)-C(7)-C(8)	117.2(2)	C(18)-C(13)-C(14)	118.0(2)
C(6)-C(7)-C(2)	119.0(2)	O(1)-C(14)-C(13)	118.0(2)
C(6)-C(7)-C(8)	123.8(3)	O(1)-C(14)-C(15)	121.7(2)
C(7)-C(8)-H(8)	120.2	C(15)-C(14)-C(13)	120.2(2)
C(9)-C(8)-C(7)	119.6(2)	C(14)-C(15)-H(15)	119.5
C(9)-C(8)-H(8)	120.2	C(14)-C(15)-C(16)	120.9(3)
C(8)-C(9)-H(9)	119.9	C(16)-C(15)-H(15)	119.5
C(8)-C(9)-C(10)	120.2(2)	C(15)-C(16)-H(16)	120.4
C(10)-C(9)-H(9)	119.9	C(15)-C(16)-C(17)	119.1(3)
N(1)-C(10)-C(9)	121.5(2)	C(17)-C(16)-H(16)	120.4
N(1)-C(10)-C(11)	115.8(2)	O(2)-C(17)-C(16)	118.6(3)
C(9)-C(10)-C(11)	122.7(2)	O(2)-C(17)-C(18)	121.5(3)
C(10)-C(11)-H(11)	116.6	C(18)-C(17)-C(16)	119.8(3)
C(12)-C(11)-C(10)	126.8(2)	C(13)-C(18)-H(18)	119.1
C(12)-C(11)-H(11)	116.6	C(17)-C(18)-C(13)	121.9(3)
C(11)-C(12)-H(12)	117.6	C(17)-C(18)-H(18)	119.1

Table 5. Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for 90. The anisotropic displacement factor exponent takes the form: $-2\pi^2 [h^2 a^{*2} U^{11} + \dots + 2 h k a^* b^* U^{12}]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
O(1)	42(1)	32(1)	21(1)	-1(1)	2(1)	7(1)
N(1)	26(1)	25(1)	24(1)	0(1)	3(1)	-2(1)
O(2)	42(1)	44(1)	27(1)	2(1)	-4(1)	11(1)
C(2)	23(1)	24(1)	26(1)	0(1)	4(1)	-4(1)
C(3)	32(2)	27(1)	26(1)	-2(1)	4(1)	-5(1)
C(4)	38(2)	28(1)	36(2)	-6(1)	13(1)	-4(1)
C(5)	31(2)	28(1)	43(2)	-4(1)	10(1)	-1(1)
C(6)	30(2)	30(1)	33(2)	3(1)	4(1)	-1(1)
C(7)	24(1)	25(1)	28(1)	1(1)	5(1)	-4(1)
C(8)	30(2)	25(1)	23(1)	3(1)	2(1)	-2(1)
C(9)	27(1)	26(1)	23(1)	-1(1)	3(1)	-3(1)
C(10)	26(1)	23(1)	22(1)	0(1)	4(1)	-5(1)
C(11)	27(1)	26(1)	21(1)	1(1)	2(1)	-3(1)
C(12)	27(1)	26(1)	22(1)	2(1)	1(1)	-1(1)
C(13)	26(1)	23(1)	24(1)	-1(1)	3(1)	-4(1)
C(14)	28(1)	24(1)	23(1)	0(1)	2(1)	-3(1)
C(15)	33(2)	31(1)	24(1)	-2(1)	6(1)	0(1)
C(16)	29(1)	26(1)	33(2)	2(1)	5(1)	2(1)
C(17)	28(1)	29(1)	26(1)	4(1)	0(1)	-3(1)
C(18)	27(1)	27(1)	24(1)	-3(1)	4(1)	-2(1)

Table 6. Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for 90.

	x	y	z	U(eq)
H(1)	7890(90)	1774(13)	3720(30)	83(14)
H(2)	13360(100)	1971(14)	8760(30)	97(16)
H(3)	570	4244	9206	34
H(4)	-2738	5015	9206	40
H(5)	-4968	5468	7560	40
H(6)	-3795	5144	5933	37
H(8)	-1040	4405	4903	32
H(9)	2352	3642	4991	31
H(11)	6163	2974	7490	30
H(12)	5288	2851	5249	31
H(15)	11610	1305	4751	35
H(16)	14085	1087	6480	35
H(18)	9345	2343	7761	31

Table 7. Torsion angles [°] for 74.

O(1)-C(14)-C(15)-C(16)	175.9(2)	C(11)-C(12)-C(13)-C(14)	178.7(3)
N(1)-C(2)-C(3)-C(4)	-179.3(2)	C(11)-C(12)-C(13)-C(18)	-2.5(4)
N(1)-C(2)-C(7)-C(6)	179.8(2)	C(12)-C(13)-C(14)-O(1)	1.9(4)
N(1)-C(2)-C(7)-C(8)	0.3(4)	C(12)-C(13)-C(14)-C(15)	-179.4(2)
N(1)-C(10)-C(11)-C(12)	172.5(3)	C(12)-C(13)-C(18)-C(17)	-177.6(2)
O(2)-C(17)-C(18)-C(13)	176.7(3)	C(13)-C(14)-C(15)-C(16)	-2.7(4)
C(2)-N(1)-C(10)-C(9)	-3.0(4)	C(14)-C(13)-C(18)-C(17)	1.3(4)
C(2)-N(1)-C(10)-C(11)	177.5(2)	C(14)-C(15)-C(16)-C(17)	0.7(4)
C(2)-C(3)-C(4)-C(5)	-0.2(4)	C(15)-C(16)-C(17)-O(2)	-177.7(2)
C(2)-C(7)-C(8)-C(9)	-1.7(4)	C(15)-C(16)-C(17)-C(18)	2.3(4)
C(3)-C(2)-C(7)-C(6)	0.6(4)	C(16)-C(17)-C(18)-C(13)	-3.3(4)
C(3)-C(2)-C(7)-C(8)	-179.0(2)	C(18)-C(13)-C(14)-O(1)	-176.9(2)
C(3)-C(4)-C(5)-C(6)	-0.3(4)	C(18)-C(13)-C(14)-C(15)	1.7(4)
C(4)-C(5)-C(6)-C(7)	0.9(4)		
C(5)-C(6)-C(7)-C(2)	-1.1(4)		
C(5)-C(6)-C(7)-C(8)	178.5(3)		
C(6)-C(7)-C(8)-C(9)	178.7(3)		
C(7)-C(2)-C(3)-C(4)	0.0(4)		
C(7)-C(8)-C(9)-C(10)	0.9(4)		
C(8)-C(9)-C(10)-N(1)	1.5(4)		
C(8)-C(9)-C(10)-C(11)	-179.0(2)		
C(9)-C(10)-C(11)-C(12)	-7.0(4)		
C(10)-N(1)-C(2)-C(3)	-178.7(2)		
C(10)-N(1)-C(2)-C(7)	2.1(4)		
C(10)-C(11)-C(12)-C(13)	179.4(2)		

Table 8. Hydrogen bonds for TCD1648b [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
O(1)-H(1)...N(1)#1	0.990(10)	1.776(17)	2.735(3)	162(4)
O(2)-H(2)...O(1)#2	0.988(10)	1.988(19)	2.938(3)	161(4)
C(18)-H(18)...O(1)#2	0.95	2.48	3.109(3)	123

Symmetry transformations used to generate equivalent atoms:

#1 $x+1/2, -y+1/2, z-1/2$ #2 $x+1/2, -y+1/2, z+1/2$

Compound 76 (Q28)

A specimen of $C_{19}H_{15}NO_2$, approximate dimensions 0.184 mm x 0.299 mm x 0.505 mm, was used for the X-ray crystallographic analysis. The X-ray intensity data were measured ($\lambda = 0.71073 \text{ \AA}$) on a Bruker D8 Quest ECO with an Oxford Cryostream Cryosystem low temperature device using a MiTeGen micromount. See Table 1 for collection parameters and exposure time.

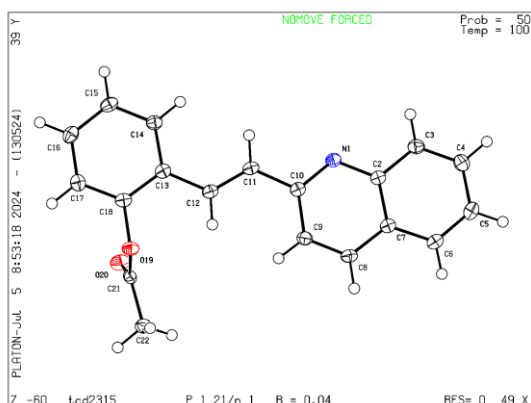
A total of 1930 frames were collected. The total exposure time was 1.07 hours. The integration of the data using a monoclinic unit cell yielded a total of 24777 reflections to a maximum θ angle of 28.30° ($0.75 \text{ \AA}$ resolution), of which 3567 were independent (average redundancy 6.946, completeness = 99.7%, $R_{\text{int}} = 2.55\%$, $R_{\text{sig}} = 1.94\%$) and 2996 (83.99%) were greater than $2\sigma(F^2)$. The final cell constants of $a = 10.9000(3) \text{ \AA}$, $b = 5.77440(10) \text{ \AA}$, $c = 22.9247(5) \text{ \AA}$, $\beta = 96.9966(7)^\circ$, volume = $1432.16(6) \text{ \AA}^3$, are based upon the refinement of the XYZ-centroids of 9923 reflections above $20 \sigma(I)$ with $6.926^\circ < 2\theta < 56.60^\circ$. Data were corrected for absorption effects using the Multi-Scan method (SADABS). The ratio of minimum to maximum apparent transmission was 0.944. The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.7040 and 0.7457.

The structure was solved with the SHELXT structure solution program using Intrinsic Phasing and refined with the SHELXL refinement program using Least Squares minimisation within the OLEX2 Software Package, using the space group $P2_1/n$, with $Z = 4$ for the formula unit, $C_{19}H_{15}NO_2$. The final anisotropic full-matrix least-squares refinement on F^2 with 200 variables converged at $R1 = 4.04\%$, for the observed data and $wR2 = 10.69\%$ for all data. The goodness-of-fit was 1.036. The largest peak in the final difference electron density synthesis was $0.314 \text{ e}^-/\text{\AA}^3$ and the largest hole was $-0.263 \text{ e}^-/\text{\AA}^3$ with an RMS deviation of $0.044 \text{ e}^-/\text{\AA}^3$. On the basis of the final model, the calculated density was 1.342 g/cm^3 and $F(000)$, 608 e^- .

Table 1: Data collection details for 76

Axis	dx/mm	2 θ /°	ω /°	ϕ /°	χ /°	Width/°	Frames	Time/s	Wavelength/Å	Voltage/kV	Current/mA	Temperature/K
Omega	61.260	19.02	198.15	255.00	54.76	0.50	386	2.00	0.71073	50	20.0	100
Omega	61.260	19.02	198.15	153.00	54.76	0.50	386	2.00	0.71073	50	20.0	100
Omega	61.260	19.02	198.15	306.00	54.76	0.50	386	2.00	0.71073	50	20.0	100
Omega	61.260	19.02	198.15	204.00	54.76	0.50	386	2.00	0.71073	50	20.0	100
Omega	61.260	19.02	198.15	102.00	54.76	0.50	386	2.00	0.71073	50	20.0	100

Structure Tables



A colourless, block-shaped crystal of tcd2315 was mounted on a MiTeGen micromount with NVH immersion oil. Data were collected from a shock-cooled single crystal at 100(2) K on a Bruker D8 Quest ECO three-circle diffractometer with a sealed X-ray tube using a graphite monochromator and a Bruker PHOTON III C7 detector. The diffractometer was equipped with an Oxford Cryostream 800 low temperature device and used Mo K_{α} radiation ($\lambda = 0.71073 \text{ \AA}$). All data were integrated with SAINT and a multi-scan absorption correction using SADABS was applied.^[1,2] The structure was solved by dual methods using XT and refined by full-matrix least-squares methods against F^2 by XL using Olex2.^[3-5] All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms were refined isotropic on calculated positions using a riding model with their U_{iso} values constrained to 1.5 times the U_{eq} of their pivot atoms for terminal sp^3 carbon atoms and 1.2 times for all other carbon atoms. Crystallographic data for the structures reported here have been deposited with the Cambridge Crystallographic Data Centre.^[6] CCDC 2315151 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures. This was generated using FinalCif.^[7]

Table 1. Crystal data and structure refinement for tcd2315

CCDC number	
Empirical formula	$\text{C}_{19}\text{H}_{15}\text{NO}_2$
Formula weight	289.32
Temperature [K]	100(2)
Crystal system	monoclinic
Space group (number)	$P2_1/n$ (14)
a [Å]	10.9000(3)
b [Å]	5.77440(10)
c [Å]	22.9247(5)
α [°]	90
β [°]	96.9966(7)
γ [°]	90
Volume [Å ³]	1432.16(6)
Z	4
ρ_{calc} [gcm ⁻³]	1.342
μ [mm ⁻¹]	0.087
$F(000)$	608
Crystal size [mm ³]	0.505×0.299×0.184
Crystal colour	colourless
Crystal shape	block
Radiation	Mo K_{α} ($\lambda=0.71073 \text{ \AA}$)
2 θ range [°]	4.36 to 56.60 (0.75 Å)
Index ranges	$-14 \leq h \leq 14$ $-7 \leq k \leq 7$ $-30 \leq l \leq 30$
Reflections collected	24777
Independent reflections	3567 $R_{\text{int}} = 0.0255$ $R_{\text{sigma}} = 0.0194$

Completeness to $\theta = 25.242^\circ$	99.8 %
Data / Restraints / Parameters	3567/0/200
Absorption correction $T_{\min}/T_{\max}$ (method)	0.7040/0.7457 (multi-scan)
Goodness-of-fit on F^2	1.036

Final R indexes [$I \geq 2\sigma(I)$]	$R_1 = 0.0404$ $wR_2 = 0.0998$
Final R indexes [all data]	$R_1 = 0.0505$ $wR_2 = 0.1069$
Largest peak/hole [$\text{e}\text{\AA}^{-3}$]	0.31/−0.26

Table 2. Atomic coordinates and U_{eq} [$\text{\AA}^2$] for 76.

Atom	x	y	z	U_{eq}
N1	0.91943(9)	0.49281(17)	0.36995(4)	0.0174(2)
C2	0.92275(10)	0.3536(2)	0.32183(5)	0.0163(2)
C3	0.99024(11)	0.4297(2)	0.27630(5)	0.0201(2)
H3	1.031963	0.574401	0.279783	0.024
C4	0.99568(12)	0.2960(2)	0.22732(5)	0.0235(3)
H4	1.041354	0.348697	0.197159	0.028
C5	0.93399(12)	0.0802(2)	0.22121(5)	0.0226(3)
H5	0.937706	-0.010287	0.186865	0.027
C6	0.86889(11)	0.0016(2)	0.26470(5)	0.0195(2)
H6	0.828181	-0.143983	0.260564	0.023
C7	0.86201(10)	0.1363(2)	0.31579(5)	0.0165(2)
C8	0.79583(11)	0.0642(2)	0.36215(5)	0.0190(2)
H8	0.753935	-0.080392	0.359936	0.023
C9	0.79271(11)	0.2034(2)	0.40986(5)	0.0193(2)
H9	0.748480	0.156867	0.441123	0.023
C10	0.85642(10)	0.42006(19)	0.41257(5)	0.0166(2)
C11	0.85718(10)	0.5767(2)	0.46298(5)	0.0181(2)
H11	0.905526	0.713549	0.462737	0.022
C12	0.79547(10)	0.5431(2)	0.50926(5)	0.0181(2)
H12	0.747839	0.405294	0.509338	0.022
C13	0.79413(10)	0.6978(2)	0.55984(5)	0.0169(2)
C14	0.86368(10)	0.9033(2)	0.56712(5)	0.0186(2)
H14	0.912852	0.947852	0.537522	0.022
C15	0.86243(11)	1.0422(2)	0.61618(5)	0.0204(2)
H15	0.910584	1.179727	0.620090	0.024
C16	0.79028(11)	0.9799(2)	0.65989(5)	0.0213(3)
H16	0.789518	1.074643	0.693724	0.026
C17	0.71964(11)	0.7797(2)	0.65397(5)	0.0202(2)
H17	0.670133	0.736632	0.683562	0.024
C18	0.72203(10)	0.6432(2)	0.60444(5)	0.0171(2)
O19	0.65523(7)	0.43440(14)	0.60020(4)	0.01919(19)

O20	0.48292(8)	0.62346(15)	0.56267(4)	0.0238(2)
C21	0.53479(10)	0.4443(2)	0.57567(5)	0.0167(2)
C22	0.48075(12)	0.2067(2)	0.56775(5)	0.0220(3)
H22A	0.453381	0.179613	0.525990	0.033
H22B	0.410002	0.193090	0.590130	0.033
H22C	0.543452	0.091587	0.581948	0.033

U_{eq} is defined as 1/3 of the trace of the orthogonalized U_{ij} tensor.

Table3. Anisotropic displacement parameters [$\text{\AA}^2$] for tcd2315. The anisotropic displacement factor exponent takes the form: $-2\pi^2 [h^2(a^*)^2 U_{11} + k^2(b^*)^2 U_{22} + \dots + 2hka^*b^* U_{12}]$

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
N1	0.0173(5)	0.0148(5)	0.0196(5)	0.0005(4)	0.0005(4)	0.0002(4)
C2	0.0152(5)	0.0150(5)	0.0181(5)	0.0008(4)	-0.0007(4)	0.0011(4)
C3	0.0211(6)	0.0173(6)	0.0217(5)	0.0021(4)	0.0017(4)	-0.0013(4)
C4	0.0264(6)	0.0249(6)	0.0196(5)	0.0026(5)	0.0046(5)	-0.0011(5)
C5	0.0260(6)	0.0220(6)	0.0191(5)	-0.0033(5)	-0.0001(5)	0.0019(5)
C6	0.0192(6)	0.0167(6)	0.0216(5)	-0.0017(4)	-0.0015(4)	0.0003(4)
C7	0.0146(5)	0.0152(5)	0.0188(5)	0.0006(4)	-0.0012(4)	0.0013(4)
C8	0.0177(5)	0.0147(5)	0.0241(6)	-0.0002(4)	0.0013(4)	-0.0021(4)
C9	0.0184(5)	0.0175(6)	0.0224(5)	-0.0001(4)	0.0043(4)	-0.0025(4)
C10	0.0148(5)	0.0150(5)	0.0194(5)	0.0000(4)	0.0000(4)	0.0017(4)
C11	0.0177(5)	0.0141(5)	0.0219(5)	-0.0007(4)	0.0002(4)	-0.0016(4)
C12	0.0161(5)	0.0156(5)	0.0220(5)	-0.0015(4)	0.0004(4)	-0.0006(4)
C13	0.0142(5)	0.0164(5)	0.0193(5)	0.0003(4)	-0.0013(4)	0.0019(4)
C14	0.0163(5)	0.0180(6)	0.0212(5)	0.0004(4)	0.0008(4)	-0.0002(4)
C15	0.0178(5)	0.0169(6)	0.0250(6)	-0.0019(5)	-0.0029(4)	0.0002(4)
C16	0.0205(6)	0.0213(6)	0.0212(5)	-0.0038(5)	-0.0010(4)	0.0034(5)
C17	0.0187(6)	0.0225(6)	0.0193(5)	0.0008(5)	0.0021(4)	0.0029(5)
C18	0.0142(5)	0.0150(5)	0.0213(5)	0.0018(4)	-0.0013(4)	0.0010(4)
O19	0.0179(4)	0.0151(4)	0.0242(4)	0.0023(3)	0.0011(3)	-0.0009(3)
O20	0.0203(4)	0.0182(4)	0.0320(5)	0.0008(4)	-0.0004(4)	0.0013(3)
C21	0.0172(5)	0.0192(6)	0.0141(5)	0.0008(4)	0.0040(4)	-0.0013(4)
C22	0.0255(6)	0.0191(6)	0.0212(6)	0.0019(5)	0.0024(5)	-0.0047(5)

Table 4. Bond lengths and angles for 76.

Atom–Atom	Length [Å]
N1–C2	1.3690(15)
N1–C10	1.3291(15)
C2–C3	1.4180(16)
C2–C7	1.4175(16)
C3–H3	0.9500
C3–C4	1.3698(17)
C4–H4	0.9500
C4–C5	1.4146(18)
C5–H5	0.9500
C5–C6	1.3697(17)
C6–H6	0.9500
C6–C7	1.4155(16)
C7–C8	1.4179(16)
C8–H8	0.9500
C8–C9	1.3611(16)
C9–H9	0.9500
C9–C10	1.4285(16)
C10–C11	1.4668(16)
C11–H11	0.9500
C11–C12	1.3379(16)
C12–H12	0.9500
C12–C13	1.4654(16)
C13–C14	1.4072(16)
C13–C18	1.3992(16)
C14–H14	0.9500
C14–C15	1.3828(16)
C15–H15	0.9500
C15–C16	1.3942(17)
C16–H16	0.9500
C16–C17	1.3865(18)
C17–H17	0.9500

C17–C18	1.3852(16)
C18–O19	1.4056(14)
O19–C21	1.3649(14)
O20–C21	1.1993(14)
C21–C22	1.4954(16)
C22–H22A	0.9800
C22–H22B	0.9800
C22–H22C	0.9800
Atom–Atom–Atom	Angle [°]
C10–N1–C2	118.27(10)
N1–C2–C3	118.56(10)
N1–C2–C7	122.72(10)
C7–C2–C3	118.71(10)
C2–C3–H3	119.8
C4–C3–C2	120.45(11)
C4–C3–H3	119.8
C3–C4–H4	119.7
C3–C4–C5	120.67(11)
C5–C4–H4	119.7
C4–C5–H5	119.9
C6–C5–C4	120.13(11)
C6–C5–H5	119.9
C5–C6–H6	119.9
C5–C6–C7	120.30(11)
C7–C6–H6	119.9
C2–C7–C8	117.45(10)
C6–C7–C2	119.74(10)
C6–C7–C8	122.81(11)
C7–C8–H8	120.2
C9–C8–C7	119.58(11)
C9–C8–H8	120.2

C8–C9–H9	120.2
C8–C9–C10	119.51(10)
C10–C9–H9	120.2
N1–C10–C9	122.46(10)
N1–C10–C11	115.49(10)
C9–C10–C11	122.04(10)
C10–C11–H11	117.3
C12–C11–C10	125.44(11)
C12–C11–H11	117.3
C11–C12–H12	116.8
C11–C12–C13	126.39(11)
C13–C12–H12	116.8
C14–C13–C12	123.43(10)
C18–C13–C12	120.09(10)
C18–C13–C14	116.48(10)
C13–C14–H14	119.1
C15–C14–C13	121.81(11)
C15–C14–H14	119.1
C14–C15–H15	120.1
C14–C15–C16	119.81(11)

C16–C15–H15	120.1
C15–C16–H16	120.0
C17–C16–C15	120.00(11)
C17–C16–H16	120.0
C16–C17–H17	120.4
C18–C17–C16	119.28(11)
C18–C17–H17	120.4
C13–C18–O19	118.37(10)
C17–C18–C13	122.61(11)
C17–C18–O19	118.91(10)
C21–O19–C18	117.18(9)
O19–C21–C22	110.89(10)
O20–C21–O19	122.62(11)
O20–C21–C22	126.49(11)
C21–C22–H22A	109.5
C21–C22–H22B	109.5
C21–C22–H22C	109.5
H22A–C22–H22B	109.5
H22A–C22–H22C	109.5
H22B–C22–H22C	109.5

Table 5. Torsion angles for 76.

Atom–Atom–Atom– Atom	Torsion Angle [°]
N1–C2–C3–C4	–179.83(11)
N1–C2–C7–C6	179.74(10)
N1–C2–C7–C8	–0.06(16)
N1–C10–C11–C12	176.74(11)
C2–N1–C10–C9	0.02(16)
C2–N1–C10–C11	179.66(10)
C2–C3–C4–C5	0.15(18)
C2–C7–C8–C9	0.14(16)
C3–C2–C7–C6	–0.54(16)

C3–C2–C7–C8	179.66(10)
C3–C4–C5–C6	–0.64(19)
C4–C5–C6–C7	0.54(18)
C5–C6–C7–C2	0.06(17)
C5–C6–C7–C8	179.85(11)
C6–C7–C8–C9	–179.66(11)
C7–C2–C3–C4	0.44(17)
C7–C8–C9–C10	–0.14(17)
C8–C9–C10–N1	0.06(17)
C8–C9–C10–C11	–179.56(11)
C9–C10–C11–C12	–3.61(18)

C10–N1–C2–C3	–179.73(10)
C10–N1–C2–C7	–0.01(16)
C10–C11–C12–C13	–179.53(11)
C11–C12–C13–C14	–2.55(19)
C11–C12–C13–C18	178.10(11)
C12–C13–C14–C15	–178.39(11)
C12–C13–C18–C17	178.28(11)
C12–C13–C18–O19	2.09(16)
C13–C14–C15–C16	–0.32(18)
C13–C18–O19–C21	–95.13(12)
C14–C13–C18–C17	–1.11(16)
C14–C13–C18–O19	–177.30(9)
C14–C15–C16–C17	–0.28(18)
C15–C16–C17–C18	0.17(17)
C16–C17–C18–C13	0.56(17)
C16–C17–C18–O19	176.73(10)
C17–C18–O19–C21	88.53(13)
C18–C13–C14–C15	0.98(16)
C18–O19–C21–O20	–6.43(15)
C18–O19–C21–C22	173.33(9)

Table 6. Hydrogen bonds for 76.

D–H $\cdots$ A [Å]	d(D–H) [Å]	d(H $\cdots$ A) [Å]	d(D $\cdots$ A) [Å]	<(DHA) [°]
C22–H22A $\cdots$ O20 ^{#1}	0.98	2.50	3.2166(14)	130

Symmetry transformations used to generate equivalent

atoms:

#1: 1–X, 1–Y, 1–Z;

Appendix 2: NCI screening data of pyrazinib and its amino acid prodrugs (Chapter 2) and 76 (Chapter 4)

NCI compound reference numbers:

Compound **54**: NSC 831168

Compound **63**: NSC 852920

Compound **64**: NSC 852915

Compound **65**: NSC 852916

Compound **66**: NSC 852917

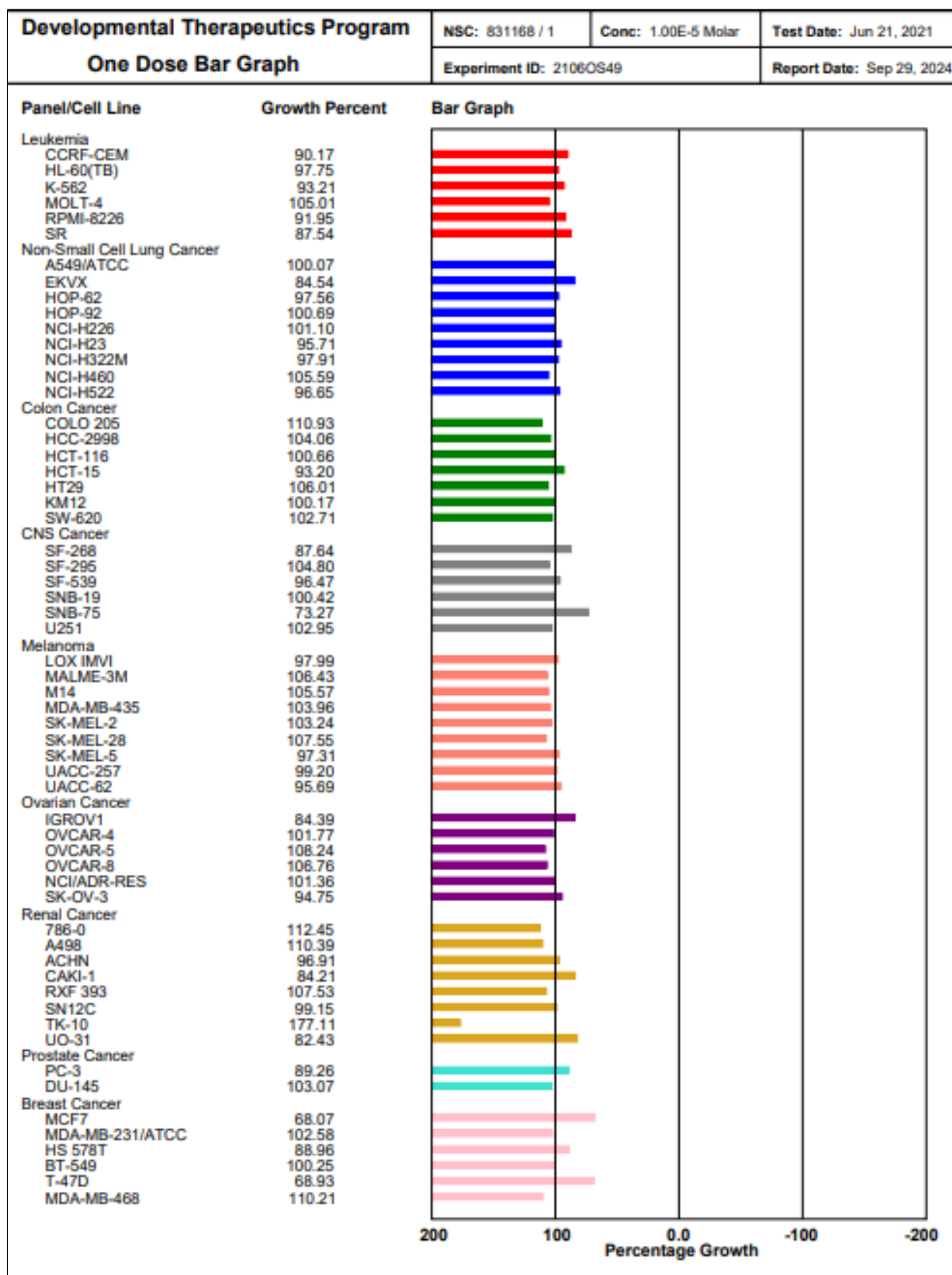
Compound **68**: NSC 852919

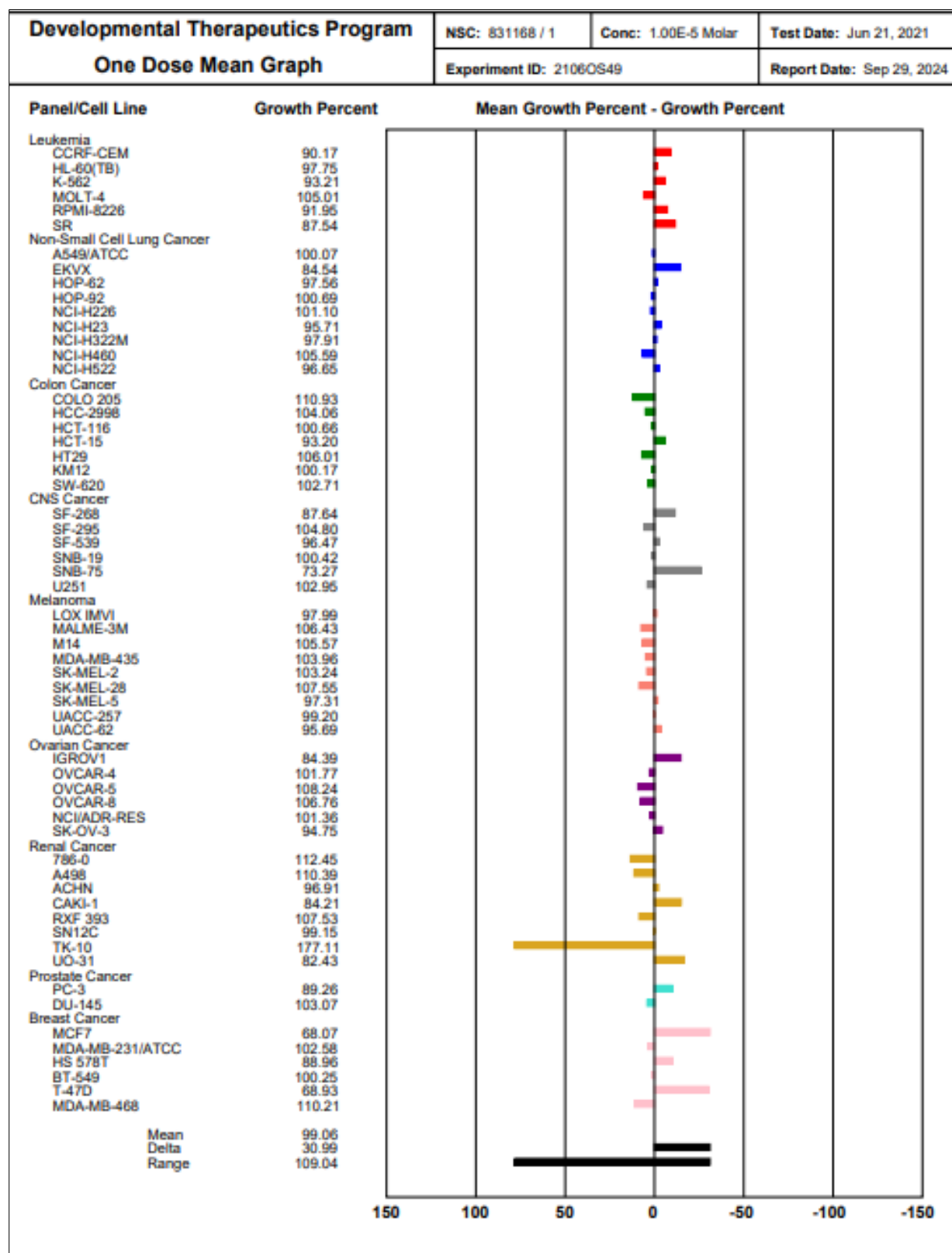
Compound **69**: NSC 852918 (data presented in section 2.6.2, chapter 2)

Compound **76**: NSC 843000

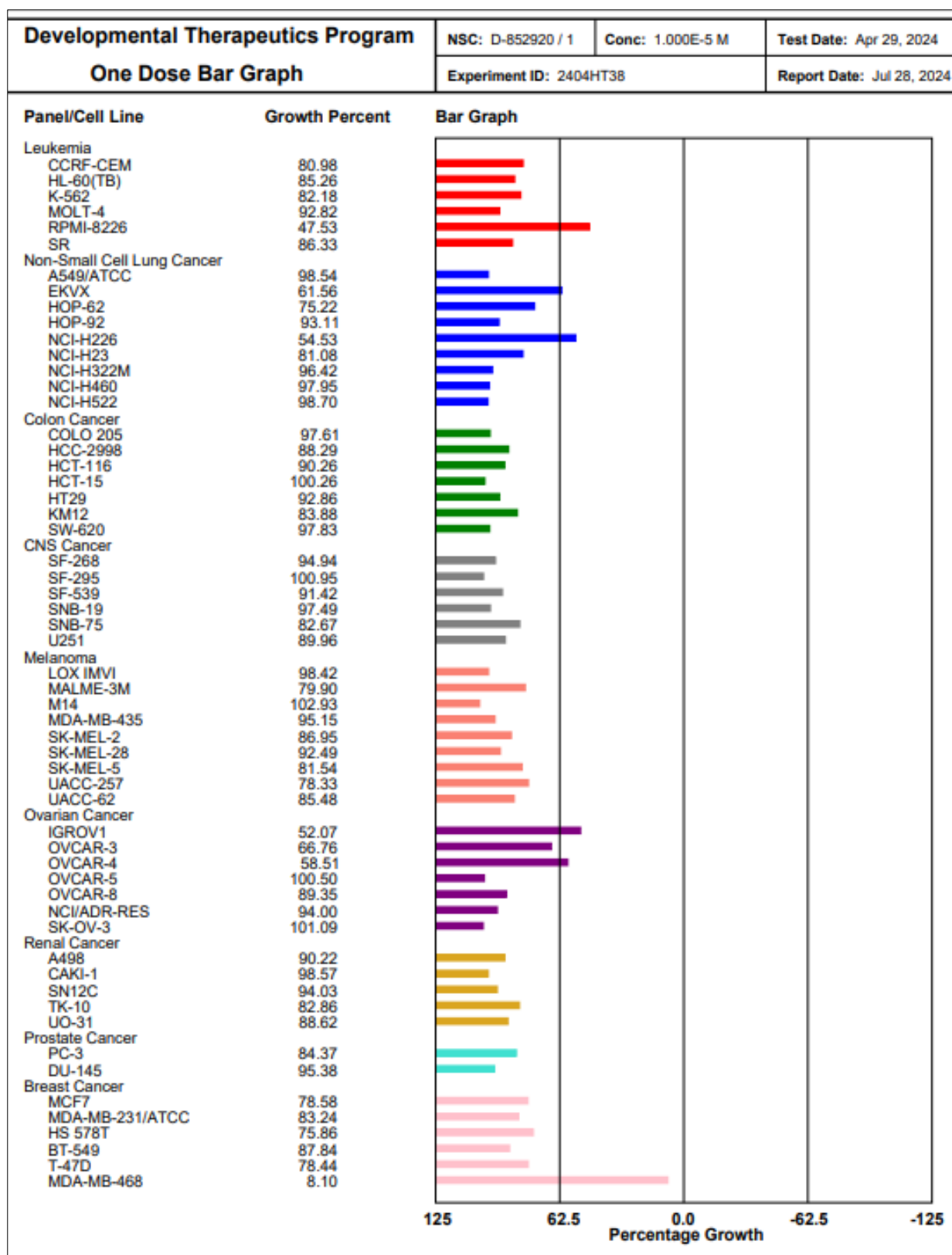
Note: For pyrazinib (**54**) and its amino acid prodrugs (**63-68**) one dose data is available and for **76**, five dose data is available.

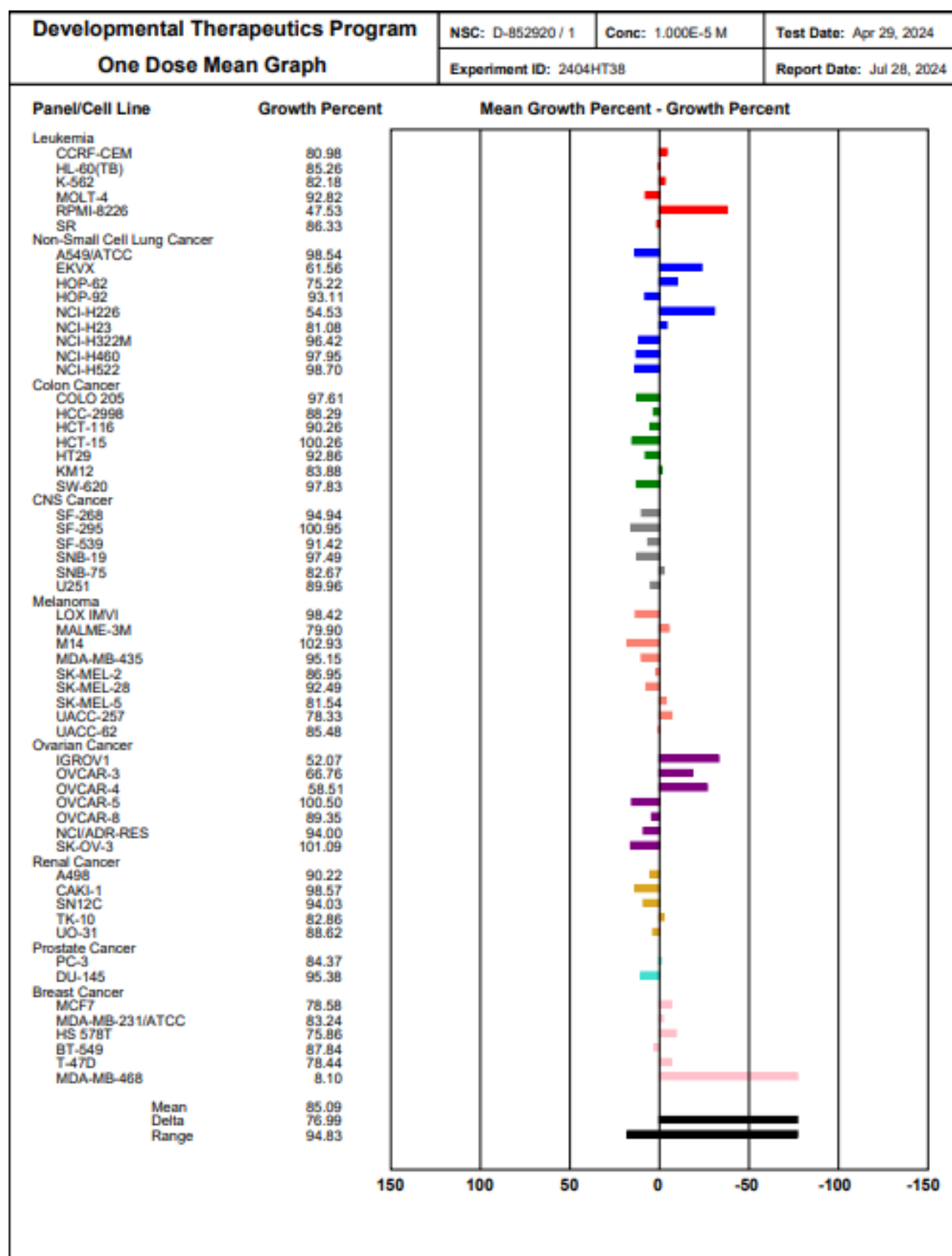
Compound 54



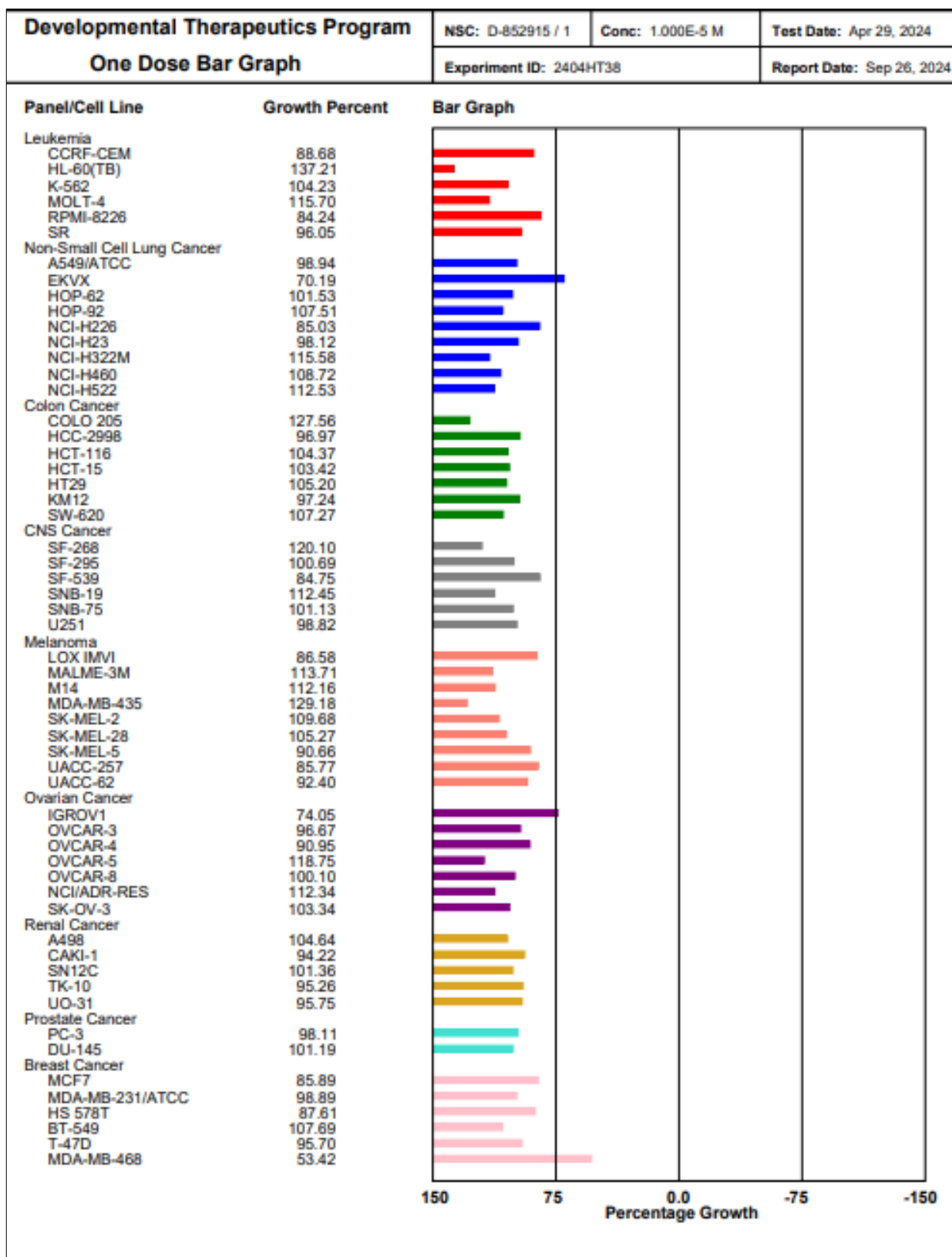


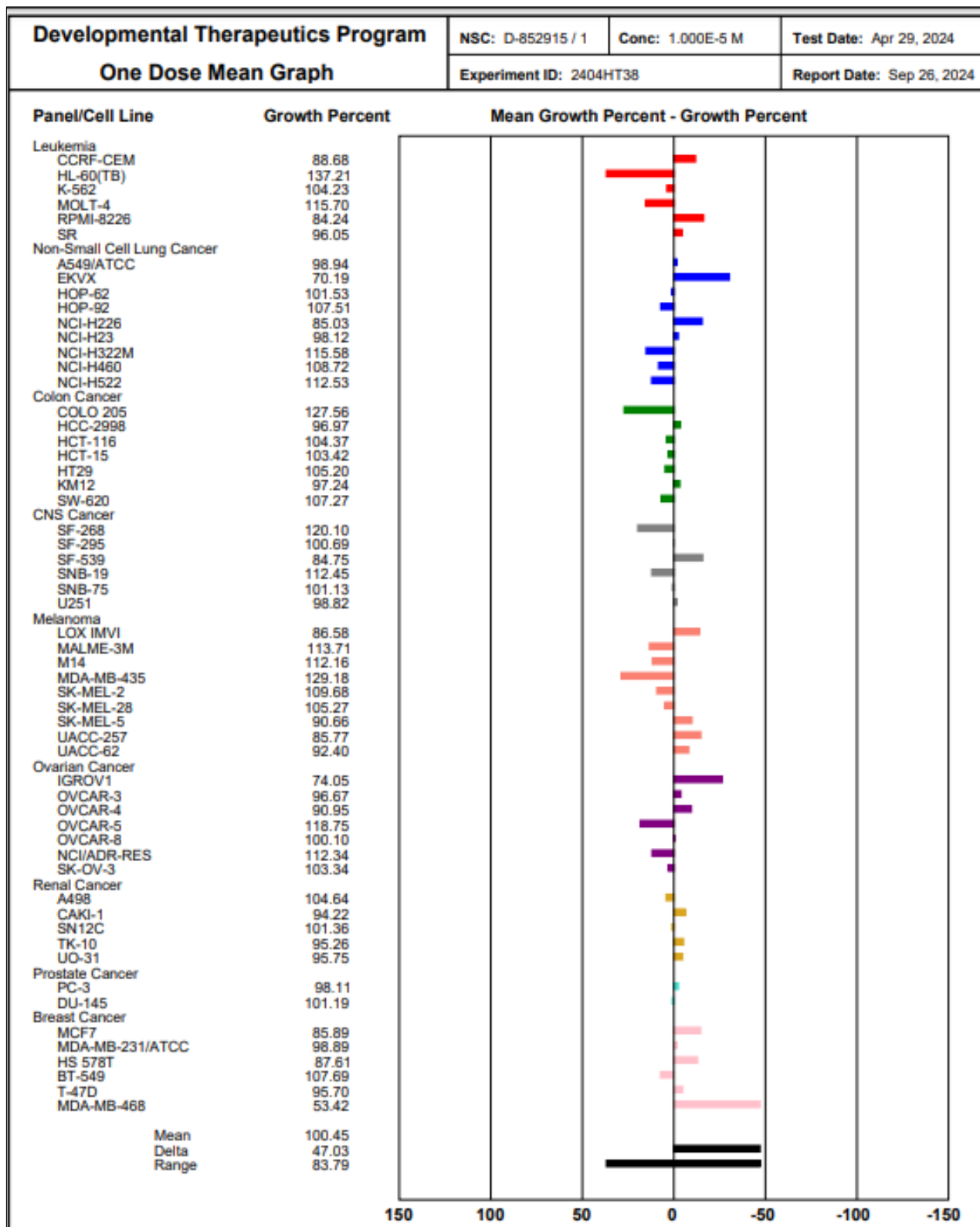
Compound 63



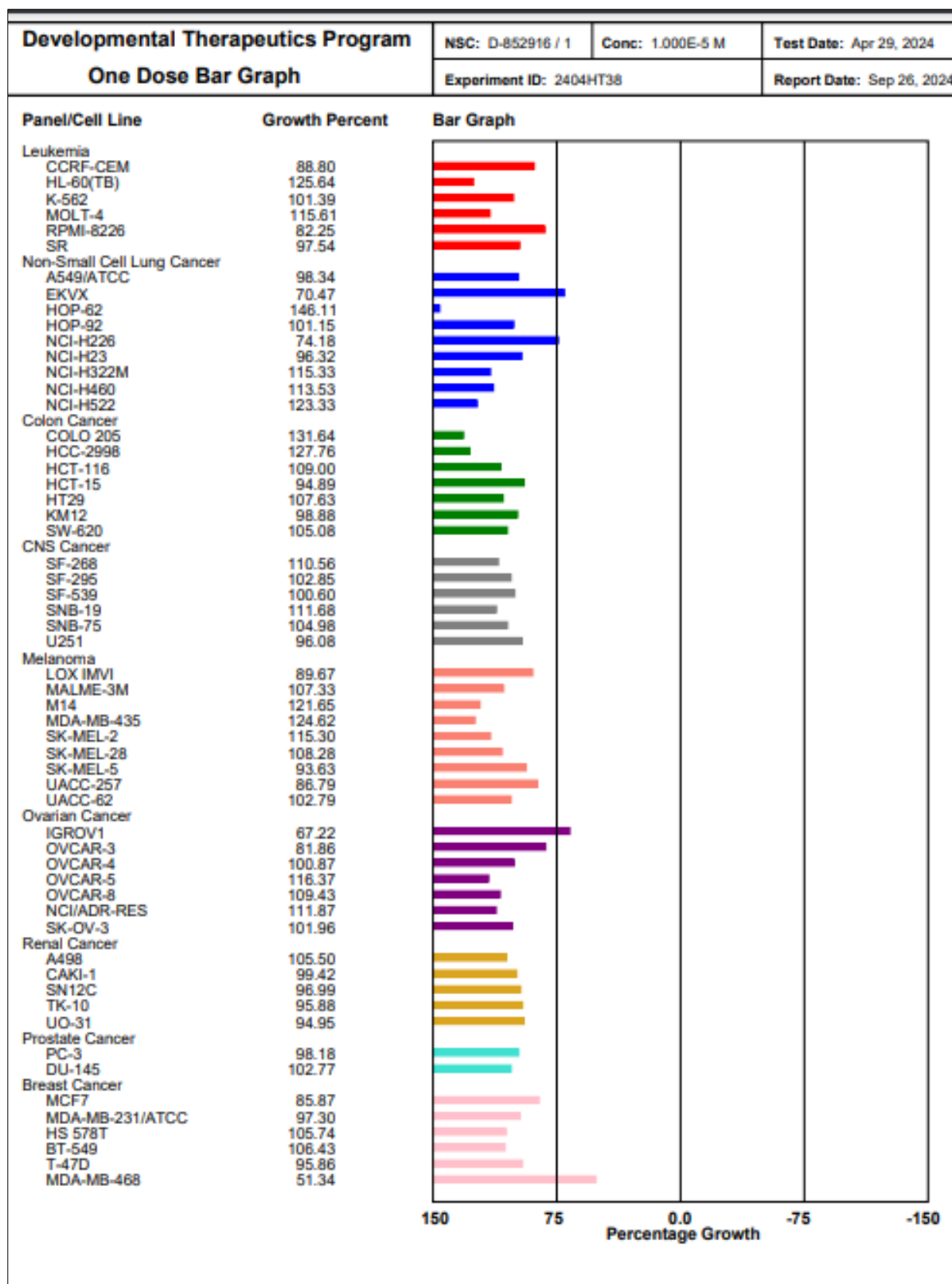


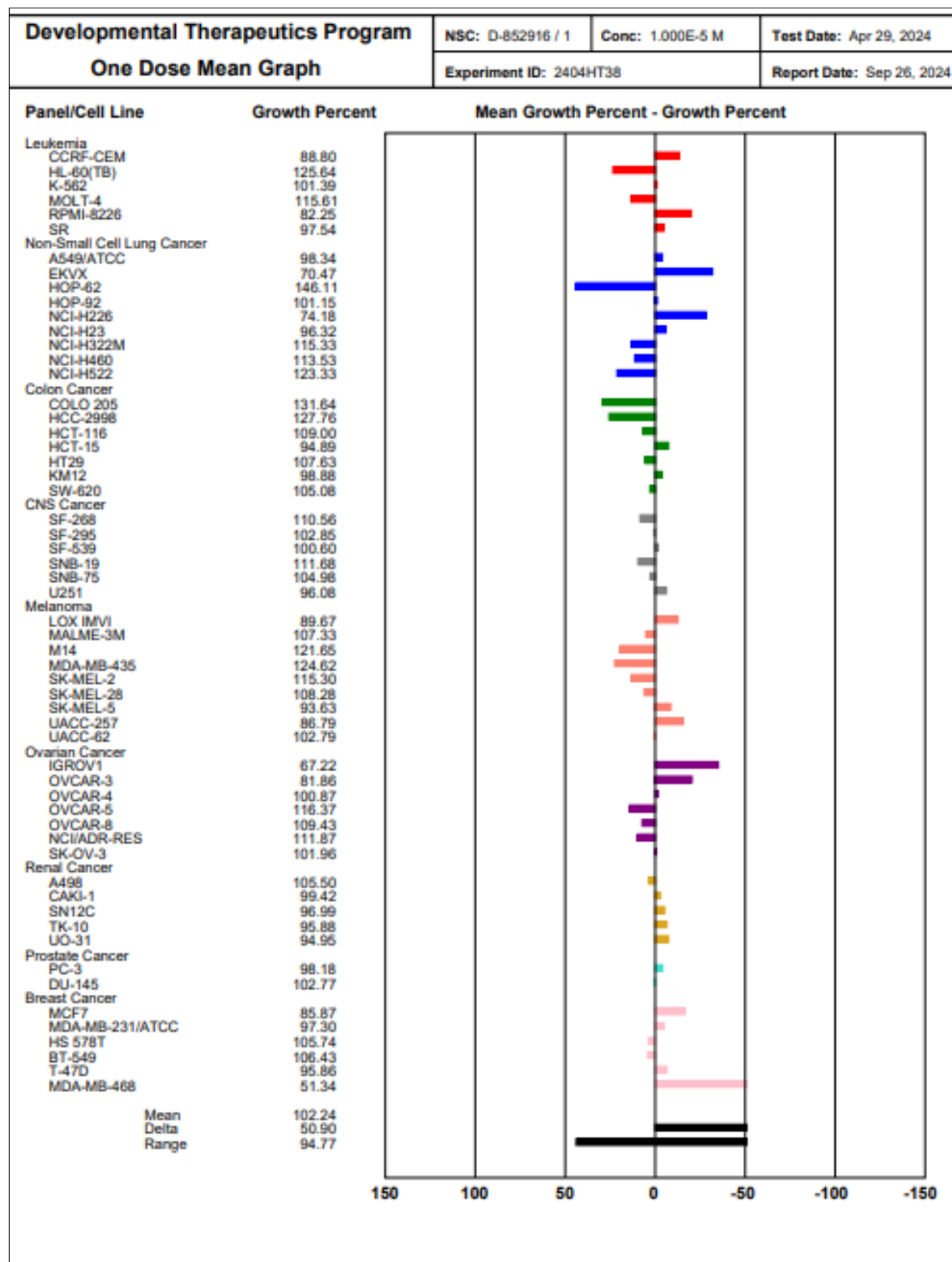
Compound 64



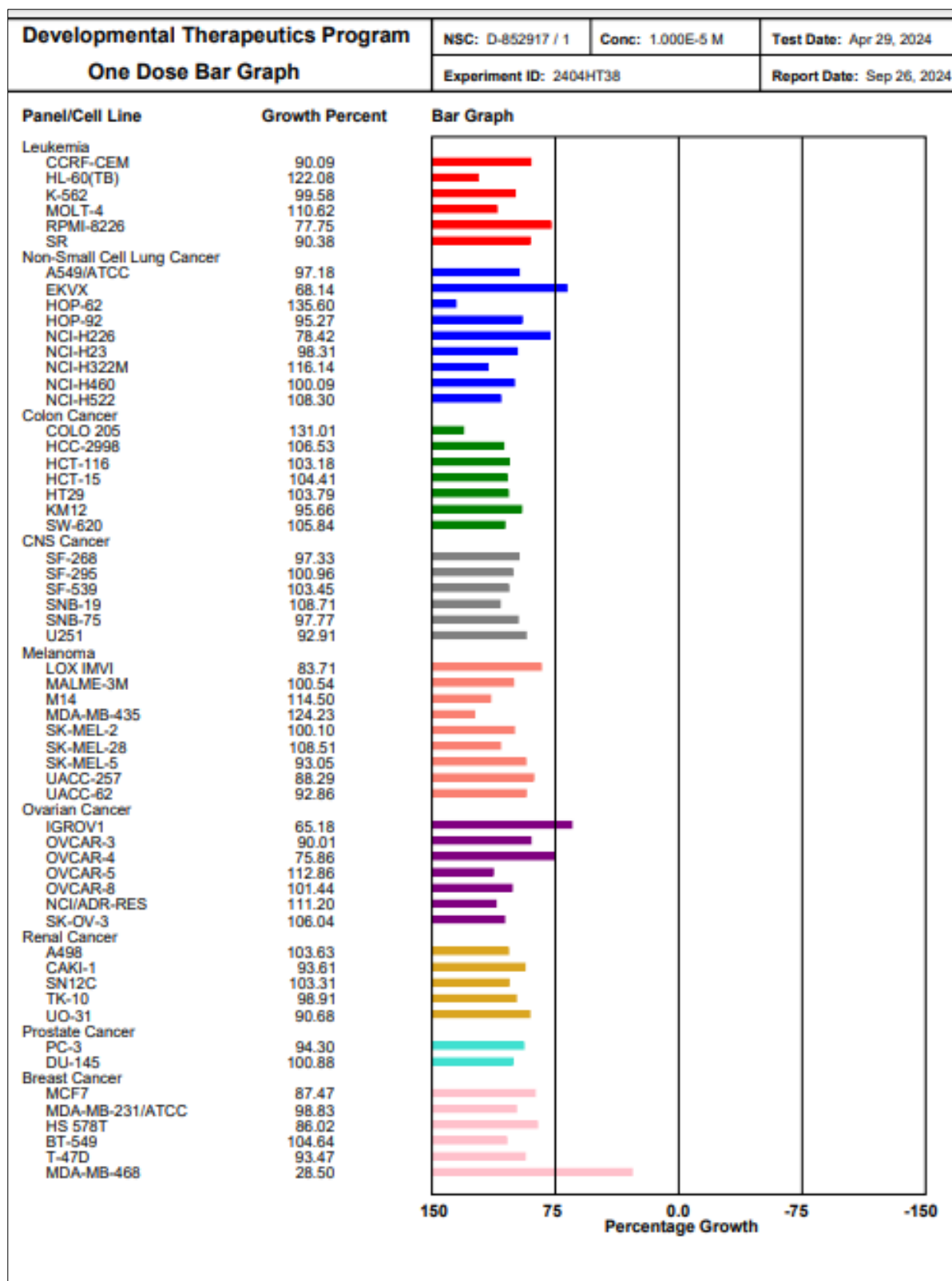


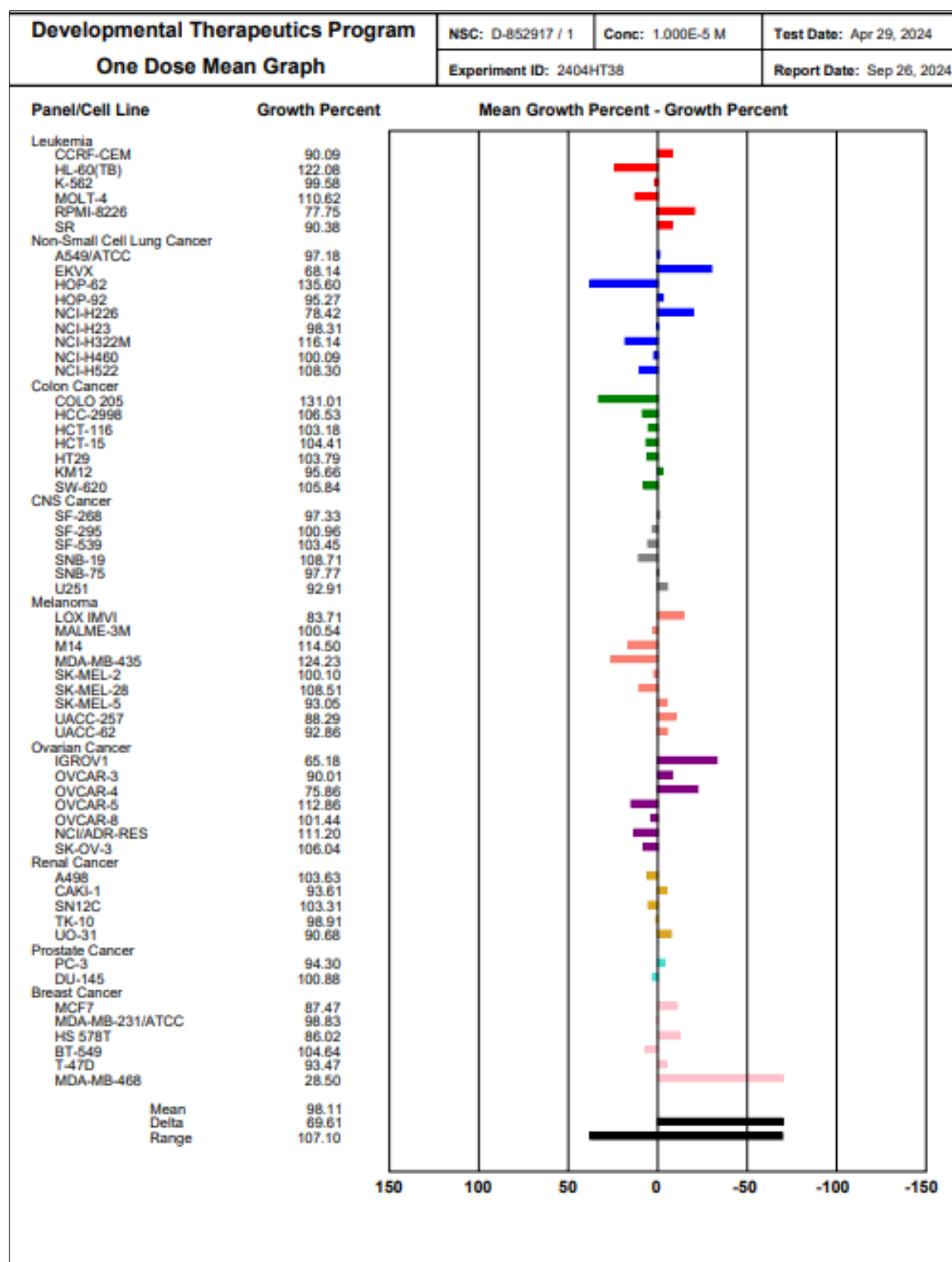
Compound 65



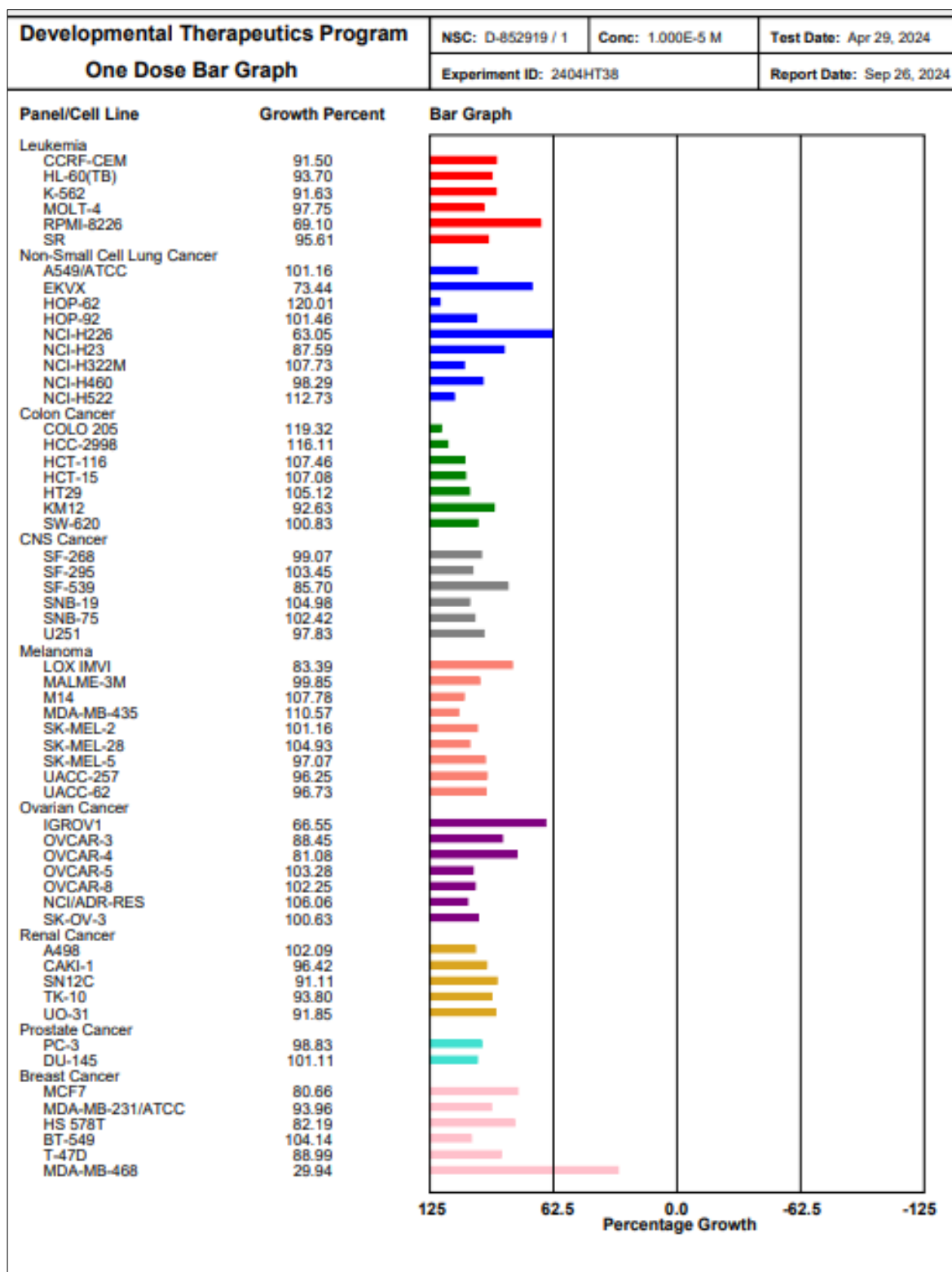


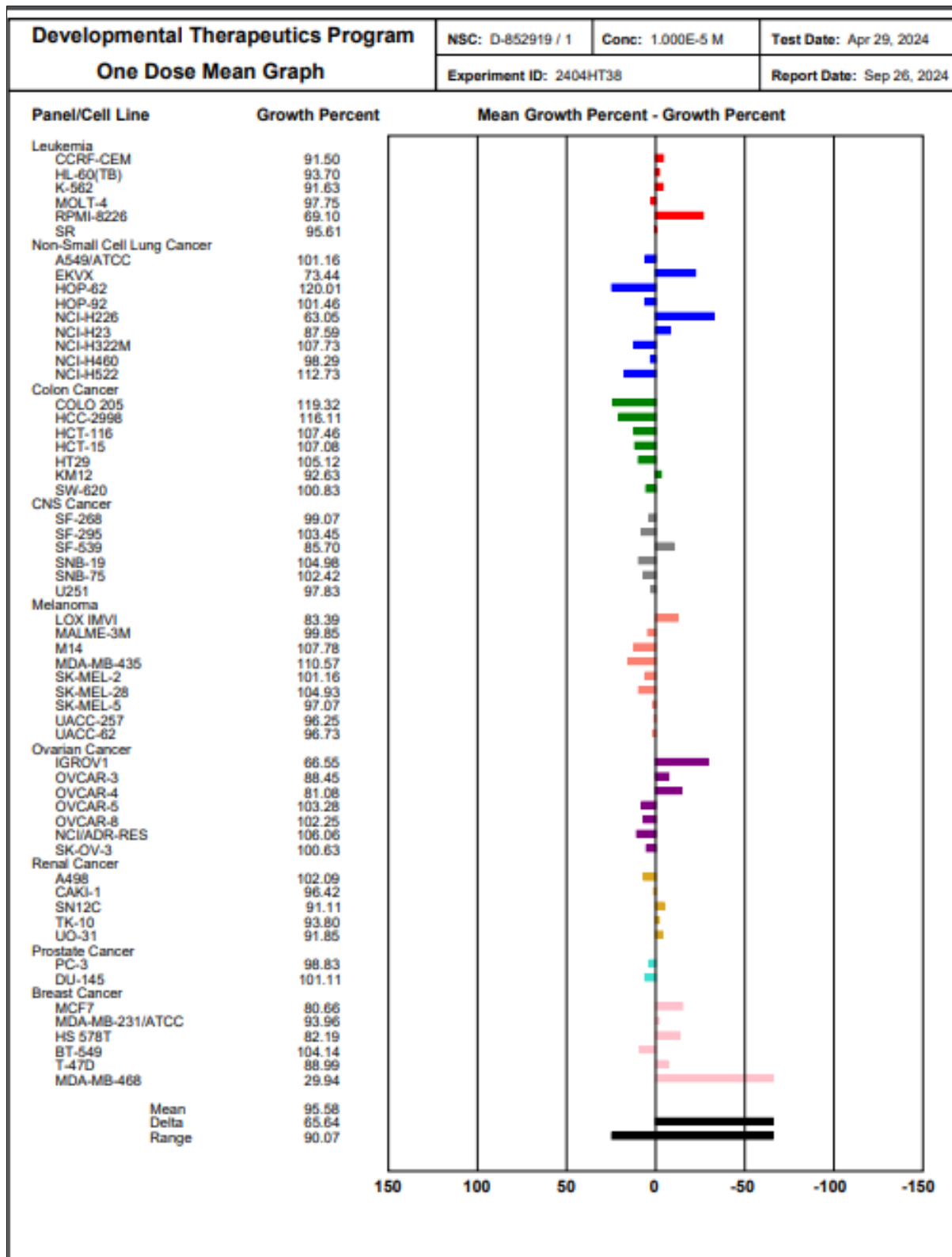
Compound 66



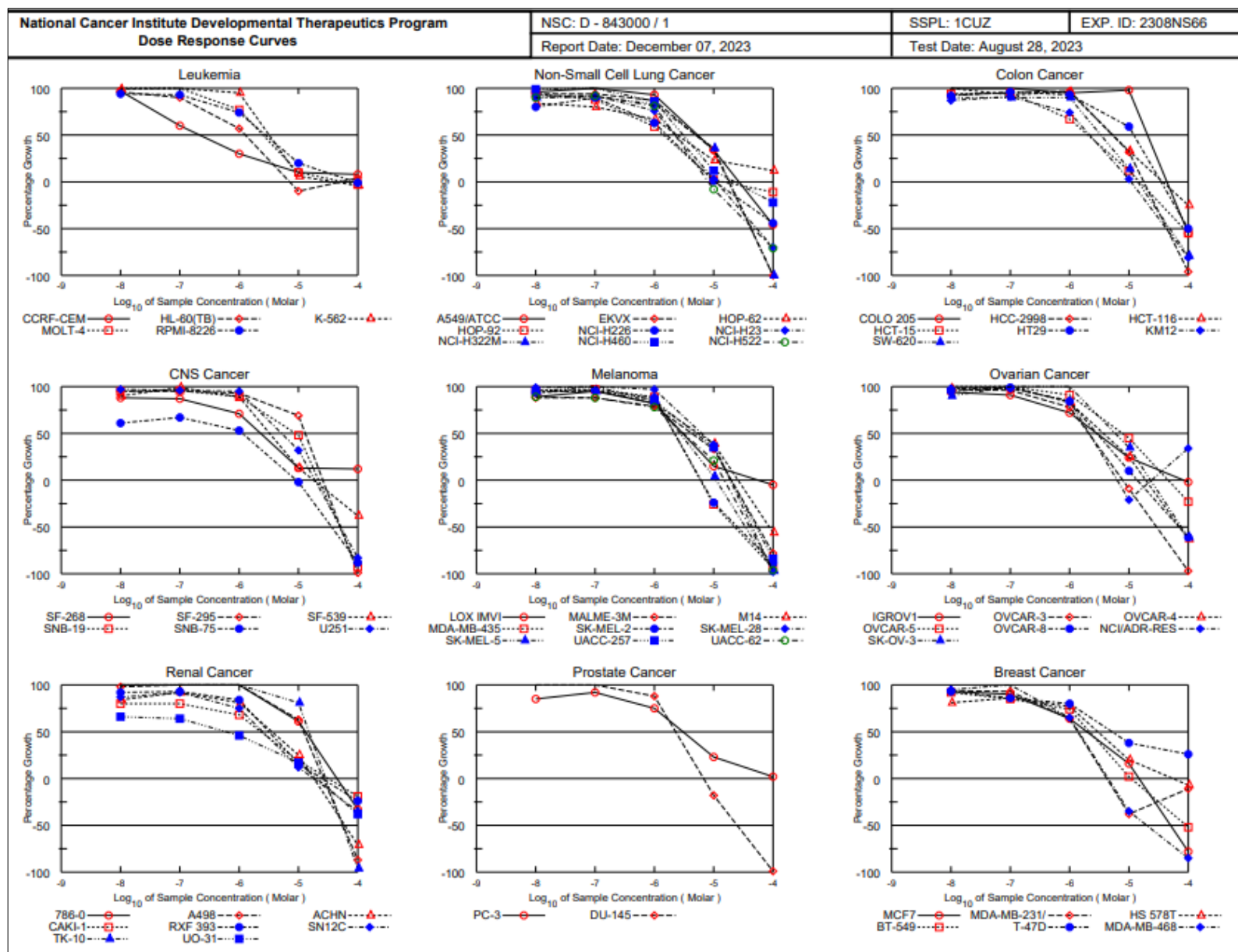


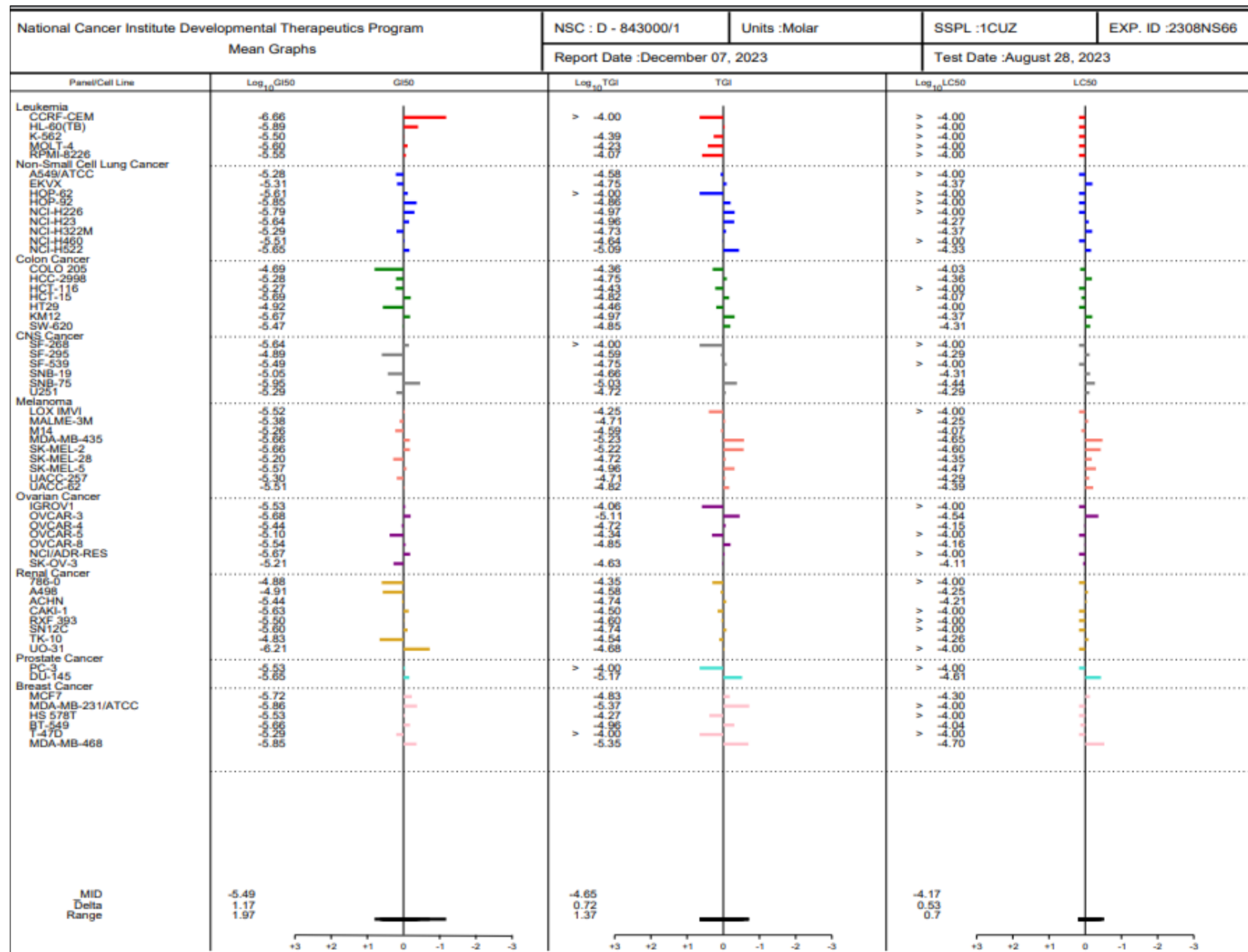
Compound 68

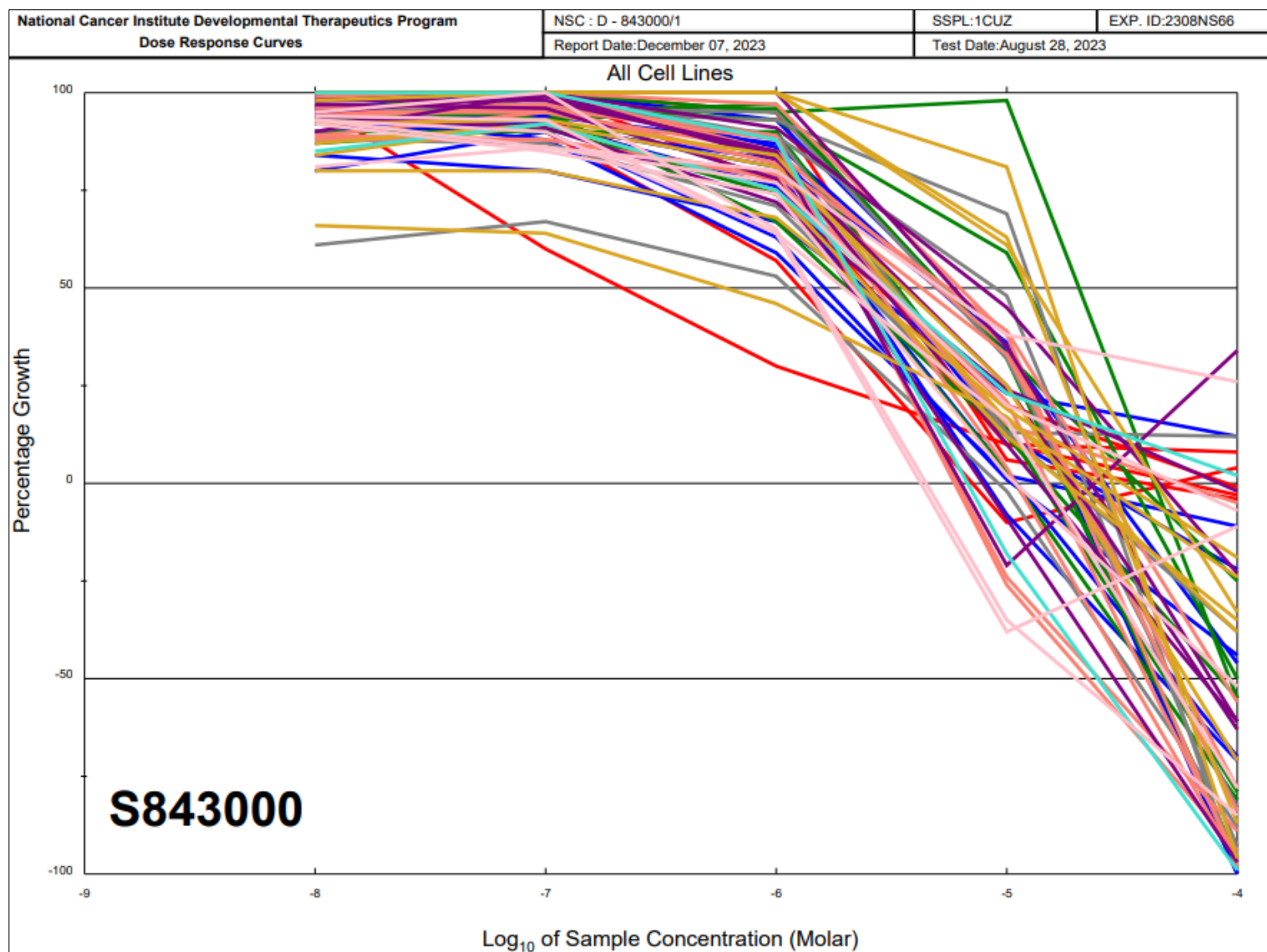




Compound 76







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