

HAMLET and synthetic derivatives as pre-operative agents in the treatment of oral and oesophageal cancer

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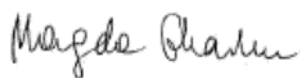
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Abstract

Oral and oesophageal cancers are aggressive tumours that are frequently diagnosed late, with high morbidity, mortality, and difficulties in surgical and therapeutic intervention. Novel imaging probes could assist in early diagnosis and alleviate treatment complications through demarcating a clear margin for resection during surgery. A further need exists for effective and selective neoadjuvant therapies that reduce tumour size before surgery. This project addresses both issues by clarifying the mechanism of action of a class of natural anti-cancer protein-fatty acid complex agents derived from milk and by creating a synthetic polymer-fatty acid derivative as a probe for cancer cell identification.

The novel probe was designed to be fluorescent and comprises natural component molecules found in milk and wheat. The probe's characteristics were investigated in SCC-9 cells - a squamous cell carcinoma of the tongue. The synthesised probe was shown to be non-toxic, and actively taken up, internalised, and expelled by cells by flow cytometry, confocal and multiphoton microscopy and fluorescence spectroscopy. An examination of the uptake mechanism revealed that the molecule is internalised *via* receptor-mediated endocytosis. The relevance of the probe for cancer cell identification was shown through inhibition of the molecule's uptake with a monoclonal antibody against the CD44 receptor, a key marker of cancer stem cell status, metastasis, and cancer progression.

HAMLET (Human Alpha-lactalbumin Made Lethal to Tumour cells) and its bovine analogue BAMLET (Bovine Alpha-lactalbumin Made Lethal to Tumour cells) are protein-fatty acid complexes that display toxicity toward cancers of different origin, while most primary non-cancer cells remain resistant. We show that metabolism is important for their selective therapeutic potential against cancer cells and that BAMLET toxicity can be modulated through metabolic changes. Differentiation of SCC-9 cells significantly increased cellular resistance, whereas adaptation to culturing in galactose conditioned medium greatly sensitized the cells to BAMLET, and the effect was reversible by glucose addition. Metabolic and cellular uptake studies suggested a link between glycolytic activity, endocytic traffic, and BAMLET sensitivity. Through endocytosis inhibition studies, macropinocytosis was shown to be BAMLET's point of entry into SCC-9 cells. This discovery could lead to further developments resulting in improved fatty-acid containing complex based neoadjuvant therapeutics.

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List of Abbreviations

2-DG	2-Deoxy-D-glucose
A ₂₈₀	Absorbance at 280 nm
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ANOVA	Analysis of variance
APS	Ammonium persulphate
ATP	Adenosine triphosphate
ATRA	<i>All-trans</i> retinoic acid
BAMLET	Bovine α -lactalbumin made lethal to tumour cells
BCA	Bicinchoninic acid
BLAGLET	β -lactoglobulin made lethal to tumour cells
C75	4-methylene-2-octyl-5-oxotetra- hydrofuran-3-carboxylic acid
CCCP	Carbonyl cyanide <i>m</i> -chlorophenyl hydrazone
CDI	1,1'-Carbonyldiimidazole
CK	Cytokeratin
COX-2	Cyclooxygenase-2
CT	Computed tomography
CuAAC	Copper-catalysed azide–alkyne cycloaddition
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DEAE FF	Diethylaminoethanol sepharose fast flow
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EAC	Oesophageal adenocarcinoma

ECAR	Extracellular acidification rate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELOA	equine lysozyme complexed with oleic acid
ESCC	Oesophageal squamous cell carcinoma
ESI-MS	Electrospray ionisation mass spectrometry
ETC	Electron transport chain
FA	Ferulic acid
FASN	Fatty acid synthase
FBS	Foetal Bovine Serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FDG	¹⁸ F-fluorodeoxyglucose
FITC	Fluorescein isothiocyanate
G ₀ phase	Resting phase
G ₁ phase	Intermediate phase (first gap)
G ₂ phase	Growth phase (second gap)
GAMLET	Goat α -lactalbumin made lethal to tumour cells
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HA	Glycosaminoglycan hialuronic acid
HAMLET	Human α -lactalbumin made lethal to tumour cells
HPV	Human papilloma virus
IC ₅₀	50 % inhibition concentration
MiR05	Mitochondria respiratory medium 05
MRI	Magnetic resonance imaging
MsCl	Methanesulfonyl chloride
mTORC1	Mammalian target of rapamycin complex 1
NIR	Near-infrared
NMR	Nuclear Magnetic Resonance
OA	Oleic acid
OCR	Oxygen consumption rate

OSCC	Oral squamous cell carcinoma
OXPPOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor receptor
PET	Positron emission tomography
PI	Propidium Iodide
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RAR	Retinoic acid receptor
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
RXR	Retinoid X receptor
S phase	Synthesis phase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SRB	Sulforhodamine B
TBAF	Tetra-n-butylammonium fluoride
TBSCl	Tert-butyldimethylsilyl chloride
TBST	Tris-buffered saline with Tween 20
TCA	Trichloroacetic acid
TEA	Triethylamine
TEMED	Tetramethylethylenediamine
THF	Tetrahydrofuran
TLC	Thin Layer chromatography
TSP	Trimethylsilyl Propanoic Acid

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1. Introduction

1.1. Oral Cancer

Oral cancer comprises malignant growths arising on the lip, tongue, mouth and oral cavity. It is the most common head and neck cancer, which is ranked as the 6th among the most widely occurring cancers worldwide and is associated with high morbidity and mortality (Hema *et al.*, 2017; Irmie *et al.*, 2018). Its prevalence is higher in South Asia, being one of the most common cancers in India, Pakistan, and Papua New Guinea in 2018 (Miranda-Filho and Bray, 2020). The rate of incidence has remained relatively stable, with cancers usually diagnosed in males over 40 years of age, suggesting that prolonged exposure to risk factors leads to an accumulation of genetic and epigenetic alterations in the cells resulting in malignant transformation (Rivera, 2015; Hema *et al.*, 2017; Irmie *et al.*, 2018). However, the number of young individuals and women being diagnosed with oral cancer has been growing in recent years (Neville and Day, 2002; Rhodus *et al.*, 2014). Despite the advancement in medicine, the mortality rate remains high, particularly in less developed areas, with a 5-year overall survival rate of approximately 55 – 60 % (Bagan *et al.*, 2010; Rhodus *et al.*, 2014; Malik *et al.*, 2016). The poor prognosis is partially a result of late diagnosis, which happens in 40 – 60 % of cases (Gigliotti *et al.*, 2019). While when diagnosed early the survival rate is estimated at 80 – 90 %, these numbers decline sharply with the advancement of the disease, to 39 % at late stages (Bagan *et al.*, 2010; Gigliotti *et al.*, 2019).

Late diagnosis is a major set-back in the prognosis for oral cancer patients. Despite the localisation of the tumours in the oral cavity – presumably a location where the disease is easily visible and noticeable – late diagnosis is common and greatly limits the treatment options and survival of the patient. Early stages of the disease are difficult to diagnose due to their asymptomatic nature and lack of biomarkers (Scully and Bagan, 2009). With the main symptom being pain, which usually occurs when the tumours reach a significant size, patients often present with advanced cancers causing substantial discomfort. Depending on the size, location, and advancement of the tumour, other symptoms may include bleeding, mobility of teeth, ear pain, as well as breathing, swallowing, and speaking difficulties (Bagan *et al.*, 2010).

The common understanding is that oral cancer could be prevented, or its incidence greatly decreased, by reducing exposure to potential risk factors. The most prevalent risk factors in oral cancer have specific geographical and social patterns. Tobacco smoking and alcohol con-

sumption have traditionally been considered the typical risk factors in western countries (Montero and Patel, 2015; Miranda-Filho and Bray, 2020). However, alcohol consumption alone has also been linked to an increase in cancer risk, even in non-smoking individuals (Montero and Patel, 2015). Tobacco and betel quid consumption are more common mainly in South Asia (Miranda-Filho and Bray, 2020). Infection with Human Papillomavirus (HPV) has also been listed as a factor in development of cancer of the mouth, such as cancer of the tongue or soft palate, and is considered responsible for the increase of oral cancer incidence among young adults (Danuthai *et al.*, 2018). Exposure to ultraviolet radiation is considered one of the main factors in development of the cancer of the lip, with a lower rate of incidence in women attributed to the use of lipstick (Rivera, 2015; Miranda-Filho and Bray, 2020). With the change of social habits and norms, cancer of the mouth is increasing in prevalence among women, historically less prone due to lower exposure to the risk factors. However, the disease is diverse and multifactorial, with genetic and other environmental factors, such as poor oral hygiene, dietary deficiencies, low fruit and vegetable consumption, and the increased inclusion of red meat in the diet playing a significant role in its onset (Neville and Day, 2002; Montero and Patel, 2015; Malik *et al.*, 2016; Hema *et al.*, 2017; Danuthai *et al.*, 2018).

1.1.1. Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) constitutes *circa* 90 % of all malignancies of the oral cavity (Rivera, 2015; Montero and Patel, 2015; Hema *et al.*, 2017; Miranda-Filho and Bray, 2020). OSCC tumours arise in the squamous epithelium of the oral cavity and can occur on the lip, tongue, gingiva, palate, floor of mouth, or buccal mucosa (Rodini *et al.*, 2017). The levels of differentiation of the tumour cells and their tendency to metastasise to the lymph nodes vary (Rivera, 2015). Of particular relevance to prevention and diagnosis is the observation that oral squamous cell carcinomas can form a range of premalignant lesions, such as leukoplakia or erythroplakia, that have a tendency to metastasise (Scully and Bagan, 2009; Bagan *et al.*, 2010). Leukoplakia is a clinical term used to describe “a white patch or plaque that cannot be characterized clinically or pathologically as any other disease”, as defined by the World Health Organisation; and therefore, does not have any histopathological significance (Neville and Day, 2002, Montero and Patel, 2015). Leukoplakias appear on the oral mucosa; however, those in the floor of the mouth, lateral tongue, and lower lip have the highest tendency to de-

velop into malignant tumours. Therefore, though not always leading to cancer, they should always be carefully treated and observed. They are usually diagnosed in middle-aged and older men; however, leukoplakias in women have been linked to a higher risk of developing oral carcinoma (Neville and Day, 2002). It has also been reported that leukoplakias in non-smokers have an increased risk to transform into malignant tumours compared to lesions in smoking patients (Neville and Day, 2002). Similarly, erythroplakia is a red lesion, that does not fall into a clinical or pathological definition of any other condition (Neville and Day, 2002; Montero and Patel, 2015). It is a soft, red plaque, often well demarcated, but can also blend into the surrounding tissue. Erythroplakia is also more common in older men and can sometimes appear as a mixture of red and white parts known as erythroleukoplakia (Neville and Day, 2002). Therefore, a thorough oral cavity exam should be carried out often, prior to experiencing symptoms, to secure an early detection (Scully and Bagan, 2009; Bagan *et al.*, 2010).

1.1.2. Therapeutic approaches

Due to the diversity of the tumours in the oral cavity, the treatment approaches for oral cancer are also diverse. A detailed treatment plan for each patient is required because of the intricate and varied background of each case. Since in a large number of cases the disease is diagnosed at late stages, the treatment must be carefully tailored by a multidisciplinary team of professionals to ensure patient's survival and wellbeing. Generally, depending on the stage of the disease, the histology of the tumour, the presence or absence of metastases, and the patient's tolerance to therapy and wishes, a suitable treatment approach is decided upon. The main therapeutic approaches adopted are excision surgery and radiation or chemotherapy, or a combination regime (Neville and Day, 2002; Rivera, 2015). High morbidity, mortality, and aggressiveness of the disease, combined with treatment difficulties, such as drug resistance of the cancer as well as side effects of harsh therapies prompt the emergence of novel therapeutic approaches and regimes.

Surgery, considered the gold standard in oral cancer therapy, is the option with the best survival rates; however, it is not always possible. The tumour must be carefully removed with resection margins, which proves not to be a trivial task. Tumours often do not have clearly demarcated edges, but instead seem to merge with the adjacent tissue, increasing the difficulty

in removing all malignant tissue. To remove all cancerous mass, surgeons may resort to broadening the resection margins, increasing the area of healthy tissue removed. In oral cancer this translates to aesthetic and functional morbidities, and necessity for reconstructive surgery (Omura, 2014). Any resection in the oral cavity entails problems with eating, speech, much lowered quality of life, and altered appearance. This can in turn cause great psychological discomfort and a sharp decline in mental and physical wellbeing of the patient, adding to the toll of the cancer itself.

Adjuvant therapy is generally applied in patients with high risk of recurrence, such as patients with large tumours or metastases. Radiotherapy is usually used as an adjuvant therapy post-surgery, as pre-operative application of radiation tends to lead to fibrosis and difficulty in operating on the scarred tissues, and therefore, is not advisable (Huang and Sullivan, 2013; Montero and Patel, 2015). Used in cancer treatment since the end of 19th century, radiotherapy employs ionizing radiation, which induces cell death through DNA damage and targets mainly rapidly dividing cells (Grubbe, 1902). Although this approach aims to exploit the proliferative characteristic of cancer cells, it also targets dividing normal cells, such as epithelia, which in turn often cause significant and dramatic side effects. The most commonly used approaches are the application of an external beam therapy, which directs the radioactive dose at the tumour from outside the body and brachytherapy, whereby radioactive material is placed near the tumour (Rivera, 2015).

The effectiveness of radiation therapy is usually enhanced by a concurrent chemotherapy treatment. Chemotherapy entails the administration of drugs that damage rapidly dividing cancer cells, thereby inducing cell death. In this approach, chemotherapeutic agents play the role of radiation sensitizers, reducing the resistance of cancer cells to ionizing radiation (Hartner, 2017). Studies have confirmed that this approach is superior to radiation alone in patients with high risk of recurrence (Hartner, 2017). Chemoradiotherapy has become more prominently used in recent years before or after surgery to increase survival rate and has showed satisfactory results, including in patients with poor prognosis, but it is also the main choice regime for non-resectable oral cancers (Huang and Sullivan, 2013; Omura, 2014; Montero and Patel, 2015; Hartner, 2017). Treatment of metastatic tumours is usually achieved through chemoradiotherapy; however, the median survival is 6-12 months (Hartner, 2017). The most commonly used chemotherapeutic agents that are radiation sensitizers are cisplatin, 5-fluorouracil, and

carboplatin. However, some reports associate post-operational chemoradiotherapy with high morbidity and complications and advise to administer lower doses of chemotherapeutics, especially cisplatin, and to apply this type of treatment only in centres with high expertise (Montero and Patel, 2015; Hartner, 2017).

Use of chemotherapeutics in oral cancer patients is not advisable as a stand-alone treatment. A metanalysis of six studies on head and neck cancers showed no benefit to employing chemotherapy; and therefore, its use is generally limited to combination with radiation therapy (Hartner, 2017).

Unfortunately, multidrug chemo-radio-resistance can occur in oral cancer and argues for concerted efforts to develop novel therapies that can effectively bypass resistance mechanisms (Naik *et al.*, 2016). Immunotherapy has been proposed as an approach for the treatment of oral cancer. Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor (EGFR) is being studied as a potential therapy agent to be applied in combination with radiation or chemoradiotherapy in oral cancer (Hartner, 2017). An interesting approach is the combination of standard therapy with epigenetic modulatory agents. As many genes associated with chemoresistance have been reported to be hypermethylated, it seems this might be a valuable improvement in sensitising cancer to traditional therapeutics (Irmie *et al.*, 2018).

The existence of therapy resistance and the heterogeneity in oral cancers is a tough challenge (Naik *et al.*, 2016). Because of the complicated background and epidemiology of OSCC, conventional diagnostic methods are not enough to ensure diagnosis at an early stage and the survival rates drop sharply with the advancement of the disease at the time of detection (Malik *et al.*, 2016). Therapy resistance, which occurs relatively often in OSCC, is one of the main reasons why resective surgery has remained the main intervention strategy (Naik *et al.*, 2016). Treatment toxicity as well as malnutrition are a genuine threat to patients' life, and both cause a great decrease in the quality of life. This calls for the development of an effective and minimally invasive neoadjuvant therapy that could ease the symptoms without introducing gruesome side effects coupled to an ability to decrease the size of the tumour in a short period of time. Ideally, a novel therapy would alleviate the debilitating symptoms and increase the quality of life of the patient as well as enable a successful and less invasive excision surgery, where the complete removal of the tumour could be ensured. Development of effective cancer

imaging agents and intraoperative cancer markers would greatly increase the success rate of surgeries and rise the survival rate of the oral cancer patients. Clear imaging of tumour borders would allow for precise removal of the malignant tissue sparing normal tissue and coincidentally preventing relapse and postoperative morbidity and disfigurement.

1.1.3. Neoadjuvant therapies in oral cancer

Typical oral cancer treatment approaches involve surgery and subsequent post operational adjuvant therapy including radiation or chemoradiotherapy (Neville and Day, 2002; Huang and Sullivan, 2013; Omura, 2014; Montero and Patel, 2015; Rivera, 2015; Hartner, 2017). Neoadjuvant therapy, which is a treatment applied prior to the primary therapy is not widely used in oral cancer. So-called induction therapy, which is an initial chemotherapeutic treatment before radiation or chemoradiotherapy is gaining prominence in oral cancer as a means to avoid metastatic spread of the disease and reduce the size of the tumour before the main treatment. It is usually a combination of chemotherapeutic agents. It allows for improved treatment efficacy and patient survival by lowering the risk of relapse (Hartner, 2017). Typical neoadjuvant chemotherapeutic agents are a combination of cisplatin and 5-fluorouracil. Other clinical trials have reported combinations of cisplatin, 5-fluorouracil and docetaxel showing higher survival rates. However, the treatment was associated with significant toxicities in cases where induction therapy was applied, leading to some treatment-related deaths (Hartner, 2017).

Reports suggest that the surgical approach, despite its impact on the patient's quality of life, has the highest survival rate, with concurrent post-operative chemoradiotherapy offering further improvements to treatment outcome (Huang and O'Sullivan, 2013). The success rate of excision surgery depends largely on the size of the tumour. Therefore, minimising the opportunity for tumour growth is important to increase the patient's chances of survival. It is vital, however, to accomplish this goal without introducing fibrosis or other complications that may prevent surgery from being carried out, or alternatively, exposing the patient to toxic side effects that could be life threatening. Tumours localised in the oral cavity, tongue, or the lip often cause malnutrition, as they prevent the patient from eating, chewing, and swallowing. Therefore, there is a desperate need for novel diagnostic techniques and new therapies mini-

mizing the symptoms and related inconvenience. Since non-operative oral cancer has lower survival rates, it is vital to develop novel, specific, and selective therapies that would effectively target cancer cells without introducing side-effects dangerous to patients' survival. By reducing the size of the tumour prior to primary therapy, the efficacy of the primary treatment could be greatly improved and concomitant disfigurement and associated psychological side-effects avoided.

1.2. Oesophageal Cancer

Oesophageal cancer is a term describing malignant growths along the oesophagus. Globally, it ranks in the top ten most commonly diagnosed neoplastic diseases and as the sixth most deadly cancer (Kato and Nakajima, 2013; Deng and Lin, 2018). Two main histological subtypes of oesophageal cancer are distinguished – oesophageal squamous cell carcinoma (ESCC) and oesophageal adenocarcinoma (EAC) – with both displaying distinct epidemiology, pathology, and clinical characteristics (Deng and Lin, 2018). ESCC and EAC differ in their geographical distribution. The most common subtype in Western Europe, North American and Australia is EAC, with its incidence rates surpassing ESCC in the recent years (Lagergren *et al.*, 2017; Deng and Lin, 2018). Eastern Asia and Eastern and Southern Africa are the regions where ESCC is the most common histological subtype of oesophageal cancer (Lagergren *et al.*, 2017; Deng and Lin, 2018). In fact, in the ‘oesophageal cancer belt’, a region from northern Iran to northcentral China with particularly high incidence of this neoplasia, 90 % of the diagnoses are ESCC (Lagergren *et al.*, 2017). Both subtypes of oesophageal cancer are significantly more prevalent in men than women and are more common in older age (Abnet *et al.*, 2018; Lagergren *et al.*, 2017).

Like oral cancer, oesophageal cancer is associated with challenging treatment strategies, poor prognosis, and a dramatic impact on the patients’ quality of life (Lagergren *et al.*, 2017). The prognosis and treatment outcome depend largely on the diagnosis time, tumour size, histology, and comorbidities. The 5-year survival rates in Europe, the USA, and China have increased from 5 % in the 1960s to 20 % in the 2000s (Lagergren *et al.*, 2017). At the moment, the estimated survival rate is 30 – 57 %, with early diagnosis associated with better outcome (Hagens *et al.*, 2020).

Since the only real diagnostic measure is endoscopy, which is considered to be invasive, cost-ineffective, and requires the patients to display symptoms justifying performance of the procedure, both types of oesophageal cancer are usually diagnosed at a late stage, when patients experience aggravating symptoms. This results in a poor prognosis and an extensive surgical therapy, which consequently further lowers the quality of life of patients (Lagergren *et al.*, 2017). New diagnostic techniques involve inflatable balls and sponges that scrape the cells from the surface of the oesophagus; however, despite a relatively high specificity for cancer

and dysplastic cells, their sensitivity of below 50 % is too low for a wide use in oesophageal cancer screening (Domper Arnal *et al.*, 2015). No established blood biomarkers for ESCC nor EAC add to the difficulty in diagnosing oesophageal cancer at an early stage. A novel diagnostic technique making early diagnosis possible and neoadjuvant therapy that allows reducing the tumour size before the surgery are, therefore, of vital importance.

1.2.1. Oesophageal squamous cell carcinoma

ESCC arises from squamous cells in the oesophageal epithelium and is usually localised in the upper and middle sections of the oesophagus. Initially, the lesions begin with squamous dysplasia, that can subsequently develop into a malignant tumour. ESCC constitutes almost 90 % of all oesophageal cancer cases diagnosed worldwide (Abnet *et al.*, 2018). The race of the patients might play a role in the epidemiology of the disease, as ESCC is predominant in eastern Asia and south-eastern Africa, suggesting geographical and cultural factors, but genetic predispositions also exist. In fact, there are reports of some single nucleotide polymorphisms that have been linked to higher risk of incidence of ESCC in Chinese and Japanese populations (Lagergren *et al.*, 2017). Furthermore, patients suffering from tylosis, an autosomal dominant skin disease, have a lifetime higher risk of developing ESCC (Domper Arnal *et al.*, 2015). However, it is the environmental factors that play the main role in ESCC epidemiology, with tobacco smoking and alcohol consumption considered to be the main risk factors of ESCC, and their effect reportedly is synergistic (Abnet *et al.*, 2018; Domper Arnal *et al.*, 2015; Castro *et al.*, 2018). Yet, exposure to polycyclic aromatic hydrocarbons from sources other than tobacco has also been linked to elevated possibility of ESCC development and is particularly high in developing countries, presumably due to ingestion of certain foods and beverages, or cooking and heating methods (Abnet *et al.*, 2018). Betel quid, widely popular in South and Southeast Asia, has been confirmed to increase the risk of ESCC, and its effect is reportedly synergistic with tobacco, with which is it often chewed (Abnet *et al.*, 2018). ESCC is found predominantly in men, implicating not only genetic bias but also possible hormonal protective effects in women (Abnet *et al.*, 2018). Poorly balanced diets with a low amount of fruit and vegetables, as well as consumption of red meat and hot beverages have also been linked to higher chances of ESCC (Domper Arnal *et al.*, 2015; Lagergren *et al.*, 2017; Abnet *et al.*, 2018). The declining incidence of ESCC in men in western countries are linked to a decrease

in smoking tobacco in these populations; however, a recent rise in ESCC cases diagnosed in women is attributed to a shift in social norms leading to an increase in tobacco smoking and alcohol consumption among women (Abnet *et al.*, 2018).

1.2.2. Oesophageal adenocarcinoma

EAC is a malignant tumour of the mucous-secreting gland cells in the distal oesophagus. Its incidence is significantly higher in western countries and in men rather than in women (Domper Arnal *et al.*, 2015; Lagergren *et al.*, 2017). The main risk factor for EAC development is the gastroesophageal reflux disease. It is capable of leading to malignant transformation directly; however, more commonly, EAC develops from a pre-malignant lesion known as Barrett's oesophagus, where the normal squamous mucosa is replaced with a specialised columnar epithelium. The cells may then progress to various grades of dysplasia and, ultimately, EAC (Domper Arnal *et al.*, 2015; Lagergren *et al.*, 2017). It is estimated that *circa* 10 % of patients with gastroesophageal reflux disease will ultimately develop Barrett's oesophagus, of which around 1 % will transform into EAC (Domper Arnal *et al.*, 2015; Huang and Yu, 2018). Obesity is associated with a higher incidence of gastroesophageal reflux disease and Barrett's oesophagus, and therefore, elevated rate of EAC development. The chronic inflammation mediated by adipocytes is recognized as an alternative mechanism of obesity induced EAC (Domper Arnal *et al.*, 2015; Lagergren *et al.*, 2017). Surprisingly, no correlation between alcohol consumption and EAC development has been recognized in multiple analyses; however, smoking tobacco is considered to be a significant risk factor (Domper Arnal *et al.*, 2015; Lagergren *et al.*, 2017; Castro *et al.*, 2018). Dietary patterns are reportedly closely connected to EAC, with consumption of red meat and processed foods increasing the probability of malignant transformation, while fruit and vegetable ingestion, *Helicobacter pylori* infection, and intake of non-steroidal anti-inflammatory drugs are considered protective (Domper Arnal *et al.*, 2015; Lagergren *et al.*, 2017; Huang and Yu, 2018). Genetic studies reported specific loci responsible for predisposition to Barrett's oesophagus, rather than EAC itself (Domper Arnal *et al.*, 2015).

1.2.3. Oesophageal Cancer Therapies

Treatment options for oesophageal cancer depend largely on tumour subtype, its size and stage, patient's condition, comorbidities, and country; however, they are generally limited. Early diagnosis increases survival rate and treatment outcome. A multidisciplinary assessment and treatment plan have been shown to improve clinical decision making and outcome (Lagergren *et al.*, 2017). In western countries, early oesophageal cancers and smaller sized tumours that have low risk of spread beyond the oesophageal mucosa and into the lymph nodes are managed by local endoscopic treatment, usually followed by ablation techniques to ensure complete removal of cancerous tissue. In patients with higher risk of disease spread and recurrence and larger regions of transformed tissue, oesophagectomy is the primary treatment regime (Dompel Arnal *et al.*, 2015). Asian countries usually adopt a more aggressive approach of oesophageal cancer treatment with oesophagectomy followed by reconstructive surgery being the gold standard in early oesophageal cancer treatment (Kato and Nakajima, 2013; Dompel Arnal *et al.*, 2015).

In more advanced stages, resective surgery is usually the preferred treatment (Lagergren *et al.*, 2017). In EAC, postoperative adjuvant chemotherapy or chemoradiotherapy is commonly used in different stages of the tumour; however, there is a shortage of large randomized trials on adjuvant therapies in ESCC, resulting in no guidelines being available for procedures applied after surgery (Tu and Hsu, 2018). Smaller studies on cisplatin and 5-fluorouracil adjuvant chemotherapy in ESCC observed risk reduction in the group of patients with lymph node metastases, yet no significant survival improvement was noted, suggesting the application of neo-adjuvant therapy instead (Tu and Hsu, 2018). Moreover, postoperative radiotherapy was reported to have a negative impact on patients' survival and is therefore not recommended. Neo-adjuvant chemoradiotherapy has reportedly improved patients' survival rates; however, more comprehensive, randomized studies are necessary to establish guidelines (Tu and Hsu, 2018). An alternative treatment approach in EAC is perioperative chemotherapy, which involves treatment around the time of operation, including pre- and post-operative chemotherapy. Several randomized trials reported improved survival rate after perioperative treatment with cisplatin and 5-fluorouracil or epirubicin and cisplatin and 5-fluorouracil, while others reported increased 3-year survival rates after chemotherapy with fluorouracil, leucovorin, oxaliplatin,

and docetaxel (Lagergren *et al.*, 2017). It is necessary to keep in mind that while aggressive adjuvant treatments increase the survival rate, the patients experience pronounced downsides and side-effects during the course of treatment (Lagergren *et al.*, 2017).

For inoperative lesions, the best choice is chemoradiotherapy, which has much better survival rate than radiotherapy or chemotherapy alone (Lagergren *et al.*, 2017; Tu and Hsu, 2018). Standard regimes consisting of radiotherapy and cisplatin and 5-fluorouracil or oxaliplatin are commonly used, and while the survival rates are similar, the levels of toxicity are different (Lagergren *et al.*, 2017). There is a growing interest in immunotherapy in oesophageal cancer, and multiple agents are currently tested in clinical trials with promising results (Kelly, 2017).

Depending on the size and histological subtype of the tumour and its location, different surgical approaches can be chosen; however, each of them often leaves the patient with significantly lower quality of life, malnutrition, and not a very optimistic survival rate. Survivors can also suffer significant pain and discomfort leading to a high chance of emotional distress. Pre- or post-operative therapies, usually displaying significant morbidity and mortality, add to the stress and suffering of the patient highlighting the dire need for an innoxious therapeutic approach.

1.2.4. Neoadjuvant therapies in Oesophageal Cancer

Employment of neoadjuvant therapy in more advanced oesophageal cancer before the main surgical intervention is necessary. It is believed to reduce the size and aggressiveness of the tumour before the oesophagectomy, which helps to decrease the size of removed tissue, prevent metastasis, spread of the disease, and is believed to improve the survival rates. Metanalysis of multiple randomized trials of neoadjuvant and chemo-radiotherapy applied prior to the surgical intervention reported improvement of patient survival. However, a study in early oesophageal cancers that were not suitable for local ablation claimed no difference in outcome between patients treated with neoadjuvant chemoradiotherapy prior to surgery and surgery alone, suggesting the importance of neoadjuvant treatment rises with the advancement of the disease (Lagergren *et al.*, 2017).

The main neoadjuvant therapeutic approaches are currently neoadjuvant chemo- and chemoradiotherapy; however, the exact therapy regime choice depends largely on the histological subtype of oesophageal cancer. Randomized trials in ESCC have reported satisfactory outcomes of neoadjuvant chemotherapy with cisplatin and 5-fluorouracil (Kato and Nakajima, 2013; Lagergren *et al.*, 2017; Deng and Lin, 2018; Tu and Hsu, 2018). EACs are less sensitive to radiotherapy; therefore, pre-operative neoadjuvant chemotherapy or chemoradiotherapy with radiation sensitising chemotherapeutics is advisable. Clinical trials tested standard platinum-fluoropyrimidine based chemotherapy in comparison to cisplatin-5-fluorouracil and cisplatin-5-fluorouracil-epirubicin-capecitabine combinations, and while the pathological response of the tumour increased with the intensity of the therapy, the survival rate was relatively unchanged; therefore, less intensive doublet chemotherapy is advised (Lagergren *et al.*, 2017). Concurrent neoadjuvant therapy with carboplatin and paclitaxel and radiation prior to surgery has proven to successfully increase the survival of patients with ESCC and EAC (Hagens *et al.*, 2020). Alternative approaches include cisplatin and oxaliplatin plus fluoropyrimidines combined with radiotherapy (Lagergren *et al.*, 2017). However, as not every tumour is radiotherapy responsive, caution is needed when choosing this regimen. There are trials reporting that chemoradiotherapy caused more deaths unrelated to disease progression. There is however no consensus if the toxicity of chemoradiotherapy may cause postoperative complications or affect the survival rate (Deng and Lin, 2018). Radiotherapy is not recommended as a pre-operative treatment without chemotherapy, particularly in EAC, where resistance to radiation is frequently observed (Lagergren *et al.*, 2017; Tu and Hsu, 2018).

It is no surprise that the more intensive the treatment, the worse the side effects that are experienced by the patient, and so it is crucial to have both the therapy results and patient's wellbeing in mind. Due to often occurring treatment resistance in oesophageal cancer, choosing the best therapeutic agent is a challenge with the patient's life at stake. It might be difficult to choose the correct radiation dose as well, as high doses are more efficient and tumouricidal, but also cause gruesome side effect for the patients. Considering all these, there is a dire need for a neoadjuvant agent bypassing the resistance of the cancer cells and causing little or ideally no side effects, and minimally impact the patient's quality of life.

1.3. Cancer imaging and diagnostic agents

In order to enhance the detection rates of cancer and facilitate early diagnosis, improved cancer detection agents are required. Cancer screening programmes have proven effective in facilitating earlier diagnosis in multiple cancers, such as breast and cervical cancers, thus greatly improving the survival rates. Similar tests are quite difficult to perform in oral and oesophageal cancers due to the difficulty in self-assessment, the asymptomatic nature of pre-malignant lesions, and a large surface area requiring screening. Moreover, the available detection techniques are unable to identify lesions until a certain degree of malignant transformation has progressed. Therefore, tissue biopsy and subsequent histological examination of the samples are the go-to standard for detection of these cancers. Effective, selective, and specific cancer imaging is also vitally important in disease staging, to determine the presence of metastases, and for intraoperative identification of the tumour margins.

Several techniques currently used in cancer imaging allow for a certain degree of detection and assessment of the lesions or tumours, mainly magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and ultrasound (van Rossum *et al.*, 2013; Drage *et al.*, 2018). CT involves use of ionising radiation and imaging contrasts to enhance the resolution of the image and is useful in detecting bone invasion. MRI is a non-invasive technique that utilizes magnetic field to image tissues. Serial magnetic pulses influence the protons in the patients' body that try to align with the magnetic field producing signals, which are then rendered into pictures illustrating tissues (Drage *et al.*, 2018). Thus, a series of images are produced without the necessity to utilise ionising radiation. Moreover, MRI boasts of a higher soft-tissue imaging resolution than CT, making the two tests complementary in many cases. Ultrasound is a relatively easy technique that is inexpensive and quick to carry out. It can be performed at the same time as a biopsy of a suspicious lesion; however, this method can only be used for imaging soft tissues (Drage *et al.*, 2018). PET is a technique usually utilising ^{18}F -fluorodeoxyglucose (FDG) radioisotope to image the malignant tissue. FDG is an analogue of glucose and is, therefore, readily internalized by the cells. This technique is based upon the principle that cancer cells are highly glycolytic and thus have a significantly higher glucose and glucose analogues uptake than normal tissues. The radioisotope is then accumulated in the tumours and the gamma rays produced by decaying fluoride isotope are detected by a gamma ray camera. Often, a CT unit registers images of the whole body to

identify the exact localisation of the malignant tissue (Drage *et al.*, 2018). FDG PET-CT displays higher sensitivity than MRI or CT; however, the radiation dose is much greater, and therefore, its use has to be justified (Drage *et al.*, 2018; Economopoulou *et al.*, 2019). Although FDG PET-CT can be used to stage the disease and monitor the treatment progression and outcome, care must be taken and the results confirmed by an alternative method due to a possibility of increased FDG uptake by non-cancerous tissues, for example as a cause of radiation-induced inflammation (van Rossum *et al.*, 2013; Economopoulou *et al.*, 2019).

Intraoperative methods to ascertain the success of tumour resection are particularly important. To ensure a better survival rate, surgeons must strive to obtain a 5 mm tumour-negative margin when resecting a malignant tumour, which proves very challenging due to relying on visual and tactile indications (Fakunerjad *et al.*, 2020). The current intraoperative standard in tumour examination and margin assessment is frozen section analysis, whereby samples of the margins are analysed by a pathologist in parallel with the surgery (Fakunerjad *et al.*, 2020). This method is not only time consuming but also prone to human and sampling error. Narrow band imaging is a technique used in detection and diagnosis as well as intraoperative assessment of tumours. It can be used in endoscopy and is based on identification of haemoglobin by the use of its absorption wavelengths, thus visualizing neoangiogenesis in solid tumours; however, it is subjective and heavily depends on properties of the examined tissue (Fakunerjad *et al.*, 2020). Other methods that can be used in endoscopy or during surgery include chromoendoscopy, electronic chromoendoscopy, confocal laser endoscopy, and optical coherence tomography (Mannath and Ragunath, 2016). However, not all of these are used outside of research setting and the quest to develop more specific, selective, and real-time methods continues.

An interesting development in cancer imaging for detection, staging, and intraoperative assessment, is the use of fluorescent molecular imaging allowing for the use of tumour-specific fluorescent dyes, fluorescently labelled antibodies, tissue autofluorescence, or fluorescent nanoparticles (Rao *et al.*, 2006; Luo *et al.*, 2011; Owens *et al.*, 2016; Hong *et al.*, 2017; Tomo *et al.*, 2019; Fakunerjad *et al.*, 2020). Development of fluorescent tumour-specific dyes and fluorescently labelled nanoparticles to visualize the malignant mass in combination with the modern imaging techniques could revolutionize the field and provide a rapid, specific, selective, and real-time detection method. Moreover, the optimal specificity could rule out the necessity

to confirm the results by alternative methods. However, development of such imaging agents is not a trivial task.

1.3.1. Characteristics of a successful cancer imaging probe

An ideal candidate for an imaging agent has to display a list of characteristics. First and foremost, the probe has to be non-toxic, so as not to introduce any harmful effects to normal tissues. The imaging agent should ideally be internalized within the malignant cells to allow for specific and detailed imaging of the tumours, as well as display bright and easily picked up fluorescence upon accumulation. Such imaging agent cannot remain in the tissues after performing the medical procedure, and so its accumulation should be only temporary, and the probe should be readily metabolised or expelled from the cells in a certain time interval afterwards. Moreover, its lack of toxicity has to be maintained in high concentration, despite accumulation in the tissues and no toxic effects should be induced upon metabolism of the molecule within the cells (Owens *et al.*, 2016).

The cancer imaging agent should be highly specific and exhibit high affinity to the malignant tissue, with minimal uptake or binding in the normal tissues, to prevent false positive staining and high signal-to-noise ratio (Owens *et al.*, 2016). The molecule should also be stable in biological environment and boast of a biologically stable, bright fluorescence, that would allow for easy *in vivo* imaging. The delivery of the probe to the target tissues should not pose much difficulty and the molecule should preferably be internalized into the cells to allow for specific imaging. The fluorescence of the markers has to be within a wavelength range that will not harm either the patient or the professional performing the imaging procedure.

There are many ways to obtain such cancer markers. To address the question of specificity, preferential uptake by the malignant tissue has to be ensured. A probe can exploit the fact that the blood vessels supplying tumours are usually leaky in comparison to non-permeable vasculature in the normal tissue, which is often used in nanoparticle imaging probes (Jenkins *et al.*, 2016). Cancer specific agents can target multiple surface receptors that are upregulated in certain cancers. An alternative method is producing markers that get activated by a stimulus, usu-

ally connected to a change in environment in the vicinity of a tumour (pH variations, presence of a certain enzyme, etc.) (Owens *et al.*, 2016).

From the point of specificity, the targeted approach to imaging probe design and development is the most appealing. Ligand-receptor binding is usually of high affinity, and therefore very specific and efficient. Due to upregulation of certain receptors in cancer tissue compared to normal tissues, it is possible to obtain a satisfactory signal-to-noise ratio. Multiple molecules are being researched for their potential as imaging probes in conjugation with fluorophores, such as glucose, folic acid, biotin, bovine serum albumin, or epidermal growth factor (EGF), in addition to natural and synthetic polymer nanoparticles (Owens *et al.*, 2016; Jenkins *et al.*, 2016).

The fluorescence spectrum of the probes is very important. Not only must the agents avoid harmful effects and injuries arising from highly energetic light wavelengths, but also a satisfactory difference between excitation and emission wavelengths (i.e. Stokes shift) must be maintained to avoid photobleaching and non-specific results. A number of studies discuss the potential of near-infrared (NIR) fluorescent agents in *in vivo* imaging; however, other approaches are also possible (Rao *et al.*, 2006; Santra and Malhotra, 2011; Jenkins *et al.*, 2016; Owens *et al.*, 2016).

An interesting combination of cancer tracking markers with anticancer therapeutics has emerged in recent years, referred to as theranostics. This approach is receiving increasing attention due to the possibility of providing an image-guided enhanced efficacy treatment; however, the field is still under research and development (Chen and Liu, 2016).

1.4. Tumouricidal protein-fatty acid complexes

1.4.1. HAMLET - Discovery, structure, and formation

α -Lactalbumin is a small metalloprotein of *circa* 14.2 kDa. Crystallographic studies revealed its structure consists of four α -helices, three antiparallel β -sheets, and four disulphide bonds (Hallgren *et al.*, 2008; Min *et al.*, 2012). To maintain its native conformation, a calcium ion is

co-ordinated into its calcium binding site (Svensson *et al.*, 2003); however, it can also interact with other divalent cations (Hallgren *et al.*, 2008). α -Lactalbumin is found in milk of all mammals and is the most abundant protein in human milk (Min *et al.*, 2012). In its native form, its general function is to help to regulate lactose synthesis as a co-enzyme (Qasba *et al.*, 2008) and to provide nutrition to the nursed infant (Min *et al.*, 2012). However, α -lactalbumin has many interesting intermediate states, where it can gain new, unusual functions.

Human milk has been believed to have additional health benefits besides nutrition, such as providing the nursing infant with antibodies and antipathogenic molecules, which led the Svanborg group to investigate its antibacterial properties. While investigating the impact of human milk on the adherence of *Streptococcus pneumoniae* to A549 lung carcinoma cells, surprisingly, induction of cancer cell death was observed (Hakansson *et al.*, 1995). Further studies revealed a fraction of human milk activated apoptosis-like cell death in cancer cells, while differentiated cells remained intact (Hakansson *et al.*, 1995). The ingredient of human milk believed to be inducing the cancer cell death has been precipitated with the casein fraction and found to consist of a monomeric and multimeric fraction. Since the monomeric fraction did not affect cancer cell survival, the multimeric fraction was believed to be the active component, responsible for cancer cell death induction (Hakansson *et al.*, 1999). Further studies revealed that toxicity was not acquired through formation of multimers and the formation of the Human α -lactalbumin Made Lethal to Tumour Cells (HAMLET) complex was identified (Svensson *et al.*, 2000).

It has been observed that α -lactalbumin in the tumouricidal complex did not undergo post-translational modifications nor secondary structure changes. Instead, it has been reported that α -lactalbumin in HAMLET was partially unfolded and went through a tertiary structure remodelling towards a molten globule-like state, wherein the secondary structure is similar to the native state but the protein lacks the well-defined tertiary structures that can be found in folded proteins (Svensson *et al.*, 2000; Min *et al.*, 2012). However, to stabilize the partially unfolded state, α -lactalbumin needs oleic acid (OA) (C18:1 – 18 carbons, monounsaturated) as a necessary co-factor to form the cytotoxic HAMLET complex (Svensson *et al.*, 2000; Svensson *et al.*, 2003; Fast *et al.*, 2005).

Consecutive extensive studies revealed that HAMLET is formed through partial unfolding of α -lactalbumin in the absence of metal ions or in low pH. Examination of Ca^{2+} binding site mutants revealed that the removal of Ca^{2+} ions induces the protein to change its tertiary conformation which grants it the ability to bind multiple OA molecules (figure 1.1) (Mossberg *et al.*, 2010b; Nakamura *et al.*, 2013). Mutant proteins with inability to bind calcium cations lost the ability to turn back to the native conformation, remaining in the unfolded state; however, that was not enough to induce cancer cell death (Svensson *et al.*, 2003), indicating that the unfolded protein itself does not possess toxicity, but might simply be the carrier (Min *et al.*, 2012).

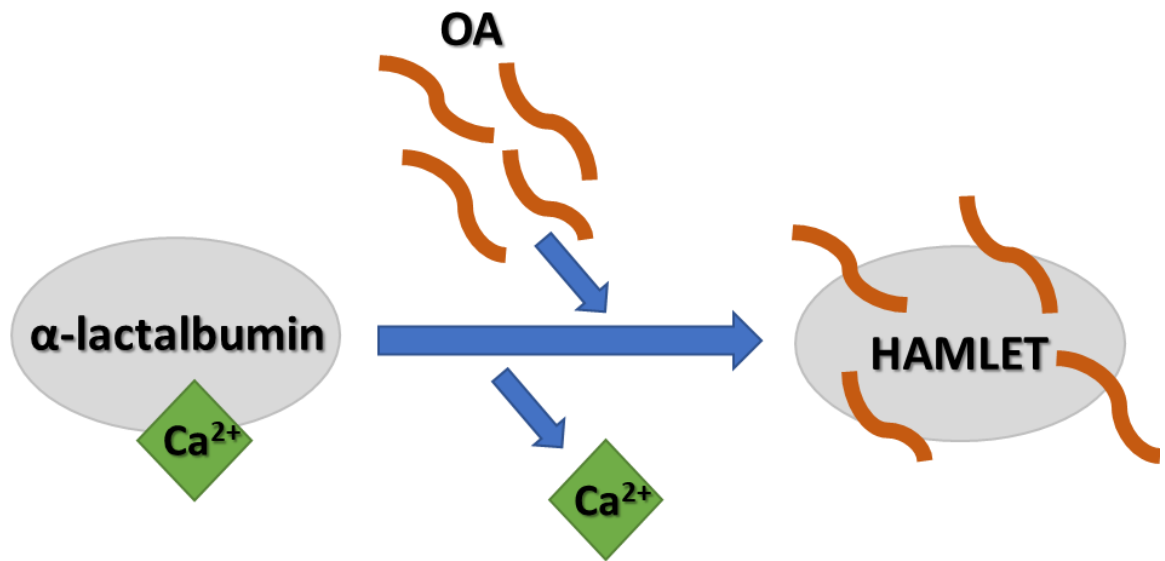


Figure 1.1. Structural components of HAMLET and its formation

Native α -lactalbumin partially unfolds after removal of the stabilizing calcium ion and binds multiple (4-8) molecules of OA.

Research on the possible fatty acid co-factors of α -lactalbumin revealed that OA monounsaturated at carbon 9 or 11 in the *cis* conformation has the ability to form active, tumouricidal HAMLET molecule, while saturated or *trans* unsaturated C18 and shorter chain fatty acids failed to form stable complexes (Svensson *et al.*, 2003b). This indicated the unfolded protein has preference for certain lengths and stereochemical configurations of fatty acids to form stable, tumouricidal complexes (Hallgren *et al.*, 2008). Moreover, studies on the interactions between the charges on the protein chains and the OA within HAMLET revealed that the positively charged lysine residues in α -lactalbumin structures are necessary to facilitate complexing OA through electrostatic interactions with the negatively charged carboxylic group of the fatty acid (Xie *et al.*, 2012). Therefore, any modification of the protein structure that substitutes positively charged residues with neutral or negative charges interrupts the OA binding capabilities of α -lactalbumin and prevents the formation of HAMLET (Xie *et al.*, 2012).

1.4.2. HAMLET derivatives

Human and bovine α -lactalbumin structures are largely homologous and both share a stabilizing Ca^{2+} binding site (Fast *et al.*, 2005). The proteins display a 76 % amino acid sequence identity and there is a “high degree of similarity” between the conformation of the native states of the human and bovine homologues (Svensson *et al.*, 2003). This fact intrigued and drove researchers to test whether bovine α -lactalbumin would also create a HAMLET-like complex under similar experimental conditions. In fact, bovine α -lactalbumin displayed analogous ability to form complexes with OA, dubbed Bovine α -lactalbumin Made Lethal to Tumour Cells (BAMLET) (Svensson *et al.*, 2003). Reportedly, the BAMLET complex was stable and closely resembled HAMLET, albeit showed a lower formation yield, suggesting less efficient OA binding by partially unfolded bovine α -lactalbumin, or that the protein is less prone to unfolding (Svensson *et al.*, 2003).

The discovery of HAMLET and BAMLET sparked interest in the possibility of modifying the proteins and the formation of complexes of other α -lactalbumin analogues and related proteins with fatty acids. Various studies reported obtaining molecules such as: GAMLET - goat α -lactalbumin derivative (Nakamura *et al.*, 2013), BLAGLET – β -lactoglobulin made lethal to tumour cells (Rath *et al.*, 2018), β -lactoglobulin-sodium oleate complex (Lišková *et al.*, 2011),

ELOA - equine lysozyme complexed with OA (Wilhelm *et al.*, 2009; Mossberg *et al.*, 2010), bovine lactoferrin-OA complex (Fang *et al.*, 2014), canine milk lysozyme-OA complex (Nakamura *et al.*, 2013), myoglobin-OA complex (Nakamura *et al.*, 2013), β 2-microglobulin-OA complex (Nakamura *et al.*, 2013), etc. In fact, HAMLET's discovery opened a new field of partially unfolded protein-fatty acid complexes study, with a new functional name of liprotides (Frislev *et al.*, 2017).

1.4.3. Tumourigenic potential and cancer selectivity of HAMLET and HAMLET-like complexes

Since their discovery, HAMLET and HAMLET-like complexes have been proven to have a broad range of anti-tumour activity, both *in vitro* and *in vivo*. Tests on over 50 cancer cell lines of different origins showed effective toxicity and cell death induction even in cell-death-resistant cancer cell lines while no such effects were observed in differentiated normal cells (Hakansson *et al.*, 1995; Svanborg *et al.*, 2003; Rath *et al.*, 2015). The lack of cell death in adjacent normal tissue has been reported in several studies in animal models and patients (Fischer *et al.*, 2004; Gustafsson *et al.*, 2004; Mossberg *et al.*, 2007; Puthia *et al.*, 2014). It is worth noting that the studies were performed over a span of over twenty years, by multiple independent research groups and were published in several articles. Still, a general consensus remains on HAMLET displaying a potent cytotoxicity in a wide range of cancer cell lines exhibiting various levels of aggressiveness and resistance to therapy. Some differences depending on the cell type and cell origin persist, with reports that lymphoma and leukaemia cell lines showed higher sensitivity and underwent cell death more rapidly than carcinoma cells (Svanborg *et al.*, 2003; Rath *et al.*, 2015). Moreover, several immortalised non-cancer cell lines also exhibited sensitivity to HAMLET suggesting a universal characteristic adopted by all cells upon immortalisation (Hakansson *et al.*, 1995; Rammer *et al.*, 2010; Xiao *et al.*, 2013; Delgado *et al.*, 2015; Rath *et al.*, 2015).

In contrast, healthy differentiated cells have been repeatedly reported to display resistance to HAMLET giving rise to the notion of HAMLET's high cancer selectivity. Most primary cells tested alongside the cancer cell lines displayed resistance to HAMLET-induced cell death in concentrations below the OA toxicity threshold (Hakansson *et al.*, 1995; Svanborg *et al.*,

2003; Rath *et al.*, 2015). However, blood and embryonic primary cells showed high sensitivity to HAMLET, indicating that along with some universal cancer-like characteristics shared by those cells, phenotype immaturity and poor differentiation status might contribute to HAMLET sensitivity (Hakansson *et al.*, 1995; Brinkmann *et al.*, 2011; Xiao *et al.*, 2013; Rath *et al.*, 2015). As a de-differentiated phenotype often coincides with higher aggressiveness and invasiveness of the tumour, the hypothesis that HAMLET targets an immature stem cell phenotype (Hakansson *et al.*, 1995) is very alluring and fuels interest and research into this molecule.

Most importantly, *in vivo* animal tests and human clinical trials showed that the tumouricidal properties and selectivity of HAMLET are maintained. Several animal models and human studies have been performed and showed promising results. In human glioblastoma xenografts in nude rats, HAMLET delayed disease progression and increased survival. Tumour cells showed induction of apoptosis, while healthy brain tissue remained intact (Fischer *et al.*, 2004). In $Apc^{Min/+}$ mice, peroral administration of HAMLET has led to drastically reduced age-related tumour development and a decrease in the number and size of tumours in mice with established tumours (Puthia *et al.*, 2014). This study also indicated that HAMLET can survive and maintain functionality within the context of the digestive tract and colon. A human placebo-controlled trial showed that HAMLET can remove skin papillomas without introducing side effects (Gustafsson *et al.*, 2004). The patients in this study were followed up two years post treatment showing very promising results. In another human trial, HAMLET was found to rapidly kill bladder cancer cells without affecting healthy adjacent tissue, leading to a reduction in tumour size (Mossberg *et al.*, 2007). Intravesical application of HAMLET pre-surgery was compared to NaCl, phosphate buffered saline (PBS), and native α -lactalbumin controls and a tumour size reduction was noted in 8 out of 9 treated patients, with an apoptotic response observed in the tumour tissue and not the adjacent healthy cells, giving hope that HAMLET therapy could in fact offer a quick, side-effect-free remedy.

Comparative analyses of HAMLET and the bovine derivative, BAMLET, demonstrate similar tumouricidal activity and specificity against cancer cells (Rammer *et al.*, 2010). Like HAMLET, BAMLET has been studied on a number of cancer cell lines, showing impressive, broad toxicity in a wide range of cancer cell types (Rammer *et al.*, 2010; Brinkmann *et al.*, 2011; Xiao *et al.*, 2013; Rath *et al.*, 2015; Rath *et al.*, 2018). Moreover, BAMLET has also displayed selectivity against cancer while sparing normal cells both *in vitro* and *in vivo* (Xiao *et al.*,

2013). Other lipotides also showed cytotoxic properties sparking interest in this field of research (Lišková *et al.*, 2011; Fang *et al.*, 2014; Frislev *et al.*, 2017).

There are reports suggesting that HAMLET could be formed in the stomach of nursing infants, as the low pH of the environment would facilitate partial unfolding of α -lactalbumin and OA can be freed from milk triacylglycerides by gastric enzymes (Nakamura *et al.*, 2013), protecting them from tumours (Svensson *et al.*, 2000; Mok *et al.*, 2007). This notion was studied further, to determine whether HAMLET and HAMLET-like compounds could be naturally formed in stomachs of infants and adults upon milk consumption, as the gastric environment conditions are in line with HAMLET formation conditions. In human milk, OA is the most abundant fatty acid, while α -lactalbumin is the most common protein (Jensen, 1999; Artym and Zimecki, 2013). α -Lactalbumin content in bovine milk is lower, with β -lactoglobulin being the most abundant protein, while OA is also present at a lower degree than in human milk (Artym and Zimecki, 2013; Mansson, 2008). Lipolytic enzymes would free OA from the triacylglycerol form, whereas pH 3 facilitates unfolding of α -lactalbumin, β -lactoglobulin, or fragmented proteins to form HAMLET-like complexes (Jensen, 1999; Svensson *et al.*, 2000; Yang *et al.*, 2006; Mansson, 2008; Tolin *et al.*, 2010). It is reported that the pH in the stomach prior to ingestion is *circa* pH 2, while after drinking milk or protein drink, it raises to pH 5-7 in adults and pH 4 and above in infants (Sullivan *et al.*, 2014; Mitchell *et al.*, 2001). Therefore, a mixture resembling milk contents, containing calcium, bovine α -lactalbumin, and ethanol-dissolved OA was exposed to pH 2.5 and then pH 7 to stimulate gastric conditions and formation of HAMLET-resembling complexes was observed (Sullivan *et al.*, 2013). A capsule endoscopy study involving a water-based α -lactalbumin, sucrose, and OA solution reported protein unfolding in the stomach; however, no cancer toxicity was observed suggesting that gastric conditions do not allow formation of a functional HAMLET-like complex (Sullivan *et al.*, 2014). However, other reports point that such water-based drink has no resemblance to the complicated composition of milk and therefore the results of the study are inconclusive (Rath *et al.*, 2015). On the other hand, a study where cancer patients were given human milk to drink as an alternative therapy reported there was no influence on disease outcome, except the placebo psychological relief of trying a new, experimental therapy (Rough *et al.*, 2009).

1.4.4. Mechanism of action and limitations

Despite its proven therapeutic potential and selective cytotoxicity, the exact mechanism of action of HAMLET is unknown. It has been reported to trigger a broad tumouricidal response that resembles apoptosis. The cells exposed to HAMLET have been observed to exhibit morphological alterations associated with apoptosis, such as nuclear condensation, DNA laddering, loss of cytoplasm, membrane blebbing (Hakansson *et al.*, 1995; Köhler *et al.*, 2001; Hallgren *et al.*, 2006; Ho *et al.*, 2017). Moreover, swelling and depolarisation of mitochondrial membranes, accompanied by cytochrome c release triggering the caspase cascade and exposure of phosphatidylserine on the outer surface of plasma membrane have been reported with further suggestions of HAMLET-induced, apoptotic-like mechanism (Köhler *et al.*, 2001; Hallgren *et al.*, 2006). However, lack of reversal by the treatment with Z-VAD pan-caspase inhibitor indicated that a caspase-independent cell death mechanism is also involved and activated upon exposure to HAMLET. This was further supported by a lack of impact of overexpressing anti-apoptotic proteins, BCL-2 and BCL-XL, on the cell death levels (Hallgren *et al.*, 2006; Ho *et al.*, 2017). Observed mitochondrial injury, decreased activation of the mammalian target of rapamycin complex 1 (mTORC1), cytoplasmic vacuolisation, and double-membrane-enclosed vesicle formation in HAMLET treated cells indicated activation of macroautophagic mechanisms that possibly contributed to tumour cell death. Moreover, suppression of Beclin-1 and Atg-5 with RNA interference inhibited the LC3 translocation and improved survival of tumour cells after exposure to HAMLET, implying a connection between autophagy and HAMLET (Aits *et al.*, 2009). The resistance of normal primary cells to HAMLET treatment indicated high tumour selectivity and suggested a possible existence of a specific, cancer-related mechanism that is activated upon exposure to HAMLET. Moreover, a bacterial study has revealed that similar apoptotic like morphological and molecular changes occur upon HAMLET exposure in prokaryotes (Hakansson *et al.*, 2011).

HAMLET kills a broad spectrum of cancer cell types and cells with an immortalized and immature phenotype *in vitro*, which might suggest that its target is a conserved mechanism or a fundamental, cancer-like characteristic (Hakansson *et al.*, 1995; Rath *et al.*, 2015). However, numerous studies have shown that HAMLET seems to interact with multiple molecular targets and cellular organelles, thus contradicting that hypothesis (Ho *et al.*, 2017). Sensitivity to

HAMLET has been reported to be linked to oncogenes such as MYC, RAS, and HIF1 α (Storm *et al.*, 2011). However, this connection does not explain the sensitivity of several non-cancer primary cells to HAMLET, nor its mechanism of action. Several reports mention multiple cellular targets of HAMLET – primarily the plasma membrane, proteasomes, lysosomes, mitochondria, and the nucleus (Köhler *et al.*, 2001; Düringer *et al.*, 2003; Gustafsson *et al.*, 2009; Ho *et al.*, 2017). It has been proposed that internalization of HAMLET into the cells is driven through protein receptor-independent pathway (Ho *et al.*, 2017); moreover, HAMLET has been shown to disturb the integrity of artificially produced membranes in physiological conditions, implying that it could potentially target the plasma membrane itself (Mossberg *et al.*, 2010b). It has been observed that HAMLET introduces plasma membrane perturbations and dysregulates the ion fluxes that subsequently activate p38 MAPK response and inhibit ERK1/2 phosphorylation, while ion flux inhibition by BaCl or amiloride protects the cells from HAMLET internalization and induction of the mentioned cell death signalling (Storm *et al.*, 2013; Nadeem *et al.*, 2015). Co-immunoprecipitation studies revealed that HAMLET binds to α -actinin-1 and α -actinin-4, implying its possible involvement in tumour cell rounding and detachment through disorganisation of the actin cytoskeleton, and potential further alterations due to α -actinin-related mechanisms (Trulsson *et al.*, 2011). A proteomic screen identified multiple nucleotide binding proteins as being cellular targets of HAMLET, including ATPases, RAS family genes, and over a hundred various kinases (Boekema, 2015; Ho *et al.*, 2015; Ho *et al.*, 2016; Ho *et al.*, 2017). Since HAMLET decreased the activity of the majority of kinases tested in the mentioned study, it has been dubbed the “pan-kinase inhibitor” (Ho *et al.*, 2016). Moreover, it reduced the activity of RAS family GTPases, further suggesting that HAMLET targets the enzymes that are typically activated in cancers (Ho *et al.*, 2016; Ho *et al.*, 2017). HAMLET’s activity in the nucleus has been reported to involve targeting histones H2B, H3, or H4, decreasing histone deacetylase activity and modifying chromatin structure (Hakansson *et al.*, 1999; Düringer *et al.*, 2003; Brest *et al.*, 2007; Sharp *et al.*, 2017). *In vitro* studies on HAMLET’s effect on 20S proteasomes revealed that HAMLET is capable of proteasome disintegration. In light of its colocalization with proteasomes in tumour cells and the decrease of proteasome activity in lysates of HAMLET treated cells, coupled with the fact that unfolded α -lactalbumin would likely be directed to the proteasomes for degradation, as usually happens with misfolded proteins, it has been suggested that proteasomes might be a further cellular target of HAMLET (Gustafsson *et al.*, 2009). A recent report implies that heat shock protein Hsp70 could be involved in vesicular uptake of HAMLET into the lysosomes

and a delayed death response of the tumour cells (Nadeem *et al.*, 2019). The rapidity of cell death induction in cancer cells as well as reports linking susceptibility to HAMLET to metabolic state and nutrient abundance in the culture medium (Mossberg *et al.*, 2007; Storm *et al.*, 2011; Ho *et al.*, 2013; Puthia *et al.*, 2014) suggest that the selective potential of HAMLET and HAMLET-like complexes might depend on the metabolic differences between cancer and differentiated cells. However, the multitude of cellular organelles and processes targeted by HAMLET seems to obscure the real initial targets and mode of action (figure 1.2).

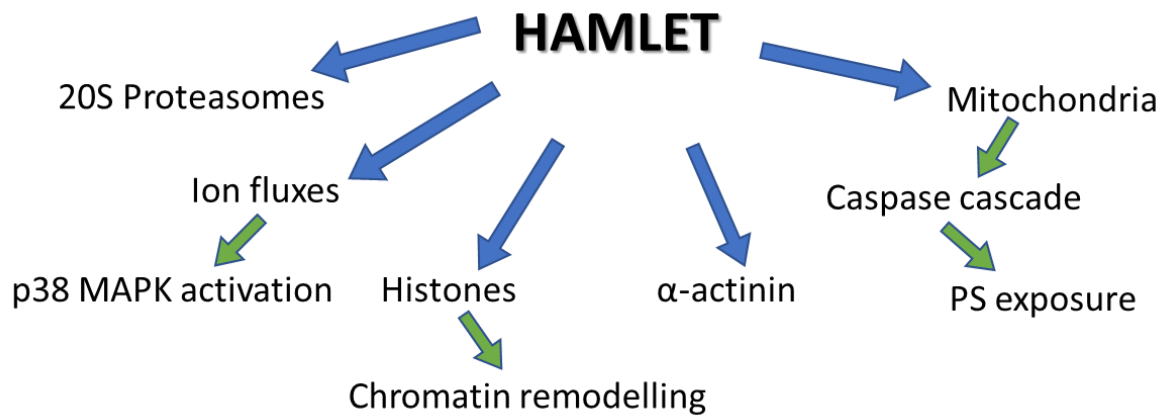


Figure 1.2. Overview of cancer cell organelles and processes affected by HAMLET

Cells exposed to HAMLET display 20S proteasome disintegration and disruption of ion fluxes, which in turn activates p38 MAPK signalling. HAMLET has been observed to target the histones and decrease histone deacetylase activity, thus modifying the chromatin structure. HAMLET binds to α -actinins indicating possible involvement in cell rounding and detachment. Mitochondrial swelling and cytochrome c release has been observed following HAMLET treatment, followed by caspase activation and phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane.

Although also containing protein and fatty acids, but structurally much larger than HAMLET in its physical dimensions, the liprotides' mechanism of action and induction of cell death in susceptible cells is unknown. It is believed to be somewhat comparable to HAMLET, as some reports indicate attacking similar targets within the cells, such as the plasma membrane, mitochondria, ion channels, proteasomes, lysosomes, etc. (Rath *et al.*, 2018). Some other studies claim that the mechanism of action of BAMLET involves activation of a caspase-independent lysosomal cell death pathway (Rammer *et al.*, 2010). There are a number of reports suggesting that BAMLET related cancer cell death can be induced by different mechanisms, such as apoptosis, necrosis, autophagy, proteasome degradation, or depolarization of the mitochondrial membrane (Hoque *et al.*, 2013). It is believed a common characteristic of HAMLET-like complexes is the delivery of the fatty acid into the cell where it unleashes its toxicity, as the partially unfolded proteins do not show toxic effects (Wen *et al.*, 2015; Delgado *et al.*, 2015). It is speculated that liprotides mediate cell death by permeabilizing the plasma membrane, as activation of a membrane repair mechanism has been observed (Frislev *et al.*, 2017); however, the exact mode of action of these molecules has yet to be deciphered.

An important draw back in HAMLET studies, possibly also contributing to the inability to pinpoint the exact mechanism of action, is the lack of reproducibility of results. The conditions used in protocols of different research groups to treat cells with HAMLET and HAMLET-like complexes differ, mentioning different time intervals of treatment, various pre-treatments of the cells or lack of them, and there is no consensus on the serum concentration in the culture media, which can greatly influence the toxicity and activity of the molecules. Moreover, as pointed by Rath *et al.* in their review (2015), the half maximal inhibitory concentrations (IC₅₀) values reported in various articles for the same cell line differ, which can be due to the aforementioned experimental protocol discrepancies or due to differences in the OA or oleate content in the HAMLET-like complex molecules (Rath *et al.*, 2015). It is believed that the toxic component of HAMLET-like complexes is the fatty acid, as the unfolded proteins do not exhibit toxicity themselves (Delgado *et al.*, 2014). The ratio of protein to fatty acid in the HAMLET-like complexes, while initially thought to be 1:1, has been proven to be different (Mossberg *et al.*, 2010; Wilhelm *et al.*, 2009; Nakamura *et al.*, 2013). Some studies mention that HAMLET forms oligomers, consisting of various (4-5) α -lactalbumin molecules binding dozens – up to 68-85 – OA molecules (Spolaore *et al.*, 2010). Others report that HAMLET and GAMLET complexes displayed heterogeneity at pH 7.5, consisting of a native protein state, a

molten globule-like state, and oligomers, with 7-8 OA molecules complexed to a single α -lactalbumin molecule (Nakamura *et al.*, 2013). It appears that the unfolded proteins can form complexes with multiple molecules of fatty acid, depending on the adopted experimental protocol and its execution, or for other unknown reasons (Wilhelm *et al.*, 2009; Nakamura *et al.*, 2013). Therefore, reproducibility of data cannot be achieved while adopting varying protocols of lipotide preparation. This stresses the necessity of clarifying, unifying, and standardizing the HAMLET-like complexes treatment, experimental, and preparation protocols, in order to achieve reproducible and trustworthy data and, possibly, be able to pinpoint the exact mechanism of action of lipotides. Here, we follow the authentic preparation protocol originally established by the Svanborg group (Hakansson *et al.*, 1999), the product of which has been shown to be monomeric in terms of the protein stoichiometry (Pettersson-Kastberg *et al.*, 2009).

Since human milk is not readily available, the production of HAMLET for this project was not possible. BAMLET shares many properties with HAMLET hence this research focuses on testing BAMLET's tumouricidal ability and how it could be exploited to enhance neoadjuvant therapies in oral and oesophageal cancers.

1.4.5. Potential of HAMLET/BAMLET as a neo-adjuvant therapeutic agent in oral and oesophageal cancers

As mentioned above, oral and oesophageal cancers are difficult to treat and often exhibit treatment resistance. The field of oral and oesophageal cancer is in great need of an efficient drug that could decrease the size of the tumour prior to surgery to maximally downsize the tumours in the oral cavity and the oesophagus. Due to chemoresistance and the aforementioned gruesome side effects, neoadjuvant therapy options are limited. However, the biggest problem is caused by patients' malnutrition and suffering because of the localisation of the tumours in the oral cavity and along the oesophagus. There is a great need for an efficient, rapidly acting drug that would not introduce further suffering to patients. HAMLET seems to be a perfect candidate. HAMLET has proven itself to be a worthwhile candidate for a neoadjuvant drug in *in vivo* studies and human trials mentioned above. The reports show that it works rapidly, efficiently, is highly tumour selective, and does not introduce side effects.

Moreover, it could be used both, topically and orally (Fischer *et al.*, 2004; Gustafsson *et al.*, 2004; Mossberg *et al.*, 2007; Puthia *et al.*, 2014).

It has been observed that neither HAMLET nor BAMLET can be applied intravenously. HAMLET-like complexes are stripped of OA by albumin (Frislev *et al.*, 2017; Rath *et al.*, 2018) as well as calcium ions (Rath *et al.*, 2018) present in blood or serum in *in vitro* cell cultures. Moreover, blood cells, such as erythrocytes, macrophages, etc., display high sensitivity to HAMLET despite not being cancer cells (Hakansson *et al.*, 1995; Rath *et al.*, 2015). Therefore, the use of HAMLET/BAMLET or other HAMLET-like complexes as neoadjuvant therapy would have to be limited to topical or oral uses. However, oral and oesophageal cancers are both cases where such applications are desirable – possibly as mouth washes or drinkable solutions. A bladder cancer trial showed that intravesically applied HAMLET rapidly introduced apoptosis and cancer cell shedding without harmful effect on adjacent healthy tissue. It has been observed that pre-surgery treatment reduced tumour size in majority of the patients in comparison to controls, proving efficacy and selectiveness of HAMLET treatment (Mossberg *et al.*, 2007). In the Apc^{Min/+} mice trial, HAMLET has proven to be active in the digestive tract, as peroral administration of HAMLET significantly reduced the number of tumours in the colon (Puthia *et al.*, 2014). These studies rise hopes that despite HAMLET's limitation in possible administration routes, it could still bring relief to patients and serve as a gentle and effective pre-operative neoadjuvant therapy. It is believed that by further examining HAMLET/BAMLET's mode of action, enhancing the effectiveness of the molecule will be possible, allowing its application as a novel neoadjuvant therapy for oral and oesophageal cancers. Ideally, the HAMLET therapy could serve as a gentle treatment efficiently reducing the size of the tumour and thereby minimise the impact that the surgery may have on the patient.

1.5. Aims of the project

This research focuses on exploring novel approaches to diagnosing/imaging and treating oral and oesophageal cancers, with the following specific aims:

1. Design a natural non-toxic HAMLET/BAMLET mimetic with fluorescent properties, that is internalized into cancer cells permitting their identification,
2. Examine the effect of modulating cancer cell metabolism on HAMLET/BAMLET sensitivity,
3. Determine the cellular properties that sensitize cells to HAMLET/BAMLET,
4. Explore how HAMLET/BAMLET uptake mechanisms relate to the uptake of the natural HAMLET/BAMLET mimetic imaging agent.

2. Materials and Methods

2.1. List of reagents

Cell culture media, media supplements, PBS and trypsin were purchased from Gibco, except DMEM/F-12 medium which was purchased from Sigma, and DMEM/F-12 without glucose and L-glutamine medium, which was purchased from Biowest. All chemicals and reagents for buffers, inhibitors and modulators for high resolution respirometry and Seahorse Flux analyser, substrates for BAMLET and organic chemical synthesis, ^{13}C labelled glucose and L-glutamine, *all-trans* retinoic acid and Nuclear Magnetic Resonance solvents were purchased from Sigma, unless stated otherwise. OA, Carbonyl Cyanide 4-(Trifluoromethoxy)phenylhydrazone, Amiloride, and Dynasore were provided by TCI Chemicals Europe. Primary antibodies were purchased from Santa Cruz Biotechnology; secondary antibodies were purchased from Promega. Alamar Blue, all Flow cytometry antibodies and Propidium Iodide were purchased from Invitrogen. Annexin V-FITC conjugate was purchased from IQ Products. The horseradish peroxidase substrate ECL was purchased from Millipore. Seahorse XFe96 analyser consumables and medium were purchased from Agilent.

2.2. Methods

2.2.1. Cell culture

2.2.1.1. Culturing of tongue cancer cell line, SCC-9

The main cell line used in this study was SCC-9, derived from a squamous cell carcinoma of the tongue. Frozen stocks were purchased from Sigma. Cells were cultured at 37 °C, in a humidified atmosphere, containing 5 % CO₂. For routine culture, DMEM/F-12 medium supplemented with 10 % Foetal Bovine Serum (FBS), 2.5 mM L-Glutamine, 0.5 mM Sodium Pyruvate, 400 ng/ml hydrocortisone, and 1 % Penicillin/Streptomycin was used. Typically, the culture medium was changed every 3 days.

2.2.1.2. Culturing of osteosarcoma cell line, U2OS

Osteosarcoma cell line, U2OS, was used as a reference cell line due to its wide use in HAMLET/BAMLET related studies. The U2OS cell line was purchased from American Type Culture Collection (VA, USA). Cells were cultured at 37 °C, in a humidified atmosphere containing 5 % CO₂. The routine culture medium was DMEM supplemented with 10 % FBS, 4 mM GlutaMAX, and 1 % Penicillin/Streptomycin. Typically, the culture medium was changed every 3 days.

2.2.1.3. Culturing of oesophageal cell line, KYSE-520

Oesophageal squamous cell carcinoma cell line, KYSE-520, was also used in the course of this study. Cells were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Cells were cultured at 37 °C, in a humidified atmosphere, containing 5 % CO₂. For routine culture, RPMI 1640 medium was used supplemented with 10 % FBS, 2 mM L-Glutamine, and 1 % Penicillin/Streptomycin. Typically, the culture medium was changed every 3 days.

2.2.1.4. Passaging of cell lines

Cells were passaged upon reaching 80 % confluency. Culture medium was gently removed from the flask and cells were washed once with pre-warmed PBS. To detach the cell from the surface of the flask a 0.25 % trypsin/EDTA solution was used at 37 °C for several minutes. Once the cells were detached, pre-warmed complete culture medium (containing FBS) was added to the cells to inactivate trypsin. Cells were subsequently pelleted by centrifugation at 250 x g for 5 min at room temperature. The cells were then re-seeded at a desired ratio depending on the experiments planned. Cells were maintained for no longer than 20 passages.

2.2.1.5. Cryopreservation of cell lines

Frozen stocks of the cell lines were prepared to ensure that the cell lines are not cultured for longer than 20 passages. Culture medium was changed 24 h before freezing. Cells were examined under the microscope to rule out contamination or morphological abnormalities. When the cells reached 80 % confluency, they were detached and centrifuged as described above. Cell pellets were resuspended in the freezing medium, consisting of 70 % of culture media of each cell line, 20 % FBS, and 10 % DMSO. Cells were frozen at the density of 1×10^6 cells per one cryovial. Vials were placed in a controlled rate freezing apparatus and placed at -80 °C for at least 24 h. Then, the vials were moved to a liquid Nitrogen container for long-term preservation. Cells were re-established from the frozen stocks by rapid thawing and immediately placing in pre-warmed medium. Cells were then pelleted by centrifugation at 250 x g for 5 min, resuspended in fresh medium and moved to a new culture flask. Following this procedure, cells were cultured as mentioned above.

2.2.1.6. Serum starvation

To ensure reproducibility in cytotoxicity assays, cells were serum starved prior to experiments. Cells were seeded at the desired density for each experiment and upon attachment, two PBS washes were performed, and then fresh, serum-free medium (0 % FBS) was added to the cells for 16 h prior to the treatment. Further experiments were performed as described below.

2.2.1.7. Differentiation of SCC-9 cells

All-trans retinoic acid (ATRA) treatment was used to induce differentiation in SCC-9 cells. ATRA is highly sensitive to light, oxygen, and temperature; therefore, caution was used when preparing working stocks to minimise exposure to adverse conditions. ATRA was purchased from Sigma in powdered form and reconstituted in DMSO to achieve a solution of 30 mg/ml. Aliquots of 100 µl were then placed in glass vials and sealed under nitrogen gas to ensure removal of oxygen from the vials. The vials were then placed in the dark at -20 °C for long-term storage. Before each differentiation procedure, a new vial was opened and a further 900 µl of DMSO was added to achieve a working solution of 3 mg/ml which was then aliquoted into 100 µl volumes. These working stock aliquots were stored in the dark, at -20 °C, for no longer than a week. The optimal ATRA concentration for differentiation induction was determined through titration. Cells were treated with a range of concentrations of ATRA for 5 days and subsequently the expression of cytokeratin 5 and 14, differentiation markers of epithelial cells, was assessed by Western Blot. SCC-9 cells were treated with 30 µM ATRA added into standard culture medium for 5 days before experiments were performed.

2.2.1.8. Mitochondrial uncoupling in SCC-9 cells

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a mitochondrial uncoupling agent, was used to investigate the impact of mitochondrial uncoupling on BAMLET sensitivity. CCCP was purchased from Sigma and an initial 1 mM stock was prepared in ethanol, aliquoted and stored at -20 °C, protected from light. The appropriate concentration of CCCP to use in BAMLET cytotoxicity assays was determined through assessment of cytotoxicity of CCCP on SCC-9 cells, described below. SCC-9 cells were treated with 0.4 µM CCCP added to standard culture medium for 72 h before the experiments were performed.

2.2.1.9. Fatty acid synthase inhibition in SCC-9 cells

4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75), a fatty acid synthase (FASN) inhibitor, was used to investigate the impact of exogenous fatty acid metabolism on BAMLET sensitivity. C75 was purchased from Santa Cruz Biotechnology and an initial 4 mM

stock was prepared in ethanol, aliquoted and stored at -20 °C. The appropriate concentration of C75 to use in BAMLET cytotoxicity assay was determined through assessment of cytotoxicity of C75 on SCC-9 cells, described below. SCC-9 cells were treated with 1 µM CCCP added into serum-free medium for 16 h before the experiments were performed.

2.2.1.10. Galactose adaptation of SCC-9 cells

For glucose deprivation studies, the SCC-9 cells were slowly adapted to galactose in culture medium, by gradual replacement of glucose with galactose over a course of 3 weeks. Cells were grown in DMEM/F-12 medium without glucose, supplemented with 10 % dialysed FBS, 2.5 mM L-Glutamine, 0.5 mM Sodium Pyruvate, 400 ng/ml hydrocortisone, and 1 % Penicillin/Streptomycin. For the first week, the ratio of glucose to galactose was 50 %:50 % (8.75 mM:8.75 mM), for the second 25 %:75 % (4.375 mM:13.125 mM) and on the third week, the cells were moved to a 100 % galactose medium (17.5 mM). Cells were further cultured in the medium containing galactose and passaged as described above.

2.2.1.11. Galactose adaptation of U2OS cells

For glucose deprivation studies, the U2OS cells were slowly adapted to galactose in medium, by gradual replacement of glucose with galactose over a course of 3 weeks. Cells were grown in DMEM medium without glucose, supplemented with 10 % dialysed FBS, 4 mM Gluta-MAX, and 1 % Penicillin/Streptomycin. For the first week, the ratio of glucose to galactose was 50 %:50 % (12.5 mM:12.5 mM), for the second 25 %:75 % (6.25 mM:18.75 mM), and on the third week the cells were moved to a 100 % galactose medium (25 mM). Cells were further cultured in the medium containing galactose and passaged as described above.

2.2.1.12. Galactose adaptation of KYSE-520 cells

For glucose deprivation studies, the KYSE-520 cells were slowly adapted to galactose in medium, by gradual replacement of glucose with galactose over a course of 3 weeks. Cells were grown in RPMI 1640 medium without glucose, supplemented with 10 % dialysed FBS, 2 mM L-Glutamine, and 1 % Penicillin/Streptomycin. For the first week, the ratio of glucose to ga-

lactose was 50 %:50 % (5.555 mM:5.555 mM), for the second 25 %:75 % (2.777 mM:8.332 mM) and on the third week, the cells were moved to a 100 % galactose medium (11.11 mM). Cells were further cultured in the medium containing galactose and passaged as described above.

2.2.3. Cytotoxicity Assays

Cytotoxicity assays were performed to assess the toxicity of certain compounds to cells. Cells were detached and pelleted by centrifugation as mentioned above. Next, the cell pellet was resuspended in fresh medium and 10 μ l of the cell suspension was loaded onto a haemocytometer (Kova International). The number of cells was determined by calculating the number of cells in three squares in the haemocytometer and multiplying the average by the dilution factor. The result was the number of cells in 1 ml of the suspension. For cytotoxicity assays, cells were plated on clear, flat-bottomed 96-well plates at 5×10^3 cells/well. Cells were left in routine medium to attach and the medium was changed to medium without FBS to serum starve the cells for 16 h before performing the assay. In the serum starvation assay and for FASN inhibition assay, control plates were not serum starved but cultured in 10 % FBS medium until treatment. After 16 h of serum starvation, cells were treated with a range of concentrations of the tested compounds, which were prepared immediately prior to the experiment. Cells were treated with 100 μ l solution containing the culture medium with or without FBS and the desired concentration of the tested compounds.

2.2.3.1. NF2792 and intermediates cytotoxicity assays

NF2792 and its intermediate compounds' toxicity was assessed on SCC-9 cells. For OA, NF2790, and NF2791 cytotoxicity assays, an initial 5 mM stock was prepared in DMSO, aliquoted and stored at -20 °C. Subsequent dilutions for the cytotoxicity assays were carried out in serum-free medium. For NF2792, HA700, and HA-FA-Pg-3F cytotoxicity assays, 5 mM stocks of the were prepared in MilliQ H₂O, aliquoted and stored at -20 °C. Next, dilutions for the cytotoxicity assays were carried out in serum-free medium. The concentrations ranged from 0.24 μ M to 100 μ M. In each cytotoxicity assay, a vehicle control with the appropriate concentration of the diluent was prepared (0.1 % v/v DMSO, MilliQ H₂O). Each concentration

of compound was added in triplicate. After 24 h at 37 °C and a humidified atmosphere of 5 % CO₂, cell viability was assessed using Alamar Blue viability assay (see section 2.2.5) in comparison to vehicle control values. IC₅₀ values were determined using GraphPad 6 software from 3 independent biological replicates of each experiment.

2.2.3.2. BAMLET cytotoxicity assays

The cytotoxicity of BAMLET was assessed on SCC-9, U2OS, and KYSE-520 cells under various conditions. For these assays, an initial 10 mg/ml (72 mM) stock was prepared in MilliQ H₂O, and dilutions ranging from 0.001 mg/ml (72 nM) to 1 mg/ml (72 µM) were prepared using MilliQ H₂O and serum-free medium. A vehicle control with the appropriate volume of MilliQ H₂O added to the medium was prepared for each assay. Each concentration of BAMLET was added in triplicate. After 24 h at 37 °C and a humidified atmosphere of 5 % CO₂, cell viability was assessed using Alamar Blue viability assay (see section 2.2.5) relative to vehicle control values. IC₅₀ values were determined using GraphPad 6 software from 3 independent biological replicates of each experiment.

2.2.3.3. BAMLET time-dependent cytotoxicity assays

Time-dependent cytotoxicity of BAMLET was assessed in SCC-9 cells. For these assays, an initial 10 mg/ml (72 mM) stock was prepared in MilliQ H₂O, and dilutions ranging from 0.001 mg/ml (72 nM) to 1 mg/ml (72 µM) were prepared using MilliQ H₂O and serum-free medium. A vehicle control with the appropriate volume of MilliQ H₂O added to the medium was prepared for each assay. Each concentration of BAMLET was added in triplicate. Cells were treated with BAMLET for 30 min, 1 h, 2 h, 3 h, and 6 h, and then washed twice with PBS before fresh serum-free medium was added. Cells were incubated at 37 °C and a humidified atmosphere of 5 % CO₂ until 24 h elapsed from the initial treatment with BAMLET. Cell viability was then assessed using Alamar Blue viability assay (see section 2.2.5) in comparison to vehicle control values. The IC₅₀ values were determined using GraphPad 6 software from 3 independent biological replicates of each experiment.

2.2.3.4. CCCP cytotoxicity assay

The appropriate concentration of CCCP to use in BAMLET cytotoxicity assay was determined through a CCCP cytotoxicity assay on SCC-9 cells. One aliquot of the 1 mM stock was diluted in standard culture medium. The concentrations ranged from 0.01 μ M to 3 μ M. In each cytotoxicity assay, a vehicle control with the appropriate concentration of the diluent was prepared (0.1 % v/v ethanol). Cells were not serum starved prior to treatment. Each concentration of compound was added in triplicate in standard culture medium. After 72 h at 37 °C and a humidified atmosphere of 5 % CO₂, cell viability was assessed using Alamar Blue viability assay (see section 2.2.5) and compared to vehicle control values. The IC₅₀ values were determined using GraphPad 6 software from 3 independent biological replicates of each experiment. The CCCP concentration used in BAMLET cytotoxicity assays with prior mitochondrial uncoupling was 0.4 μ M.

2.2.3.5. C75 cytotoxicity assay

The appropriate concentration of C75 to use in BAMLET cytotoxicity assay was determined through assessment of cytotoxicity of C75 on SCC-9 cells. A 4 mM stock was diluted in serum-free medium to generate concentrations ranging from 0.1 μ M to 50 μ M. In each cytotoxicity assay, a vehicle control with the appropriate concentration of the diluent was prepared (0.1 % v/v ethanol). Cells were not serum starved prior to treatment. Each concentration of compound was tested in triplicate in serum-free medium to serum starve the cells during FASN inhibition. After 16 h at 37 °C and a humidified atmosphere of 5 % CO₂, cell viability was assessed using Alamar Blue viability assay (see section 2.2.5) and related to vehicle control values. The IC₅₀ values were determined using GraphPad 6 software from 3 independent biological replicates of each experiment. The C75 concentration used in BAMLET cytotoxicity assays after FASN inhibition was 1 μ M.

2.2.3.6. Amiloride cytotoxicity assay

Amiloride, an inhibitor of sodium-proton exchange and macropinocytosis was used to investigate its effect on endocytosis in SCC-9 cells. The appropriate concentration of amiloride to

use in further assays was determined through assessment of cytotoxicity of amiloride on SCC-9 cells. An initial 40 mM stock was prepared in DMSO, aliquoted and stored at -20 °C. Subsequent dilutions for the cytotoxicity assays were carried out in serum-free medium. The concentrations ranged from 1 µM to 1 mM. In each cytotoxicity assay, a vehicle control with the appropriate concentration of the diluent was prepared (0.1 % v/v DMSO). Cells were serum starved prior to the treatment. Each concentration of compound was added in triplicate. After 2 h at 37 °C and a humidified atmosphere of 5 % CO₂, cell viability was assessed using Alamar Blue viability assay (see section 2.2.5) and related to vehicle control values. The IC₅₀ values were determined using GraphPad 6 software from 3 independent biological replicates of each experiment. The concentration of amiloride used in further assays was 1 mM.

2.2.3.7. Dynasore cytotoxicity assay

Dynasore, an inhibitor dynamin GTPase activity was used to investigate its effect on endocytosis. The appropriate concentration of dynasore to use in further assays was determined through assessment of cytotoxicity of dynasore on SCC-9 cells. An initial 30 mM stock was prepared in DMSO, aliquoted and stored at -20 °C. Subsequent dilutions for the cytotoxicity assays were carried out in serum-free medium. The concentrations ranged from 1 µM to 1 mM. In each cytotoxicity assay, a vehicle control with the appropriate concentration of the diluent was prepared (0.1 % v/v DMSO). Cells were serum starved prior to the treatment. Each concentration of compound was added in triplicate. After 2 h at 37 °C and a humidified atmosphere of 5 % CO₂, cell viability was assessed using Alamar Blue viability assay (see section 2.2.5) and related to vehicle control values. The IC₅₀ values were determined using GraphPad 6 software from 3 independent biological replicates of each experiment. The concentration of dynasore used in further assays was 100 µM.

2.2.4. Proliferation Assay

Assays to determine proliferation were performed to investigate if cellular differentiation induced by ATRA could influence the rate of proliferation of SCC-9 cells. Cells were detached and counted as mentioned above. For proliferation assays, clear, flat-bottomed 96-well plates were used. Both differentiated and control (untreated) cells were plated at 5 x 10³ cells/well.

Cells were then cultured in routine culture medium for 24 h and 48 h. The experiments were performed in 3 independent biological replicates. Then, their viability was assessed by Alamar Blue viability assay (see section 2.2.5) and Sulforhodamine B (SRB) colorimetric assay (see section 2.2.6).

2.2.5. Alamar Blue Viability Assay

Alamar Blue (Invitrogen) is a reagent used to quantify cellular metabolic activity, which also allows a determination of cell viability. Alamar blue contains resazurin, present in the reagent solution, which is a nonfluorescent blue dye that can be chemically reduced into pink fluorescent resorufin by metabolically active cells. Measuring fluorescence at a single point can be used to determine cell viability. At the end of the cytotoxicity and proliferation assay incubation period, 10 μ l of Alamar Blue was added to each well containing 100 μ l medium with the tested compounds. Plates were then incubated for 3.5 h at 37 °C in the dark. Fluorescence levels were measured using a SPECTRAmax Gemini Microplate Spectrofluorometer (Molecular Devices) with an excitation wavelength set at 544 nm, and emission at 590 nm. The fluorescence level of the medium was subtracted from the samples as a blank. Cell viability was calculated as a percentage relative to cells treated with appropriate vehicle (which were arbitrarily set to a value of 100 %). The average data from 3 independent biological replicates was plotted using GraphPad Prism 6 software.

2.2.6. Sulforhodamine B colorimetric Assay

The SRB assay is widely used to assess cell density after various treatments. In this study, SRB colorimetric assay was performed to investigate if ATRA induced differentiation influenced the rate of proliferation of SCC-9 cells. The assay is based on the stoichiometric binding of SRB to proteins under mildly acidic conditions, and subsequent extraction under basic conditions, providing a measure of absorbance that correlates to cell mass.

At the end of incubation time for the proliferation assay, 6 μ l of 50 % Trichloroacetic acid (TCA) was added to each well to achieve a final concentration of 10 % and the plates were incubated at 4 °C for 1 h. Medium with TCA was then removed and the wells were washed 3

times with 0.5 x PBS. 100 μ l of 0.4 % SRB solution in 1 % acetic acid was added to dry plates and plates were incubated at room temperature for 30 min. Wells were then washed 3 times with 1 % acetic acid to remove unbound SRB. When the plates dried, 200 μ l of 10 mM Tris pH 9.5 was added to each well. Plates were then incubated on a shaker until the dye was fully resolved. The absorbance was measured at 540 nm on SPECTRAmax PLUS 384 Microplate Spectrophotometer (Molecular Devices). The absorbance level of the medium was subtracted from the samples as a blank. Cell proliferation of differentiated cells was calculated as a percentage of control cells (which were arbitrarily set to 100 %). The average data from 3 independent biological replicates was plotted using GraphPad Prism 6 software.

2.2.7. Seahorse Metabolic Flux analysis

The metabolism of SCC-9, U2OS, and KYSE-520 cells was examined by Seahorse XFe96 analyser (Agilent). It allows for real-time simultaneous measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in the medium above a monolayer of adhered cells in a 96-well plate. The OCR corresponds to mitochondrial respiration rate and the ECAR to glycolysis rate. At 24 h before running the assay, cells were detached as mentioned above, counted and seeded on the Seahorse 96-well plates at the density of 3×10^4 cells/well for SCC-9 cells, 4×10^4 cells/well for U2OS, and 5×10^4 cells/well for KYSE-520 cells. According to the manufacturer's protocol, the Seahorse cartridge was hydrated in MilliQ H₂O overnight in a non-CO₂ 37 °C incubator. At 1 h prior to running the assay, the MilliQ H₂O in the utility plate was replaced with pre-warmed Seahorse XF Calibrant buffer and placed in a non-CO₂ 37 °C incubator. On the morning of the assay, the culture medium was removed from the Seahorse plate that contained the cells and replaced with assay medium, and the plate was placed in the non-CO₂ 37 °C incubator for 45 min. Then, the working stocks dilutions of the reagents used in the assay were prepared in the assay medium and loaded into the injection ports on the hydrated cartridge (mentioned in Table 1.1 – 1.6). The plate layout and assay protocol were set up on the Seahorse XFe96 analyser computer, and the cartridge and utility plate were placed in the Seahorse XFe96 analyser, and the calibration protocol was initiated.

After the completion of the calibration procedure, the utility plate was replaced with the cell plate and the assay was run for roughly 40 min. After the assay, the medium from the cell plate was removed and the plate was stored at -80 °C for cell number determination. Final analysis of the data was performed using the Wave (Agilent) and GraphPad Prism 6 software.

2.2.7.1. Mitochondrial stress test

Mitochondrial stress tests were performed to study mitochondrial function in the cells. The analyser measures the real-time oxygen consumption rate in response to injections of inhibitors and modulators of the electron transport chain, providing information on the state and activity of the mitochondria of the investigated cells. Preparation of the reagents used in the mitochondrial stress test is shown in Tables 1.1, 1.3, and 1.5.

2.2.7.2. Glycolysis stress test

Glycolysis stress tests were performed to study glycolytic function in the cells. The analyser measures the real-time extracellular acidification rate in response to injections of inhibitors and modulators of glycolysis, providing information on the state and activity of the mitochondria of the investigated cells. Preparation of the reagents used in the mitochondrial stress test is shown in Tables 1.2, 1.4, and 1.6.

Table 2.1. Reagent preparation for Mitochondrial stress test in SCC-9 cells

Mitochondrial stress test reagent preparation									
Port	Reagent	Volume in well	Final concentration in well	Dilution factor	Injected volume	Stock concentration	Working stock concentration	Volume of stock	Volume of Seahorse medium
A	Oligomycin	180 µl	1 µM	10 x	20 µl	2 mM	10 µM	5 µl	995 µl
B	FCCP	200 µl	0.125 µM	10 x	22 µl	1 mM	1.25 µM	1.5 µl	1198.5 µl
C	Rotenone/	222 µl	0.1 µM/4µM	10 x	25 µl	0.9 mM/5 mM	1 µM/40 µM	1.5 µl /	1337.7 µl
	Antimycin A							10.8 µl	

Table 2.2. Reagent preparation for Glycolysis stress test in SCC-9 cells

Glycolysis stress test reagent preparation									
Port	Reagent	Volume in well	Final concentration in well	Dilution factor	Injected volume	Stock concentration	Working stock concentration	Volume of stock	Volume of Seahorse medium
A	Glucose	180 μ l	10 mM	10 x	20 μ l	1.1 M	100 mM	100 μ l	900 μ l
B	Oligomycin	200 μ l	1 μ M	10 x	22 μ l	2 mM	10 μ M	6 μ l	1194 μ l
C	2-Deoxyglucose	222 μ l	30 mM	10 x	25 μ l	Pure solid form	300 mM	64 mg	1300 μ l

Table 2.3. Reagent preparation for Mitochondrial stress test in U2OS cells

Mitochondrial stress test reagent preparation									
Port	Reagent	Volume in well	Final concentration in well	Dilution factor	Injected volume	Stock concentration	Working stock concentration	Volume of stock	Volume of Seahorse medium
A	Oligomycin	180 μ l	2 μ M	10 x	20 μ l	2 mM	20 μ M	10 μ l	990 μ l
B	FCCP	200 μ l	0.5 μ M	10 x	22 μ l	1 mM	5 μ M	6 μ l	1194 μ l
C	Rotenone/ Antimycin A	222 μ l	0.1 μ M/4 μ M	10 x	25 μ l	0.9 mM/5 mM	1 μ M/40 μ M	1.5 μ l / 10.8 μ l	1337.7 μ l

Table 2.4. Reagent preparation for Glycolysis stress test in U2OS cells

Glycolysis stress test reagent preparation									
Port	Reagent	Volume in well	Final concentration in well	Dilution factor	Injected volume	Stock concentration	Working stock concentration	Volume of stock	Volume of Seahorse medium
A	Glucose	180 μ l	10 mM	10 x	20 μ l	1.1 M	100 mM	100 μ l	900 μ l
B	Oligomycin	200 μ l	2 μ M	10 x	22 μ l	2 mM	20 μ M	12 μ l	1188 μ l
C	2-Deoxyglucose	222 μ l	30 mM	10 x	25 μ l	Pure solid form	300 mM	64 mg	1300 μ l

Table 2.5. Reagent preparation for Mitochondrial stress test in KYSE-520 cells

Mitochondrial stress test reagent preparation									
Port	Reagent	Volume in well	Final concentration in well	Dilution factor	Injected volume	Stock concentration	Working stock concentration	Volume of stock	Volume of Seahorse medium
A	Oligomycin	180 μ l	2 μ M	10 x	20 μ l	2 mM	20 μ M	10 μ l	990 μ l
B	FCCP	200 μ l	0.125 μ M	10 x	22 μ l	1 mM	1.25 μ M	1.5 μ l	1198.5 μ l
C	Rotenone/ Antimycin A	222 μ l	0.1 μ M/4 μ M	10 x	25 μ l	0.9 mM/5 mM	1 μ M/40 μ M	1.5 μ l / 10.8 μ l	1337.7 μ l

Table 2.6. Reagent preparation for Glycolysis stress test in KYSE-520 cells

Glycolysis stress test reagent preparation									
Port	Reagent	Volume in well	Final concentration in well	Dilution factor	Injected volume	Stock concentration	Working stock concentration	Volume of stock	Volume of Seahorse medium
A	Glucose	180 μ l	10 mM	10 x	20 μ l	1.1 M	100 mM	100 μ l	900 μ l
B	Oligomycin	200 μ l	2 μ M	10 x	22 μ l	2 mM	20 μ M	12 μ l	1188 μ l
C	2-Deoxyglucose	222 μ l	30 mM	10 x	25 μ l	Pure solid form	300 mM	64 mg	1300 μ l

2.2.8. Hoechst 33258 dsDNA quantitation

To normalize the Seahorse metabolic flux data for cell number, FluoReporter Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes) was used. The kit contains Hoechst 33258 fluorescent dye, which emits upon binding to A-T rich regions of double stranded DNA. According to the manufacturers' protocol, a cell number standard curve for each cell line was prepared. Cells were seeded on 96-well plates, as mentioned above, at densities of 1×10^3 , 5×10^3 , 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , and 5×10^4 cells/well. After attachment, medium was aspirated, and plates were frozen at -80°C . Next, the plates were thawed, and 100 μl MilliQ H_2O was added to each well. Plates were then placed at 37°C for one hour and after that frozen at -80°C . To read the fluorescence, the plates were thawed, 100 μl of Hoechst 33258 in TNE buffer (1:400) was added. Fluorescence levels were measured using a SPECTRAmax Gemini Microplate Spectrofluorometer (Molecular Devices) with an excitation set at 360 nm and emission at 460 nm. The fluorescence values were plotted in GraphPad Prism 6 software and cell number standard curves for each cell line were graphed. Cell plates from the Seahorse metabolic flux experiments were processed following the same protocol and the obtained fluorescence values were fitted to the standard curves to determine cell number in each well.

2.2.9. High Resolution Respirometry

The respiratory rate of SCC-9 cells was determined by high resolution respirometry. It allows for real-time measurement of oxygen flux in suspended cells in an airtight chamber of the Oxygraph (OROBOROS Instruments). Cells were cultured, differentiated and detached as mentioned above. After centrifugation, the cell pellet was resuspended in mitochondria respiratory medium 05 (MiR05), counted and injected at 2 million cells/ml (4 million cells/chamber) into the chambers of the Oxygraph. For the respirometry measurements, the basic respiration protocol was used to monitor the basal OXPHOS rate of the cells. Experiments and results were analysed using DatLab7 software.

X	4Rot	5Ama
E	3U	
P	1ce	
L	2Omy	

Figure 2.1. The basic respiration protocol for high resolution respirometry

Additions were: **1ce** – injection of the cells [2×10^6 cells/ml] and measurement of the basal respiratory rate of the cells, **2Omy** – Oligomycin [0.0025 mM] to measure leak respiration, **3U** – titrations of Uncoupler [1 titration: 0.0005 mM] to show the maximal capacity of the mitochondria, **4Rot** – Rotenone [0.0005 mM] to inhibit mitochondrial complex I, and **5Ama** – Antimycin A [0.0025 mM] to inhibit mitochondrial complex III and thus the cellular respiration and measure the background Oxygen consumption.

X	5Rot	6Ama
E	4U	
P	1ce	2Glc
L	3Omy	

Figure 2.2. The basic protocol developed for galactose adapted cells

Consecutive additions were: **1ce** – injection of the cells [2×10^6 cells/ml] and measurement of the basal respiratory rate of the cells, **2Glc** – Glucose [10 mM] was added to see the reaction of the glucose starved mitochondria to addition of glucose, **3Omy** – Oligomycin [0.0025 mM] to measure leak respiration, **4U** – titrations of Uncoupler [1 titration: 0.0005 mM] to show the maximal capacity of the mitochondria, **5Rot** – Rotenone [0.0005 mM] to inhibit mitochondrial complex I, and **6Ama** – Antimycin A [0.0025 mM] to inhibit mitochondrial complex III and thus the cellular respiration and measure the background Oxygen consumption.

2.2.10. Western Blotting

2.2.10.1. Protein extraction

For protein extraction, 2×10^5 cells were seeded in a 6-well dish. For differentiation studies, cells were treated with increasing concentrations of ATRA for 5 days. For apoptosis studies after BAMLET treatment, cells were pre-treated with either vehicle or endocytosis inhibitors, amiloride or dynasore, for 30 min and then treated either with vehicle or 20 μ M BAMLET for 6 h. At the end of each treatment period, medium was removed, and the cells were washed with PBS. 100 μ l of cold RIPA buffer (Sigma) containing protease inhibitor cocktail tablet (Roche) was added to each well and incubated for 15 min on ice. Cells were then scraped, and the lysates were transferred to 1.5 ml tubes and centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatants were moved to a fresh tube and kept on ice. The protein concentration of each sample was measured using the bicinchoninic acid assay (BCA) assay (Thermo Fisher). Samples were then standardized at 1 μ g/ml with 4 x SDS loading buffer (8 % SDS, 30 % glycerol, 0.25 M Tris, 10 % β -mercaptoethanol, 1 % bromophenol blue, H₂O), and boiled at 95 °C for 3 min. Tubes were then left to cool down in room temperature and stored at -20 °C until SDS-Page gel electrophoresis.

2.2.10.2. Bicinchoninic acid assay

As described in the manufacturer's protocol, the BCA working reagent was prepared by adding 50 parts of BCA reagent A with 1 part of BCA reagent B. Standard solutions with known concentrations of bovine serum albumin (BSA) were prepared from a 2 mg/ml stock solution as described in table 2.7. 200 μ l of the working reagent was pipetted into each well on a 96-well plate and 25 μ l of the standard or sample solutions were then added. The plate was wrapped in aluminium foil to protect from light, shaken on a plate shaker for 30 s, and incubated at 37 °C for 30 min. After the incubation period, an absorbance at 562 nm was read using a SPECTRAmax PLUS 384 Microplate Spectrophotometer (Molecular Devices). The values obtained from the standard solutions allowed for plotting a standard curve, which was then used to determine the protein concentration in the unknown samples.

Table 2.7. Preparation of BSA standards for the BCA assay

Vial	Volume of diluent [μ l]	Volume and source of BSA [μ l]	Final concentration of BSA [μ g/ml]
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B	750
E	325	325 of vial C	500
F	325	325 of vial E	250
G	325	325 of vial F	125
H	400	100 of vial G	25
I	400	0	0

2.2.10.3. SDS-PAGE Gel Electrophoresis

A 10 % resolving polyacrylamide gel with a 4 % stacking polyacrylamide gels were used for the separation of proteins from cell lysate samples. Gels were prepared immediately prior to electrophoresis. 20 μ g of each cell lysate sample was loaded into each well. A pre-stained protein weight ladder was used to determine the molecular size of the proteins. The gel was run in running buffer (25 mM Tris base, 190 mM glycine, 0.1 % SDS) at 70 V for 20 min to allow the proteins to migrate through the stacking gel and, consequently, at 120 V until fully separated. When the separation was complete, the stacking gel was removed, and the proteins from the resolving gel were blotted onto a polyvinylidene difluoride (PVDF) (Merck) membrane. Prior to setting up the wet transfer, the PVDF membrane was activated by submerging in methanol for 10 s and then washed in transfer buffer (25 mM Tris base, 192 mM glycine, 10 % methanol). The transfer was set up by placing 3 sheets of Whatman paper, the gel, membrane, and further 3 sheets of Whatman paper, removing air bubbles and placing the sandwich in the tank, with the gel facing the cathode and the membrane facing the anode. The transfer was run for 16 h at 140 mA. After completion of the transfer, to avoid non-specific binding, the membrane was blocked in 5 % non-fat milk in TBST buffer (20 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1 % Tween 20) for 1 h in room temperature. The primary antibody was then added to the 5 % milk-TBST solution and the membrane was incubated for 1 h at room tempera-

ture or at 4 °C overnight (o/n) (see table 2.4). The membrane was washed in TBST 3 times for 15 min. The membrane was then incubated with horseradish peroxidase conjugated secondary antibodies (anti-rabbit 1:2500, anti-mouse 1:5000) for 1 h in room temperature, and then washed in TBST 3 times for 15 min. The membranes were then visualized using a horseradish peroxidase substrate (Millipore) on Universal Hood III ChemiDoc Imaging System with Image Lab Software (BioRad).

Table 2.8. Composition of polyacrylamide gels for SDS-Page electrophoresis

Component	4 % stacking gel	10 % resolving gel
30 % acrylamide/ bis-acrylamide solution	660 µl	3.3 ml
1 M Tris HCl, pH 6.8	630 µl	---
1.5 M Tris HCl, pH 8.8	---	2.5 ml
10 % SDS	50 µl	100 µl
H ₂ O	3.63 ml	4.0 ml
TEMED	5 µl	5 µl
10 % APS	25 µl	100 µl

Table 2.9. Antibodies used in Western Blotting

Antibody	Supplier	Isotype	Type	Primary antibody dilution and incubation
Cytokeratin 14 #sc-53253	SantaCruz Biotechnology	Mouse	Monoclonal	1:5000, RT, 1 h
Cytokeratin 5 #sc-32721	SantaCruz Biotechnology	Mouse	Monoclonal	1:1000, 4 °C, o/n
Caspase 3 #9662S	Cell Signalling	Rabbit	Polyclonal	1:1000, 4 °C, o/n
GAPDH #6C5	Calbiochem	Mouse	Monoclonal	1:5000, RT, 1 h

2.2.11. Nuclear Magnetic Resonance

The Nuclear Magnetic Resonance (NMR) experiments were performed on an Agilent Technologies 400 MHz NMR spectrometer by Nicola Relitti at the University of Siena. The studied compounds were vacuum dried and subsequently diluted in deuterated chloroform (CDCl_3) – NF2790 and NF2791, and heavy water (D_2O) – NF2792. The tubes were loaded into the NMR spectrometer and the probe was set to ^1H or ^{13}C frequencies. 1D ^1H , ^{13}C and 1D ^{13}C spectra were obtained. The data allowed for qualitative analysis of the synthesized chemical molecules to assess their structural characteristics.

2.2.12. BAMLET preparation – liquid chromatography

BAMLET complex was prepared using liquid chromatography method (Pettersson-Kastberg, *et al.*, 2009) on a Dionex Ultimate 3000 Pump (Thermo Fisher Scientific Inc) with the help of Anna Hastings and Daniel Fortunati. This method is an adaptation of the original method (Hakansson *et al.*, 1999) providing an authentic version of the protein-fatty acid complex. Tris buffer A (10 mM Tris pH 8.5) and Tris buffer B (10 mM Tris 1 M NaCl pH 8.5) were prepared, filtered and attached to the Dionex pump. The lines were purged to eliminate trapped air bubbles. Next, a HiTrap DEAE FF column was attached and flushed with Tris buffer A for at least 30 min at a flow rate of 1 ml/min. 247 μl of OA was dissolved in 500 μl ethanol and 5 ml Buffer A. To obtain an emulsion, the mixture was then sonicated at a duty cycle of 0.5 s and an output control of 50 % for 5 min in 30 s blasts, at 4 °C. The OA emulsion was then filtered through a 0.2 μm filter and loaded onto the column at a rate of 1 ml/min. Unbound OA was removed by washing the column with a continuous NaCl gradient using a combination of Tris buffer A and B (A:B ratio going from 100 %:0 % to 0 %:100 % gradually). Prior to loading the protein solution, the lines were washed with Tris buffer A at a flow rate of 1 ml/min. The protein solution was prepared by dissolving 33 mg/ml of bovine α -lactalbumin in 5 ml of Tris buffer A, filtered through a 0.2 μm filter, and loaded onto the column at a flow of 1 ml/min. The same continuous NaCl gradient was applied as before and the BAMLET complex was collected upon elution from the column in high salt conditions, confirmed by the change in absorbance at 280 nm. The eluted fractions were then dialysed at 5 °C for 48 h using Snake-skin tubing (Thermo Fisher) to remove excess salt in the solution resulting from the high-salt

elution. Dialysed samples were aliquoted and frozen using liquid nitrogen before being lyophilised to obtain a pure protein complex sample. The lyophilised samples were stored at -20 °C.

2.2.13. Confocal Microscopy

SCC-9 cells were seeded onto an 8 chamber μ -slide with cover slip base at a density of 8×10^4 cells/chamber 24 h prior to the experiment. Cells were left to attach and then serum starved for 16 h before treatment. Each chamber of the slide was treated with 5 μ M, 10 μ M, 20 μ M, and 50 μ M of NF2792 in serum-free medium, with the control cells left untreated, and incubated for 2 h. Cells were then washed twice with PBS, and medium was then changed to fresh serum-free DMEM/F-12 medium, to remove excess compound. Cells were then visualized using Leica Confocal Microscope with the help of Gavin McManus. The samples were analysed at 40 x magnification, at an excitation in ultraviolet spectrum and emission between 400 nm and 500 nm. The fluorescence on internalized NF2792 was compared to untreated control.

To visualize the outlines of the cells and show internalization of NF2792, 0.1 μ M fluorescein was added to the medium immediately prior to visualizing. Cells were then visualized using Leica Confocal Microscope at 40 x magnification with the help of Gavin McManus. The samples were analysed at an excitation in ultraviolet spectrum and emission between 400 nm and 500 nm for NF2792 and an excitation at 495 nm and emission between 500 nm and 550 nm for fluorescein. The fluorescence on internalized NF2792 was compared to untreated control.

2.2.14. Multiphoton Microscopy

SCC-9 cells were seeded on a 6-channel ibidi VI 0.4 μ -slide at a density of 5×10^4 cells/channel. After attachment, cells were serum starved for 16 h prior to treatment. Cells were then treated with 20 μ M of NF2792 in serum-free medium for 2 h. Cells were then washed twice with PBS and fresh serum-free DMEM/F-12 medium was then added, to remove excess compound. Cells were then visualized using a custom upright (Olympus BX61WI) laser multiphoton microscopy system equipped with titanium:sapphire laser (Chameleon Ultra, Coherent®, USA) and water-immersion 25 x objective (Olympus, 1.05NA) by Dr. Gavin

McManus, with an excitation of 730 nm and emission between 397 nm and 550 nm. The fluorescence of internalized NF2792 was compared to untreated control.

2.2.15. Endocytosis studies in SCC-9 cells

To study the endocytic processes in SCC-9 cells under various conditions, fluorescent endocytosis tracers were used. The cells were seeded on a 6-well plate at a density of 3×10^5 cells/well. They were then serum starved for 16 h prior to the treatment. Next, the cells were treated with the endocytosis tracers as described below. After the incubation period, cells were washed twice in PBS and twice in MilliQ H₂O. As buffers used for cell lysis could interfere with the fluorescence of the tracer molecules, 100 μ l of MilliQ H₂O was added to each well, the cells were scraped, the solution was placed in a 1.5 ml tube, and frozen at -80 °C. Cell solutions were then thawed and passed through a 25-gauge needle 10 times and centrifuged for 15 min at 16,000 rpm. The supernatants were then moved to a 96-well plate and an absorbance at 280 nm and fluorescence at the wavelengths described below was read. The fluorescence values were subsequently corrected for the absorbance at 280 nm (A_{280}) values, measured using a SPECTRAMax PLUS 384 Microplate Spectrophotometer (Molecular Devices), as it is the optical absorption for proteins.

2.2.15.1. 70 kDa Dextran uptake studies in SCC-9 cells

70 kDa Dextran is a hydrophilic polysaccharide, that can be labelled with various fluorescent dyes, allowing for its visualisation or fluorescence reading in live cells. It is often used in neuronal tracing, to monitor cell division, hydrodynamic properties of the cytoplasmic matrix, or studies of endocytosis. It is reported that the 70 kDa Dextran enters the cells via clathrin- and dynamin-dependent process, largely through mannose receptor, in addition to macropinocytosis (Li *et al*, 2015). In the study of SCC-9 cells, 70 kDa Dextran labelled with Texas Red fluorescent dye was used. 70 kDa Dextran-Texas Red was purchased from Invitrogen, reconstituted in MilliQ H₂O and 1 mM stock was stored at 4 °C. First, a saturation curve was performed to determine the concentration of 70 kDa Dextran-Texas Red to be used in the experiments. The cells were seeded and serum starved as described above. Further dilutions of the 70kDa Dextran-Texas Red stock solution were performed in serum-free medium. Then, the cells were

treated with 70 kDa Dextran-Texas Red concentrations ranging from 5 nM to 2 μ M for 1 h. A vehicle control with the appropriate volume of MilliQ H₂O added to the medium was prepared for each assay. The cells were then collected and processed as described above, and the fluorescence levels were measured using SPECTRAmax Gemini Microplate Spectrofluorometer (Molecular Devices) at an excitation at 590 nm and emission at 615 nm. The fluorescence values were then corrected for A₂₈₀ values measured using SPECTRAmax PLUS 384 Microplate Spectrophotometer (Molecular Devices). The absorbance and fluorescence values for the solvent were subtracted from the samples as a blank. Then, a saturation curve of three separate experiments was plotted in GraphPad Prism 6 software. The concentration of 70 kDa Dextran-Texas Red used in further experiments was 1 μ M.

Subsequent Dextran uptake studies in SCC-9 cells under different conditions were performed as described above. Cells were treated with 1 μ M 70 kDa Dextran-Texas Red and incubated for 1 h either at 37 °C or 4 °C to distinguish uptake from non-specific binding to the plasma membrane. Three independent experiments were performed for each condition.

2.2.15.2. Lucifer Yellow uptake studies in SCC-9 cells

Lucifer Yellow is a fluorescent dye, allowing for its visualisation in live cells. It is often used in neurobiology (Arends and Jacquin, 1993) and to investigate macropinocytosis (Swanson *et al*, 1985). For the uptake studies of SCC-9 cells, Lucifer Yellow was purchased from Biotium, reconstituted in MilliQ H₂O and 1 mM stock was stored at 4 °C. First, a saturation curve was performed to determine the concentration of Lucifer Yellow to be used in the experiments. The cells were seeded and serum starved as described above. Further dilutions of Lucifer Yellow stock solution were performed in serum-free medium. Then, the cells were treated with Lucifer Yellow concentrations ranging from 1 μ M to 30 μ M for 1 h. A vehicle control with the appropriate volume of MilliQ H₂O added to the medium, was prepared for each assay. The cells were then collected and processed as described above, and the fluorescence levels were measured using a SPECTRAmax Gemini Microplate Spectrofluorometer (Molecular Devices) at an excitation of 425 nm and emission of 540 nm. The fluorescence values were then corrected for A₂₈₀ values measured using SPECTRAmax PLUS 384 Microplate Spectrophotometer (Molecular Devices). The absorbance and fluorescence values for the solvent were sub-

tracted from the samples as a blank. Next, a saturation curve of three separate experiments was plotted in GraphPad Prism 6 software. The concentration of Lucifer Yellow used in further experiments was 15 μ M.

Subsequent uptake studies of Lucifer Yellow by SCC-9 cells under different conditions were performed as described above. Cells were treated with 15 μ M Lucifer Yellow and incubated for 1 h either at 37 °C or 4 °C to distinguish uptake from non-specific binding to the plasma membrane. Three independent experiments were performed for each condition.

2.2.15.3. NF2792 uptake studies in SCC-9 cells

NF2792 exhibits fluorescence properties upon aggregation within the cells. NF2792 was re-constituted in MilliQ H₂O at a concentration of 60 μ M. The absorbance spectrum of NF2792 was measured using a SPECTRAmax PLUS 384 Microplate Spectrophotometer (Molecular Devices) and the fluorescence spectra was measured using a SPECTRAmax Gemini Microplate Spectrofluorometer (Molecular Devices). The values were then blanked for the absorbance and emission of the solvent. The spectra were plotted on GraphPad Prism 6 software. The excitation maximum was at 325 nm and the emission at 445 nm.

For the uptake studies in SCC-9 cells under different conditions, the cells were seeded and serum starved as described above. Further dilutions of NF2792 stock solution were performed in serum-free medium. Then, the cells were treated with 5 μ M NF2792 for 1 h either at 37 °C or 4 °C to distinguish uptake from non-specific binding to the plasma membrane. A vehicle control, with the appropriate volume of MilliQ H₂O added to the medium, was prepared for each assay. The cells were then collected and processed as described above, and the fluorescence levels measured using a SPECTRAmax Gemini Microplate Spectrofluorometer (Molecular Devices) with an excitation at 325 nm and emission at 445 nm. The fluorescence values were then corrected for A₂₈₀ values measured using a SPECTRAmax PLUS 384 Microplate Spectrophotometer (Molecular Devices). The absorbance and fluorescence values for the solvent were subtracted from the samples as a blank. Three independent experiments were performed for each condition.

2.2.15.4. Endocytosis inhibition studies in SCC-9 cells

For initial endocytosis inhibition studies, amiloride, an inhibitor of sodium-proton exchange and macropinocytosis, and dynasore, an inhibitor of dynamin GTPase activity, were used. Optimal concentrations were determined through cytotoxicity assays described above. Cells were seeded and serum starved as described above. Next, cells were either left untreated, or treated with either 1 mM amiloride, 100 μ M dynasore, or vehicle (0.1 % DMSO) for 30 min. Then, either 5 μ M NF2792, 1 μ M 70 kDa Dextran-Texas Red, or 15 μ M Lucifer Yellow were added to the cells for 1 h in conjunction with the inhibitors. Amiloride could not be studied together with NF2792, because their fluorescence spectra overlapped. Cells were incubated either at 37 °C or 4 °C to distinguish uptake from non-specific binding to the plasma membrane. The cells were then collected and processed, and the fluorescence and A_{280} levels measured as described above. Absorbance and fluorescence values for the solvent were subtracted from the samples as a blank. Three independent experiments were performed for each condition.

Since NF2792 is a hyaluronic acid derivative, and CD44 is a hyaluronic acid receptor, the effect of blocking CD44 on the uptake of NF2792 was investigated. An unconjugated IM7 rat monoclonal anti-CD44 antibody and an unconjugated rat IgG2b kappa monoclonal isotype control antibody were purchased from Invitrogen. First, a saturation curve for the blocking antibody was performed. Cells were seeded as described before, and then treated with either an appropriate volume of MilliQ H₂O as vehicle or a range of concentrations (0.5 μ g/ml – 7.5 μ g/ml) of the anti-CD44 and isotype control antibodies for 30 min and co-treated with 5 μ M NF2792 for a further 1 h at 37 °C. The cells were then collected and processed, and the fluorescence and A_{280} levels were measured as described above. The absorbance and fluorescence values for the solvent were subtracted from the samples as a blank. Three independent experiments were performed for each condition. Inhibition of uptake was observed at 2.5 μ g/ml.

Finally, cells were seeded as described before, and then were either left untreated or treated with either an appropriate volume of MilliQ H₂O as vehicle or 2.5 μ g/ml of the anti-CD44 and isotype control antibodies for 30 min and subsequently co-treated with 5 μ M NF2792 for a further 1 h at 37 °C or 4 °C to distinguish uptake from non-specific binding to the plasma membrane. The cells were then collected and processed, and the fluorescence and A_{280} levels

were measured as described above. The absorbance and fluorescence values for the solvent were subtracted from the samples as a blank. Three independent experiments were performed for each condition.

2.2.16. Flow cytometry

2.2.16.1. Annexin V/Propidium Iodide staining

Annexin V/Propidium Iodide (PI) double staining was used to analyse apoptotic levels in SCC-9, U2OS and KYSE-520 cells after serum starvation and SCC-9 cells after NF2792 treatment. This double staining method allows for a theoretical distinguishing of early apoptotic from late apoptotic cells. Apoptotic cells express phosphatidylserine on the outer surface of the plasma membrane. Annexin V is a phospholipid-binding protein that has a high affinity to phosphatidylserine and so stains the apoptotic cells. PI is a fluorescent intercalating agent that stains nucleic acids. PI does not permeate intact plasma membranes, but since late apoptotic cells lose the integrity of their plasma membrane, PI is able to permeate them. Thus, early apoptotic cells stain positive for Annexin V and negative for PI, while late apoptotic cells stain positive for both, Annexin V and PI. In this study, Annexin V-FITC (IQ Products) and PI (Invitrogen) were used. Cells were seeded on 6-well plates, at a density of 1.5×10^3 cells/well. In the serum starvation apoptosis studies, cells were either serum starved for 16 h, cultured in their standard culture medium, or treated with 100 μ M cisplatin in serum-free medium, or an appropriate volume of MilliQ H₂O as a vehicle control. In the NF2792 apoptosis studies, cells were serum starved for 16 h and treated for 24 h either with 5 μ M NF2792, 100 μ M cisplatin (Sigma), or an appropriate volume of MilliQ H₂O as a vehicle control. In the BAMLET uptake inhibition studies, cells were serum starved for 16 h and then were either left untreated or treated with 1 mM amiloride or 100 μ M dynasore for 30 min. Next, cells were treated either with 0.3 mg/ml (20 μ M) BAMLET or an appropriate volume of MilliQ H₂O for 6 h. After the treatment, cells were harvested as described above, pellets were washed with PBS and stained with Annexin V-FITC (1:25 dilution in Annexin V binding buffer; Invitrogen) on ice for 20 min. Then, cells were centrifuged for 5 min at 250 x g, supernatant removed, and 1:400 PI dilution in Annexin V binding buffer was added to the cells. Cells were then analysed by flow cytometry using BD Accuri™ C6 (BD Biosciences) software, reading 10,000 events per sam-

ple. Further analysis of the data was performed using FlowJo 10.6 software. Cell populations were gated accordingly, and percentages of early apoptotic and late apoptotic cell populations from three independent experiments were plotted in GraphPad Prism 6 software.

2.2.16.2. Cell cycle analysis

Cell cycle analysis was performed using PI/RNase staining buffer staining which allows for analysis by flow cytometry. The method is based on the fact that distinct phases of cell cycle differ in the DNA content within the cell. As PI can also stain RNA, the presence of ribonucleases is necessary to ensure staining is specific to DNA. SCC-9, U2OS, and KYSE-520 cells were seeded on 6-well plates at a density of 1.5×10^5 cells/well. Cells were then either serum starved for 16 h or cultured in their standard culture medium. Cells were then harvested as described previously. Pellets were washed in PBS and then suspended in 300 μ l of PBS. Cells were then fixed by slow, dropwise addition of 700 μ l of cold absolute ethanol while vortexing. Cells were then stored at -20 °C for 24 h. Next, they were centrifuged for 5 min at 500 x g and washed in PBS to remove the ethanol. The cells were then suspended in 500 μ l of PI/RNase binding buffer (Invitrogen), and analysed by flow cytometry using BD Accuri™ C6 (BD Biosciences) software reading 10,000 events per sample. Further analysis of the data was performed on FlowJo 10.6 software. Cell populations were gated accordingly, and percentages of cells in G0/G1, S and G2 phases of three independent experiments were plotted in GraphPad Prism 6 software.

2.2.16.3. CD44 staining

CD44 is a receptor generally overexpressed on the surface of cancer stem cells, also in head and neck squamous cell carcinoma (Prince *et al.*, 2007). It has also been reported to be the hyaluronic acid receptor (Aruffo *et al.*, 1990). The level of expression of CD44 in SCC-9 cells was studied by flow cytometry. A FITC conjugated IM7 rat monoclonal anti-CD44 antibody and a FITC conjugated rat IgG2b kappa monoclonal isotype control antibody were purchased from Invitrogen. The cells were grown in standard culture medium and harvested and counted as described above. 1.5×10^3 cells were washed in PBS and then stained with either the anti-CD44 antibody or isotype control antibody (1:400 dilution in 1 % FBS in PBS) on ice for 20 min. Cells were then centrifuged for 5 min at 250 x g and resuspended in 100 μ l of 1:400 PI

diluted in 1 % FBS in PBS. Cells were then analysed by flow cytometry using BD Accuri™ C6 (BD Biosciences) software reading 10,000 events per sample. Further analysis of the data was performed using FlowJo 10.6 software. Cell populations from three independent experiments were gated accordingly, and CD44 positive populations were plotted versus isotype control.

2.2.17. Organic Synthesis

The organic synthesis of hyaluronic acid derivative with an ester bond was performed during a 5-week secondment to the University of Siena, Italy. The designed molecule was a derivative of polymers grafted by Cappelli group at the aforementioned university (Cappelli *et al.*, 2018). The chemical synthesis included inserting an ester bond between OA and glycol linker. Subsequent steps of click chemistry reactions between the OA-glycol and ferulic acid and hyaluronic acid were based on Cappelli's method. All the electrospray ionisation mass spectrometry (ESI-MS) analyses to confirm the mass and NMR studies to confirm the structure of the molecules were performed by Nicola Relitti at the University of Siena. A detailed description of the steps of each chemical reaction involved in the process of synthesis of the ester bond derivative can be found in Chapter 3.2.

2.2.18. Statistical analyses

GraphPad Prism 6 software was used for statistical analyses of experimental data. Results show means $\pm$ standard deviation (SD) of three independent experiments. For comparisons of two values, Student t-test was used. For comparison of two or more groups, a One-way ANOVA followed by Bonferroni multiple comparison test was performed. For comparison of two or more groups with two independent variables, a Two-way ANOVA test followed by Bonferroni multiple comparison test was performed. Significant values were marked as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and n.s. was used to mark not significant values.

3. Generation and validation of a synthetic HAMLET derivative for tumour imaging

3.1. Introduction

This project is funded through the Marie Skłodowska Curie ITN TRACT programme that is focused on facilitating the discovery of novel biomarkers and treatment strategies for oral and oesophageal cancers. As mentioned in Chapter 1, because of the limited understanding of the background and epidemiology of these cancers, conventional diagnostic methods are not enough to ensure detection at an early stage (Domper Arnal *et al.*, 2015; Malik *et al.*, 2016). Therefore, still too often, the disease is diagnosed at a late stage, when the tumours are large and require an extensive surgery or are not resectable (Nam *et al.*, 2020; Lagergren *et al.*, 2017). The difficulty in treating these cancers arises from the aggressiveness of both, the disease itself and the treatment strategy, as well as the unfortunate localisation of the tumours in the oral cavity or oesophagus. Patients undergoing treatment are inclined to suffer great pain and difficulty eating, swallowing, breathing, speaking, all of which severely decrease their quality of life during treatment and post-surgery.

The impact that oral and oesophageal cancers have on the patient's life could be greatly reduced by narrowing the window of diagnosis. An effective screening test or successful diagnostic technique that is capable of detecting early and very early stages of oral and oesophageal cancers has yet to be developed. Both these cancer types lack prominent and specific biomarkers that enable effective screening tests to be implemented for clinical use. Commonly, oral and oesophageal cancer patients are diagnosed after exhibiting various levels of impediment to swallowing or breathing (Economopoulou *et al.*, 2019). As these cancers are highly aggressive and progress quickly, obtaining an early diagnosis is crucial for the patient's survival (Domper Arnal *et al.*, 2015; Ettinger *et al.*, 2019). These limitations call for the development of a novel and highly effective screening and detection technique that can identify transformed cells at an early stage of the disease and that can facilitate early diagnosis and treatment.

Surgery remains the main therapeutic strategy for oral and oesophageal cancers (Ettinger *et al.*, 2019; Yang *et al.*, 2020). As the patients are typically diagnosed at quite a late stage of the disease, the amount of tissue that needs to be removed tends to be large and healthy tissue margins are difficult to estimate for the surgeons and pathologists (Fakurnejad *et al.*, 2020). Moreover, it is important to ensure complete removal of the tumour tissue with a sufficient

margin, to avoid relapse. Currently employed intraoperative margin assessment by frozen sample analysis faces difficulties with pathologist-surgeon information relay, as well as high risk of false interpretation (Fakurnejad *et al.*, 2020).

With the development of cancer detection techniques, labelled cancer imaging molecules for *in vivo* imaging have emerged (Liu *et al.*, 2018; Fakurnejad *et al.*, 2020). Such agents are typically non-toxic molecules or antibodies with fluorescent or radioactive properties that aid in distinguishing the localisation of the tumour within the body or tumour mass in a tissue. Their principal application is the ability to differentiate between healthy and cancer tissue during surgery, where the imaging agent is taken up by the malignant cells but not by the normal cells, thus allowing for an accumulation of the labelled molecule within the tumour. Since the uptake of such molecules by the healthy cells could still happen on a low level, it is important that the imaging agents are not only non-toxic to healthy cells but eventually expelled from the cells. An example of such a molecule is ¹⁸F-fluorodeoxyglucose PET, which has been successfully used in surgery allowing for precise resection of all tumour tissue with minimal healthy tissue margins (Economopoulou *et al.*, 2019).

The limitations of these imaging agents are the means and time of administration – usually the molecules are administered intravenously, and an amount of time is given to ensure sufficient accumulation. In the case of oral and oesophageal cancer, an ideal agent would be administered orally, for instance as a mouthwash or a drink, enter the tumour cells within a short span of time and ensure enough fluorescence to be analysed with a probe or an endoscope. Such a technique could not only allow detection of the malignant tissue for diagnostic purposes but also greatly assist in the successful resection of all the tumour mass with minimal removal of healthy marginal tissue. Therefore, a cancer diagnostic dye would assure minimal impact on the patient's appearance and quality of life.

To test natural compounds with the potential to function as imaging agents, Cappelli *et al.* (2018) have designed a hyaluronan based graft polymer that exhibits inducible fluorescence properties following its physical aggregation when accumulated in sufficient quantities. The molecule itself consists of ferulic acid (FA), which is a derivative of hydroxycinnamic acid, glycosaminoglycan hyaluronic acid (HA), and OA connected to the backbone of the molecule by a biocompatible ethylene glycol linker.

FA is universally present in plants, where it plays an important role in maintaining the structure of the cell wall *via* cross-linking of polysaccharides (Mélida *et al.*, 2010). Through this activity, FA residues can influence various plant cell wall characteristics, such as its coherence and stress response, but can also affect its synthesis (Mélida *et al.*, 2010). Significantly, FA possesses an intriguing ability to fluoresce. It has been reported to be the main fluorophore present in plants exhibiting blue-green fluorescence upon excitation with ultraviolet light (Meyer *et al.*, 2003). Modern science is searching for novel biomarkers and imaging agents with favourably large Stokes shifts, to increase detection success, overcoming the noise and self-quenching possibility during imaging (Ren *et al.*, 2018). FA showed a desirable Stokes shift of circa 100 nm (Inokuchi *et al.*, 2016), which could be further modulated by polarity and pH of the solvent (Meyer *et al.*, 2003). The excitation wavelength of FA in the UV region could be overcome by multiphoton microscopy, which was specifically designed for life imaging in tissues and live animals with minimal adverse effects of high energy wavelengths (Zipfel *et al.*, 2003; Larson 2011). The intricate design involves producing signals of lower energy photons in the visible spectrum, where two or more of those photons reach the fluorophore at the same time and the sum of their energy is sufficient to excite it (Larson, 2011). Such technology allows for successful *in vivo* deep tissue imaging and could effectively bypass the limitations of imaging agents excitable by low wavelengths, exciting them with non-harmful near infrared visible light instead.

HA is widely present in human body, mainly in the extracellular matrix (Chen, C. *et al.*, 2018), epithelial, connective, and neural tissues (Huang and Huang, 2018). It plays essential roles in cell adhesion, proliferation, and migration (Cohen *et al.*, 2004). Moreover, HA is taken up by the cell *via* receptor-mediated endocytosis through CD44 – a HA receptor (Aruffo *et al.*, 1990; Hua *et al.*, 1993; Yu *et al.*, 2016). Interestingly, many types of cancer cells exhibit an overexpression of CD44 on their plasma membranes, with oral and oesophageal cancers among them (Gotoda *et al.*, 2000; Christopoulos *et al.*, 2006; Chen *et al.*, 2014; Yu *et al.*, 2016; Qin *et al.*, 2019); it has also been reported to be linked to cancer progression (Wang *et al.*, 2009; Chen, C. *et al.*, 2018), metastasis (Ozer *et al.*, 1997; Sebanjo and Chellaiah, 2017), and to be a stem cell marker in various cancer cell types (Cho *et al.*, 2015). Due to CD44 overexpression in cancer cells, it has been proposed to be a good therapeutic target for new anticancer agents; however, as HA is important for proper cell function, targeting CD44 dis-

rupts skin healing and proliferation (Yu and Stamenkovic, 1999). There are also reports suggesting that HA plays a role in cancer progression through the activation of cytoskeleton and matrix metalloproteinase signalling (Bourguignon *et al.*, 2014). The overexpression of CD44 suggests there could be a preferential uptake of HA by cancer cells relative to normal cells, and as such, agents containing a HA backbone could function as successful drug carriers. Their potential for antitumour drug delivery has been extensively studied with some molecules entering clinical trials (albeit unsuccessful) and there is hope for development of novel HA based transport technology to be industrialised (Huang and Huang 2018).

OA is a member of a large group of long chain fatty acids that are universally found in plants and animals. It is an ω -9 monounsaturated fatty acid that is commonly found in cell membrane lipids (Casado-Díaz *et al.*, 2019). It is widely present in food, most prominently in vegetable oils, such as olive oil (Owen *et al.*, 2000), and is also found in human adipose tissue (Kokatnur *et al.*, 1979). Its omnipresence could be linked to a plethora of functions OA can modulate in the organism, such as cell signalling (Hostetler *et al.*, 2005), inflammation (Calder, 2012), and the immune response (Miles and Calder, 1998; Sales-Campos *et al.*, 2013), and the function of cell membranes (Sales-Campos *et al.*, 2013). Like other fatty acids, OA can also function as an energy source (Gimeno *et al.*, 2003). However, fatty acids in their free form exhibit toxicity and ingestion of free fatty acids has been linked to many adverse effects, such as glucose intolerance and diabetes (Plötz *et al.*, 2017).

In their studies, Cappelli and colleagues (2018) grafted several HA macromolecule backbones with various densities of FA residues coupled with ethylene glycol covalently bound to OA. Thanks to the presence of the fatty acid, the polymer molecules were amphiphilic and exhibited the ability to self-assemble and form aggregates in an aqueous environment. An important feature of a successful imaging agent would be aggregation induced emission, wherein the molecule would emit significantly better detectable fluorescence upon forming aggregates relative to when it is uniformly distributed in solution (Luo *et al.*, 2001; Qian and Tang, 2017). Cappelli (2018) reports that the polymers with FA fluorophore do in fact exhibit the aggregation induced emission activity, proving to be an interesting candidate for further studies. Cappelli suggests that due to the presence of OA, the polymers had the capabilities to interact with lipids in the cellular membranes. Internalization of the molecules was confirmed on NIH3T3 cells by fluorescent microscopy and PANC-1 pancreatic cancer cell lines by confocal micros-

copy, and the molecules showed no toxicity in NIH3T3 fibroblast and HC chondrocyte cell lines (Cappelli *et al.*, 2018).

We speculate that the internalization of the molecule could be facilitated through interaction with the CD44 receptor by means of its affinity to the polymer's HA backbone, as opposed to the interaction of OA with the membrane lipid. The lack of toxicity exhibited by these molecules could be linked to the stable and rigid amide bond between OA and the rest of the molecule, which does not release the cytotoxic potential of OA. It is also speculated that the exposure of the methyl group of the OA could account for the lack of toxicity of the molecule despite the presence of a potentially toxic fatty acid moiety. As the carboxylic group is the chemically and biologically active group of OA, capable of binding and complexing with proteins (Xie *et al.*, 2012), the polymer derivatives, which would expose the methyl group, are not expected to show similar properties to OA, with the exception of lipophilicity. However, the stable amide bond between OA and the glycol linker could also impede the exocytosis or recycling of the large, rigid molecules. Therefore, this project involved creating a new hyaluronan-based derivative wherein the amide bond is replaced by an ester bond. Such a structural change would grant the new molecule some degree of flexibility as the ester bond rotation and conformation could be influenced by the solvent. Hopefully, the altered charge distribution among the moieties within the molecule could also enhance its fluorescence and increase its Stokes shift, raising its imaging capacity. The ester bond has a potential to be split by esterase enzymes within the cell. Conceivably, this would release the component parts of the molecule which could then be metabolised or recycled by the cell or, alternatively, easily expelled. This property of the molecule would increase its potential as an imaging agent, since its long-term accumulation within the cell is not desired and it is envisioned that hydrolysis could affect efficient release of the molecule after the medical procedure. Possibly, esterase enzymes could release OA from the fluorescent molecule by reinstating the carboxylic group, which in turn could prove toxic for the cancer cells. Therefore, aside from the chemical synthesis of the new molecule, the investigation of its structure and fluorescent spectrum, the project also comprised characterisation of cytotoxicity as well as its uptake and release patterns in SCC-9 squamous cell carcinoma of the tongue cell line.

3.2. Results

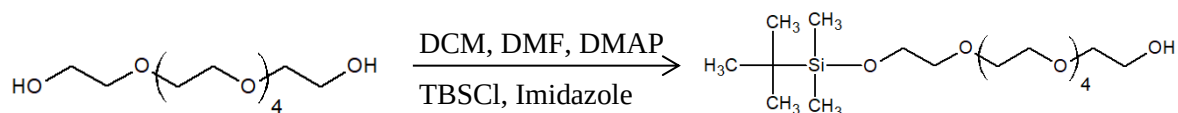
3.2.1. Generating an hyaluronan-based polymer derivative with oleic acid bound *via* an ester bond

The Cappelli group have designed a synthetic route (2018) to generate derivatives wherein OA was bound to the glycol linker and HA-FA backbone through an amide bond. This project aimed for replacing the amide bond with an ester bond to grant the new derivative more structural flexibility with the intention of increasing its fluorescence and facilitating the recycling and release of the molecule from the cell. The newly designed molecule could still benefit from a stable covalent bond that facilitated the uptake of the molecule through CD44 receptor or lipid interactions with the membrane. The synthetic route has been redesigned to first include the ester bond between OA and the glycol linker. Subsequently, a ferulic acid moiety and ferulic acid-hyaluronic acid backbone was attached to the OA-glycol linker residue by means of click chemistry. The intermediate molecules were characterised by ESI-MS and NMR by Nicola Relitti at the University of Siena to confirm their structures. Since the fluorescent properties of the new derivative were vital to its proposed use in cancer uptake and release studies, the excitation and emission spectra were studied, and maxima were confirmed before performing *in vitro* experiments.

3.2.1.1. Synthesis of the derivative with an ester bond and its intermediates

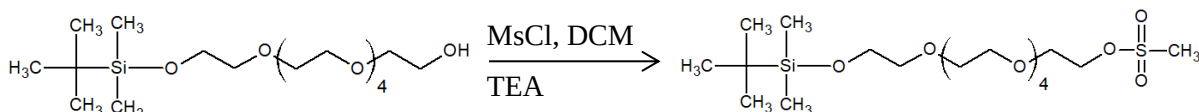
Generation of the final molecule, NF2792 (figure 3.1), was achieved through a series of chemical reactions (figure 3.2), including steps involving click chemistry methods. Along with the final product, the individual chemical components and two intermediate products from the synthetic route (molecules NF2790 and NF2791) were purified and prepared for *in vitro* tests to allow a full understanding of the properties of NF2792 (all tested molecules are listed in table 3.1).

3.2.1.1.1. Tert-butyldimethylsilyl ether – protection of the alcohol



The initial step involved the protection of the μ -alcohol residue on the hexaethylene glycol molecule by reacting with tert-butyldimethylsilyl chloride (TBSCl). 1 equivalent of hexaethylene glycol was added to a 25 ml flask which was placed on ice and the solvents were added at 0 °C. 0.05 equivalents of 4-Dimethylaminopyridine (DMAP), 3 of imidazole and 0.9 of TBSCl were weighed and added to the flask in this order. Ice was removed after 20 min, and the reaction was run for 24 h under inert atmosphere. Thin layer chromatography (TLC) was performed on a silica plate to ensure the presence of the product. The product was then washed in brine. Dry sodium sulphate was added to remove water impurities and the mixture was then filtered. The product was washed three times with chloroform to remove dimethylformamide (DMF) and purified on a silica column with the eluents being: ethyl acetate and then ethyl acetate:methanol 50:1. Collected fractions were visualised by TLC on silica plates. The mass of the product was confirmed by ESI-MS. Further checks by NMR were performed to confirm the product was the desired compound. Product was then evaporated and prepared for further reactions.

3.2.1.1.2. Mesylation



The α -alcohol group on the glycol needed to react with methanesulfonyl chloride (MsCl) in order to make it accessible for further reactions. 1 equivalent of product from reaction 2.18.1. was prepared in a flask on ice. 10 ml dichloromethane (DCM) and 2 equivalents of triethylamine (TEA) and 2 of MsCl were added at 0 °C. The ice was removed after 10 min, and the reaction was run for 2 h under inert atmosphere. After 2 h, more solvent was added to the flask, and the solution was moved to an extraction flask and washed in brine. Dry sodium sulphate was added to remove water impurities and the solution was filtered on a cotton filter. Product was visualised by TLC and ESI-MS, then evaporated and prepared for the following reaction.

3.2.1.1.3. Demesylation



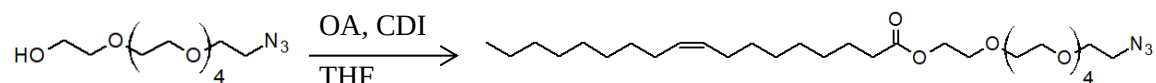
The mesylated glycol was reacted with NaN_3 under the presence of acetonitrile (CH_3CN) to introduce the azide group in place of the mesylated residue. 1 equivalent of product from reaction 2.18.2. was placed in a flask in an oil bath with the condenser on. 0.5 ml of DMF and 10 ml of acetonitrile were added. 2.8 equivalents of NaN_3 were weighed and added to the reaction mix. The reaction was run overnight (circa 18 h) under inert atmosphere at 85 °C. TLC on silica plates was performed to ensure the presence of the product. Product was purified on a silica column with the eluents being: petroleum ether:ethyl acetate 1:1, and petroleum ether:ethyl acetate 1:2. Collected fractions were visualised by TLC, and mass of the product was confirmed by ESI-MS and NMR. Product was washed in chloroform to remove traces of DMF and evaporated and prepared to use in the following reactions.

3.2.1.1.4. Removing tert-butyldimethylsilyl group



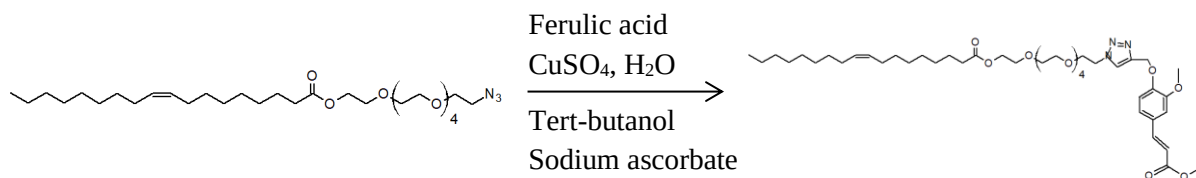
After introducing the α -azide group, the μ -alcohol group can be freed from the protective tert-butyldimethylsilyl group. A flask with 1 equivalent of product from reaction 2.18.3. was placed on ice. 10 ml of tetrahydrofuran (THF) was added and then 2 equivalents of tetra-n-butylammonium fluoride (TBAF) were added drop wise. The ice was removed after 15 min and the reaction was run under inert atmosphere for 2 h. TLC was performed to ensure no more starting material was present in the flask. The reaction was then quenched, and the product was washed in brine. Dry sodium sulphate was added to remove traces of water, and the solution was filtered. Next, the product was purified on a silica column with the eluents being ethyl acetate, ethyl acetate:methanol 20:1, ethyl acetate:methanol 10:1. The collected fractions were visualised on TLC silica plates, and product's mass was confirmed by ESI-MS and NMR. The product was evaporated and prepared for further reactions.

3.2.1.1.5. Introduction of an ester bond between the glycol and oleic acid



Achieving glycol with an μ -alcohol and α -azide groups, allows for introducing an ester bond between the glycol and OA. 1 equivalent of OA and 1,1'-Carbonyldiimidazole (CDI) were dissolved in 3 ml of THF. The flask was placed in an oil bath and heated to 90 °C and run under inert atmosphere overnight. Then, the solvent was evaporated, and the reaction mixture was checked by NMR that confirmed 50 % efficacy in activating the OA. After that, the activated OA, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) and product of reaction 2.8.4. were placed in a flask and the reaction was run under inert atmosphere overnight. The product was confirmed by TLC, and purified on a silica column with eluents being petroleum ether:ethyl acetate 5:1, petroleum ether:ethyl acetate 4:1, petroleum ether:ethyl acetate 3:1, petroleum ether:ethyl acetate 2:1, petroleum ether:ethyl acetate 1:1, petroleum ether:ethyl acetate 1:2. Collected fractions were confirmed by TLC, ESI-MS, and NMR. The compound was labelled NF2790 and prepared for further reactions and for shipping to Dublin.

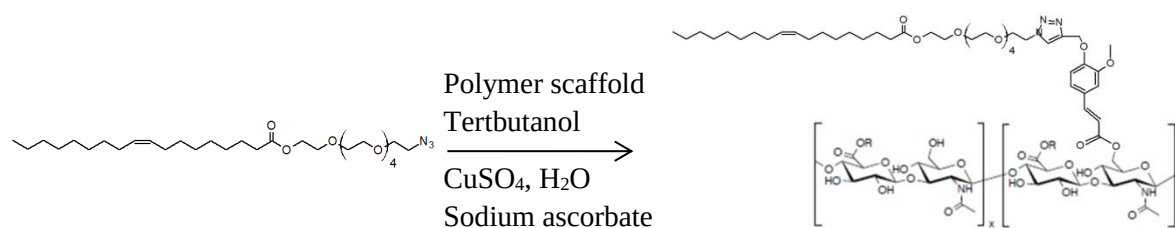
3.2.1.1.6. Click chemistry with ferulic acid residue



A click chemistry approach was adopted to couple NF2790 and ferulic acid (FA) moiety. Click chemistry is a term covering a wide range of high yielding and relatively selective reactions between compatible molecules, that can be performed in easily removable solvents and whose by-products can be removed without resorting to chromatographic methods (Kolb *et al.*, 2001). The Copper-catalysed azide–alkyne cycloaddition (CuAAC) is one of the most effective click chemistry reactions designed to date (Hein and Fokin, 2010). The use of the copper catalyst greatly increases the reaction rate and allows it to be performed at room temperature in comparison to the classical click chemistry reactions performed at high temperatures (Hein and Fokin, 2010).

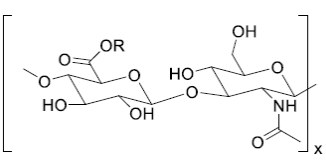
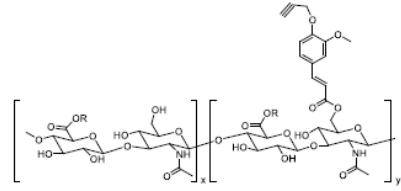
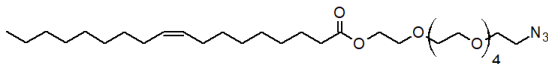
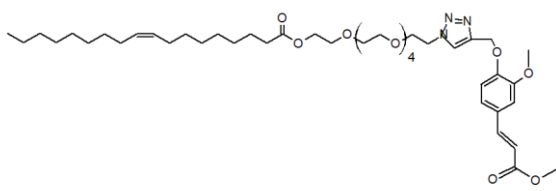
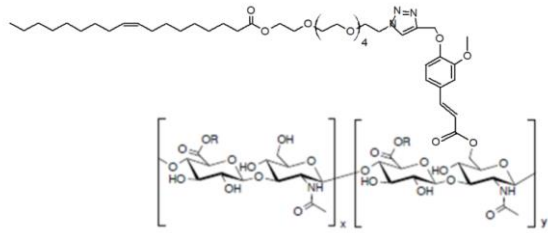
To achieve a CuAAC coupling between NF2790 with a FA in mild conditions, the catalyst must be prepared beforehand. A 1.4 ml 50:50 solution of H₂O and tert-butanol was prepared. Then, 250 µl of CuSO₄ x 5 H₂O and 250 µl of sodium ascorbate were added. The solution was left to react under inert atmosphere in room temperature for 1 h. Then, the click chemistry reaction was prepared by preparing a flask with NF2790 and FA diluted in 1.3 ml of tertbutanol and 0.7 ml of H₂O. 0.6 ml of the catalyst was added, and the reaction was run for 24 h under inert atmosphere. The presence of the product was confirmed by TLC and ESI-MS. The reaction was quenched by adding ammonium hydroxide and the solution was extracted in DCM three times. H₂O was removed by addition of sodium sulphate. Filtered solution was purified on a silica column with the eluents being ethyl acetate, ethyl acetate:methanol 50:1, ethyl acetate:methanol 20:1, ethyl acetate:methanol 10:1. The collected fractions were visualized on TLC, ESI-MS, and NMR. The product was labelled NF2791 and prepared for further reactions and for shipping to Dublin.

3.2.1.1.7. Click chemistry with polymer scaffold



The CuAAC coupling was performed between NF2790 and the hyaluronic acid backbone with a ferulic acid residue (HA-FA-Pg-3F) in mild conditions. The catalyst was prepared as mentioned above. In a separate flask, the polymer scaffold and NF2790 were diluted in 2.5 ml of H₂O. 0.5 ml of the catalyst was, added, and the reaction was run under inert atmosphere overnight. The reaction was quenched using 100 mg of QuadraSil MP (Sigma) to scavenge the copper in the mixture. The solids were then filtered on a filter paper and H₂O was evaporated. The solid product – light brown glassy film was then washed in acetone for 2 h to remove any residual starting material. It was then dissolved in H₂O to allow the polymer to swell and spread. The suspension was then washed in acetone to remove the impurities. The polymer was then retrieved by centrifugation (4,800 rpm, 3 min, 4 times). The product was visualised by the NMR, labelled NF2792, and prepared for shipping to Dublin.

Table 3.1 Compounds synthesised in the University of Siena, Italy and their component parts
 HA8700 is the hyaluronic acid molecule. HA-FA-Pg-3F is the hyaluronic acid polymer coupled with ferulic acid. NF2790 consists of a hexaethylene glycol group bound to OA by an ester bond. NF2791 incorporates a hexaethylene glycol group bound to OA by an ester bond, linked to ferulic acid. These two compounds were intermediate reaction products in the process of synthesis of the polymer molecule. NF2792 is the derivative of hyaluronic acid polymer coupled with ferulic acid, glycol residue and OA bound by an ester bond.

Compound name	Structure	Molecular weight	Solubility
HA8700		8,700	H ₂ O
HA-FA-Pg-3F		10,000	H ₂ O
NF2790		571.80	DMSO
NF2791		818.06	DMSO
NF2792		12,232	H ₂ O

3.2.1.2. Structural characterization of NF2792 and its intermediates

To confirm the effectiveness of the chemical reactions and the structure of the products along the synthetic chemistry route, ^1H and ^{13}C NMR analyses were performed on the final molecule NF2792 and two intermediate products of the synthesis, NF2790 and NF2791. As the intermediate products display poor water solubility, their NMR analysis was performed in CDCl_3 as solvent. Due to good water solubility of NF2792, the solvent for the analyses of the final product of the synthesis was deuterated water (D_2O).

NF2790 was the first molecule to be obtained. After a series of steps protecting the μ -alcohol group and introducing an α -azide group in the hexaethylene glycol, it was reacted with OA. The μ -alcohol group attacks the carboxylic group of the OA to form an ester bond, through a classic esterification reaction. The ^1H NMR analysis (figure 3.3) confirmed the predicted structure of the molecule showing a combination of signal patterns typical of OA and the hexaethylene glycol, thus confirming that the esterification reaction had occurred.

Next, the NF2791 molecule was synthesised by click chemistry – through a CuAAC coupling between NF2790 and FA. To confirm the occurrence of the click chemistry reaction, an ^1H NMR spectrum of NF2791 (figure 3.4) was compared to ^1H NMR spectrum of NF2790 (figure 3.3). The appearance of the peaks in the distant region of the NMR spectrum, typical of aromatic groups, confirmed the presence of FA in the reaction product. Similarly, the ^{13}C NMR spectrum of NF2791 (figure 3.5) showed the presence of carbon atoms from the aromatic rings in the FA moiety, affirming the effectiveness of the CuAAC coupling.

NF2792 was the final molecule to be synthesised. It was also obtained through a click chemistry approach – a CuAAC coupling between NF2790 and a previously published HA-FA backbone, HA-FA-Pg-3F. The ^1H NMR analysis (figure 3.6) showed a combination of expected signals from all the constituent parts of the complex. The near region of the ^1H NMR spectrum was dominated by signal patterns typical of OA and HA, while the middle part of the spectrum displayed a pattern comprised of HA and glycol residue peaks. The distal region of the ^1H NMR spectrum contained signals that are attributable to the aromatic rings in the FA moiety, confirming the predicted structure of the NF2792 molecule. The ^{13}C NMR analysis (figure

3.7) provided further affirmation of the structure of the obtained molecule, demonstrating a sequence of peaks attributable to all the constituent parts of the complex molecule.

3.2.1.3. Optical properties of NF2792

As reported previously, FA and the molecules containing the HA-FA backbone exhibit interesting fluorescence properties (Meyer *et al.*, 2003). Moreover, the fluorescence of FA can be further modified by the pH, polarity of the solvent, and presumably, by the chemical structure of the molecule it is incorporated into (Cappelli *et al.*, 2018). To test the extent by which the ester bond within the NF2792 can influence the fluorescence of the polymer, the excitation and emission spectra of NF2792 were examined.

The absorption and emission spectra of the molecule were studied in MilliQ H₂O due to good water solubility of NF2792. In aqueous solution, the excitation maximum (figure 3.8) was observed at 325 nm with a shoulder at 295 nm suggesting the absorbance spectrum of the polymeric molecule is dominated by the FA moiety. The emission maximum of NF2792 in MilliQ H₂O (figure 3.9) was detected at 445 nm, as expected of the FA moiety in a solvent of high polarity. The observed Stokes shift of 120 nm (figure 3.10) was slightly higher than ferulic acid alone (Inokuchi *et al.*, 2016) and previously published polymeric molecules (Cappelli *et al.*, 2018), which is a very desirable characteristic of a potential imaging agent. Moreover, both the absorbance and emission were increasing with the concentration of the solution (figure 3.8 and figure 3.9), indicating the fluorescence of the molecule is enhanced on accumulation. As would be desirable for a successful imaging agent, the data suggests the molecule would need to accumulate within the cell in order to obtain a better level of image resolution.

3.2.2. *In vitro* studies of the effect of NF2792 on oral cancer cell survival

3.2.2.1. Cytotoxicity of NF2792 and its intermediates

An evaluation of the toxicity of NF2792, the HA-FA polymeric derivative with an ester bond, was determined using an SCC-9 squamous cell carcinoma tongue cell line by means of an Alamar Blue viability assay. SCC-9 cells were exposed to a range of concentrations of

NF2792 and the intermediate products of its chemical synthesis, NF2790 and NF2791, for 24 h. To investigate the influence of various component parts of the final molecule on its toxicity, HA (HA8700), HA-FA backbone (HA-FA-Pg-3F), and OA (TCI Chemicals) were also tested on SCC-9 cells (for chemical structures and properties see table 3.1).

The results of the cytotoxicity studies in SCC-9 cells are presented in figure 3.11. As could be assumed—considering HA's properties influencing cell adhesion and proliferation—HA-8700 did not exhibit toxicity in SCC-9 cells. Moreover, at the concentration of 100 μ M, cell viability surpassed 100 % compared to vehicle treated control possibly due to the beneficial effect of HA in mediating cell attachment. The HA-FA polymer backbone, HA-FA-Pg-3F, was likewise not toxic, and also showed over 100 % viability at the concentration of 100 μ M, indicating the FA moiety does not obstruct the advantageous effect of HA on cell function and proliferation. As expected, OA had a negative impact on the viability of SCC-9 cells, with an IC_{50} value of 19.7 μ M. Interestingly, the intermediate compounds incorporating OA and hexaethylene glycol – NF2790 and OA, hexaethylene glycol, and FA – NF2791 presented significantly higher toxicity in SCC-9 cells. NF2790 showed an IC_{50} value of 4.7 μ M and NF2791 a value of 2 μ M. It is speculated that the azide group in NF2790 and the alcohol group in NF2791 could be responsible for higher reactivity and toxicity of these two compounds. It is also possible that the ester bond is split by the esterases within the cell liberating OA which could prove cytotoxic. In contrast, the final product of the synthesis, NF2792, exhibited no observable toxicity in SCC-9 cells after 24 h incubation. At 100 μ M concentration, the viability of the cells was over 80 % compared to the vehicle treated control. The lack of toxicity suggests that the structural changes in the polymeric molecule after introduction of the ester bond do not impact its functionality as an imaging agent. The flexibility and different charge distribution within the chemical bonds of the constituent parts of the molecule seem not to impede the cellular viability. However, in comparison to HA-8700 and HA-FA-Pg-3F, NF2792 displayed a modest decrease in viability at concentrations above 25 μ M. It is speculated that it could be due to a number of different reasons. The size of the molecule and the rigidity of the polymer backbone may obstruct the metabolism of the HA by the cells, and therefore cells could not benefit from its proliferative and adhesion promoting properties. Another reason could be a low level of hydrolysis of OA from esterase activity. The low levels of freed OA could be explained by either difficulty of the access to the ester bond because of the secondary structure interactions limiting or almost completely obstructing the enzymatic ac-

cess, or poor esterase activity in SCC-9 cells. Nevertheless, the 80 % cell viability at the concentration of 100 μ M was a satisfactory observation and could be successfully exploited in cancer imaging; therefore, NF2792's impact on SCC-9 cells was further analysed.

3.2.2.2. Cell survival and apoptosis induction after NF2792 treatment

Next, the effect of NF2792 on SCC-9 cell viability was investigated. The viability and cell survival of SCC-9 cells after 24 h treatment with 5 μ M NF2792 was compared to the effect of cisplatin and vehicle treatment (figure 3.12.C). Since the SCC-9 cells have been reported to show a resistance to cisplatin treatment (Elias *et al.*, 2015), a concentration of 100 μ M was chosen to observe a growth inhibitory effect. As seen in figure 3.12.C, NF2792 did not reduce cell viability comparing to a vehicle treated control as measured by the Alamar Blue assay, whereas cisplatin treatment caused a significant decrease in viability compared to both, vehicle and NF2792 treated cells ($p < 0.01$, Student t-test). The results obtained suggest the working concentration of NF2792 does not impact cell survival in SCC-9 cells and NF2792 could potentially be a candidate live imaging agent.

Subsequently, the induction of apoptosis in SCC-9 cells by a 24 h treatment with 5 μ M NF2792 was investigated by Annexin V/PI double staining. The test is based on the principle that apoptotic cells invert phosphatidylserine from the inside to the outside leaflet of their plasma membrane. Annexin V has strong affinity to phosphatidylserine; therefore, apoptotic cells will stain Annexin V positive. In late stages of apoptosis, the plasma membrane loses its integrity, and becomes penetrable. This, in turn, allows PI, an intercalating agent, to enter the cell and stain the nucleic acids, thus late apoptotic cells will stain positive for both, Annexin V and PI. The NF2792 effect was contrasted to vehicle and 100 μ M cisplatin for comparison (figure 3.12.A and 3.12.B). As seen in figure 3.12.B, SCC-9 cells treated with cisplatin presented significantly increased levels of Annexin V and PI positive staining ($p < 0.001$, Two-way ANOVA), indicating an induction of programmed cell death. NF2792 treated SSC-9 cells, by contrast, displayed basal levels of Annexin V and PI staining, comparable to the level exhibited by the vehicle treated control, suggesting no induction of apoptosis. The observed lack of cell death induction confirms the non-toxic nature of NF2792 for SCC-9 cells, and its potential for use as a live cell imaging agent.

3.2.2.3. Confocal microscopy studies on NF2792 in SCC-9 cells

The polymers comprising an amide bond between OA and the derivatised hyaluronic acid have previously been shown to be internalized into the cytoplasm of NIH3T3 and PANC-1 cell lines in work carried out by Andrea Cappelli's group (2018). However, the capacity for the molecules to be internalised by SCC-9 cells was unknown. To investigate the uptake of the polymer intermediates, confocal studies on NF2792 were performed.

The confocal data in figure 3.13 shows aggregates of the NF2792 molecule within the cellular boundaries of SCC-9 cells. The cells were incubated with the compound for 2 h at various concentrations, which shows a relatively effective rate of uptake. The polymeric molecule exhibited satisfactory fluorescence properties, despite the fact that the confocal microscope can only emit excitation wavelengths above 400 nm. To prove that the molecule in fact entered the cells, instead of binding to the outer plasma membrane, confocal studies with fluorescein medium staining were performed. 0.01 % fluorescein sodium salt was added to the medium immediately before confocal imaging to visualise the outlines of the cells and the extracellular space. With the medium emitting green fluorescence, the interior of the cells was dark. As seen in figure 3.14, the blue fluorescence of NF2792 coincides with the dark cellular interior. The 3-dimensional images and sections demonstrate accumulation of NF2792 molecules within the cells. The blue fluorescence appears to be localised in various units distributed within the cells, supporting the theory that NF2792, like its amide bond derivatives, is able to form aggregates and this in turn induces its emissive properties. Cappelli *et al.* reported that the hyaluronan polymeric derivatives were localised in the cell cytoplasm; however, fig. 3.13 and 3.14 suggest that the molecules are accumulated in discrete cellular vesicles and prevented from dispersing into the cytoplasm. Arguably, localisation within the cytoplasm would lead to a more uniform distribution of the molecule, thus limiting its fluorescence, whereas accumulation in vesicles could promote aggregation.

3.2.2.4. Multiphoton imaging of NF2792 in SCC-9 cells

The excitation maximum in the ultraviolet spectrum is not ideal for a live cell and tissue imaging agent. Intraoperative and diagnostic use of UV induced fluorescent imaging agents would

not be safe nor achievable. The photodamage introduced to the cell would be too high a price to pay. However, the Stokes shift of 120 nm is a promising characteristic that could be otherwise exploited. There are existing techniques designed to reduce the adverse effects of live imaging and long exposure of tissues to high energy light wavelengths. Multiphoton imaging allows to overcome the limitations of an agent with a UV excitation wavelength – by exposing the sample to multiple lower energy photons. When two or more of those photons reach the fluorophore at the same time and the sum of their energy is enough to transfer it to the excited state, the fluorescence occurs (Larson, 2011). The low energy light does not have harmful side effects to the imaged tissue, and because of the long wavelength of the light, it can penetrate deeper into a thick sample, allowing for imaging in tissue samples and live animals (Zipfel *et al.*, 2003). To test if NF2792 could be excited by longer wavelengths, multiphoton imaging was performed on SCC-9 cells treated with NF2792.

Figure 3.15 demonstrates that NF2792 can indeed be successfully visualised by multiphoton microscopy. In fact, the quantity of visualised molecule was much greater than when visualised with confocal microscope, with the samples treated with identical concentration of NF2792 and incubated for the same time interval. This confirms that the excitation wavelength emitted by the confocal microscope was nearly out of range of NF2792 absorption spectrum and, therefore, only a portion of the actual internalised molecule was visualised due to very weak excitation and emission. However, multiphoton microscopy succeeded at demonstrating the full effectiveness of internalisation of NF2792 into SCC-9 cells. Unfortunately, due to the low resolution of the available microscope, it is not possible to visualise the exact cellular compartments where the NF2792 molecule is incorporated and deposited.

3.2.3. Study of NF2792 uptake patterns in SCC-9 cells

3.2.3.1. Time dependent NF2792 uptake and release in SCC-9 cells

After confirming the lack of toxicity of the NF2792 molecule and its internalisation in SCC-9 cells, its uptake patterns were investigated. As the microscopy studies showed a relatively effective rate of internalisation, a time course uptake study was performed to examine how fast SCC-9 cells can amass detectable amounts of NF2792 within them. The experiments were

performed at 37 °C and 4 °C, to distinguish between active internalisation and energy-independent passive binding or diffusion.

At 37 °C a detectable NF2792 fluorescence was observed in cellular lysates within 30 min of treatment (figure 3.16.A). A steady linear growth of fluorescence was observed in the successive time points until 24 h. Meanwhile, in the lysates from samples treated at 4 °C only a basal fluorescence was observed (figure 3.16.B), suggesting very low membrane binding in these conditions, possibly due to lipophilic properties of the NF2792 molecule. The results indicate that NF2792 is actively internalised into the cell in an energy dependent manner. This finding discards the possibility of lipophilic interactions with the plasma membrane as the main uptake mechanism and points to one or more of endocytic pathways as the “means of entry” of NF2792 into SCC-9 cells. Since the internalisation is only observed at 37 °C, the mechanism is most likely a regulated cellular process, driven by ATP and, therefore, cannot occur at 4 °C, when cellular machinery does not function.

The release of NF2792 from SCC-9 cells was examined to assess its recycling by the cells and to investigate further into its potential as a live cell imaging agent. Cells were treated with NF2792 for 24 h, then washed and incubated in fresh medium for various incubation periods. Next, cells were harvested and lysed, and the fluorescence of NF2792 in cell lysates was assessed. Interestingly, a decrease in NF2792 fluorescence in SCC-9 cell lysates compared to cells harvested immediately after the 24 h incubation period was detectable as soon as 1 h after the medium was changed to a fresh one (figure 3.17). A linear decrease was observed over the course of successive timepoints, and the fluorescence was scarce and hardly detectable after 24 h of incubation in medium without NF2792. The result obtained indicates that the molecule is fully released from SCC-9 cells within a maximum timeframe of 24 h. Alternatively, NF2792 could be metabolised within the cell, thus introducing modifications to the structure of the molecule that would stop its fluorescence properties. The active uptake pattern could suggest an active release pattern; however, further experimentation is required to explain this phenomenon in detail.

3.2.3.2. NF2792 uptake mechanism in SCC-9 cells

The mechanism of uptake of NF2792 in SCC-9 cells was a fundamental question in need of clarification. HA is an important cell metabolite, vital to stabilize cellular functions; therefore, unsurprisingly, cells express receptors to recruit it, CD44 being the main receptor (Aruffo *et al.*, 1990; Hua *et al.*, 1993; Yu *et al.*, 2016). Since in Cappelli's research (2018) the point of entry of the HA-FA polymeric derivatives was not defined, but merely proposed to be facilitated through the lipid interactions with the cellular membranes due to OA presence, it was a vital question to be investigated into.

Dextran is a polysaccharide derivative of D-glucose of various lengths (3 to 2000 kDa). Due to the relative ease of conjugating with stable fluorophores and their commercial availability, dextrans have become widely used as endocytic markers. They are reportedly used as a marker of macropinocytosis (Wang *et al.*, 2014) and general fluid-phase endocytosis (Shurety *et al.*, 1998); however, they have also been proven to be the ligands of the mannose-receptor (Sallusto *et al.*, 1995; Ampel *et al.*, 2005), indicating their abilities to enter through clathrin-coated vesicles *via* clathrin-mediated endocytosis (Rappaport *et al.*, 2014). Similarly, lucifer yellow is a fluorescent hydrophilic dye used as a marker of macropinocytosis (Griesinger *et al.*, 2002; Harasztosi and Gummer, 2019). Since it is an anion, it cannot passively diffuse through the plasma membrane (Davis *et al.*, 2010) and therefore its internalisation requires active cellular uptake machinery. The two fluorescent endocytosis markers were chosen to examine the endocytosis pathways of SCC-9 cells and to compare them against the pattern of NF2792 uptake.

The cells were exposed to increasing concentrations of 70 kDa dextran-Texas Red conjugate and lucifer yellow lithium salt for 1 h to establish the working concentration at which the fluorescence of lysates becomes detectable (figure 3.18). The uptake of these two compounds in SCC-9 cells was quite pronounced, with 70 kDa dextran-Texas Red's fluorescence being easily readable at 1 μ M final concentration in the medium. Lucifer yellow's fluorescence is reportedly easier to bleach (Griesinger *et al.*, 2002); therefore, the cells were treated with 15 μ M final concentration in the culture medium to obtain a steady, detectable fluorescence signal. Since endocytic processes are necessary to ensure cell survival and inhibitors of endocytosis

can also have off-target effects, it was vital to establish an optimal concentration and incubation time for endocytosis inhibition assays. Therefore, optimal concentrations of amiloride, an inhibitor of sodium-proton exchange and macropinocytosis (Koivusalo *et al.*, 2010), and dynasore, a non-competitive inhibitor of GTPase activity of dynamin-1 and 2 obstructing dynamin-dependent uptake pathways, including clathrin-mediated endocytosis (Macia *et al.*, 2006; Kirchhausen *et al.*, 2009; Preta *et al.*, 2015) were determined in SCC-9 cells through a cytotoxicity assay after 2 h of treatment (figure 3.19).

Next, the endocytic pathways in SCC-9 cells were inhibited through treatment with the corresponding inhibitors for 30 min. Then, the cells were co-treated with 70 kDa dextran-Texas Red and lucifer yellow fluorescent dyes for 1 h to distinguish which endocytic pathways are responsible for their respective internalisation into SCC-9 cells. As presented in figure 3.20, both endocytic markers are incorporated into the cells through different processes. Dextran-Texas Red's fluorescence was significantly lowered in comparison to vehicle treated cells (0.1 % DMSO) after both amiloride and dynasore treatment, indicating multiple possible entrance pathways through both macropinocytosis and clathrin-mediated endocytosis, in line with previous literature reports. Since dextran is a glucose derivative, it can enter the cells through the mannose receptor, bypassing inhibition of macropinocytosis by amiloride. Similarly, after dynasore driven inhibition of clathrin-mediated endocytosis through the mannose receptor, the molecule can successfully enter the cell through macropinocytosis. In the case of lucifer yellow, the inhibition studies showed that the molecule's main entry route into the cell is *via* macropinocytosis, as indicated by a significant decrease in fluorescence in SCC-9 cell lysate after amiloride treatment when compared to non-treated cells. In contrast, dynasore treatment did not decrease the uptake of lucifer yellow into the cells and its fluorescence in cell lysates. SCC-9 cells were also exposed to both endocytic markers at 4 °C to confirm that their internalisation is an energy-dependent process as opposed to passive and energy-independent diffusion through or binding to the plasma membrane. In fact, a negligible level of fluorescence in the lysates treated at 4 °C was observed, suggesting no energy-independent internalisation.

After performing initial confirmatory endocytic studies in SCC-9 cells, an analysis of the NF2792 endocytic pathway was pursued. Unfortunately, due to fluorescence overlap, amiloride could not be used alongside NF2792 to examine macropinocytosis in NF2792 uptake. However, as seen in figure 3.21, NF2792 internalisation pattern seems to be dynamin related,

as its fluorescence in SCC-9 cell lysates was significantly decreased after dynasore treatment and clathrin-mediated endocytosis inhibition. As in previous studies, cells treated at 4 °C displayed no fluorescence proving that NF2792 is internalised in an energy dependent manner and not through a passive, nonspecific process. This result points to the HA receptor being the main entryway of NF2792 into SCC-9 cells. However, since dynasore can non-specifically inhibit all dynamin related endocytosis and therefore could have some off-target effects, this study does not provide a definitive proof for the HA receptor being the principal NF2792 uptake mechanism.

3.2.3.3. CD44 mediated endocytosis of NF2792

To prove that NF2792 enters SCC-9 cells through clathrin-mediated endocytosis by means of the CD44 receptor, SCC-9 cells were first examined to establish their CD44 expression levels. CD44 has been found in epithelial tissue (Huang and Huang, 2018) and to be a cancer stem cell marker (Cho *et al.*, 2015); moreover, it has been reported to be overexpressed in oral and oesophageal cancers (Christopoulos *et al.*, 2006; Yu *et al.*, 2016; Ludwig *et al.*, 2019; Qin *et al.*, 2019). In fact, our flow cytometry studies have confirmed high levels of expression of CD44 in SCC-9 cells, with nearly 99 % of the cell population staining positive compared to isotype control (figure 3.22).

Monoclonal blocking antibodies were previously reported to inhibit CD44 function and recognition of HA (Zheng *et al.*, 1995; Amash *et al.*, 2016; Chen *et al.*, 2018); therefore, an anti-CD44 monoclonal antibody was chosen to examine its ability to block CD44 mediated endocytosis of NF2792. Following the previously performed endocytosis studies on SCC-9 cells with 70 kDa dextran-Texas Red and lucifer yellow, SCC-9 cells were exposed to various concentrations of an unconjugated IM7 rat monoclonal anti-CD44 antibody and an unconjugated rat IgG2b kappa monoclonal isotype control antibody for 30 min. Next, cells were co-treated with NF2792 for further 1 h. Cells were then washed and lysed, and the fluorescence levels of NF2792 were assessed to determine whether antibody mediated blocking of the CD44 receptor had occurred and at which concentration. As illustrated in figure 3.23, the CD44 monoclonal antibody elicited a pronounced decrease of NF2792 fluorescence in SCC-9 cell lysates at 2.5 µg/ml, suggesting that the bulk of NF2792 is endocytosed through the HA receptor, CD44.

This has been further confirmed, by exposing cells pre-treated with either CD44 blocking antibody, isotype control or vehicle to NF2792 at 37 °C and 4 °C which showed a decrease in NF2792 uptake after exposure to CD44 blocking antibody at 37 °C (figure 3.24). These results further demonstrate that the uptake of NF2792 in SCC-9 is facilitated through the CD44 receptor due to its affinity to the HA backbone of the molecule. As CD44 is reportedly overexpressed in various types of cancer, this mode of internalisation might suggest preferential and higher uptake of NF2792 by the cancer cells, thus making it a very promising imaging agent in the detection and surgical removal of cancerous tissue.

3.3. Discussion

Cappelli *et al.* (2018) reported synthesising a series of fluorescent polymeric probes derived from natural substances that have an interesting potential in cancer imaging. Following this report on new HA derived bioprobes (2018) and questioning the usefulness of the rigidity of the molecules' backbones, the synthetic approach has been redesigned to include an ester bond between the OA and the hexaethylene glycol linker to introduce more flexibility to the structure. The natural origin of the compound's component moieties, their reported lack of toxicity and interesting fluorescence properties (induced upon aggregation) suggest the molecules could be promising bioprobes with an ability to be exploited as cancer imaging agents. It was believed that by introducing certain structural changes, the molecule's characteristics could be improved upon, increasing its potential as an imaging probe.

Much like Cappelli's compounds (2018), the new molecule, NF2792, incorporates OA, which presumably grants it the self-assembling and aggregating properties due to its hydrophilicity. An important feature of NF2792 is the FA moiety, responsible for its fluorescent properties. The excitation and emission wavelengths of FA can be modulated by the pH and polarity of the solvent, allowing it to achieve a very satisfying Stokes shift, desirable in an imaging bioprobe. In fact, NF2792, presumably due to the ester bond within its structure and slightly altered intramolecular electrical charge distribution, boasts of a Stokes shift of 120 nm in aqueous solutions. Its fluorescence increases with concentration, and seemingly is induced by aggregation of the molecule within the cell, as suggested by clusters of fluorescence seen under confocal microscopy. The molecule can be successfully visualised by multiphoton microscopy, indicating its possible use in a thick tissue sample and *in vivo* tissue imaging.

The main backbone of the molecule is HA, which reportedly has a high affinity to CD44, its cellular receptor (Aruffo *et al.*, 1990; Hua *et al.*, 1993; Yu *et al.*, 2016). HA is important in maintaining cellular function and homeostasis and promotes cell adhesion and proliferation (Cohen *et al.*, 2004). Therefore, it was expected that NF2792 would not exhibit toxicity in SCC-9 squamous cell carcinoma of the tongue cell line. In fact, HA and the HA-FA backbone, showed an increased cell viability post 24 h treatment, suggesting HA could affect cell viability through facilitating cell adhesion and proliferation (Cohen *et al.*, 2004). The two immediate

synthetic precursors of NF2792, namely NF2790 and NF2791, showed significantly low IC_{50} values. It is speculated that the azide group in NF2790 and the alcohol group in NF2791 could be responsible for higher reactivity and toxicity of these two compounds in SCC-9 cells. In case of NF2792, SCC-9 cells displayed 80 % cell viability after treatment with 100 μ M NF2792 for 24 h, what is in line with Cappelli's results (2018). The 80 % viability at a high concentration suggests no direct toxic effects; however, there could be some low level of cell death induction. The ester bond within the structure of the molecule could be a target of esterase enzymes within the cells. Esterases perform a hydrolysis reaction on ester bonds, where they split them to form alcohol and carboxylic groups – exactly the opposite of the esterification reaction that initially produced the bond. Through this process, they could free the OA from the molecule and release its toxic effects in the cells. Presumably, the rigid bonds in the polymer backbone and the chemical modifications to the constituent parts of the molecule prevent the toxicity from being released within the cells. However, had NF2792 been hydrolysed inside the cell, the OA would have been released from the polymeric molecule, which in turn could possibly induce cytotoxicity. It is not clear if the esterase driven hydrolysis is happening in SCC-9 cells – the slight decrease in cell viability after 24 h treatment with NF2792 in comparison with HA-8700 and HA-FA-Pg-3F might suggest either the occurrence of hydrolysis, or that the rigid backbone does not release the HA's ability to enhance cell adhesion and proliferation. It is difficult to assume the efficacy of esterases, but most probably they would not facilitate freeing OA from all NF2792 molecules. It is highly improbable for a high amount of OA to have been freed from NF2792 by the esterases – the molar mass of OA is 282.468 g/mol, which constitutes *circa* 2 % of the molar mass of NF2792 (12,322 g/mol); however, there is a 1:1 ratio of OA in every NF2792 molecule. This suggests that a high level of hydrolysis reaction at a high concentration of NF2792 would lead to a high intracellular concentration of OA, which in turn would induce high level of cell death. As mentioned before, OA showed an IC_{50} value of 19.7 μ M in SCC-9 cells. That is to say, at the NF2792 concentration of 100 μ M, the concentration of prospective liberated OA would most probably be enough to have a significant effect on cell viability, which was not observed here. Furthermore, cell survival and apoptosis induction studies showed no detrimental effect of NF2792 on SCC-9 cells. NF2792 treated cells displayed basal apoptotic activity in par with vehicle treated cells, in contrast to cisplatin treated cells, which showed a significant apoptosis induction. The observations lead to a conclusion that NF2792 is not toxic to SCC-9 cells at concentrations up to 100 μ M and could be considered as a non-toxic bioprobe.

Intracellular uptake of NF2792 has been confirmed in SCC-9 cells by confocal and multiphoton microscopy. Further uptake studies have been performed on cell lysates utilising the fluorescence and stability of the molecule. NF2792 showed a time dependent increase in its fluorescence, with detectable fluorescence after 30 min from treatment, suggesting a relatively rapid and effective internalisation. Moreover, studies performed at 4 °C displayed no fluorescence – a clear indication that the molecule is internalised in an active, energy dependent process as opposed to passive diffusion or plasma membrane binding. The release study showed the fluorescence of cell lysates gradually decreases over time and is negligible after a maximum time of 24 h. This would suggest an effective release or recycling and expulsion of NF2792, which is a desirable property in a prospective bioprobe.

The uptake pattern and pathway has been further studied in detail with the help of two fluorescent endocytic markers, 70 kDa dextran-Texas Red and lucifer yellow. Using two endocytosis inhibitors, amiloride to inhibit macropinocytosis and dynasore to inhibit dynamin related clathrin-mediated endocytosis, the two pathways could be distinguished. NF2792 followed the dynamin related pathway and opened the question whether CD44 is in fact facilitating its uptake through its affinity to the HA backbone of the molecule. A monoclonal CD44 antibody has been used to inhibit CD44 mediated endocytosis in SCC-9 cells after affirming their high levels of expression of CD44. In fact, the monoclonal antibody succeeded at inhibiting NF2792 uptake into the cells, which was as a dramatic decrease of fluorescence in comparison to vehicle and isotype control treated samples. This suggests that NF2792 uptake is driven through its affinity to CD44 receptor. As CD44 is a marker of cancer stem cells and is often overexpressed in cancer cells of various types, it is speculated that cancer cells would internalise significantly higher levels of NF2792 in comparison to healthy cells. This in turn would enable the usage of NF2792 as a cancer imaging agent. It is envisioned that through the efficient and preferential uptake of it by the cancer cells, it would aid in tumour localisation in a patient. Possibly, after much needed further validation, it could be used as a tool to diagnose and localise tumours in patients; however, much more research is needed to confirm its release from the cells and where it is localised within the cells post internalisation. It is vital to determine its effect on normal, healthy cells and possibly how well would it function *in vivo* in a model organism.

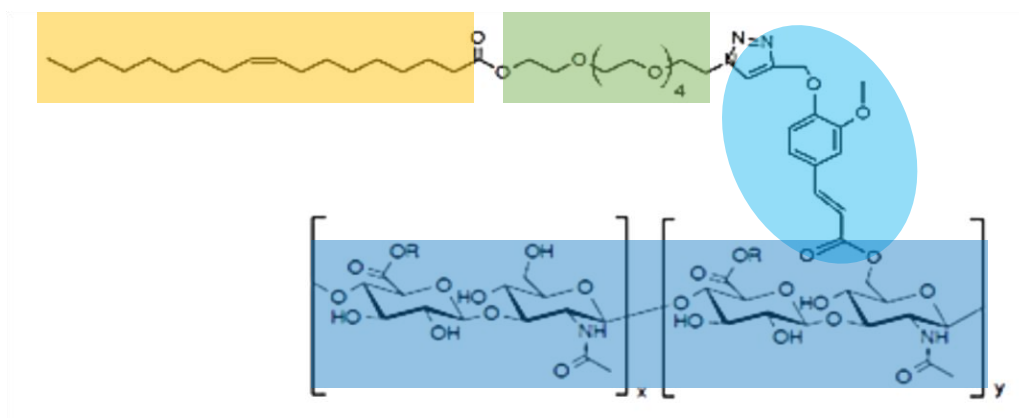


Figure 3.1. Chemical structure of NF2792

NF2792 is the derivative of hyaluronic acid (navy blue) coupled with ferulic acid (blue), glycol component (green) and OA (yellow) bound by an ester bond. Ferulic acid part has fluorescent properties induced by aggregation. Representation adapted from Cappelli *et al.*, 2018.

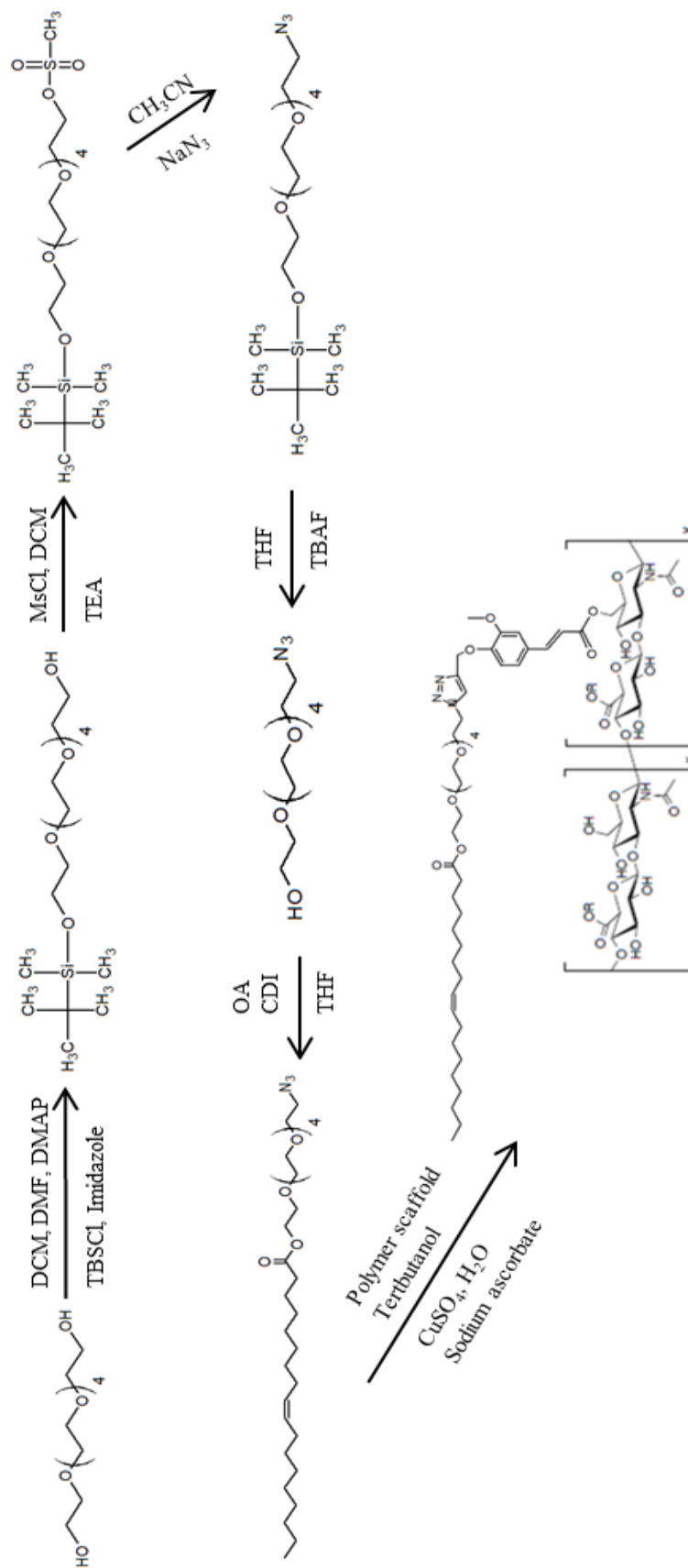


Figure 3.2. Synthesis of NF2792

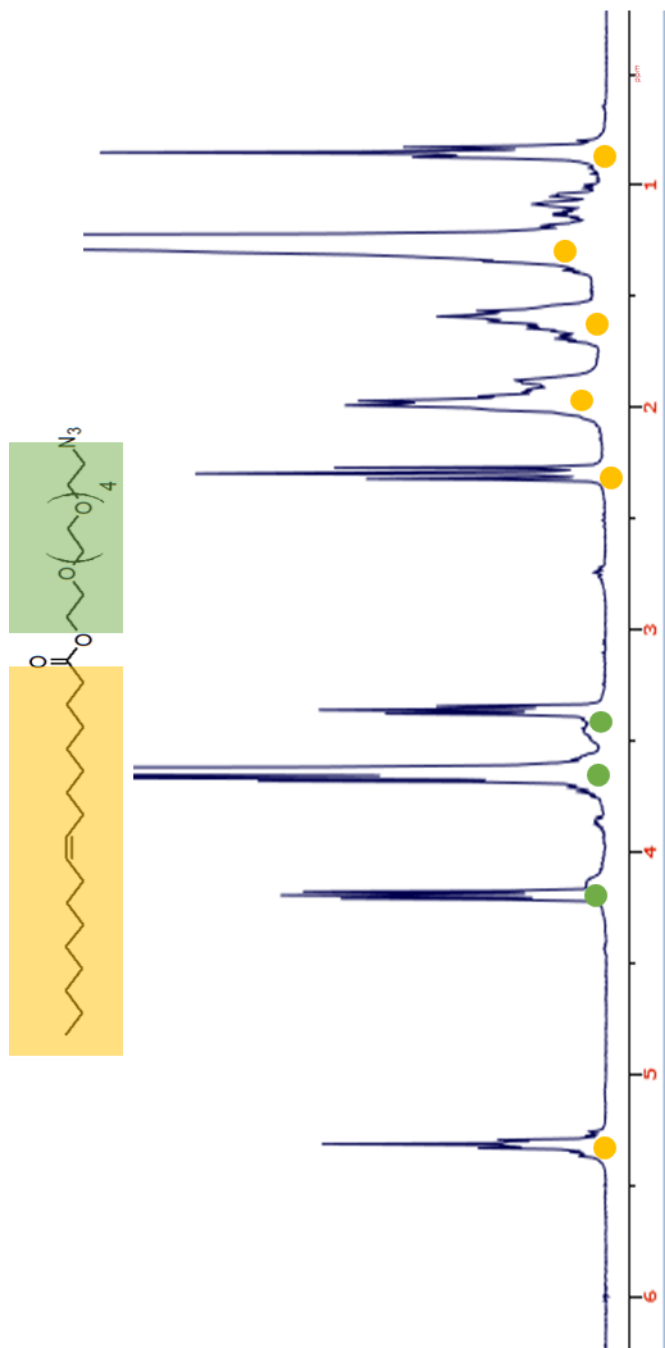


Figure 3.3. ^1H NMR spectrum of NF2790 (in CDCl_3)

Yellow dots in the NMR profile indicate the chemical shift of the functional groups in the OA moiety, whereas the green dots mark the glycol component. Representation adapted from Cappelli *et al.*, 2018.

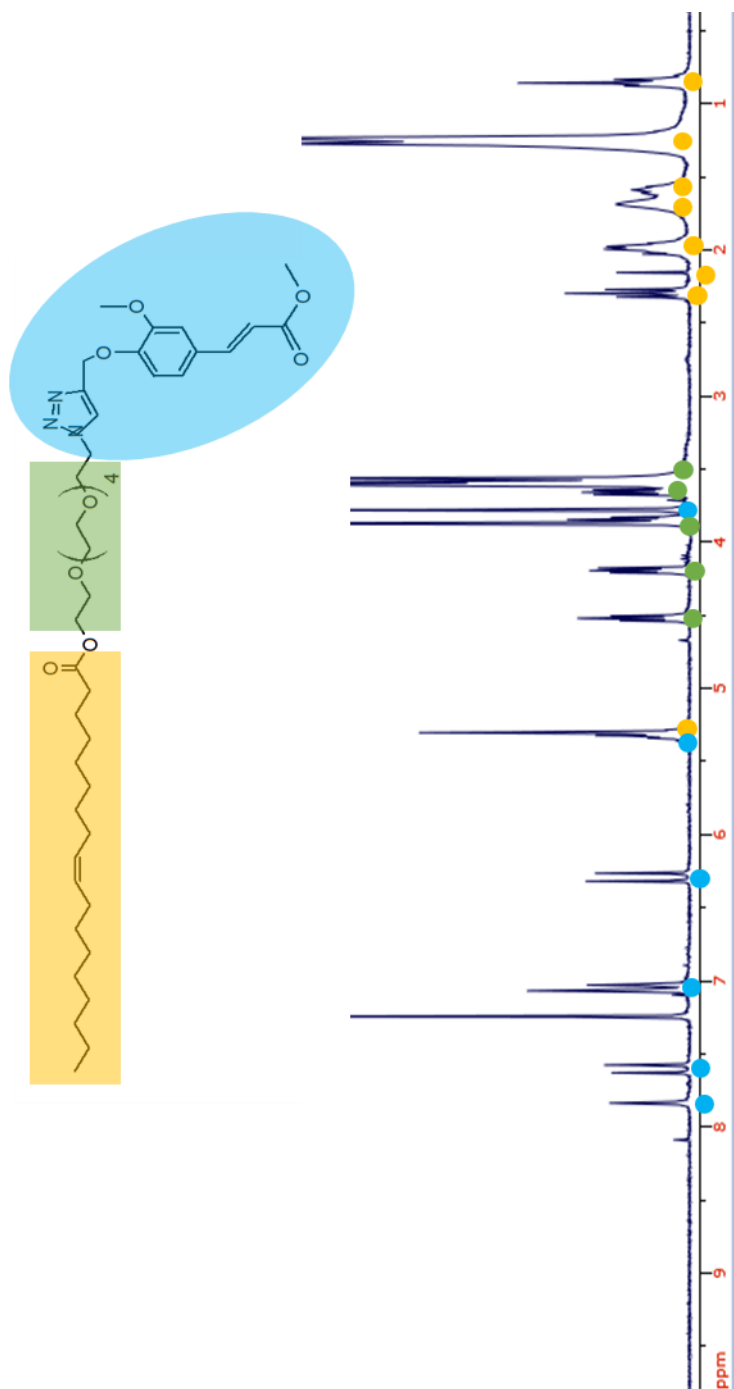


Figure 3.4. ^1H NMR spectrum of NF2791 (in CDCl_3)

Yellow dots in the NMR profile indicate the chemical shift of the functional groups in the OA moiety, green dots mark the glycol component, and the blue dots correspond to the ferulic acid residue. Representation adapted from Cappelli *et al.*, 2018.

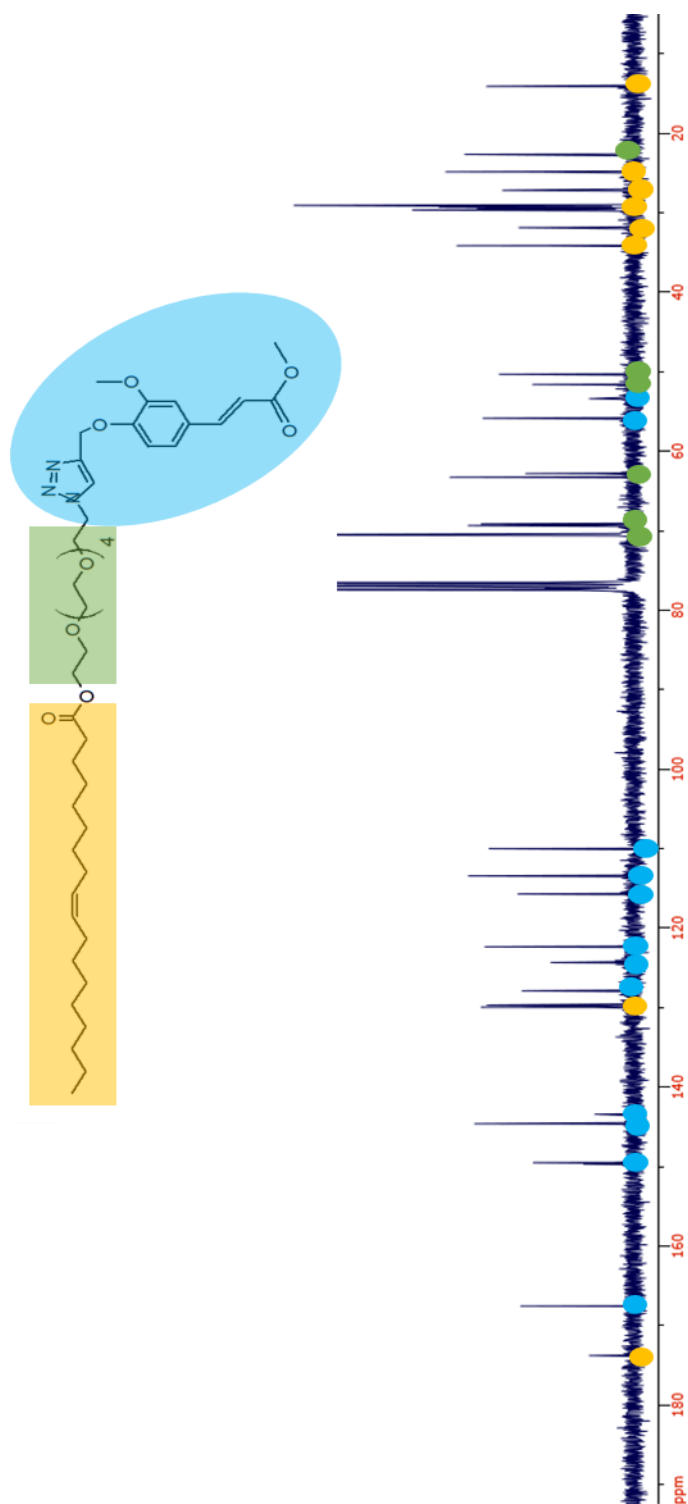


Figure 3.5. ^{13}C NMR spectrum of NF2791 (in CDCl_3)

Yellow dots in the NMR profile indicate the chemical shift of the carbon atoms in the OA moiety, green dots mark the glycol component, and blue dots correspond to the ferulic acid residue. Representation adapted from Cappelli *et al.*, 2018.

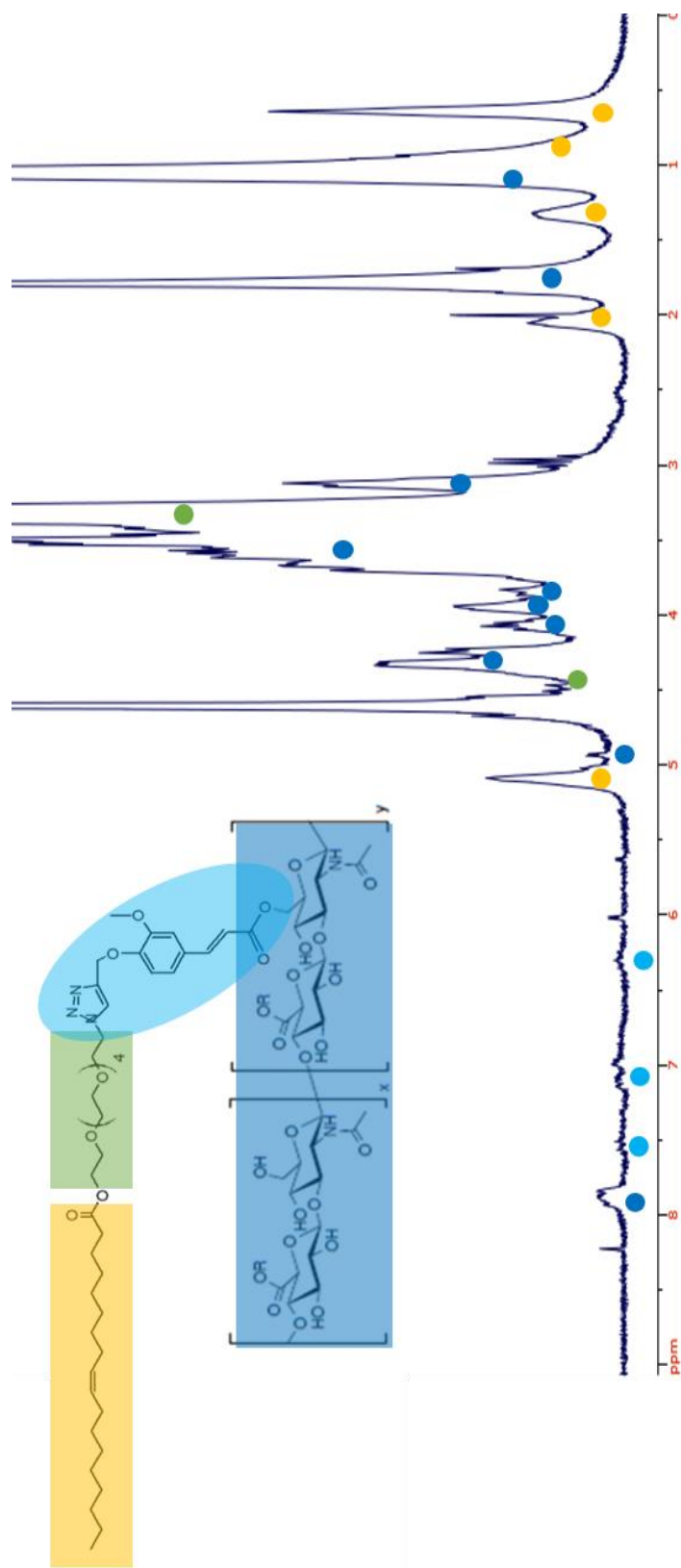


Figure 3.6. ^1H NMR spectrum of NF2792 (in D_2O)

Yellow dots in the NMR profile indicate the chemical shift of the functional groups in the OA moiety, green dots mark the glycol component, blue dots correspond to the ferulic acid residue, and navy-blue dots point to the hyaluronic acid chains. Representation adapted from Cappelli *et al.*, 2018.

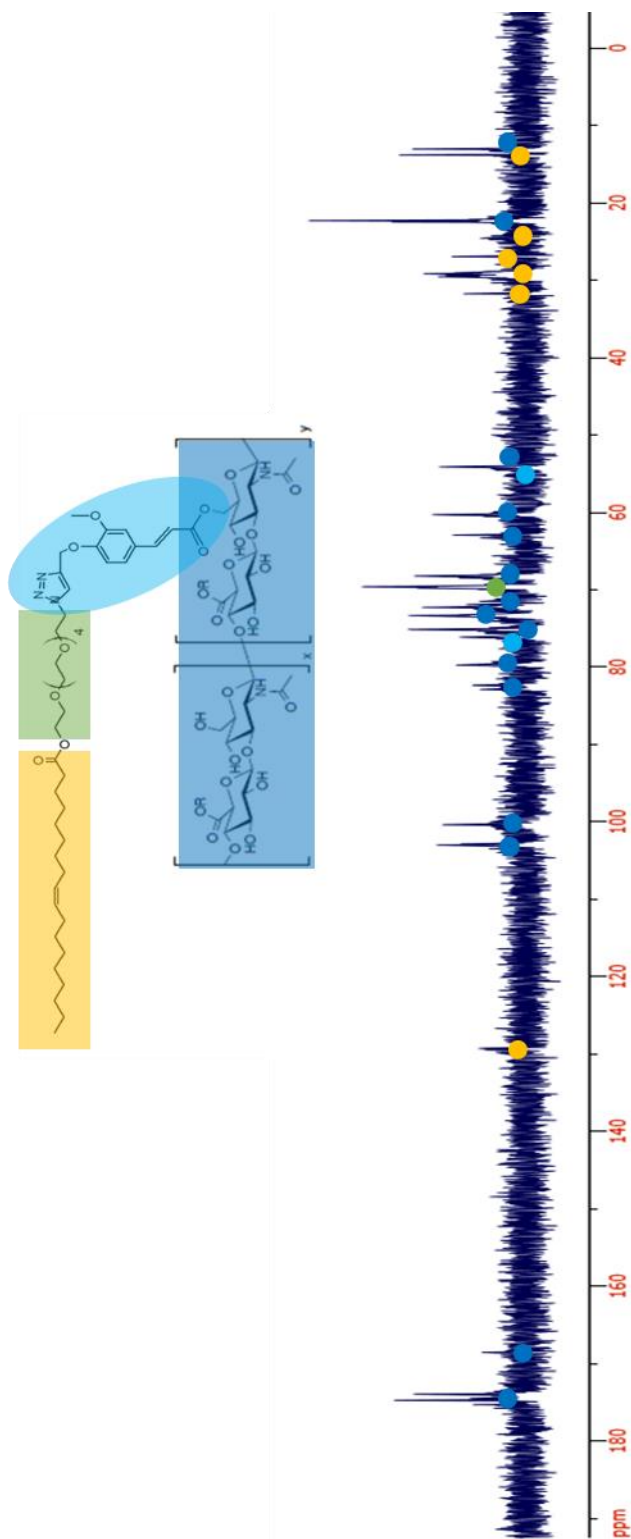


Figure 3.7. ^{13}C NMR spectrum of NF2792 (in D_2O)

Yellow dots in the NMR profile indicate the chemical shift of carbon atoms in the OA moiety, green dots mark the glycol component, blue dots correspond to the ferulic acid residue, and navy-blue dots point to the hyaluronic acid chains. Representation adapted from Cappelli *et al.*, 2018.

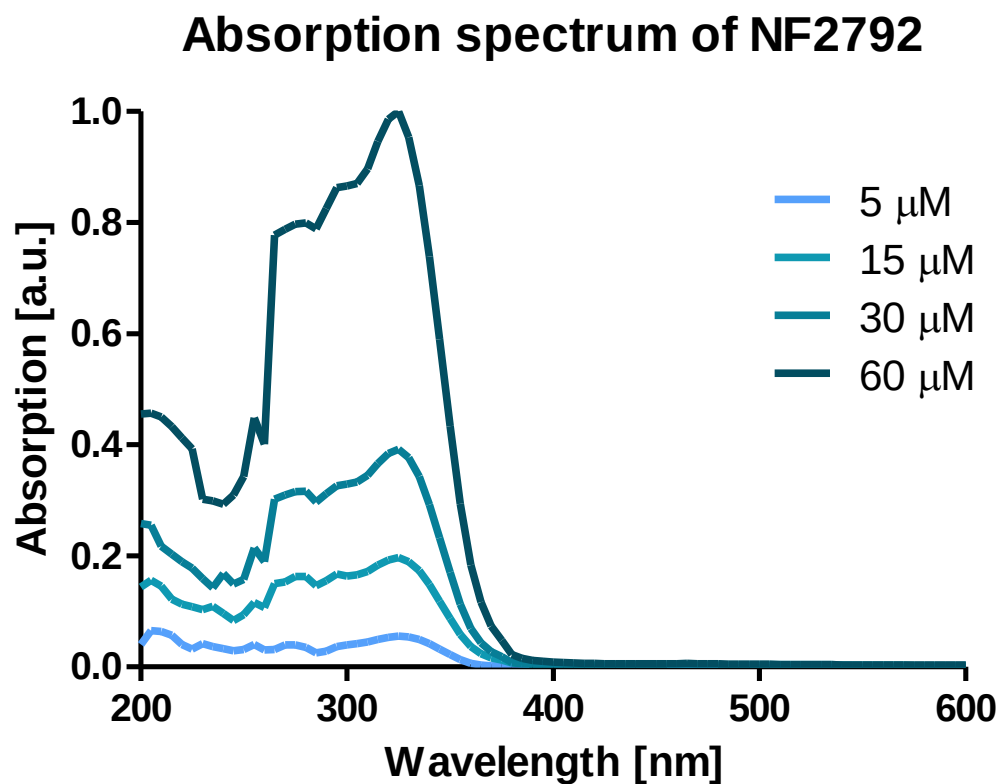


Figure 3.8. Absorption spectrum of NF2792

Normalized absorption of NF2792 measured in MilliQ H₂O at the concentration of 5 μM , 15 μM , 30 μM , and 60 μM (as indicated). a.u. – arbitrary units.

Fluorescence spectrum of NF2792 at an excitation of 325 nm

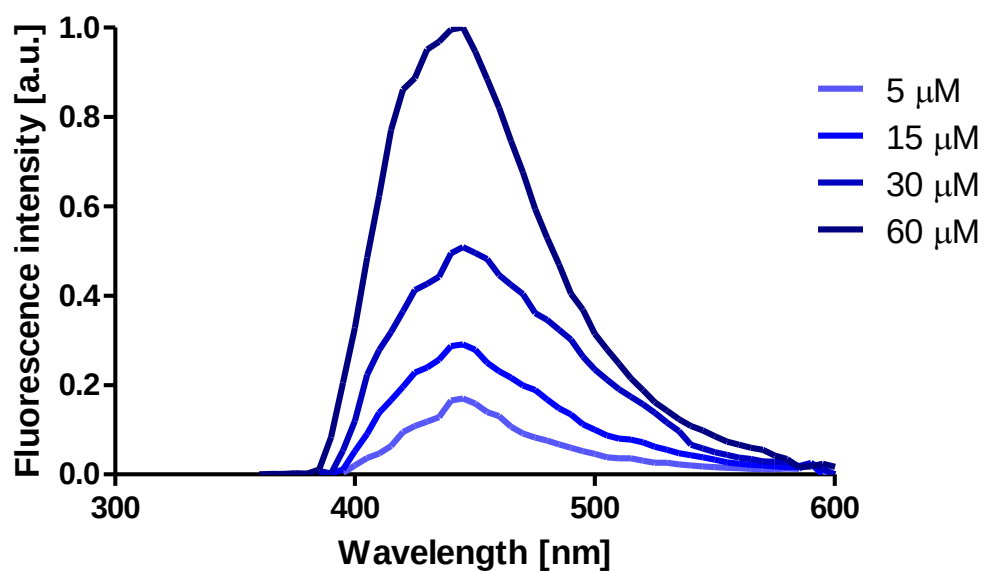


Figure 3.9. Emission spectrum of NF2792

Normalized emission at an excitation of 325 nm spectra of NF2792, measured in MilliQ H₂O at the concentration of 5 μM, 15 μM, 30 μM, and 60 μM (as indicated). a.u. – arbitrary units.

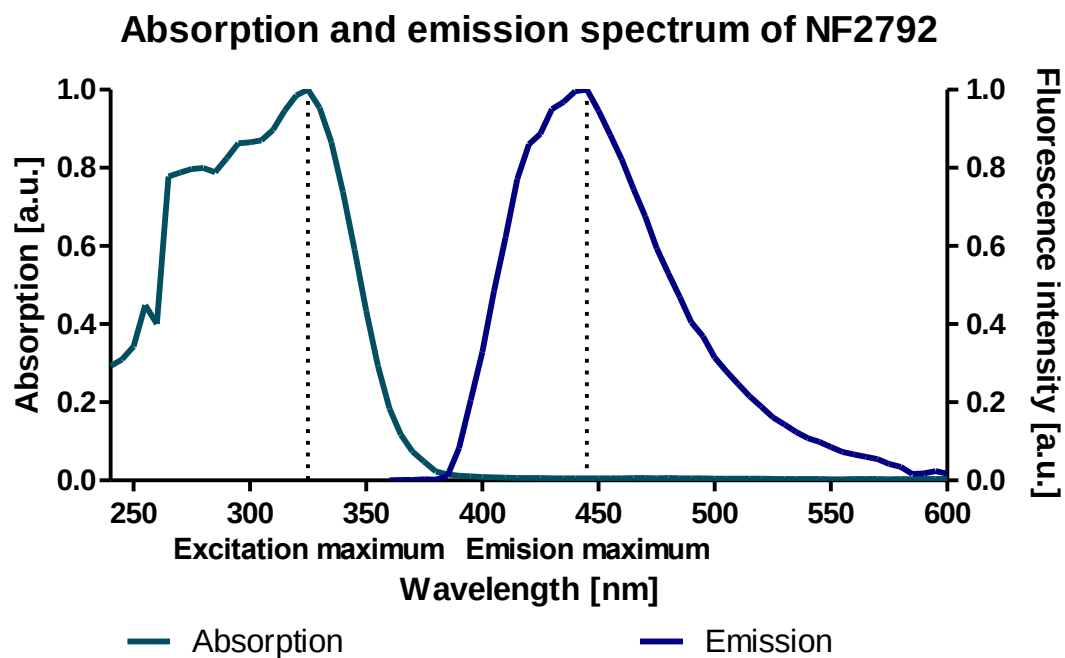


Figure 3.10. Absorption and emission spectrum of NF2792

Normalized absorption (green) and emission at 325 nm (blue) spectra of NF2792, measured in MilliQ H₂O at the concentration of 60 μ M. a.u. – arbitrary units.

Viability assays of SCC-9 cells with NF2792 and its intermediates

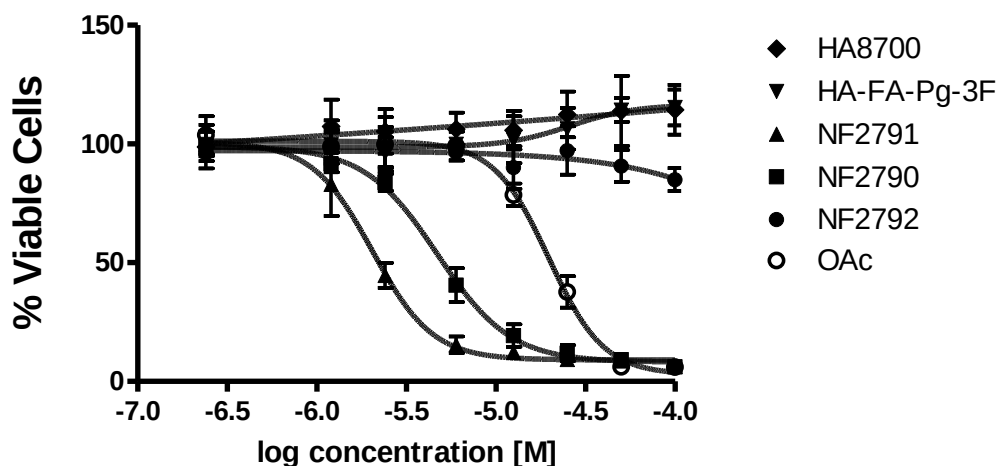


Figure 3.11. Cell viability of SCC-9 cells after 24 h exposure to different concentrations of NF2792 molecule and its intermediates

NF2790 and NF2791 are intermediate products of chemical synthesis of NF2792, HA-FA-Pg-3F is a polymer chain with a ferulic acid residue, and HA-8700 is hyaluronic acid. Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well and serum starved for 16 h prior to treatment with a range of concentrations of the polymer intermediates for 24 h. The viability of the cells was assessed by Alamar Blue assay relative to a vehicle control (0.1 % volume of MilliQ H₂O or DMSO). The concentrations are shown in a log scale. Values represent the mean $\pm$ SD of three independent experiments. IC₅₀ values were as follows: 4.7 μ M for NF2790, 2 μ M for NF2791, 19.7 μ M for OA. Cell viability was 85 % for 100 μ M NF2792, 115 % for 100 μ M HA-FA-Pg-3F, and 114 % for 100 μ M HA8700.

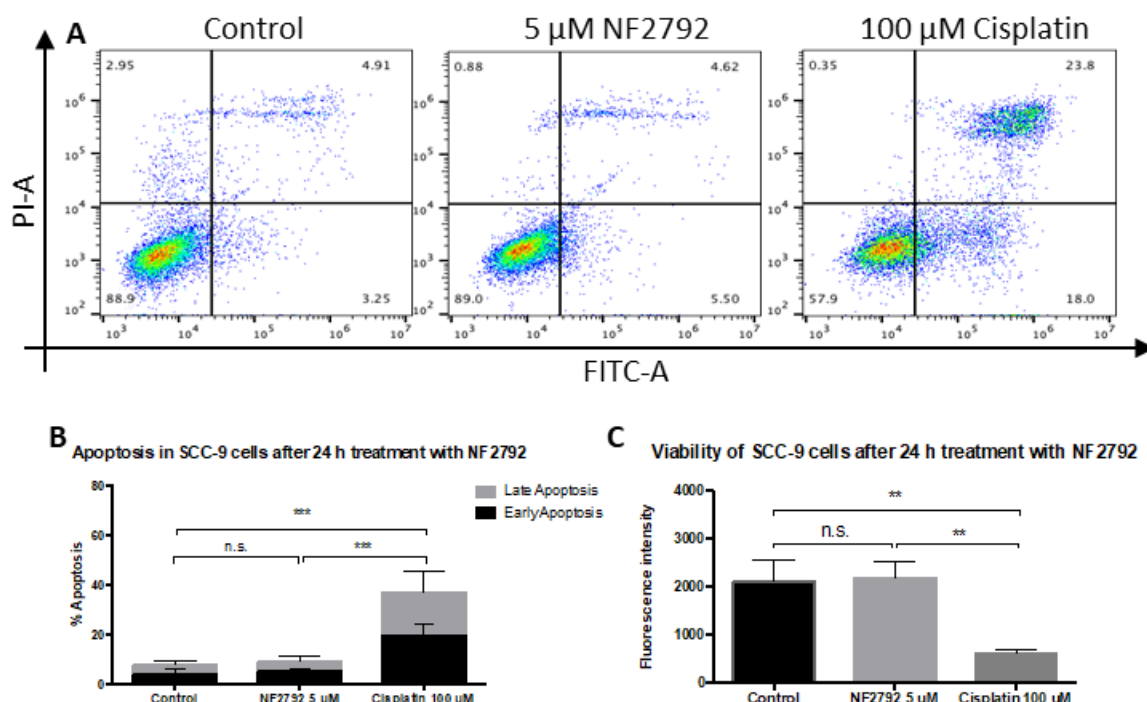


Figure 3.12. Analysis of NF2792 toxicity in SCC-9 cells

A – Representative of three independent flow cytometry experiments. Cells were seeded on a 6-well plate at a density of 1.5×10^5 cells/well and serum starved for 16 h prior to treatment with either 0.1 % volume of MilliQ H₂O as vehicle control, 5 μM NF2792 or 100 μM cisplatin as a positive control. After 24 h of treatment, cells were harvested, stained with Annexin V antibody and propidium iodide, and analysed by flow cytometry. B – Quantitative apoptosis levels assessment after treatment, means $\pm$ SD of three independent experiments. Two-way ANOVA, *** $p < 0.001$, n.s. – not significant. C – Viability of SCC-9 cells after 24 h treatment with NF2792. Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well and then serum starved for 16 h prior to treatment with either 0.1 % volume of MilliQ H₂O as vehicle control, 5 μM of NF2792 or 100 μM cisplatin as a positive control. After 24 h exposure, the viability of cells was assessed by Alamar Blue assay. Values shown on the graph are fluorescence intensity means of three independent experiments. Student t-test, ** $p < 0.01$, n.s. – not significant.

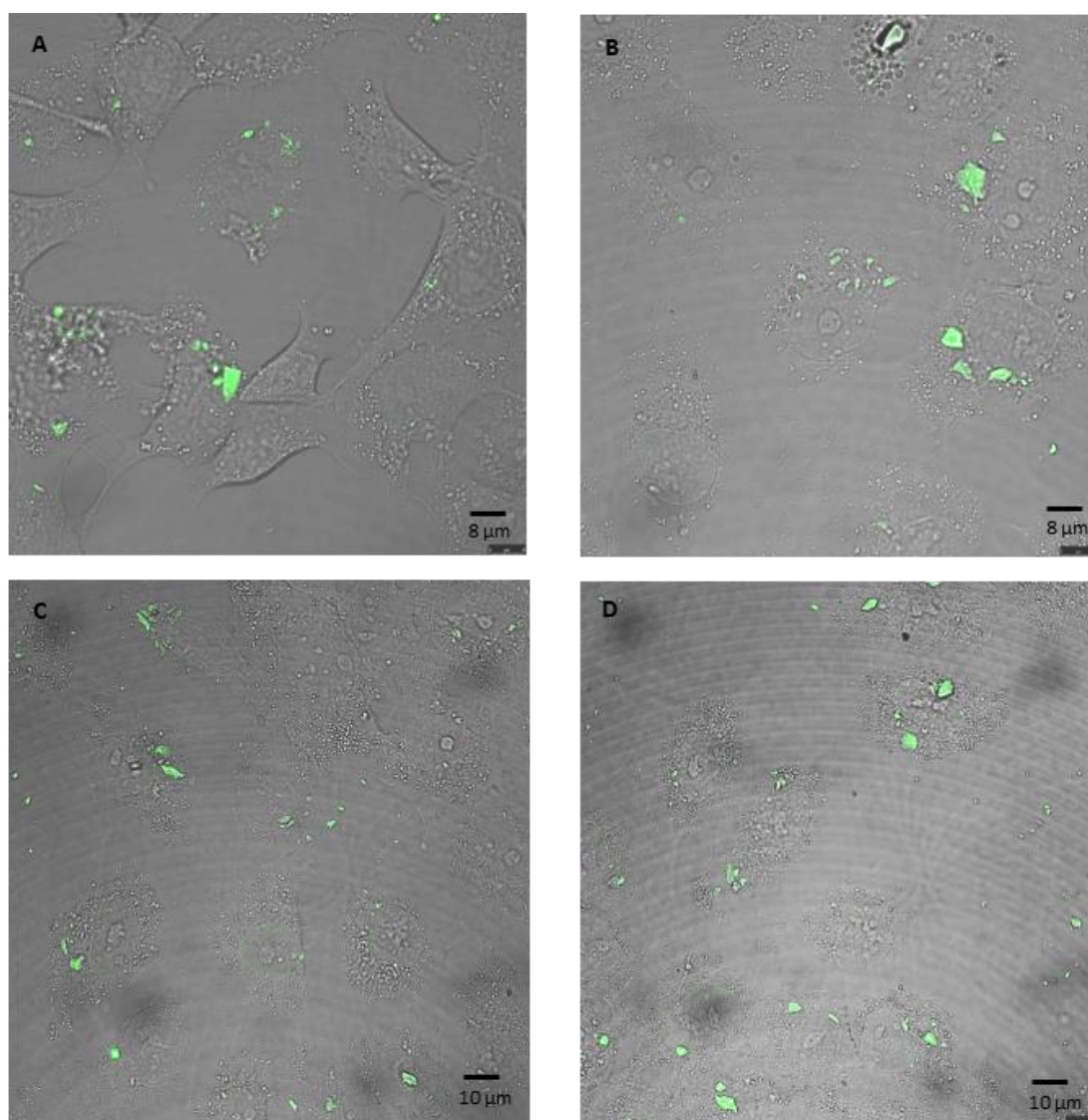


Figure 3.13. Confocal microscopy images showing internalization of NF2792 into SCC-9 cells

Cells were seeded on a 8 chamber glass slide at a density of 9×10^4 cells/chamber and then serum starved for 16 h before treatment with A – 5 μ M, B – 10 μ M, C – 20 μ M, and D – 50 μ M NF2792 in serum-free medium and incubated for 2 h. Prior to imaging on a confocal microscope cells were washed twice with PBS and fresh serum-free medium was added. Superimposition of brightfield and emission between 400-500 nm.

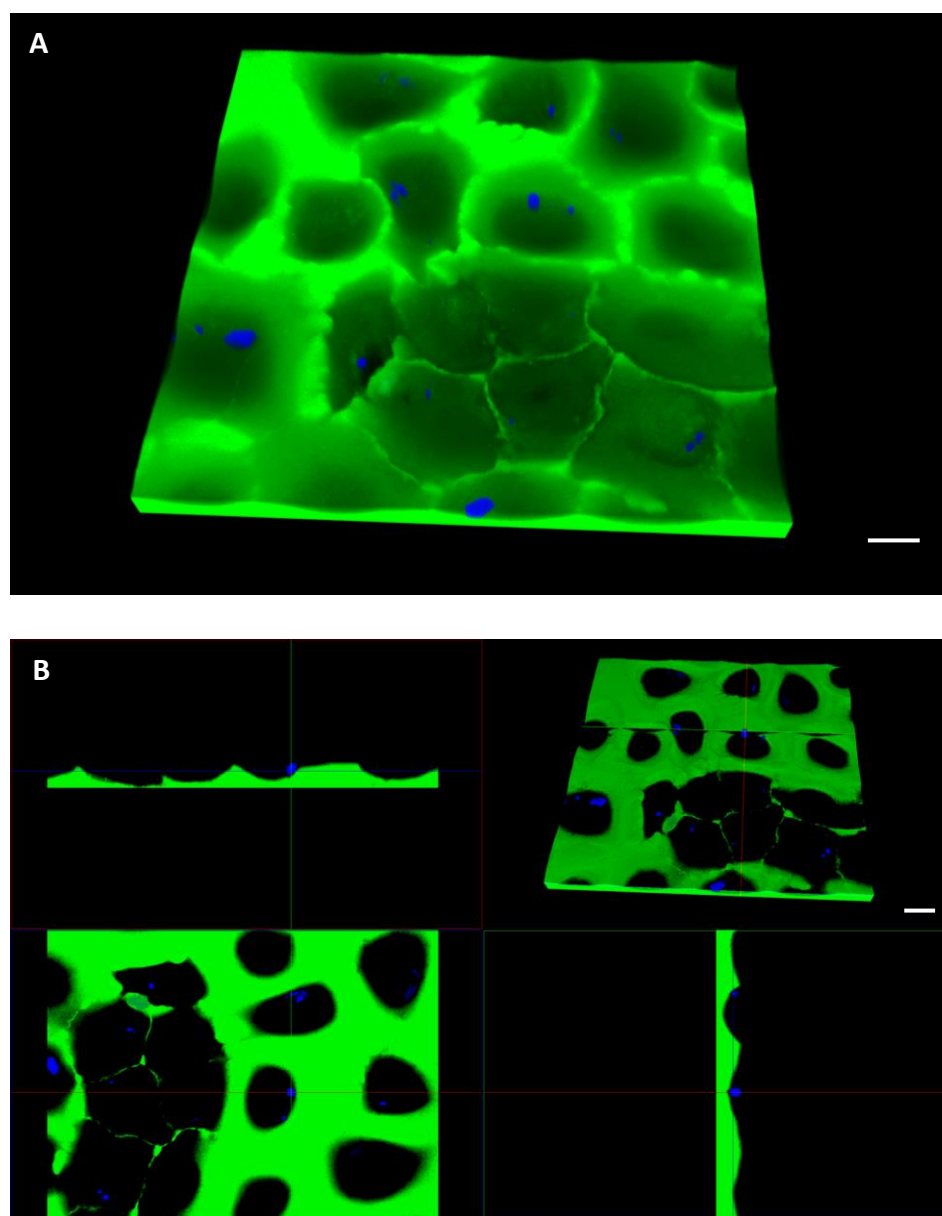


Figure 3.14. 3D confocal images showing internalization of NF2792 into SCC-9 cells

Cells were seeded on an 8-chamber glass slide at a density of 9×10^4 cell/chamber and then serum starved for 16 h. Subsequently, NF2792 was added at the concentration of 20 μM in serum-free medium and incubated for 2 h. Prior to the visualisation by confocal microscopy, cells were washed twice with PBS and fresh serum-free medium was added to the cells. Immediately prior to imaging, a concentration of 0.1 μM fluorescein was added to the medium to allow visualisation of the cell outlines. A – Bird's eye view, B – Sagittal view. Bar is 20 μm .

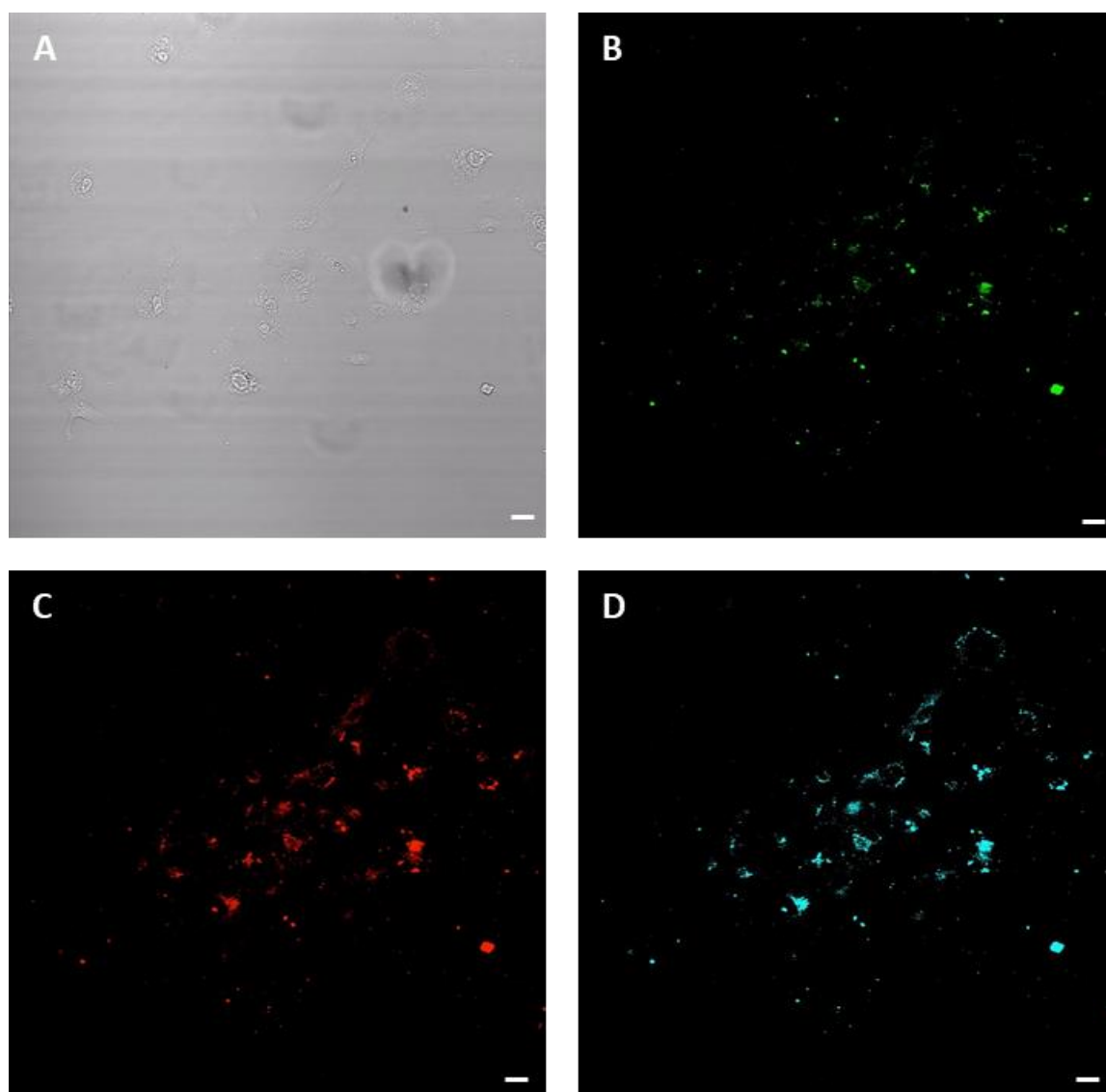


Figure 3.15. Multiphoton microscopy images showing internalization of hyaluronan polymer derivative NF2792 into the SCC-9 cells compared to untreated control

Cells were plated on a 6-channel ibidi VI 0.4 μ -slide (5×10^4 cells/channel) and after attachment, were serum starved for 16 h. Next, cells were treated with 20 μ M NF2792 in serum-free medium for 2 h. Prior to the visualisation, cells were washed twice with PBS and the medium was changed to fresh serum-free medium. A - brightfield. B – D – An excitation wavelength: 730 nm. Emission: B – 397-420 nm, C – 455-490 nm, D – 500-550 nm. Bar is 20 μ m.

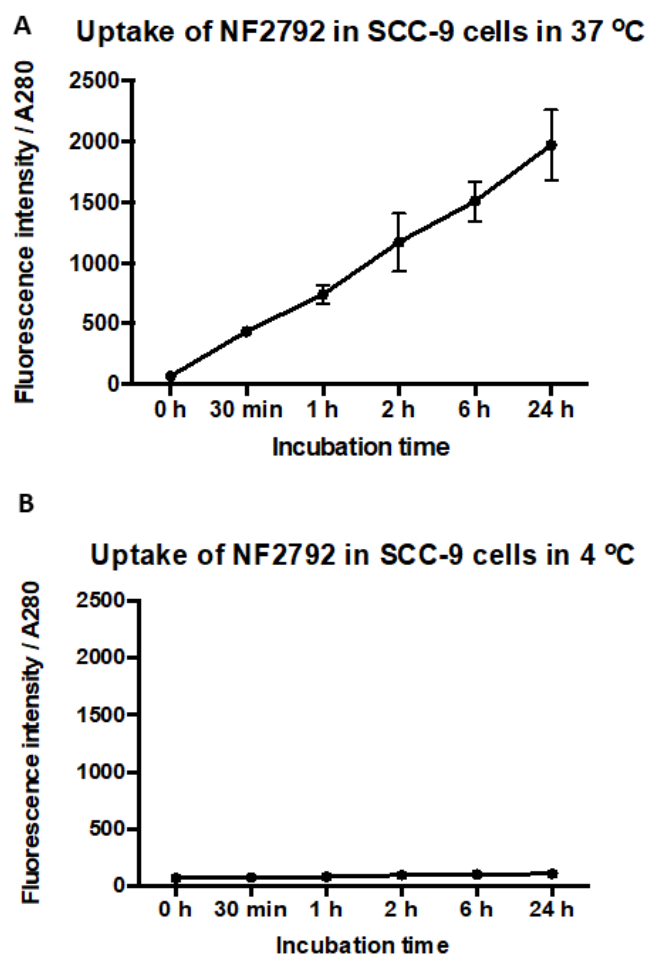


Figure 3.16. Analysis of time-dependent uptake of NF2792 by SCC-9 cells

Cells were seeded on a 6-well plate at 3×10^5 cells/well and serum starved for 16 h prior to the treatment, and then treated with 5 μ M NF2792 for 24 h, 6 h, 2 h, 1 h, and 30 min at A – 37 °C, B – 4 °C. Then, cells were washed twice with PBS and lysed. Fluorescence of the cell lysates was analysed at an excitation of 325 nm and emission of 445 nm. Fluorescence values were corrected for the absorbance at 280 nm. Results show the mean $\pm$ SD of three independent experiments.

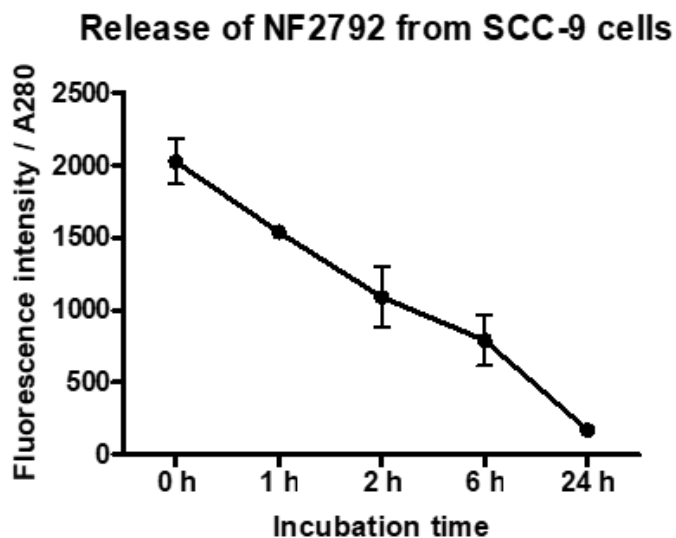


Figure 3.17. Analysis of time-dependent exocytosis of NF2792 by SCC-9 cells

Cells were seeded on a 6-well plate at 3×10^5 cells/well and serum starved for 16 h prior to the treatment, and then treated with 5 μ M NF2792 for 24 h. Subsequently, the medium was removed, cells washed twice with PBS and fresh medium was added for 24 h, 6 h, 2h, and 1 h. Then, cells were washed twice with PBS and lysed. Fluorescence of the cell lysates was analysed at an excitation of 325 nm and emission of 445 nm. Fluorescence values were corrected for the absorbance at 280 nm. Results show the mean $\pm$ SD of three independent experiments.

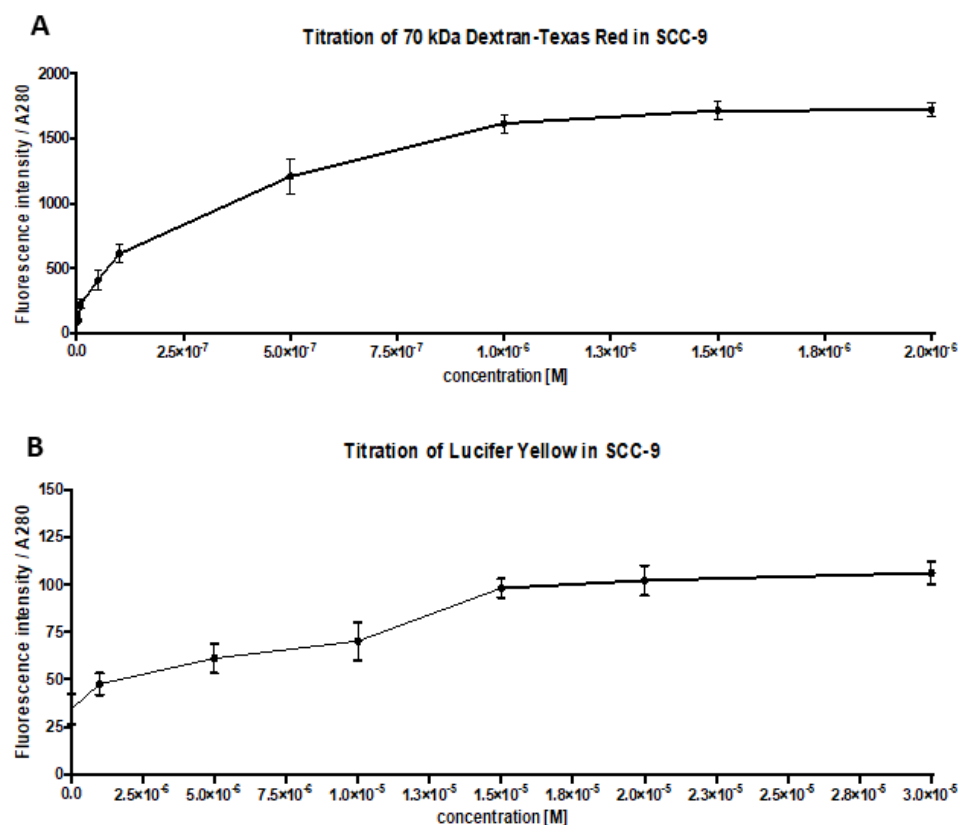


Figure 3.18. Concentration dependent uptake of endocytic markers

A – Concentration dependent uptake of 70 kDa Dextran-Texas Red in SCC-9 cells. Cells were seeded on a 6-well plate at 3×10^5 cells/well and serum starved for 16 h prior to the treatment, and then treated with a range of concentrations of 70 kDa Dextran-Texas Red for 1 h. Then, cells were washed twice with PBS and lysed. Fluorescence of the cell lysates was analysed at an excitation of 590 nm and emission of 615 nm. Fluorescence values were corrected for the absorbance at 280 nm. Results show the mean $\pm$ SD of three independent experiments. The saturation concentration was established to be 1 μ M. B – Concentration-dependent uptake of Lucifer Yellow in SCC-9 cells. Cells were seeded on a 6-well plate at 3×10^5 cells/well, serum starved for 16 h prior to the treatment, and then treated with a range of concentrations of Lucifer Yellow for 1 h. Next, cells were washed twice with PBS and lysed. Fluorescence of the cell lysates was analysed at an excitation of 425 nm and emission of 540 nm. Fluorescence values were corrected for the absorbance at 280 nm. Results show the mean $\pm$ SD of three independent experiments. The saturation concentration was established to be 15 μ M.

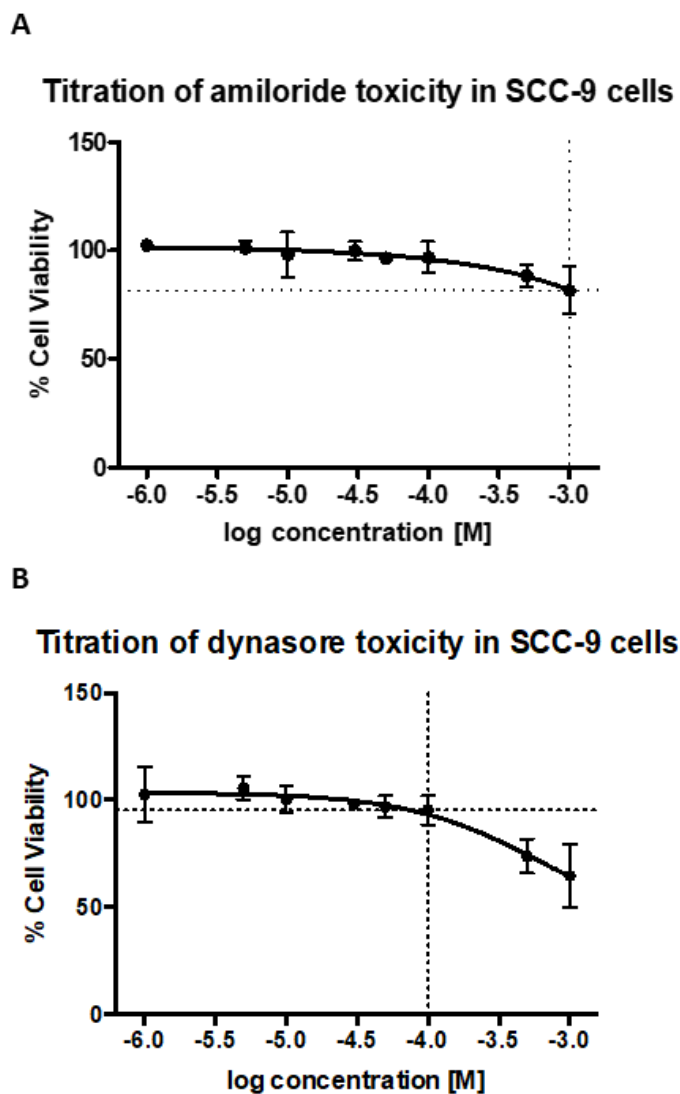


Figure 3.19. Toxicity evaluation of uptake inhibitors in SCC-9 cells

Viability of SCC-9 cells after exposure to A – amiloride, B – dynasore. Cells were seeded on a 96-well plate at the density of 5×10^3 cells/well and serum starved for 16 h. Then, cells were treated with a range of concentrations of uptake inhibitors for 2 h. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Values represent the mean $\pm$ SD of three independent experiments. Cell viability was above 80% at concentrations of 1 mM for amiloride and 100 μ M for dynasore, as indicated.

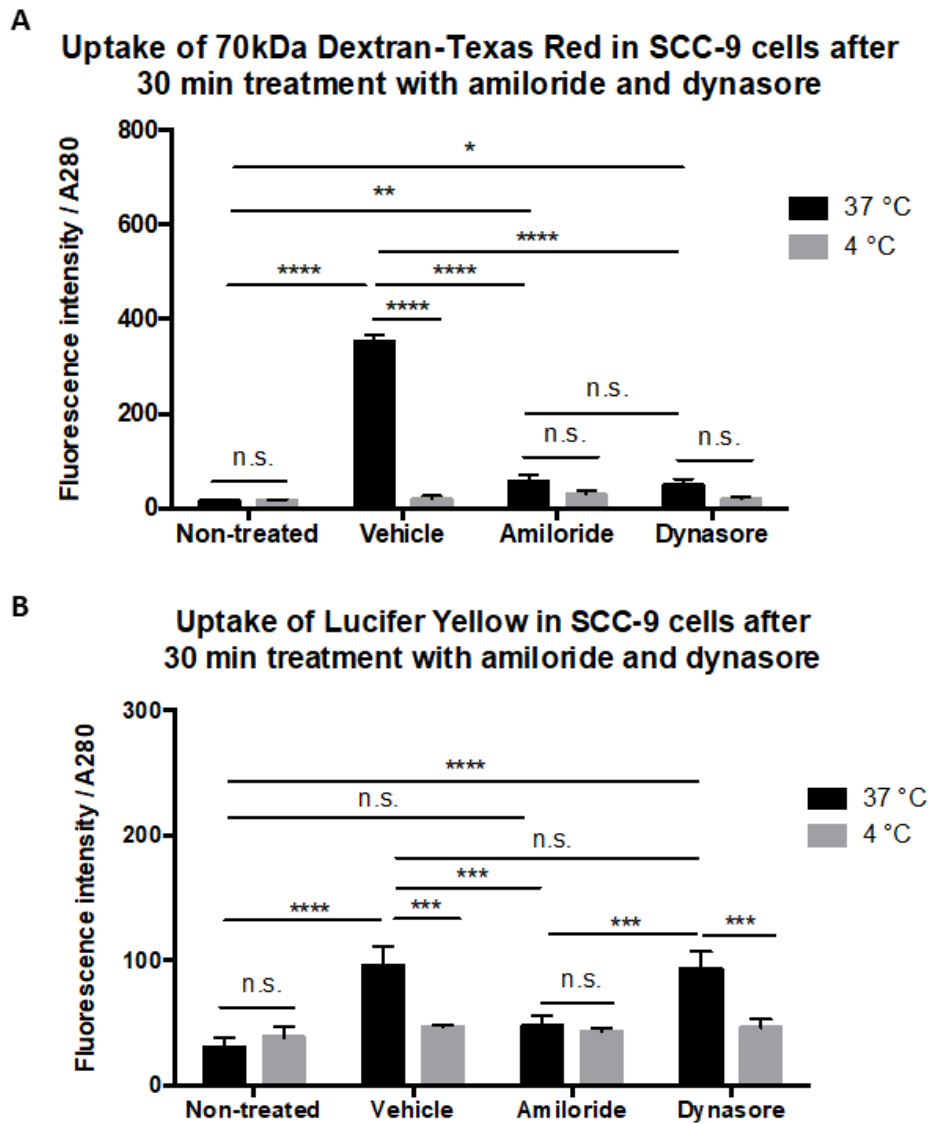


Figure 3.20. Inhibition of endocytosis pathways in SCC-9 cells

Cells were seeded on a 6-well plate at 3×10^5 cells/well and serum starved for 16 h prior to the treatment, and then treated with 1 mM Amiloride or 100 μ M Dynasore or 0.1% DMSO as vehicle for 30 min and then co-treated with A – 1 μ M 70 kDa Dextran-Texas Red, B – 15 μ M Lucifer Yellow for 1 h at 37 °C and 4 °C to distinguish uptake from unspecific binding to plasma membrane. Next, cells were washed twice with PBS and lysed. Fluorescence values were corrected for the absorbance at 280 nm. Results show the mean $\pm$ SD of three independent experiments. Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. – not significant.

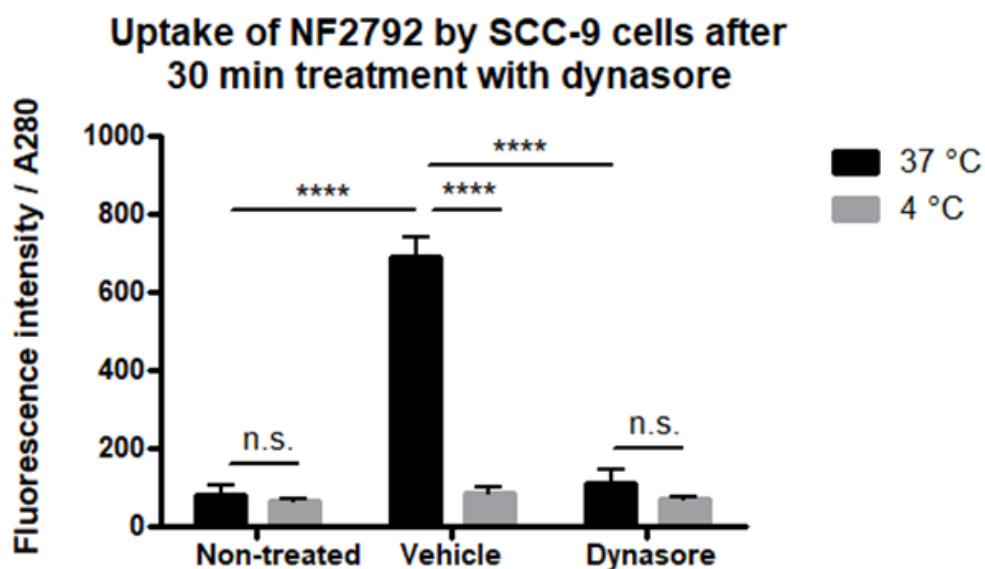


Figure 3.21. Inhibition of dynamin related endocytosis of NF2792 in SCC-9 cells

Cells were seeded on a 6-well plate at a density of 3×10^5 cells/well, and serum starved for 16 h. They were then treated with 100 μ M dynasore or 0.1 % DMSO as vehicle for 30 min and co-treated with 5 μ M NF2792 for 1 h at 37 °C and 4 °C to distinguish uptake from non-specific binding to plasma membrane. Then, cells were washed twice with PBS and lysed. Fluorescence of the cell lysates was analysed at an excitation of 325 nm and emission of 445 nm. Fluorescence values were corrected for the absorbance at 280 nm. Results show the mean $\pm$ SD of three independent experiments. Two-way ANOVA, **** $p < 0.0001$, n.s. – not significant.

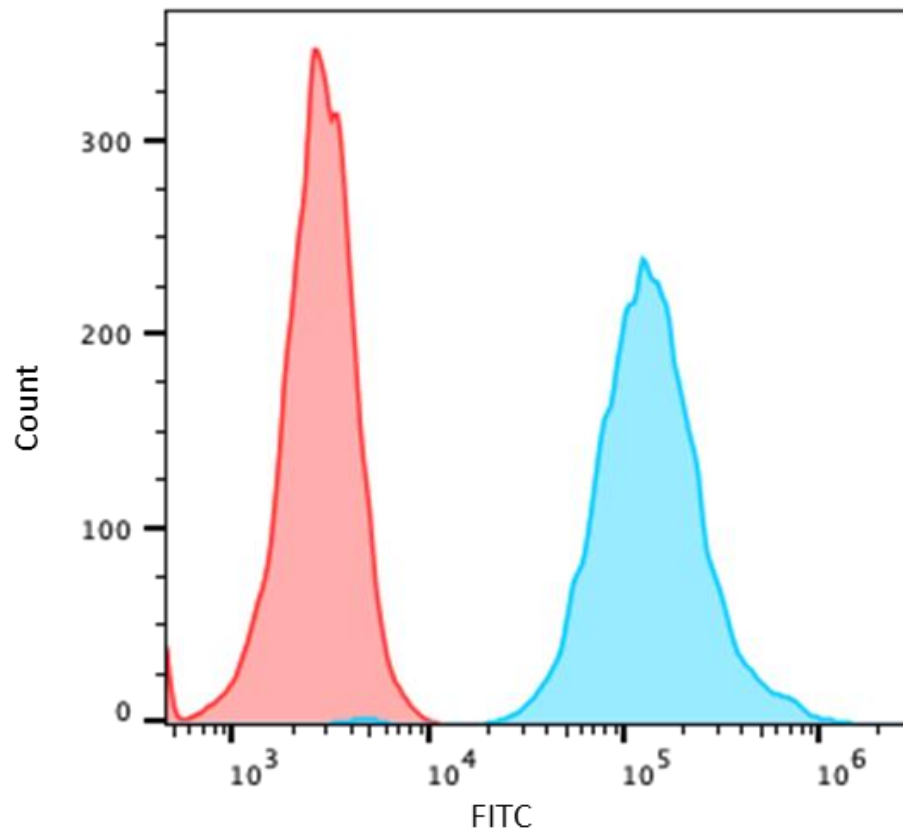


Figure 3.22. Expression of CD44 receptor in SCC-9 cells

A representative histogram of three independent experiments, showing the level of expression of CD44 in SCC-9 cells (blue) compared to isotype control (red). Cells grown in normal culture medium were washed and harvested. 1.5×10^5 cells were stained with either anti-CD44 antibody or isotype control and analysed by flow cytometry.

Titration of CD44 blocking antibody and NF2792 uptake in SCC-9

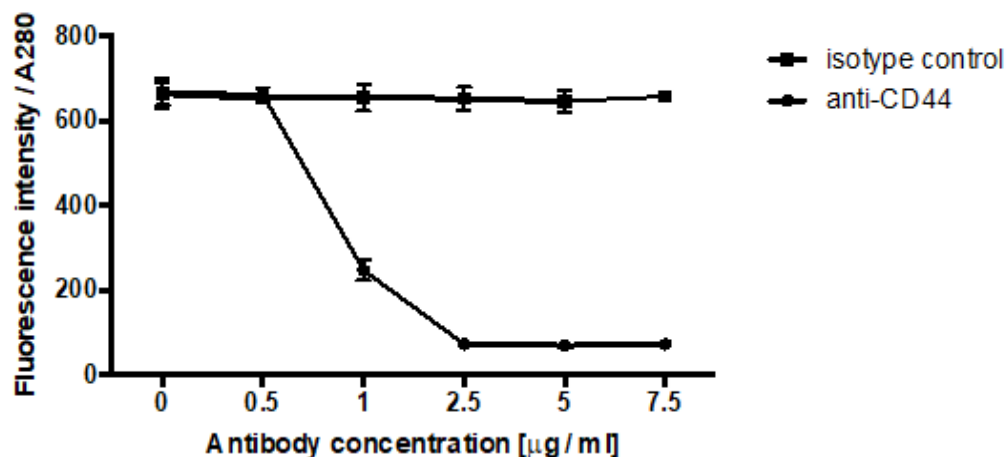


Figure 3.23. Titration of CD44 blocking antibody

Cells were seeded on a 6-well plate at a density of 3×10^5 cells/well, and serum starved for 16 h. Then, cells were treated with different concentrations of CD44 blocking antibody for 30 min and then co-treated with 5 μM NF2792 for 1 h. Next, cells were washed twice with PBS and lysed. Fluorescence of the cell lysates was analysed at 325 nm excitation and 445 nm emission. Fluorescence values were corrected for the absorbance at 280 nm. Results show the mean $\pm$ SD of three independent experiments. Complete inhibition of uptake was observed at ≥ 2.5 $\mu\text{g}/\text{ml}$ of CD44 blocking antibody.

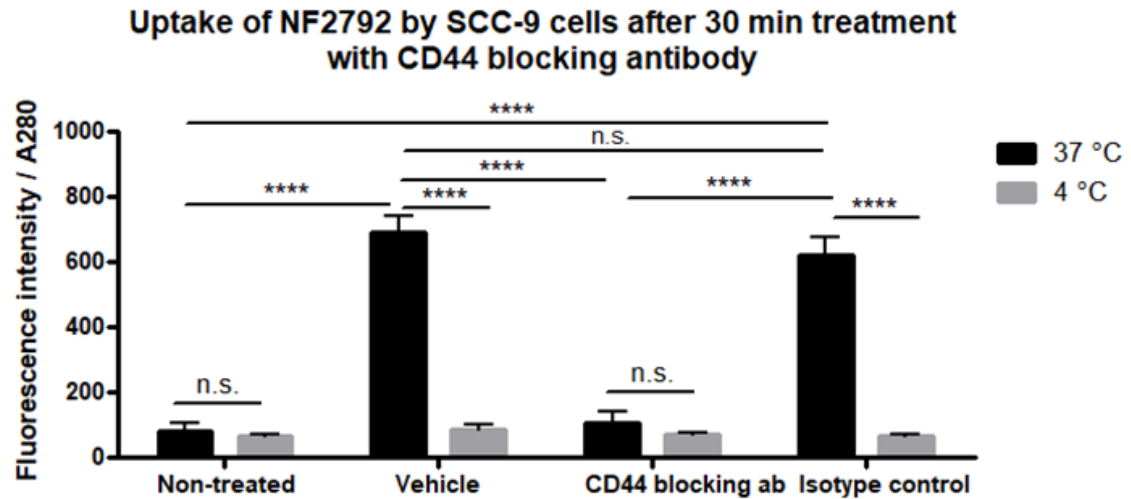


Figure 3.24. Inhibition of CD44 receptor mediated endocytosis of NF2792 in SCC-9 cells
Cells were seeded on a 6-well plate at a density of 3×10^5 cells/well, and serum starved for 16 h. They were then treated with 2.5 $\mu\text{g/ml}$ antiCD44 antibody for 30 min and co-treated with 5 μM NF2792 for 1 h at 37 °C and 4 °C to distinguish uptake from non-specific binding to plasma membrane. Subsequently, cells were washed twice with PBS and lysed. Fluorescence of the cell lysates was analysed at an excitation of 325 nm and emission of 445 nm. Fluorescence values were corrected for the absorbance at 280 nm. Results show the mean $\pm$ SD of three independent experiments. Two-way ANOVA, **** $p < 0.0001$, n.s. – not significant.

4. Impact of metabolic manipulation on the sensitivity of SCC-9 cells to BAMLET

4.1. Introduction

In order to preserve homeostasis and to ensure their survival, cells adapt to changes in external signals and growth conditions. Oftentimes, the cells and tissues of an organism can experience a shortage of nutrients during various states of disease, during a scarcity of food, or due to other circumstances such as ischaemia-reperfusion injury. It is understood that in solid tumours, where proliferation of the neoplastic mass is more rapid than neoangiogenesis, tumour cells are subject to a sharp decline in both nutrient and oxygen supply. The response to nutrient deprivation depends on the cell type and the favoured source of energy; however, typically, starvation triggers mobilization of energy stores and reduction of cell function, activity, and metabolism (Caro-Maldonado and Muñoz-Pinedo, 2011). Yet, in some cases cells are not able to overcome such adversity and activate a cell death response.

Cell culture media are typically designed to contain selected nutrients at specific concentrations. Sera contain a lot of proteins, amino acids, lipids, growth factors, etc, that are necessary to ensure healthy cell growth; however, their exact content is unknown and is believed to be batch specific and variable. Drug and compound toxicity tests must provide reproducible and trustworthy data; therefore, optimal experimental conditions have to be carefully prepared. It can be achieved by removing all unknown variables from the cellular medium, and as the formulation of the sera is unknown, it is common to treat cells in serum-free conditions (Pirkmajer and Chibalin, 2011). Thus, it is not surprising that there is a number of literature reports highlighting the necessity to perform HAMLET/BAMLET cytotoxicity assays in the absence of serum in the medium (Tolin *et al.*, 2010; Spolaore *et al.*, 2010; Frislev *et al.*, 2017; Nadeem *et al.*, 2019). Since HAMLET/BAMLET-like molecules are complexes of a partially unfolded protein and fatty acid, their effect could not be easily distinguished from other serum components in a cellular toxicity assay performed with serum in the medium. Moreover, the complexing of protein and fatty acid can be reversed thus inactivating the molecules by the presence of calcium ions (Hoque *et al.*, 2015; Rath *et al.*, 2018) or albumin in the medium (Frislev *et al.*, 2017).

Serum starvation is a method commonly employed in toxicity studies to ensure homogenous, reproducible data. It is believed to induce cell cycle arrest by leading the cells to synchronise in a quiescent G_0/G_1 state, thus synchronising the proliferating cell population (Chen *et al.*,

2012; Kim *et al.*, 2016). This in turn ensures a uniform response to the treatment, eliminating the possible cell cycle phase specific response. Moreover, it is generally applied before performing an assay in serum-free conditions to prepare the cells for the change in environment and to avoid stress response obscuring the results. This step seems necessary in light of reports of changes in cell metabolism and activity under serum starvation, such as an increased lactate secretion in fibroblasts (Golpour *et al.*, 2014) or an increased insulin action in L6 myoblasts (Ching *et al.*, 2010). However, serum starvation has been reported to trigger apoptosis in some cell types, such as luteal, endothelial, and tongue squamous epithelial cells (Goyeneche *et al.*, 2006; Gama Sosa *et al.*, 2016; Huang *et al.*, 2018), while no such effect has been observed in others, such as HFF fibroblasts, primary human dermal fibroblasts, or adipose stem cells (Gilroy *et al.*, 2001; Chen *et al.*, 2012). Hence, establishing a valid serum starvation protocol for the cell line under investigation and defining its impact on cellular homeostasis is vital to a successful cell cytotoxicity study.

HAMLET and HAMLET-like complexes have proven to be effective against cancer cells derived from tumours of varying origins. The absence of reported side effects of HAMLET has been attributed to its components being natural molecules, present in our daily diet. OA has been reported to be the tumouricidal component of HAMLET (Ho *et al.*, 2013). It is one of the most common fatty acids occurring naturally in various animal and vegetable fats and oils. It is also an important component of the cell membrane. α -Lactalbumin is a milk protein found in most lactating mammals and its function is to help regulate lactose synthesis (Qasba *et al.*, 2008; Chaudhuri *et al.*, 2016).

HAMLET has previously been shown to selectively kill cancer cells and spare proximal differentiated cells (Svanborg *et al.*, 2003; Gustafsson *et al.*, 2005; Mossberg *et al.*, 2010; Puthia *et al.*, 2014). *In vitro* studies performed by various research groups show that susceptibility to HAMLET arises from the immortalization of non-cancer cell lines (Rath *et al.*, 2015). It suggests that HAMLET could be targeting a common characteristic shared by all immortalized cells. Generally, primary cells in culture show greater resistance, in agreement with the above hypothesis; however, primary embryonic and blood cells displayed significant sensitivity (Rath *et al.*, 2015). The exact mechanism of action of HAMLET and HAMLET-like molecules is unknown. However, there have been reports linking it to the metabolic state of the cells (Storm *et al.*, 2011; Ho *et al.*, 2013; Puthia *et al.*, 2014). Since an obvious difference be-

tween cancer and normal cells is their metabolic activity, the selective potential of HAMLET/BAMLET complexes may be attributable to this differential cell property.

ATRA is the main active metabolite of vitamin A. It plays an important role in embryonic development, as well as in the processes of cell growth, proliferation, development and metabolism (Zhu *et al.*, 2015). ATRA and its derivatives, known collectively as retinoids, are ligands of nuclear retinoic acid receptors (RARs), binding ATRA with high affinity, and retinoid X receptors (RXRs) binding other, naturally occurring isomers (Evans, 1988; Chambon, 1996). In order to achieve biological activity, a RAR-RXR heterodimer must be formed, which in turn activates gene promoters and induces ATRA-mediated signalling (Costantini *et al.*, 2020).

ATRA is a widely used differentiation agent that has also been reported to exhibit anticancer properties. Through transcriptional changes in gene expression, it is able to successfully encourage terminal differentiation of cancer cells and, in turn, inhibit their proliferation and growth, induce apoptosis, alter metabolism and reduce metastatic transformation. These effects have been observed in various cancers such as non-small cell lung carcinoma (Lokshin *et al.*, 1999), acute promyelocytic leukaemia (Huang *et al.*, 1988; Castaigne *et al.*, 1990), oral cancer (Hayashi *et al.*, 2001), osteosarcoma (Luo *et al.*, 2010), hepatic progenitor cells (Hu *et al.*, 2019), hepatocellular carcinoma cells (Cui *et al.*, 2016), and squamous cell carcinomas (Beere and Hickman, 1993). There are reports on clinical trials using retinoic acid as the sole active agent on solid tumours of the lung, stomach, breast, liver, and colon cancers (Costantini *et al.*, 2020). Moreover, ATRA has been reported to induce differentiation in normal cells as well (Hansen *et al.*, 2000). Studies on various cell types suggest ATRA can modulate metabolic function during differentiation (Hu *et al.*, 2019; Terao *et al.*, 2019) driving the remodelling of the cancer cell phenotype.

The metabolism of cancer cells either supports survival in a hostile environment or can allow for growth and proliferation at pathological levels driven by changes in growth signalling pathways (e.g. AKT), oncogenes (e.g. MYC), tumour suppressor genes (e.g. p53) and activation to hypoxic conditions (e.g. HIF1 α) (DeBerardinis and Chandel, 2016). Due to increased glycolytic flux, the glycolytic intermediates are used for biosynthesis. TCA cycle intermediates are also utilized in biosynthesis. Coupled to these changes in glycolysis, glutaminolysis

and pyruvate carboxylation provide anaplerotic flux to replenish the TCA cycle (Hsu and Sabatini, 2008). Cancer cells grow in a competitive, nutrient-depleted environment among large numbers of other cells, and therefore, utilize many fuels and nutrients for survival (Vander Heiden and DeBerardis, 2017). Differentiated cells on the other hand do not need to alter and enhance their metabolism and do not have a need for large biomass synthesis due to their comparatively lower rate of proliferation (van Horssen *et al.*, 2013) (figure 4.1).

Despite being used as a standalone anticancer therapeutic, ATRA provides an interesting opportunity to alter the gene expression and reduce the invasive potential of cancer cells as a differentiation-inducing pre-treatment to BAMLET administration. Establishing the link between differentiation driven changes in metabolism and HAMLET/BAMLET sensitivity may help elucidate the mechanistic underpinning for its tumour selectivity.

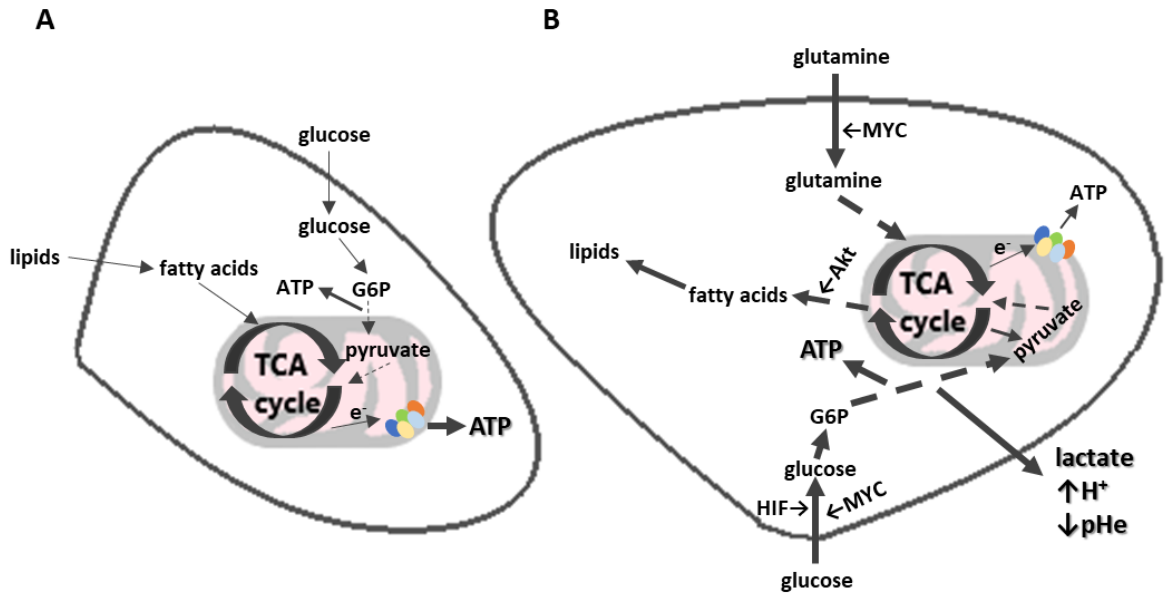


Figure 4.1. Metabolism of differentiated and non-differentiated cancer cells

A – Differentiated cell metabolism: glucose is taken up and metabolised in both glycolysis and oxidative phosphorylation to produce ATP. Lipids are also taken up and incorporated into the TCA cycle. B – Cancer cell metabolism: glycolysis is upregulated compared to the differentiated cell and is the main source of energy. This results in the production of large quantities of lactate that are excreted from the cell and promoting the acidification the extracellular environment. Oxidative phosphorylation plays a secondary role in ATP production. Glutamine is used to resupply the TCA cycle in a process called glutaminolysis. Pyruvate carboxylation is another anaplerotic process that supplies the TCA cycle.

Mitochondria are key players in cellular energy metabolism, and their functionality is important to preserve homeostasis within the cell. By performing oxidative phosphorylation (OXPHOS), they produce the majority of the ATP needed for the cell to survive, grow, proliferate, and perform its functions. Impaired mitochondrial activity has been observed in metabolic diseases such as diabetes (Wang *et al.*, 2010; Cheng and Ristow, 2013; Busiello *et al.*, 2015). Otto Warburg, upon formulating his Warburg effect hypothesis, suggested that increased aerobic glycolytic rate in cancer cells may be caused by dysfunctional mitochondria (Warburg, 1956). Later, it has been suggested that it is the high rates of glycolysis in rapidly proliferating cells, including cancer cells, that have an inhibitory effect on OXPHOS, rather than a mitochondrial defect, a hypothesis known as the Crabtree effect (Crabtree, 1929). Recently, it has been reported that the Crabtree effect may arise from the competition between OXPHOS and glycolysis for ADP and inorganic phosphate (Baffy, 2017).

Mitochondrial uncoupling is a state whereby the ATP synthesis through the electron transport chain is disturbed (Cadenas, 2018). In the course of OXPHOS, mitochondrial complexes I, III, and IV pump protons into the mitochondrial intermembrane space to generate a membrane potential. These return to the mitochondrial matrix in accordance with their concentration and pH gradient driving ATP synthesis *via* the activity of the F_1F_0 ATP synthase. Protons can, however, return to the matrix through an ATP synthase independent pathway, thus making the system not completely coupled. When the ATP synthase dependent flow of protons is disturbed in a process called proton leak, the energy needed to produce ATP is dissipated before the ATP synthase can use it. Therefore, when mitochondria are uncoupled, ATP production stalls, generating heat instead (Alasadi *et al.*, 2018; Cadenas, 2018).

Mitochondrial uncoupling is a process happening in nature, facilitated by the uncoupling proteins. However, several reports indicate that chemical induction of mitochondrial uncoupling and induction of proton leak is beneficial to cells in that it reduces the production of reactive oxygen species (ROS), a phenomenon which was observed in brain, skeletal muscle, and heart (MacLellan *et al.*, 2005; Tahara *et al.*, 2009; Toime and Brand, 2010). However, when uncoupled, cells are deprived of the ATP produced by OXPHOS. To overcome this obstacle, cells must therefore resort to an alternative energy supply pathway, thus remodelling their energy metabolism. Since cancer cells have large energy needs, it is interesting to investigate the ef-

fect of constant treatment with sublethal concentrations of chemical uncouplers and the impact it might have on BAMLET sensitivity.

It is established that cancer cells have increased energy and nutrient demands. Their rapid rate of proliferation requires large amounts of ATP production and a powerful metabolism, but also a constant, high production rate of membrane components to maintain the pace of growth. For that reason, cancer cells are generally characterised by an increased demand for fatty acids. A rise in *de novo* lipid production is often observed in such cells in comparison to normal cells, excluding the lipogenic tissues, such as liver or adipose tissue (Menendez and Lupu, 2007; Li and Cheng, 2014). Lipids play an important role in cell signalling, and fatty acid oxidation generates energy and produces many molecules vital for cell growth and homeostasis (Corbet and Feron, 2017). Moreover, in case of increased oxidative stress, the surge in fatty acid synthesis allows the cells to modify the composition of the membranes to contain ROS resistant saturated fatty acids (Rysman *et al.*, 2010; Pavlova and Thompson, 2016), illustrating one of the many adaptive mechanisms in cancer cells. Understandably, the enzymes participating in *de novo* fatty acid synthesis have been selected as targets in anticancer therapies (Vander Heiden *et al.*, 2011; Goswami and Sharma-Walia, 2016). However, cancer cells do not only rely on *de novo* synthesis, since lipids can also be transported into the cell from the exterior. Fatty acid uptake is a tightly regulated process, that is particularly important to the cancer cells in times of metabolic stress or hypoxia (DeBerardinis and Chandel, 2016).

FASN, is an enzyme responsible for the *de novo* production of long chain saturated fatty acids, mostly palmitate and, in a lower ratio, myristate and stearate from acetyl-CoA and malonyl-CoA (Jump, 2009). FASN was found to be upregulated in various cancers (Pavlova and Thompson, 2016) and suggested as a prognostic marker in aggressive breast cancers (Kuhajda *et al.*, 1994; Vazquez *et al.*, 2016). Several studies observed that inhibition of FASN can have anticancer properties *in vitro* and *in vivo*, further accentuating the importance of this enzyme in cancer cells (Bentebibel *et al.*, 2006; Goswami and Sharma-Walia, 2016). As it is an upstream enzyme for various elongases and desaturases - enzymes producing longer chain and mono- and polyunsaturated fatty acids (Jump, 2009), the inhibition of FASN deprives cells of a wider range of fatty acids that are normally utilised by the cell in a number of processes. It is understood that cells have to upregulate the uptake of extracellular fatty acids in order to survive the shortage and to supply enough lipids for membrane synthesis, fatty acid oxidation,

and lipid signalling. Since HAMLET contains several molecules of OA complexed to an α -lactalbumin molecule, it was speculated that inhibition of FASN would induce an increased uptake of HAMLET to fulfil the cellular demand for lipids, which would in turn sensitize the cells further. It is particularly interesting to test the chemical inhibitors of FASN in the setting with HAMLET as they were previously mentioned to have anticancer properties themselves (Bentebibel *et al.*, 2006; Goswami and Sharma-Walia, 2016).

An alternative method of modulating metabolism is to alter the supply of energy substrates. Serum starvation mentioned above is only one of the possible nutrient restrictions. Most cells rely on glucose as the main energy source to fulfil their energy needs and fuel their metabolism. Furthermore, cancer cells have been reported to heavily rely on glycolysis and high glucose utilisation (Pavlova and Thompson, 2016). Mainly in solid tumours, the cells experience a shortage of nutrients due to a lack of supply, as the tumour growth is usually more rapid than neoangiogenesis (DeBerardinis and Chandel, 2016). Therefore, the supply of glucose is much lower, and the cells must reorganise their metabolism to adapt to the environment, as glycolysis is no longer a readily available option.

To enact this kind of situation *in vitro*, one of the possible scenarios is glucose starvation. However, by completely depriving the cells of carbohydrates, their growth and homeostasis would be jeopardised. Therefore, the glucose in the medium can be replaced with a different carbohydrate that would be also not readily available for glycolysis. Substituting glucose in the media with galactose should enhance OXPHOS, since galactose does not provide a net gain of ATP following glycolytic consumption (i.e. glucose provides a positive yield of 2 ATP molecules per unit consumed by the cell). This ensures that the cell relies exclusively on the mitochondria for ATP generation and results in the cell shifting metabolism to an aerobic state (Gohil *et al.*, 2010) (figure 4.2).

The mode of introduction of such change is of vital importance. Short glucose deprivation could be overcome by energy stores or result in an acute stress response. To fully examine the effect of metabolic changes on HAMLET sensitivity and understand the underlying causes, a careful and methodical approach is necessary. A gradual decrease of glucose concentration with simultaneous increase of galactose content in the medium, ensures that the cells have enough time to respond to the change in their environment and remodel their metabolism to

meet their needs. This way, the metabolic differences are stable and reproducible. Observing how such a pronounced metabolic change would affect the sensitivity of cancer cells to HAMLET/BAMLET could provide an invaluable insight into HAMLET's mode of action and mode of entry into the cell.

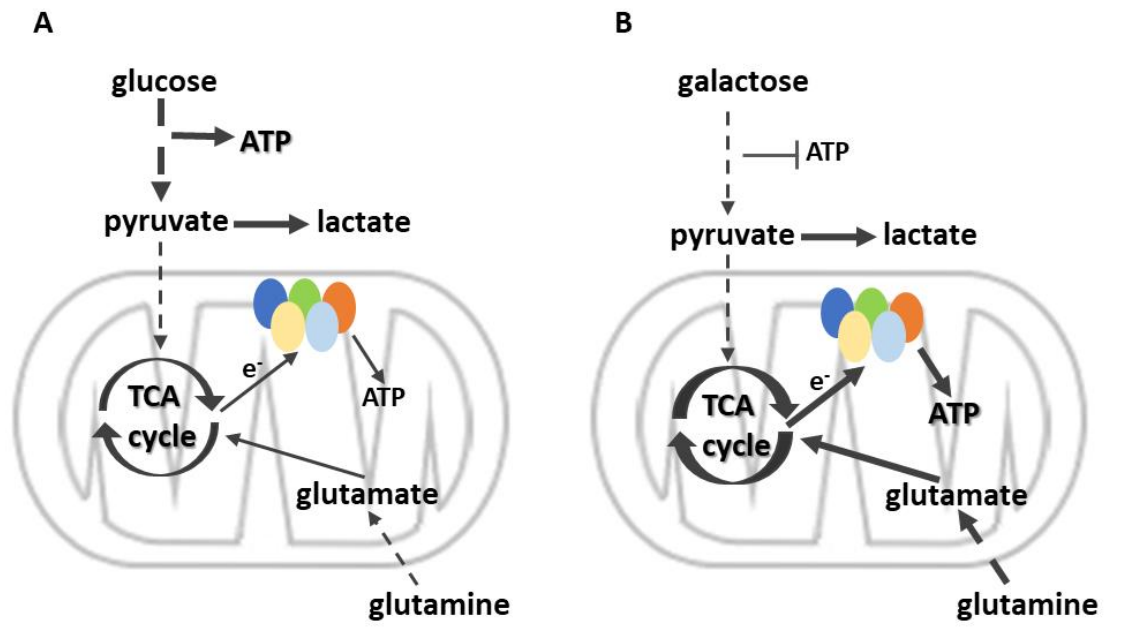


Figure 4.2. Glucose and galactose metabolism

A – In the presence of glucose, the cell gets its energy from both glycolysis (turning glucose into pyruvate) and oxidative phosphorylation. B – In the case of substitution of glucose with galactose, the transformation of galactose into pyruvate is not energetically profitable, and the cell shifts its metabolism to rely mostly on oxidative phosphorylation (ETC – electron transport chain) for producing the ATP.

4.2. Results

4.2.1. Effect of serum starvation on BAMLET sensitivity

It is established that HAMLET/BAMLET complexes cannot come in contact with serum without a decrease in activity and most studies understandably recommend performing the assays in a serum-free medium. Therefore, 0 % FBS medium has been elected as the experimental condition to be adhered to during the treatment period. As such a rapid decrease in serum content in the culture medium would likely result in a stress response from the cells, an acclimatization period has been decided upon. A 16 h serum starvation period has been adopted, as an incubation period that would allow the cells to adjust to the new growth conditions, induce the cell cycle arrest but not trigger an apoptotic response in the starved cells, as could occur after a longer period of starvation.

Initially, three cell lines have been selected for the study to investigate the sensitivity of different cancer cell types to BAMLET. A squamous cell carcinoma of the tongue cell line, SCC-9, has been used as an *in vitro* model of oral cancer. KYSE-520, an oesophageal squamous cell carcinoma cell line, has been chosen as a representative of oesophageal cancer. An osteosarcoma cell line, U2OS, has been chosen as a control cell line, due to its previous appearance in HAMLET related studies (Xie *et al.*, 2012).

As expected, 16 h of serum starvation did not induce cell death in the tested cell lines (figure 4.3). All three cell lines have been incubated in 0 % FBS medium for 16 h and tested for the possible induction of an apoptotic response with Annexin V/PI double staining. Upon examination by flow cytometry, no significant increase in apoptotic cells was observed in comparison to control cells, whereas a cisplatin treated comparative sample displayed a significantly increased apoptotic response. Moreover, the adopted serum starvation protocol induced a significant increase in the proportion of cells in the G₀/G₁ phase and a simultaneous decrease in cells in S and G₂ phases of the cell cycle (figure 4.4). To that end, the proposed serum starvation protocol has been adopted for subsequent cytotoxicity assays.

In the next step, all three tested cell lines were examined as per their sensitivity to BAMLET with and without previous serum starvation. As seen in figure 4.5, in the case of each of the investigated cell lines, serum starvation significantly increased cell sensitivity to BAMLET thus decreasing the IC_{50} values. The largest decrease has been observed in U2OS cell line which displayed a mean IC_{50} value decline from 7.2 μM in cells without serum starvation prior to BAMLET treatment to 3 μM with prior 16 h in 0 % FBS incubation, demonstrating a ~2.4-fold rise in sensitivity. In KYSE-520 cells, the least sensitive of the three, the mean IC_{50} values after serum starvation decreased from 20 μM to 8.8 μM , displaying a ~2.3-fold increase in sensitivity. The SCC-9 cells exhibited a ~1.7-fold increase in sensitivity, with the mean IC_{50} value dropping from 2.5 μM to 1.5 μM in cells that were serum starved prior to BAMLET treatment. The observed increase in sensitivity could be explained by the homogeneity of the cell population in serum starved cells, which could result in a more uniform response to BAMLET treatment. Alternatively, the increase in sensitivity to BAMLET could be driven by enhanced uptake of the constituent parts of BAMLET, as cancer cells have been reported to take up large quantities of fatty acids and albumin to fuel their metabolism. Serum depleting conditions could enhance the uptake of BAMLET due to its natural origin.

It is unknown why SCC-9 cells showed the highest sensitivity to BAMLET among the three cell lines examined (figure 4.6). There are reports linking the cellular response to HAMLET-like complexes to the cell type, and the differentiation status of the cell. It is speculated, that due to some unknown metabolic characteristic of SCC-9 cells, their sensitivity to BAMLET is the highest, and for this reason this cell line has been chosen for further detailed studies on metabolism and BAMLET cytotoxicity.

4.2.2. Effect of induced differentiation of SCC-9 cells by *all-trans* retinoic acid on BAMLET sensitivity

As mentioned above, HAMLET-like complexes exhibit different levels of cytotoxicity in cancer and immortalized cells relative to normal differentiated somatic cells (Ho *et al.*, 2012). It has also been proposed that the immortalization of normal cells influences HAMLET sensitivity (Rath *et al.*, 2015). Other studies revealed that differentiation influences HAMLET and

BAMLET susceptibility in PC12 cells (Lišková *et al.*, 2011; Harte *et al.*, 2015). Therefore, this project endeavoured to investigate this phenomenon in SCC-9 cells.

4.2.2.1. Differentiation of SCC-9 cells

Interestingly, ATRA is reported to modulate the growth and differentiation of epithelial cells *in vivo* (Lippman *et al.*, 1987) suggesting that the SCC-9 cell line would be an interesting target for ATRA treatment.

Cytokeratins are a large group of filamentous proteins that are expressed in epithelial cells. They are divided into type I acidic (CK9-28) and type II basic (CK1-8 and CK71-74) cytokeratins (Schweizer *et al.*, 2006) that constitute obligatory heteropolymers consisting of one type I and one type II cytokeratin assembled in 1:1 molar ratio (Herrmann and Aebi, 2004). Expression of cytokeratins differs among epithelial cells, with stratified squamous epithelium expressing cytokeratins 5 and 14 (Nelson and Sun, 1983). Their levels decrease with the progress of differentiation (Alam *et al.*, 2011).

To establish the optimal conditions for ATRA mediated induction of differentiation, the SCC-9 cells were exposed to various concentrations of ATRA for 5 days, and the expression levels of cytokeratins 5 and 14 were assessed by Western blot. The expression of both cytokeratin 5 and cytokeratin 14 was downregulated after 5 days of 30 μ M ATRA treatment indicating a transcriptional remodelling and a change of phenotype of the cells (figure 4.7). As mentioned above, the downregulation of the pair of cytokeratins 5 and 14 is a marker of differentiation of stratified epithelial cells, confirming that the ATRA treatment has indeed succeeded in inducing the differentiation of SCC-9 cells.

To further confirm the differentiated state of the cells after adopting this ATRA treatment protocol, proliferation assays were performed. As the proliferation rate of differentiated cells is generally lower than cancer, stem, or undifferentiated cells, it has been expected that SCC-9 cells after ATRA treatment will exhibit a deceleration of cell division rate. Therefore, the non-differentiated control SCC-9 cells and SCC-9 cells treated with 30 μ M ATRA for 5 days were plated and incubated in full medium for 24 h and 48 h to assess their proliferation rates. Ala-

mar blue viability assay confirmed lower cell activity after 24 h and 48 h incubation period in ATRA-induced cells in comparison to control non-differentiated cells (figure 4.8.A). However, due to the nature of the fluorophore present in the Alamar blue assay and its need to be turned over by the cellular machinery, the change in its fluorescence could be assigned to either a change in cell number or in cell metabolic activity. To further establish the proliferation rate status, SRB protein quantification dye was employed to examine cell numbers in differentiated versus non-differentiated cells after 24 h and 48 h growth periods. It displayed a significant decrease in the proliferation rate of the ATRA-induced SCC-9 cells after 48 h incubation comparing to the non-differentiated control cells (figure 4.8.B), with no significant differences after 24 h incubation. Thus, these results confirmed a decreased proliferation rate in cells treated with 30 μ M ATRA for 5 days, further suggesting that the applied protocol is successful. To that end, based on the differentiation marker expression profile being in accordance with the previously published reports, and the cell proliferation rate data demonstrating a significant decrease, the 5-day 30 μ M ATRA treatment was adopted as the protocol to induce differentiation in SCC-9 cells and applied henceforth in the project.

4.2.2.2. BAMLET sensitivity of SCC-9 cells after *all-trans* retinoic acid induced differentiation

After establishing the differentiation protocol for SCC-9 cells, the impact of differentiation on BAMLET sensitivity was investigated. In agreement with the previously published data, ATRA-induced differentiation of SCC-9 cells resulted in a significant decrease in their sensitivity to BAMLET versus non-differentiated cells (figure 4.9.A). The IC₅₀ value of SCC-9 cells differentiated through ATRA induction was 2-fold higher (2 μ M) than in the case of control non-differentiated SCC-9 cells (1 μ M) (figure 4.9.B), supporting the notion that differentiation status of the cell influences its susceptibility to BAMLET. This raises the question as to why and how differentiation may impact BAMLET sensitivity. It is understood that ATRA signaling affects multiple cellular processes through induction of differentiation and maturation of the cell. Inhibition of proliferation, modulation of expression patterns, and metabolic modifications are fundamental changes to the cell achieved through ATRA stimulation (Liu *et al.*, 2005; Mercader *et al.*, 2007; Lu *et al.*, 2008; Orfali *et al.*, 2020). ATRA treatment has been reported to induce cell death in multiple cancer cell lines (Hu *et al.*, 2019; Costantini *et al.*,

2020) indicating that the treatment prohibits cancer cells from evading apoptosis. The questions how this cellular remodelling affects BAMLET sensitivity and whether BAMLET susceptibility fluctuations are dependent on the metabolic state of the cell remain open.

4.2.2.3. Impact of *all-trans* retinoic acid induced differentiation on metabolism of SCC-9 cells

It has been reported that ATRA treatment stimulates oxidative metabolism in various tissues, such as liver, skeletal muscle and white adipose tissue (Ribot *et al.*, 2019). To assess the impact of ATRA induced differentiation on the metabolic state of SCC-9 cells, their oxygen consumption and extracellular acidification rates were studied.

The mitochondrial activity and oxygen consumption were initially studied by high resolution respirometry, whereby the SCC-9 cells were treated with various stimulants and inhibitors of the mitochondrial electron transport chain to investigate the state of their mitochondria and the rate at which they perform OXPHOS. During the course of the experiment, the cells were injected into the chambers of the Oxygraph, to determine the basic mitochondrial respiratory rate. There, they were stirred in suspension at a constant rate in a glucose-deprived medium, in which they mostly use the endogenous substrates for respiration. Next, oligomycin was injected, to block the ATP synthase and prevent the OXPHOS of ADP into ATP. However, it does not stop the flow of protons completely, as some can still diffuse into the mitochondrial matrix in a process called the proton leak. Subsequent addition of CCCP boosted the flow of protons. CCCP is a so-called mitochondrial uncoupler – an ion carrier that transports the protons through the mitochondrial membrane at a high rate. Upon multiple titrations of CCCP, the mitochondria reach the maximal capacity of the Electron Transport Chain. Rotenone is an inhibitor of the mitochondrial complex I and its addition decreases the mitochondrial activity. Antimycin A inhibits the mitochondrial complex III, effectively stopping the mitochondrial activity altogether (figure 4.10).

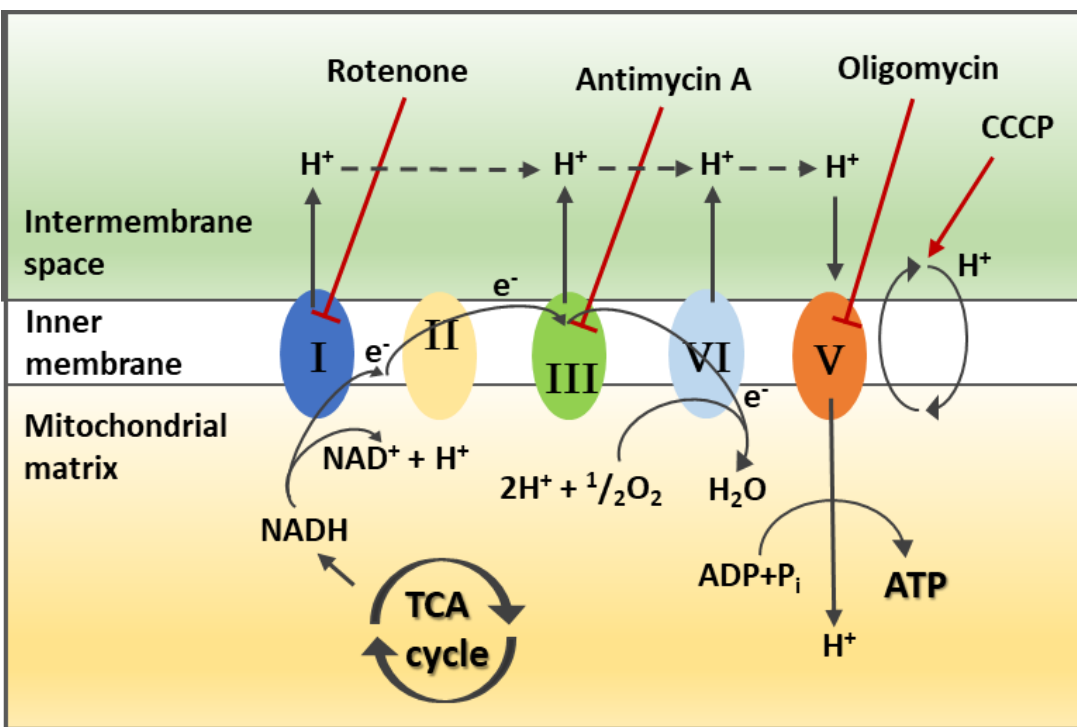


Figure 4.10. A schematic representation of the structure of a mitochondrion and the mitochondrial oxidative phosphorylation system showing places of action of the inhibitors and stimulants added during high resolution respirometry experiments

Oligomycin inhibits ATP and measures proton leak. CCCP uncouples the mitochondria and shows the maximal capacity of the electron transport chain. Rotenone is an inhibitor of mitochondrial complex I, and Antimycin A inhibits the mitochondrial complex III.

Interestingly, contrary to the expected results, high resolution respirometry revealed no significant differences in oxygen consumption rate between ATRA differentiated and non-differentiated SCC-9 cells (figure 4.11). The Oxygraph allows for a two-point calibration, thus eliminating a lot of background noise and accounting for a sensitive, stable signal of oxygen flow rate. However, as the OROBOROS machine analyses cells in suspension, the adherent cells used in this study are not in their optimal state, and therefore may exhibit different respiration patterns than under normal culture conditions. Therefore, this data has been further confirmed by an oxygen consumption rate study using the Seahorse Metabolic Flux Analyser, whereby cells were grown in a monolayer in 96-well plates, allowing for the optimal culture conditions of adherent cells to be maintained.

The cells were cultured in their typical culture medium, without addition of glutamine or sodium pyruvate; however, glucose was available in the medium. The basal level of oxygen consumption was measured initially, next the plates were treated with sequential injections of oligomycin to measure the oxygen consumption during mitochondrial leak conditions, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), a mitochondrial uncoupler to observe the maximal capacity of the mitochondria of SCC-9 cells, and then rotenone and antimycin A to measure the oxygen consumption rate after the inhibition of mitochondrial complex I and III (figure 4.12).

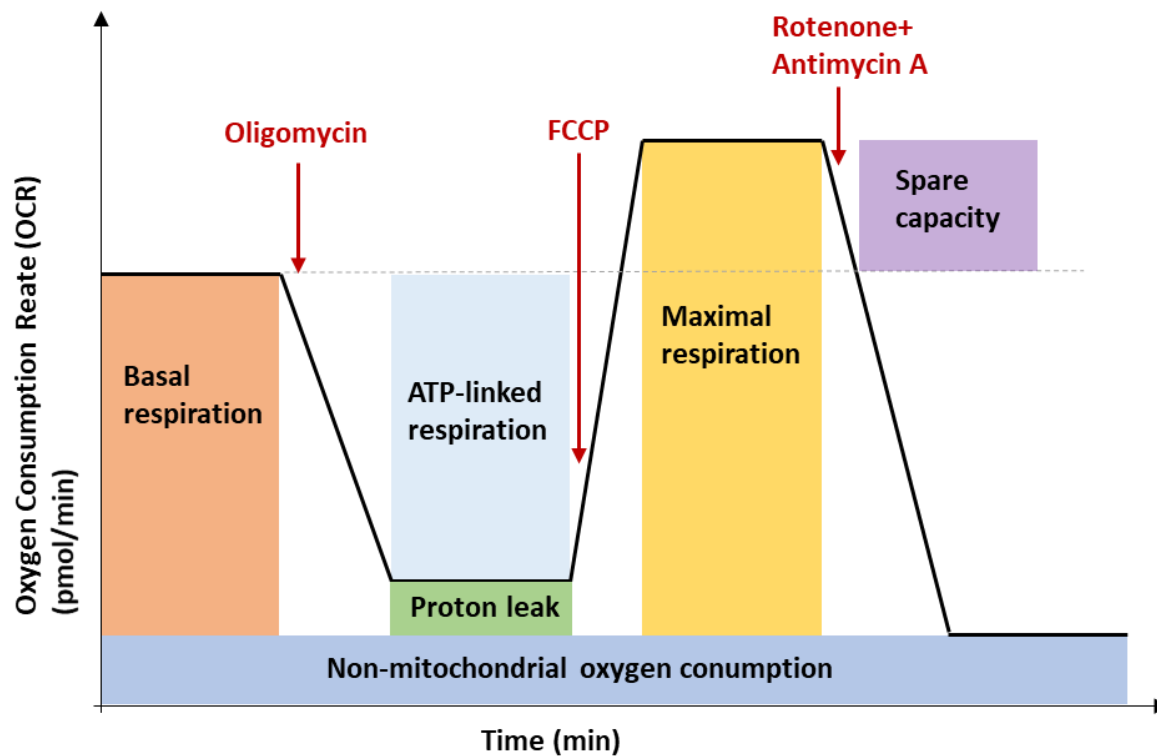


Figure 4.12. Mitochondrial stress test protocol schematic

Oligomycin inhibits ATP and allows measurement of proton leak. FCCP uncouples the mitochondria and reveals the maximal capacity of the electron transport chain. Rotenone is an inhibitor of mitochondrial complex I, and Antimycin A inhibits the mitochondrial complex III to show the non-mitochondrial oxygen consumption.

The Seahorse Metabolic Flux Analyser allows for measurement of oxygen consumption rate (OCR) of adherent cells in conditions as close as possible to the typical cell culture. The measurements also revealed no significant difference between basal and stimulated OXPHOS between ATRA differentiated and non-differentiated SCC-9 cells (figure 4.13.A and figure 4.14.A). These results confirm that ATRA-induced differentiation does not influence mitochondrial activity and oxidative metabolism in SCC-9 cells.

Subsequently, the extracellular acidification rate (ECAR) of ATRA-differentiated and non-differentiated cells was examined by the Seahorse Metabolic Flux Analyser. ECAR is assumed to be the measure of glycolysis rate, as by-products of glycolysis (namely lactate) excreted from the cells are largely responsible for increase of acidity of the culture medium.

As mentioned above, the Seahorse Metabolic Flux Analyser allows for assessing the oxygen consumption rate by measuring the oxygen concentration decrease over time in the medium above a layer of cells in a culture plate. However, the fluorescent probes in the Seahorse cartridges have the ability to simultaneously measure the pH of the medium as well. By setting the pH of the medium at 7.4 before the experiment and careful measurement of pH fluctuations in the medium during the experiment, the extracellular acidification rate can be assessed. Cells are cultured in their respective medium without any exogenous substrates to measure the non-glycolytic acidification rate. Then the main energy substrate, such as glucose is added, and glycolysis rate is measured. Next, oligomycin is injected into the wells to measure the glycolytic capacity when the OXPHOS does not produce ATP. Finally, the addition of excess 2-Deoxy-D-glucose (2-DG) inhibits the glycolysis related extracellular acidification through competitive inhibition of glucose-6-phosphate production from glucose at the initial stages of glycolysis. The final measurements are ultimately corrected for the non-glycolytic acidification values (figure 4.15).

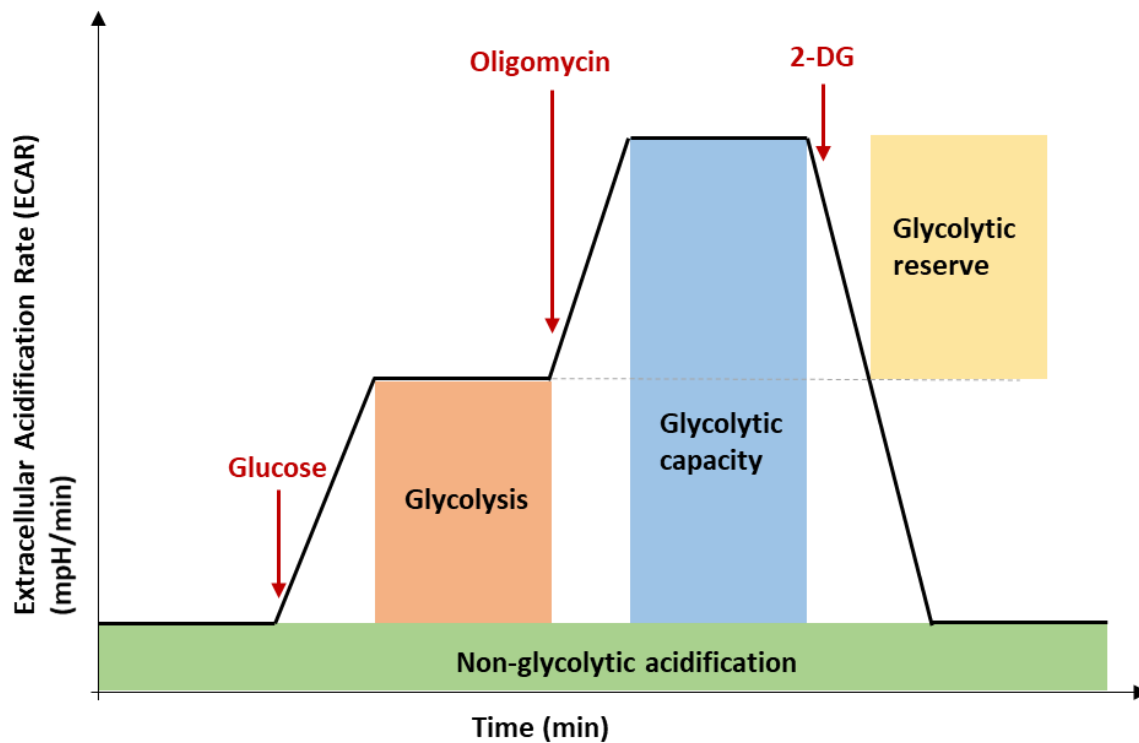


Figure 4.15. Glycolysis stress test protocol schematic

Glucose provides a substrate for glycolysis to take place. Oligomycin inhibits production of ATP from oxidative phosphorylation in order to determine the glycolytic capacity. 2-DG inhibits the glycolysis-related extracellular acidification through competitive inhibition of glucose-6-phosphate production from glucose at the initial stages of glycolysis.

SCC-9 cells after ATRA-induced differentiation show significant increase in glycolysis in comparison to non-differentiated SCC-9 cells (figure 4.13.B and figure 4.14.B). This suggests a heavier reliance of the cells on glycolysis than oxidative respiration, a conclusion in contrast with the previously published data. However, as seen in figure 4.14.B, the acidity of the medium in wells with ATRA treated cells is generally higher throughout the assay, also at the stages where non-glycolytic acidification is measured. This observation could indicate a possible presence of ATRA metabolites in the medium that could interfere with the measurements by changing the pH of the medium irrespectively of the glycolysis stimulation.

The above results indicate an inversely proportional relationship between glycolysis and BAMLET sensitivity in SCC-9 cells – cells showing higher glycolysis rate have lower sensitivity to BAMLET treatment. This result is in line with previous studies on HAMLET/BAMLET wherein it was reported that glycolysis is the key to HAMLET/BAMLET sensitivity in cancer cells (Storm *et al.*, 2011). It is important to note that in this project, the metabolic state of the cells was modulated and measured before BAMLET treatment, and therefore the data represents the metabolic state of the cells that could influence changes in sensitivity or resistance to BAMLET. Whereas in previously published studies, the metabolism of the cells was often assessed after treatment, clearly illustrating a response to the treatment (Storm *et al.*, 2011). Nonetheless, more research is needed to establish a profound understanding of HAMLET/BAMLET sensitivity and discover if it is correlated to the metabolic state of the cell.

4.2.3. Effect of mitochondrial uncoupling on BAMLET sensitivity of SCC-9 cells

There have been multiple studies linking mitochondrial uncoupling to cancer therapies. It has been observed that targeting the uncoupling proteins facilitating the natural mitochondrial uncoupling can increase the effect of chemotherapy by raising the ROS levels in the cancer cell leading to oxidative injury (Baffy, 2017). In other cases, the uncoupling protein UCP2 has been associated with promoting chemoresistance in colon cancer HCT116 cells (Derdak *et al.*, 2008). Likewise, the chemical mitochondrial uncouplers have also been reported to affect cancer cell expression profile and growth. CCCP, a commonly used mitochondrial uncoupler has displayed toxicity in bladder carcinoma cell line MGH-U1, and in murine mammary sarcoma

cells EMT-6 (Newell and Tannock, 1989; Pathania *et al.*, 2009). Other chemical mitochondrial uncouplers, such as FCCP, have been observed to induce apoptosis in tumour cells (Kurosu *et al.*, 2007) and to decrease HIF-1 targeted gene expression in PC-3 and DU-14 prostate cancer cells (Thomas and Kim, 2007).

To further address the question if metabolic modulation in SCC-9 cells could affect BAMLET sensitivity, mitochondrial uncoupling was investigated. Since the toxicity of mitochondrial uncouplers is cell line specific (therefore mitochondrial uncouplers have to be initially titrated in metabolic assays), a toxicity test was performed to plot the toxicity curve of CCCP in SCC-9 cells. The goal was to avoid shock reaction and brief temporal metabolic changes and to ensure a stable metabolic remodelling and a possible effect on gene transcription. To achieve these aims, the cells have been treated with various concentrations of CCCP for 72 h with daily replenishment of medium and CCCP. Then, the viability of the cells was quantified with Alamar Blue assay, and a concentration of 0.4 μ M at which over 70 % of cells were viable was chosen as the working protocol (figure 4.16). Understandably, the constant exposure to CCCP caused mitochondrial uncoupling at a level where the cells could still maintain their function and therefore apoptosis was not induced.

After that, it was examined if the metabolic remodelling of SCC-9 cells induced by the treatment with a chemical mitochondrial uncoupler impacts their sensitivity to BAMLET. Cells were exposed to 0.4 μ M CCCP for 56 h in full medium and then for 16 h in serum-free medium to incorporate serum starvation into the mitochondrial uncoupling protocol. Next, the cells were exposed to a range of concentrations of BAMLET in serum-free medium, with the presence of 0.4 μ M CCCP for 24 h with the control cells not exposed to any mitochondrial uncoupling prior to the assay. The resulting viability curve (figure 4.17.A) demonstrates the lack of effect of 72 h CCCP treatment on BAMLET sensitivity of SCC-9 cells. As seen on the IC₅₀ bar graph comparison (figure 4.17.B), the cells exposed to CCCP for 72 h prior to BAMLET treatment show an IC₅₀ value very similar to that of control cells, not treated with CCCP in advance. This result could indicate that mitochondrial uncoupling did not have a dramatic effect on SCC-9 cells metabolism and homeostasis; therefore, the effect on BAMLET sensitivity was negligible.

4.2.4. Effect of fatty acid synthase inhibition on BAMLET sensitivity of SCC-9 cells

4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid, known as C75, is one of the chemical inhibitors of FASN. Its advantage over others is that it is an irreversible inhibitor, competitive against all three substrates of FASN: acetyl-CoA, malonyl-CoA, and NADPH (Bentebibel *et al.*, 2006). It has been proposed as an antiobesity drug, as it affects the appetite in patients through modulation of metabolism of the neurons in the hypothalamus (Loftus *et al.*, 2000; Hu *et al.*, 2003). C75 has also displayed antitumour properties. It has been reported to induce apoptosis in cancer cells *via* FASN inhibition induced increase in malonyl-CoA and dysregulation of lipid metabolism (Li *et al.*, 2001).

The extent of dependence of SCC-9 cells on fatty acid synthesis is unknown; therefore, it was not possible to predict the effects of C75 treatment on the viability and homeostasis of SCC-9 cells. For that reason, a toxicity test was performed to establish the concentration of C75 that would not introduce toxic effects in SCC-9 cells. Through a study of manufacturer's protocol and published reports, a 16 h incubation period was chosen for the C75 treatment to allow sufficient time for FASN activity inhibition without induction of apoptosis. This time point was also possible to coincide with the serum starvation period, minimising the timespan of exposure of the cells to harmful factors. The cells were treated with a range of C75 concentrations in serum-free medium, and after 16 h their viability was assessed with Alamar blue assay. The concentration chosen for further assays was 1 μ M, at which cell viability was *circa* 90 % (figure 4.18).

Next, the effect of C75 treatment on the sensitivity of SCC-9 cells to BAMLET was assessed. The cells were cultured for 16 h in serum-free medium with or without the addition of 1 μ M C75 for FASN inhibition prior to the exposure to various concentrations of BAMLET in serum-free medium for further 24 h. Next, the cell viability was assessed with Alamar blue assay with comparison to untreated cells. The resulting viability curves revealed no dramatic effect of C75 treatment on BAMLET sensitivity in SCC-9 cells (figure 4.19). The IC_{50} value of control cells that were not treated with C75 was 1 μ M, while the cells treated with 1 μ M C75 for 16 h showed an IC_{50} value of 0.7 μ M and the difference was not statistically significant. It is

not clear if the lack of sensitization of SCC-9 cells through the C75 treatment was due to an alternative fatty acid pathway through which SCC-9 cells could bypass the inhibitory effect of C75. An upregulation of other metabolic pathways could also help to compensate for the lack of fatty acid synthesis. An alternative explanation to the observation could be that the SCC-9 cells are sensitive to the FASN inhibition which results in cell death and the lack of toxicity of 1 μ M C75 was caused by a very little degree of FASN inhibition at that concentration; therefore, no significant sensitization to BAMLET could be observed.

4.2.5. Effect of galactose adaptation on BAMLET sensitivity of SCC-9 cells

Literature reports suggest that even short periods of glucose deprivation sensitize cells to HAMLET-like complexes (Storm *et al.*, 2011). The studies also mention feeding cells with glycolysis inhibitors, such as 2-deoxy-D-glucose, as enhancing the toxicity of HAMLET linking it to a decrease of ATP in the cells (Storm *et al.*, 2011). In this study, to avoid stress and shock reactions to sudden changes in nutrient content in the culture media, SCC-9 cells were gradually adapted to the absence of glucose in the medium, that was progressively being replaced in the medium with galactose, until a stable metabolic change has been achieved. Therefore, the physiological shift that the cell must undergo to utilise galactose involves a new stable metabolic and transcriptional state that facilitates experiments to pinpoint the exact physiological factor responsible for BAMLET sensitivity. At the same time, it allows to avoid transient non-specific stress reactions and compensatory metabolic adaptations that are unrelated to the effects of BAMLET.

The galactose adaptation indeed sensitised SCC-9 cells to BAMLET as illustrated in figure 4.20. The difference is very interesting, as the IC₅₀ value dropped *circa* -fold to 0.4 μ M from 1.5 μ M in control conditions (figure 4.8). It is known that glucose deprivation introduces dramatic metabolic changes in the cells. The careful method of galactose adaptation adopted in this project ensured that the modulations of cellular metabolism were stable and reproducible. Therefore, it was possible to study what factors influence the change in sensitivity to BAMLET under galactose adaptation in more detail and a full study of galactose adaptation effect on BAMLET sensitivity is pursued in the following chapter.

4.3. Discussion

It is generally understood that HAMLET/BAMLET complexes need to be administered in FBS free conditions, because albumin present in FBS can result in their deactivation (Rath *et al.*, 2015). Therefore, to obtain consistent and reproducible results, cells need to be exposed to serum-free medium in advance. Lack of serum assures homogeneity of the cell population but also ensures the same rate of uptake. Serum starved cells need to slow down their metabolism due to a lack of lipids and growth factors, and therefore conceivably, are more predisposed to internalizing molecules found in the medium. However, there is no consensus in the HAMLET/BAMLET literature on how the serum in assays potentially impacts on experimental outcomes. Multiple reports mention treatment in serum-free medium (Tolin *et al.*, 2010; Spolaore *et al.*, 2010; Nadeem *et al.*, 2019) and subsequent addition of serum (Svensson *et al.*, 2003; Fast *et al.*, 2005; Rammer *et al.*, 2010; Mercer *et al.*, 2011; Storm *et al.*, 2013; Xiao *et al.*, 2013; Rath *et al.*, 2018), but serum starvation is not mentioned in the procedure. There are also reports mentioning a 6 h serum starvation period (Hoque *et al.*, 2013) and studies where all assays have been performed in standard culture medium, including the treatment phase (Xie *et al.*, 2012). Such lack of consensus and consistency prevents comparability and reproducibility of data between the studies. Therefore, for the purpose of this study, a uniform assay procedure has been developed and employed in order to achieve precision and clarity in experimental design.

The results obtained in this study suggest that serum starvation has a crucial effect on BAMLET sensitivity. The procedure applied prior to the exposure to BAMLET did not induce cell death mechanisms in the cells. Moreover, all three cell lines showed a higher ratio of cells in G₀/G₁ phase after starvation. An enhanced response to BAMLET treatment and an increase in its toxicity could be explained by the fact that serum starved cells have a limited supply of nutrients and growth factors and therefore would more-readily internalize a protein-fatty acid complex. More homogenous cell population ensures greater reproducibility of the results, as a higher ratio of cells are within the same cell cycle phase thereby negating the effect of metabolic differences inherent to the phases of the cell cycle. Moreover, as HAMLET/BAMLET complexes have the potential to be deactivated by serum contents, they must be administered in serum-free medium. However, if experiments are designed to cause a sudden change in the serum content of the medium concurrent with the addition of HAMLET/BAMLET it is likely

for non-specific effects on proliferation to obscure the true effect of HAMLET/BAMLET. Therefore, serum starvation provides the cells with sufficient adaptation time for them to remodel their cellular activity in response to new circumstances. Upon HAMLET/BAMLET treatment, the cells can be considered to be undergoing a single factor change in their environment and any resulting loss of cell viability may be attributed exclusively to HAMLET/BAMLET toxicity, and not to other non-specific factors. To this end, we recommend adopting a serum starvation protocol prior to cytotoxicity assays, particularly with agents not suitable to be administered with serum.

Out of the three tested cell lines, SCC-9 cells exhibited the highest BAMLET sensitivity. Although HAMLET-like complexes have been reported to exhibit higher toxicity against cells with an immature phenotype, this does not seem to explain the observed phenomenon, as according to the supplier's provided information, all the examined cell lines have moderately differentiated phenotypes. We speculate that some metabolism-related characteristic of SCC-9 cells sets them aside from the U2OS and KYSE-520; however, it is not clear what it could be. SCC-9 cells have been reported to display slight cisplatin resistance (Taveira Elias *et al.*, 2015) showing that they are otherwise a cell line not exhibiting sensitivity to therapeutics. KYSE-520, in turn, exhibited the lowest sensitivity among the examined cell lines. There are reports on overexpression of cyclooxygenase-2 (COX-2) in KYSE-520 cells (Pak *et al.*, 2009), an enzyme linked to carcinogenesis, cancer progression, and multidrug resistance (Dannenberg *et al.*, 2001; Sobolewski *et al.*, 2010). It is interesting to note that despite the fact that the tested cell lines display resistance to typically used chemotherapeutics, BAMLET showed very satisfactory toxicity levels. This result illustrates the potency of HAMLET-like complexes to treat therapy-resistant cancers and highlights its potential as a neoadjuvant agent. More research is needed to pinpoint which cellular characteristic is responsible for the differences in sensitivity to BAMLET between the investigated cell lines, involving a comparison of metabolic activity. Due to the high level of BAMLET sensitivity displayed by the SCC-9 cell line, it has been elected as the main cell line for ensuing research in this project.

The literature suggests that the selectivity of HAMLET cytotoxicity towards cancer cells and their metabolic state are connected (Storm *et al.*, 2011; Ho *et al.*, 2013). Since HAMLET and HAMLET-like complexes have been reported to spare proximal differentiated cells, while effectively killing cancer cells, the links to metabolism as the differentiating factor are obvi-

ous. For that reason, this study investigated various metabolic modifications and their impact on the BAMLET sensitivity of SCC-9 cells.

The differentiation of SCC-9 cells by ATRA had the effect of changing protein markers, and their proliferation rate. The differentiated SCC-9 cells displayed a decrease in sensitivity to BAMLET, in line with the literature reports of resistance to HAMLET-like complexes in healthy differentiated cells. To understand where the difference between undifferentiated and differentiated cancer cells stems from, a comparative examination of the metabolic profiles of SCC-9 cells in the two conditions was carried out. Interestingly, the results revealed no differences in mitochondrial respiration rate; however, a higher glycolytic activity was observed in the differentiated cells. The traditional understanding is that cancer cells have upregulated glycolysis in comparison to differentiated cells, yet the opposite has been true in the case of ATRA differentiated SCC-9 cells. Although the results obtained could indicate that increased glycolysis rate could be causing the resistance of cells to HAMLET, more research on an exhaustive panel of various cell lines would be necessary to confirm this hypothesis. Alternatively, the differentiated cells could simply have finite energy needs that they can easily satisfy, while the non-differentiated cells phenocopy cancer cells – with constantly increasing energy demand and therefore resorting to increasing nutrient uptake.

Following the standard view of highly glycolytic cancer cells, presumably, in cell lines satisfying the majority of their energetic needs with glycolysis, uncoupling of the mitochondria could be bypassed and not affect the homeostasis. Upregulation of glycolysis in cancer cells is an often-reported phenomenon (Vazquez *et al.*, 2016). SCC-9 cells have displayed a well functional glycolysis machinery and are understood to be able to compensate for the uncoupled mitochondria. It may be inferred that higher concentrations of CCCP are toxic to the cells due to off-target effects. However, uncoupling the bulk of mitochondria and cessation of oxidative phosphorylation in the cell could ostensibly deprive the cell of necessary building blocks and cause an accumulation of cytotoxic by-products. That, in turn, could disturb the homeostasis of the cell, leading to death. Therefore, the treatment with the mitochondrial uncoupler should be carried out in sub-lethal conditions, where there are some functional, not fully uncoupled mitochondria in the cells. This could in turn provide HAMLET/BAMLET with a potentially exploitable gateway to increase its toxicity in the cells. However, no such effect was observed in SCC-9 cells, where the continuous 72 h treatment with CCCP did not significantly change

their sensitivity to BAMLET. It is speculated that the sub-lethal concentration of CCCP might not induce enough mitochondrial uncoupling to induce a sufficient remodelling of cellular metabolism. However, as higher CCCP concentrations were found to induce cell death, it was not possible to explore high concentrations of CCCP in the examination of the effect on BAMLET toxicity without compounding off-target effects. A plausible explanation of the result obtained is that mitochondrial uncoupling does not influence the sensitivity to HAMLET-like complexes as its effects might incorporate separate metabolic and signalling pathways. The exact reasons for the lack of influence over BAMLET sensitivity are unknown; however, the mitochondrial uncoupling angle was not explored any further in this project.

Fatty acid synthase inhibition has been proposed to be a target for novel anticancer therapies. As the cancer cells have increased demand for energy production and substrates to satisfy the requirements for membrane synthesis during periods of rapid proliferation, in cancer the endogenous fatty acid production is often upregulated (Pavlova and Thompson, 2016). In fact, FASN has been touted as a prognostic marker in aggressive breast cancers (Kuhajda *et al.*, 1994; Vazquez *et al.*, 2016). Inhibition of FASN activity has been reported to induce apoptosis in cancer cells (Bentebibel *et al.*, 2006; Goswami and Sharma-Walia, 2016). However, inhibition of endogenous production of fatty acids could be compensated for through the upregulation of the uptake of exogenous lipids in order to satisfy cellular demand. It was speculated that BAMLET could be increasingly taken up by the cells pre-treated with C75, as a readily available fatty acid is a part of the molecule. That in turn would result in an increased internalisation of BAMLET into the cells and higher toxicity. Surprisingly, no such effect was observed in SCC-9 cells. Cells pre-treated with 1 μ M C75 for 16 h prior to BAMLET treatment showed no significant difference in IC₅₀ value compared to cells without the C75 pre-treatment. The results, though unexpected, could be explained in many ways. Possibly, the SCC-9 cells possess an alternative pathway metabolising the by-products of fatty acid synthesis not utilised by FASN, therefore reducing their toxic effects and reducing the need for exogenous lipid uptake. Another probability could be an alternative long chain fatty acid synthesis pathway, by-passing FASN inhibition by C75. A less plausible explanation is an upregulation of other metabolic pathways compensating for the lack of FASN activity in the cell. One other possible explanation could be that toxicity of C75 is not due to off-target toxic effect, but due to the fact that SCC-9 cells are very dependent on endogenous fatty acid synthesis by FASN and its inhibition is lethal. Because of the nature of the tests, the only C75 concentra-

tion possible to examine could be a sub-lethal one, so as not to overshadow the effect of BAMLET treatment. It is possible that a sub-lethal C75 concentration did not introduce full FASN inhibition, and therefore did not induce sensitisation to BAMLET in SCC-9 cells. Due to a lack of effect and no clear reason for the absence of a response to the treatment with FASN inhibitor this aspect of metabolism was not investigated further in this project.

Storm *et al.* (2011) have reported that short periods of glucose deprivation prior to treatment with HAMLET increase the cells' sensitivity. To test that observation further, in greater detail and with greater care, the final metabolic manipulation examined in this project was adapting the cells to feed on galactose. In order to avoid acute stress response by cells suddenly deprived of their main energy substrate, great caution was exercised with gradually exposing the cells to decreasing content of glucose with simultaneous gradual increase of galactose content in the culture medium. Therefore, a stable and reproducible metabolic reorganisation was ensured to satisfy the cell's energy needs through oxidative phosphorylation (Gohil *et al.*, 2010). Since in the case of ATRA-induced differentiation of SCC-9 cells, an increase in glycolytic activity coinciding with an increase of resistance to BAMLET was observed, we were interested in investigating the effect of metabolic shift towards mitochondrial respiration on BAMLET sensitivity in SCC-9 cells. This metabolic approach proved to be a success, with a nearly 4-fold increase in sensitivity in galactose adapted SCC-9 cells in comparison to cells treated in the standard culture medium with the typical glucose content. This is particularly interesting in light of lack of effect of mitochondrial uncoupling and fatty acid synthase inhibitor treatments, that should have similar, energy limiting properties. It is speculated that galactose fed cells have limited options of metabolism remodelling without the presence of glucose. They can attempt at shifting to other energy producing pathways, in turn upregulating uptake of all other energy substrates. That would result in an increased BAMLET uptake due to its composition from natural compounds and ability to pose as a nutrient. However, further metabolic profiling of galactose adapted cells needs to be performed to pinpoint what causes elevation of BAMLET sensitivity. It is believed that by comparing and contrasting the metabolic states of galactose and glucose fed cells of the same cell line it will be possible to extrapolate the cellular characteristic which accounts for HAMLET sensitivity and possibly also its cellular targets and mechanism of action. To further understand the underlying mechanisms responsible for the sensitisation of cells adapted to galactose, a more comprehensive study of this phenomenon followed.

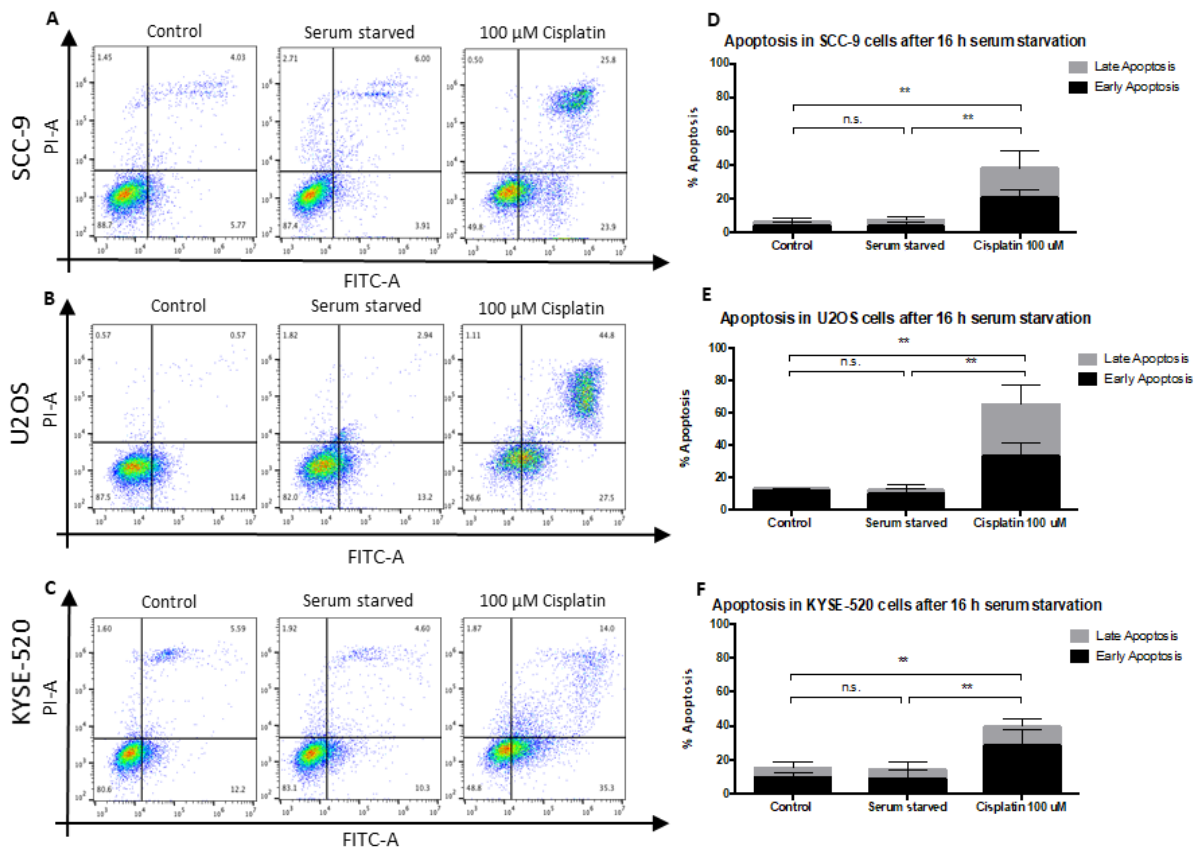


Figure 4.3. Analysis of apoptosis after 16 h serum starvation

A – C – Representative data of flow cytometry analysis with double Annexin V/PI staining after 16 h serum starvation for A – SCC-9 cells, B – U2OS cells, C – KYSE-520 cells. Cells were seeded on a 6-well plate at a density of 1.5×10^5 cells/well. After adhesion, cells were either serum starved for 16 h prior to the experiment, cultured in medium containing 10 % FBS for non-starved control, or treated with 100 μ M cisplatin for positive control. Cells were then stained with Annexin V-FITC and PI and analysed by flow cytometry. Early apoptotic cells were Annexin V positive and late apoptotic cells were Annexin V and PI positive. D – F – Quantitative apoptosis levels assessment after 16 h serum starvation in D – SCC-9 cells, E – U2OS cells, F – KYSE-520. Data generated from three independent flow cytometry experiments. Two-way ANOVA, ** $p < 0.01$, n.s. – not significant.

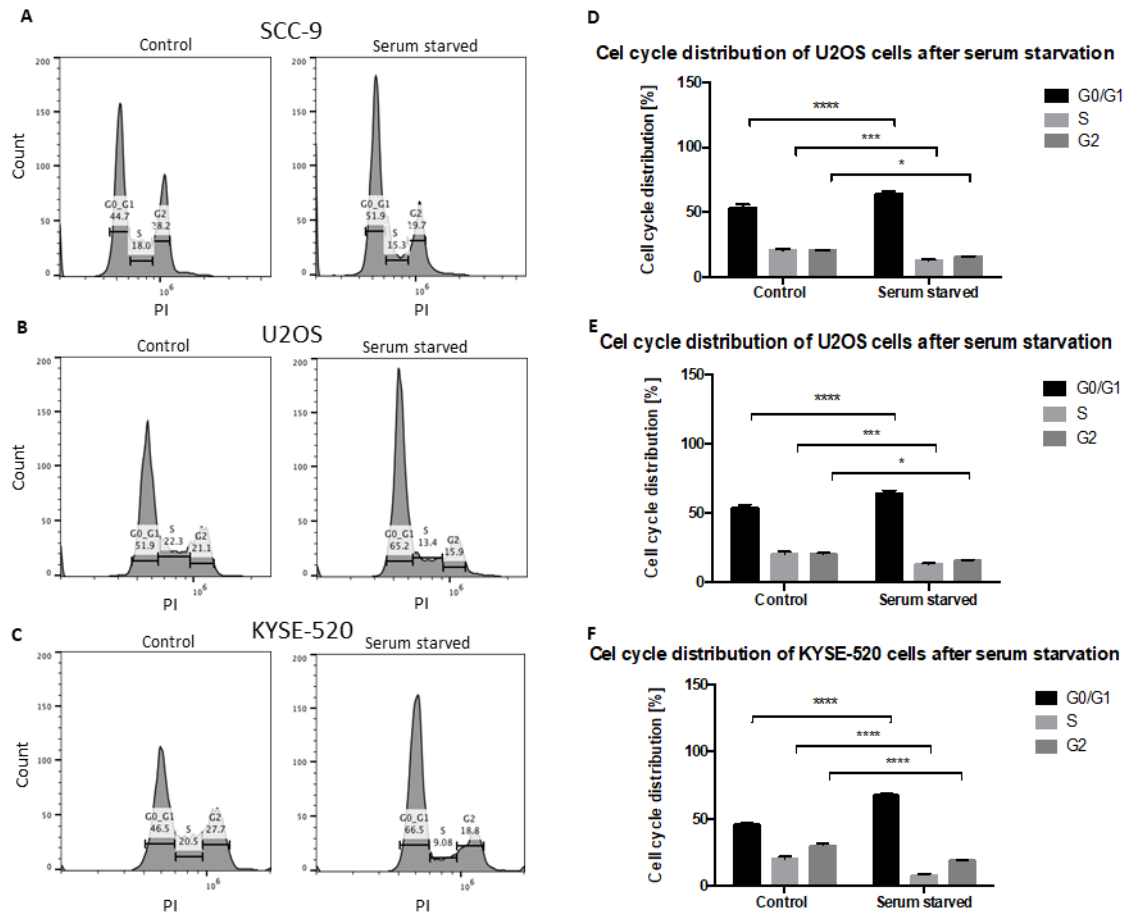


Figure 4.4. Cell cycle analysis after 16 h serum starvation

A – C – Representative data of flow cytometry with PI staining for cell cycle analysis after 16 h serum starvation of A – SCC-9 cells, B – U2OS cells, C – KYSE-520 cells. Cells were seeded on a 6-well plate at a density of 1.5×10^5 cells/well. After adhesion, cells were serum starved for 16 h prior to the experiment, while non-starved control cell were cultured in medium containing 10 % FBS. Cells were treated with 100 μ M cisplatin for 16 h as a positive control. Cells were then harvested, fixed in 70 % ethanol, and stained with PI and analysed by flow cytometry. D – F – Quantitative cell cycle analysis after 16 h serum starvation in D – SCC-9 cells, E – U2OS cells, F – KYSE-520 cells. Data generated from three independent flow cytometry experiments. Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. – not significant.

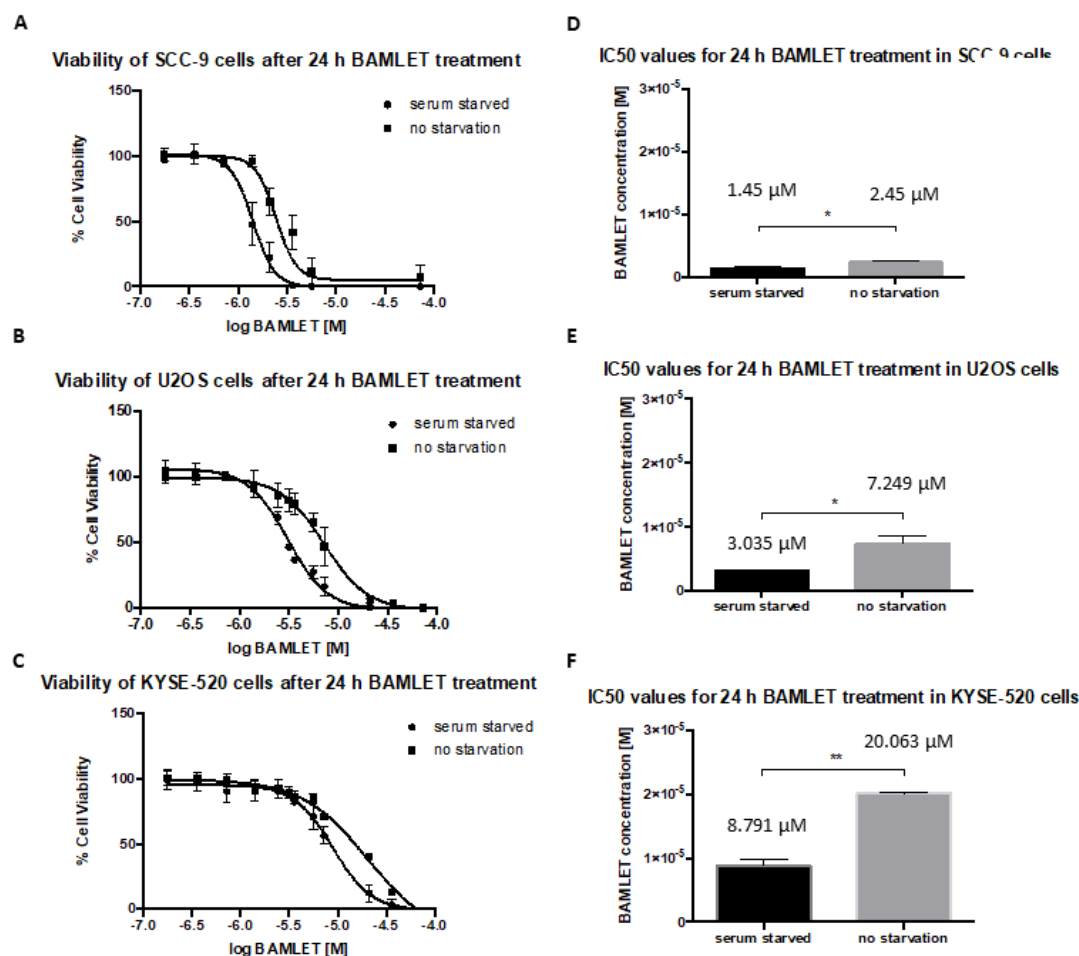


Figure 4.5. Cell viability after 24 h exposure to different concentrations of BAMLET with and without prior serum starvation

A – C – Viability curves of A – SCC-9 cells, B – U2OS cells, and C – KYSE-520 cells after 24 h exposure to BAMLET with or without prior serum starvation. Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well. Cells were serum starved for 16 h prior to the experiment, while non-starved cells were cultured in medium containing 10 % FBS until treatment. Next, various concentrations of BAMLET were administered in serum-free medium for 24 h, and the viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Values represent the mean $\pm$ SD of three independent experiments. D – F – IC₅₀ value comparison between serum starved cells and non-starved cells of D – SCC-9, E – U2OS, and F – KYSE-520 cells. Data generated from three independent experiments. Student t-test, * $p < 0.05$, ** $p < 0.01$.

IC₅₀ values for 24 h BAMLET treatment in 3 cell lines

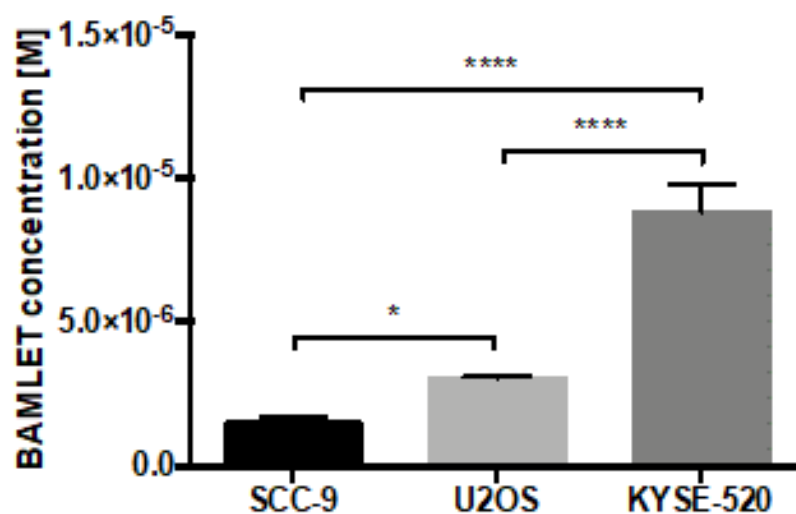


Figure 4.6. BAMLET IC₅₀ value comparison between the cell lines

Bars represent the mean $\pm$ SD of three experiments. Values shown on the graphs: SCC-9 cells – 1.5 μ M; U2OS cells – 3. μ M; KYSE-520 cells – 8.8 μ M. One-way ANOVA, * $p < 0.05$, **** $p < 0.0001$.

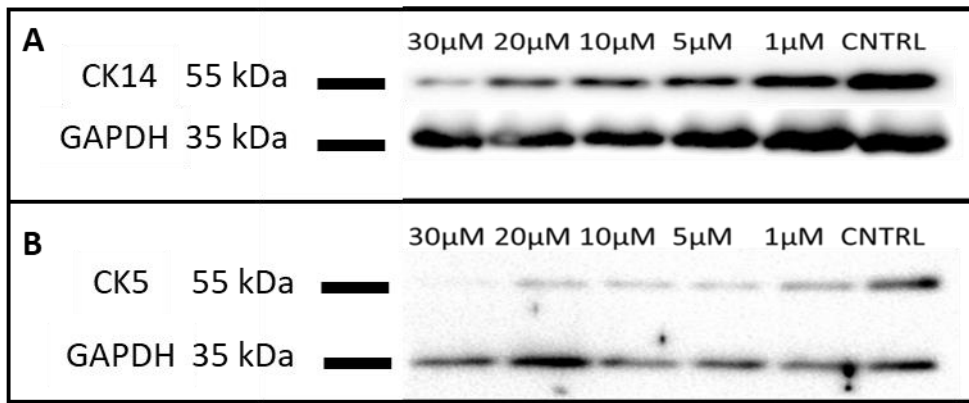
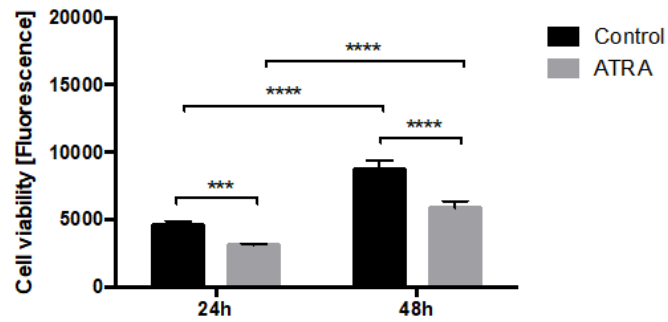


Figure 4.7. Expression of cytokeratin 14 and cytokeratin 5 in SCC-9 cells treated with different concentrations of ATRA

A representative of three independent Western Blot analyses of expression of A - cytokeratin 14 (CK14) and 5 (CK5) in SCC-9 cells upon treatment with various ATRA concentrations for 5 days. Different concentrations of ATRA were added to the normal culture medium for 5 days, control cells were untreated. 5-day treatment with 30 μ M ATRA significantly lowered the cytokeratin 5/14 expression and was chosen to be the ATRA induced differentiation procedure.

A
SCC-9 proliferation before and after differentiation / Alamar Blue assay



B
SCC-9 proliferation before and after differentiation / SRB assay

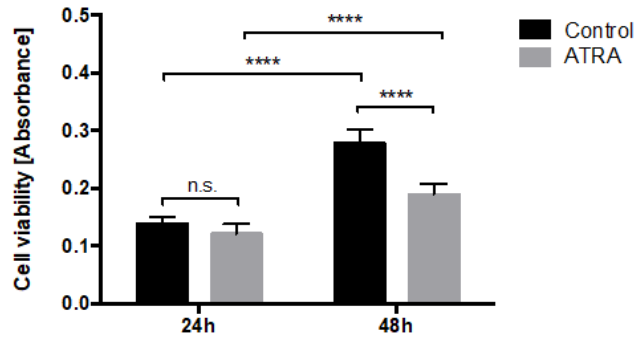
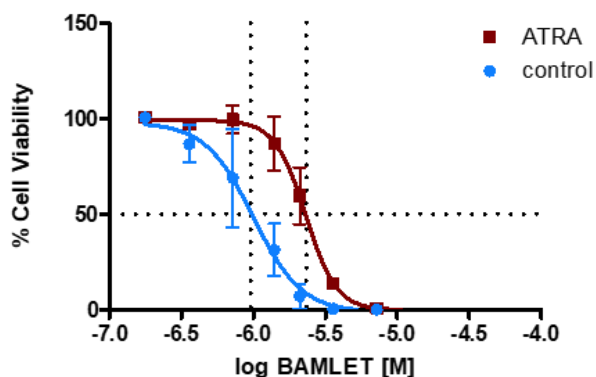


Figure 4.8. Proliferation of control and differentiated SCC-9 cells for 24 h and 48 h

Control and differentiated cells were seeded on a 96-well plate at a density of 5×10^3 cells/well. Cells were grown in standard culture medium without addition of ATRA for 24 h or 48 h. After that, their viability (and proportionally cell number) were measured by A – Alamar Blue assay and B – SRB assay. Graphs represent means $\pm$ SD of three independent experiments. Two-way ANOVA, *** $p < 0.001$, **** $p < 0.0001$, n.s. – not significant.

A

Viability of differentiated SCC-9 cells after 24 h BAMLET treatment



B

IC50 values for 24 h BAMLET treatment

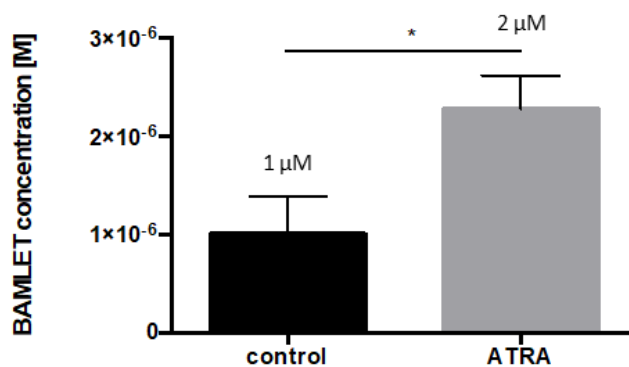


Figure 4.9. Cell viability of differentiated SCC-9 cells after 24 h exposure to different concentrations of BAMLET

A – Viability curves of control and ATRA differentiated SCC-9 cells after 24 h of BAMLET exposure. Control and ATRA differentiated SCC-9 cells were seeded on a 96-well plate at a density of 5×10^3 cells/well. Cells were serum starved for 16 h prior to the experiment. Various concentrations of BAMLET were administered in serum-free medium for 24 h. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Values represent the mean $\pm$ SD of three independent experiments. B – IC₅₀ value comparison between control and ATRA differentiated SCC-9 cells after 24 h BAMLET treatment. Data generated from three independent experiments. Student t-test, * $p < 0.05$.

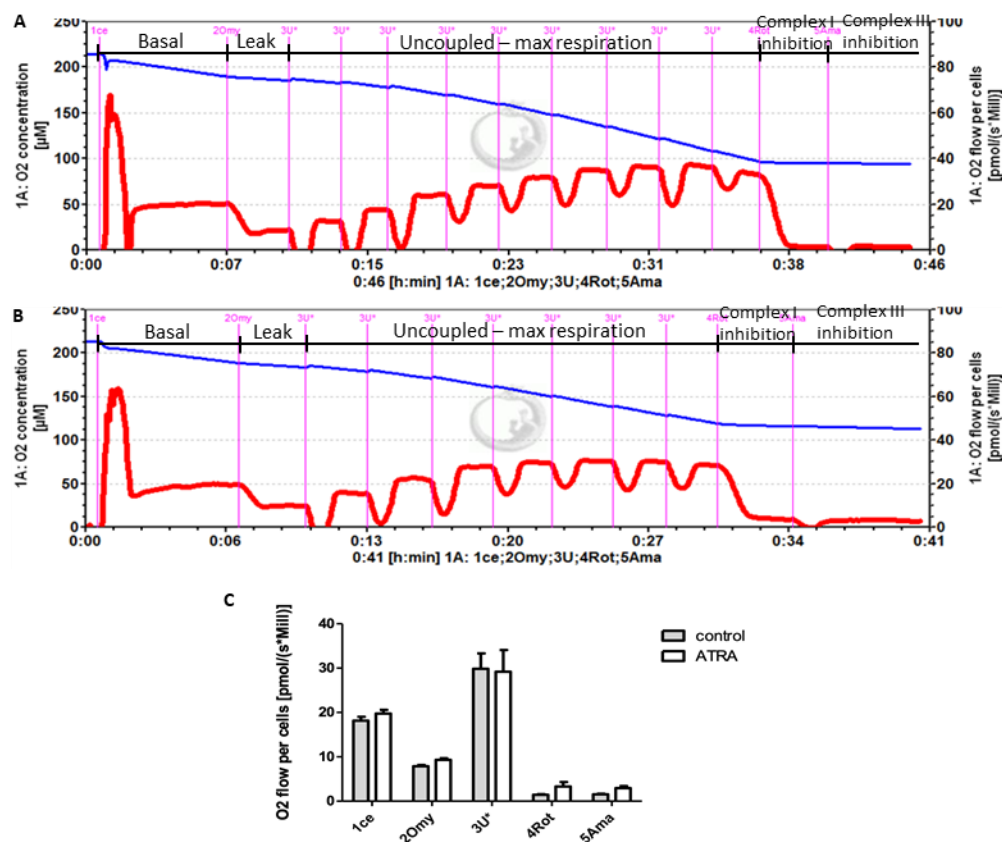


Figure 4.11. Respiration of SCC-9 cells

A representative of three independent high resolution respirometry experiments on A – control SCC-9 cells, B – differentiated SCC-9 cells. Cells were injected at 4 million cells/chamber and their respiration was measured in MiR05 medium. C – Bar graph comparison of the respiration of the control and differentiated (ATRA) SCC-9 cells. Injections: 1ce – cells – basal respiration, 2Omy – oligomycin – leak respiration, 3U – several titrations of Uncoupler – maximal respiration capacity, 4Rot – rotenone – inhibition of mitochondrial complex I, and 5Ama – Antimycin A – inhibition of mitochondrial complex III. Graph represents means $\pm$ SD of data from three independent experiments.

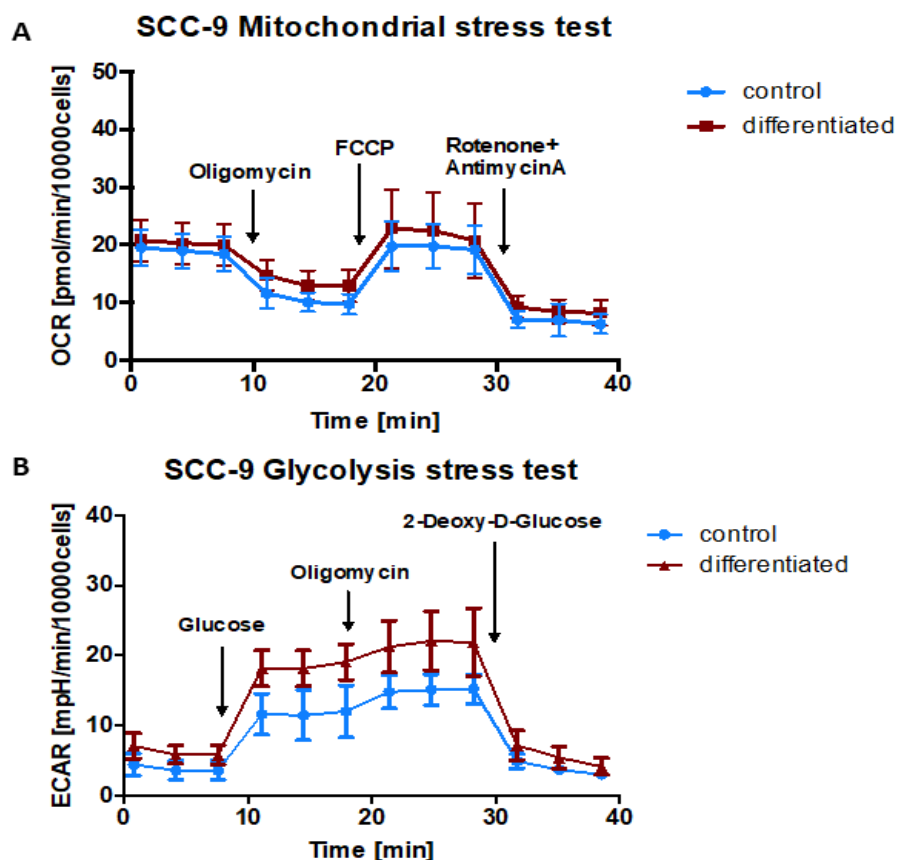


Figure 4.13. Metabolic differences in SCC-9 cells before and after differentiation

A – Mitochondrial stress test. OCR in control and differentiated SCC-9 cells with sequential injections of oligomycin (1 μ M), FCCP (0.125 μ M), rotenone (0.1 μ M) and antimycin A (4 μ M). Graph represents means $\pm$ SD of data obtained from three independent experiments. B – Glycolysis stress test. ECAR in control and differentiated SCC-9 cells with sequential injections of glucose (10 mM), oligomycin (1 μ M), and 2-deoxy-D-glucose (30 mM). Graph represents means $\pm$ SD of data obtained from three independent experiments.

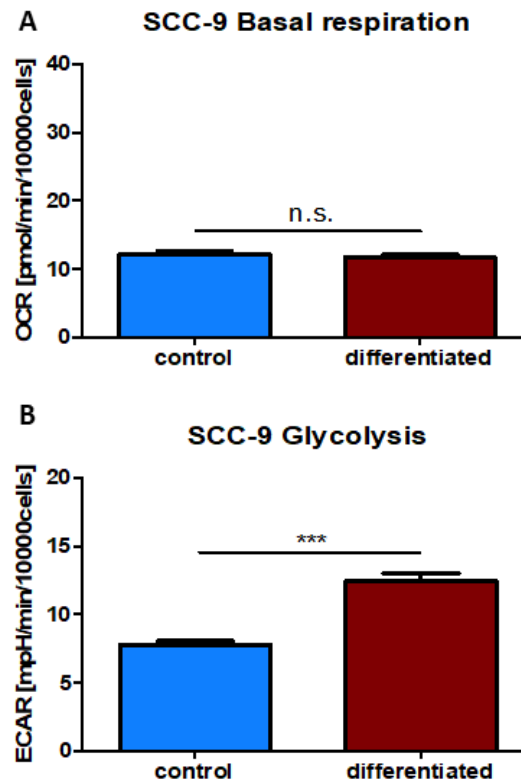


Figure 4.14. Metabolic differences in SCC-9 cells before and after differentiation

A – Basal respiration – OCR in control and differentiated SCC-9 cells before any injection. B – Glycolysis rate – ECAR in control and differentiated SCC-9 cells after injection of glucose. Graphs represent means $\pm$ SD of values obtained in three independent experiments. One-way ANOVA, *** $p < 0.001$, n.s. – not significant.

Sensitivity of SCC-9 cells to 72 h CCCP treatment

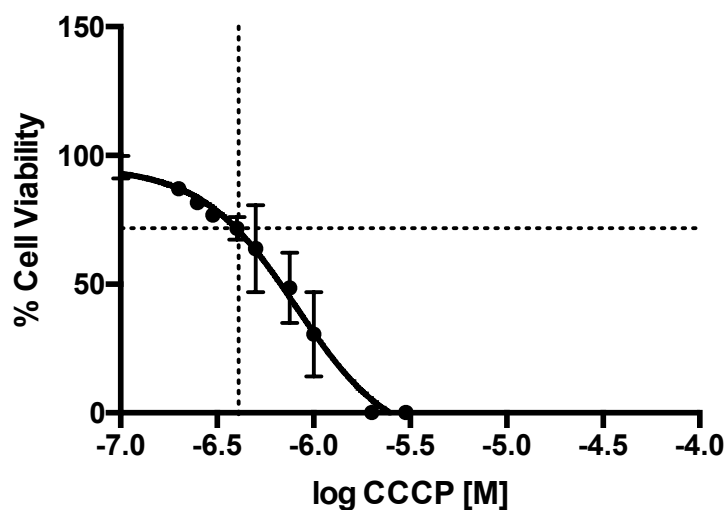


Figure 4.16. Viability of SCC-9 cells after 72 h of exposure to a range of CCCP concentrations

Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well. Cells were serum starved for 16 h prior to the experiment. Various concentrations of CCCP were administered in serum-free medium for 72 h. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Cell viability was above 70 % at concentration of $0.4 \mu\text{M}$, as indicated. Values represent means $\pm$ SD of three independent experiments.

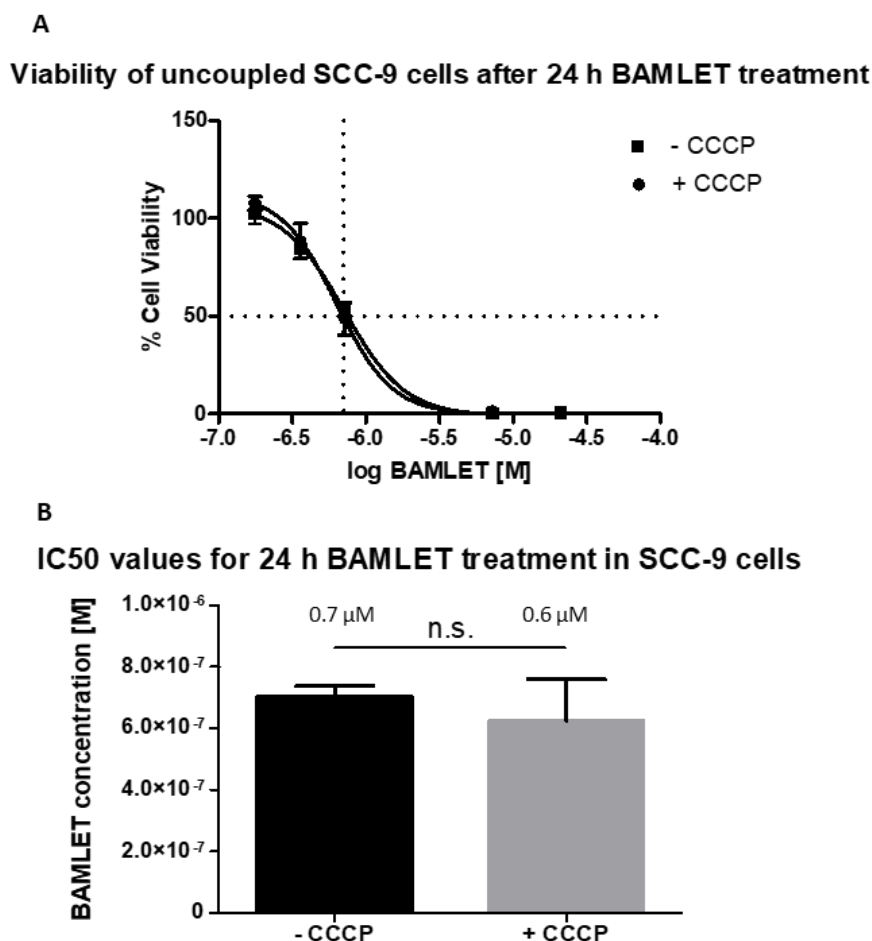


Figure 4.17. Sensitivity of SCC-9 cells to BAMLET after mitochondrial uncoupling

A – Viability curves of uncoupled, CCCP pre-treated and untreated SCC-9 cells. Uncoupled cells were treated with 0.4 μM CCCP for 72 h, while the control cells were grown in standard culture medium. They were then seeded on a 96-well plate at a density of 5×10^3 cells/well and serum starved for 16 h prior to the experiment. Various concentrations of BAMLET were administered in serum-free medium for 24 h. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Values represent means $\pm$ SD of three independent experiments. B - IC₅₀ value comparison of uncoupled and control SCC-9 cells to 24 h BAMLET treatment. Values represent means $\pm$ SD of three independent experiments. Student t-test, n.s. – not significant.

Viability of SCC-9 cells after C75 treatment

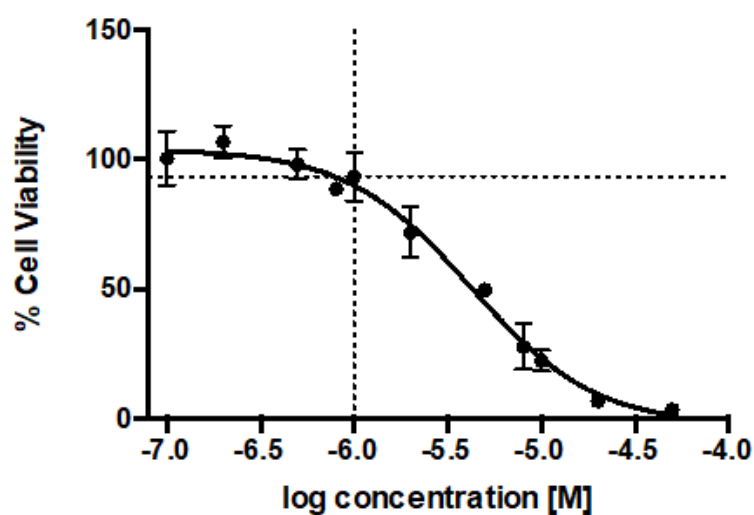


Figure 4.18. Viability curves of SCC-9 cells after 16 h of exposure to a range of C75 concentrations

Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well. Cells were treated with a range of C75 concentrations in serum-free medium for 16 h. Cells were washed twice in PBS and cultured for 24 h in fresh serum-free medium without C75. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Cell viability was above 90 % at concentration of 1 μ M, as indicated. Values represent means $\pm$ SD of three independent experiments.

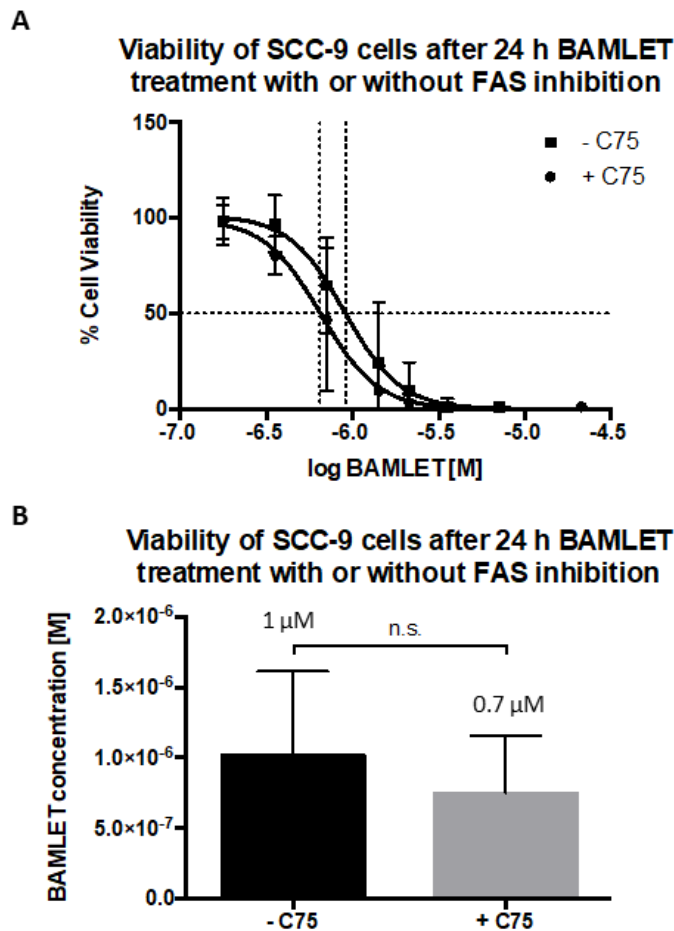


Figure 4.19. Sensitivity of SCC-9 cells to BAMLET after exposure to a FASN inhibitor

A – Viability curves of FASN inhibitor non-treated and pre-treated SCC-9 cells after 24 h BAMLET treatment. Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well and then, treated with 1 μ M C75 in serum-free medium for 16 h. Next, cells were treated with a range of BAMLET concentrations for 24 h. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Values represent means $\pm$ SD of three independent experiments. B - IC₅₀ value comparison between FASN inhibitor non-treated and pre-treated SCC-9 cells. Values represent means $\pm$ SD of three independent experiments. Student t-test, n.s. – not significant.

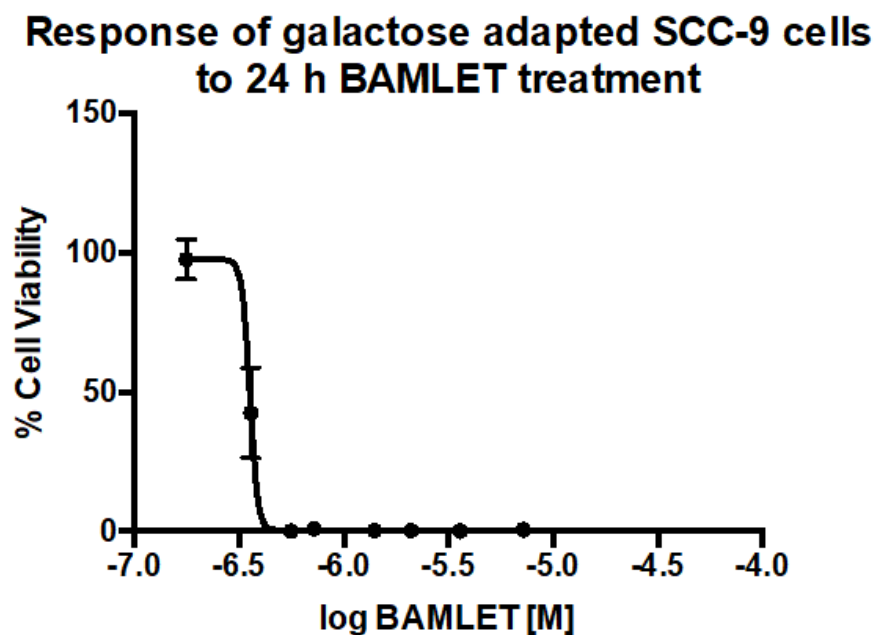


Figure 4.20. Cell viability after 24 h exposure to different concentrations of BAMLET in galactose adapted cells

Cytotoxicity curves for BAMLET in galactose adapted SCC-9 cells. Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well and serum starved for 16 h prior to treatment with a range of concentrations of BAMLET for 24 h. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Values represent the mean $\pm$ SD of three experiments. IC_{50} value was $0.4 \mu M$.

5. Impact of modulating glycolysis on the sensitivity of SCC-9 cells to BAMLET

5.1. Introduction

Science and medicine have long strived to develop a successful therapy that would be selective for malignant cancer cells and simultaneously spare the healthy proximal tissue. Despite years of research and many discoveries elucidating the hallmarks of cancer and the characteristics that differentiate tumour cells from healthy tissue, the goal of selective cancer cell eradication is still not in sight. It is clear that a deeper understanding of the fundamental molecular, metabolic, and microenvironmental differences between cancer and healthy cells is required, with the ultimate aim being the development of cancer-selective therapies that display low to no side effects to patients. However, the mechanisms contributing to carcinogenesis are often complicated and diverse. The range of genetic and epigenetic alterations that underly various cancers also vary widely (Pelicano *et al.*, 2006). One obvious defining characteristic of cancer cells is their distinct metabolism, supporting both survival in difficult environments and a high proliferation rate (DeBerardinis and Chandel, 2016). Cancers may be induced to shift from, or towards, certain metabolic dependencies based on nutrient availability and the external environmental conditions (Vander Heiden and DeBerardis, 2017). In cancer, there is an almost universally observed upregulation of glycolytic activity under aerobic conditions (Warburg, 1956), a situation that helps to meet the high ATP demand of these cells, while simultaneously ensuring the supply of metabolic intermediates required for macromolecular biosynthesis and proliferation. Clinical evidence reveals increased glycolytic rate as being a marker of tumour aggressiveness and poor patient prognosis (Detterbeck *et al.*, 2004). Some studies report that upregulation of glycolysis is likely to be responsible for drug resistance, either through the enhanced activity of the ATP-binding cassette transporter, or alternative energy-dependent mechanisms (Nakano *et al.*, 2011).

It is no surprise therefore, that the glycolytic machinery has been the focus of multiple new therapies in cancer. It has been speculated that inhibition of glycolysis could lead to cancer cell death through decreasing cellular ATP levels and a reduction in cellular biomass (Pradelli *et al.*, 2010; Nakano *et al.*, 2011). Conceivably, no such effect should be observed in normal differentiated cells as their energy metabolism is typically principally reliant on OXPHOS (van Horssen *et al.*, 2013). Multiple experimental studies have shown that the inhibition of glycolysis has therapeutic potential in cancer. Inhibition of glycolytic activity with the use of 3-bromopyruvate (a hexokinase inhibitor) facilitated a decrease in ATP production, and, more

interestingly, inactivation of the ATP-binding cassette transporter activity in multiple cell lines, thereby lowering their resistance to daunorubicin (Nakano *et al.*, 2011). A decrease of ATP levels driven by suppression of glycolytic activity with 2-DG has been reported to activate death-receptor mediated apoptosis in Jurkat, HeLa, and U937 cells through activation of 5' AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) inhibition (Pradelli *et al.*, 2010). A combined treatment of zoletarelin doxorubicin with 2-DG showed a significant increase of apoptosis in triple-negative breast cancer cells and tumour reduction in nude mice compared to a single agent therapy (Gründker *et al.*, 2019). Similar effects have been seen in HepG2 cells, where co-treatment with one of the glycolysis inhibitors, 2-DG, dichloroacetate, or 3-bromopyruvate together with doxorubicin introduced disturbances in energy metabolism and oxidative injury (Korga *et al.*, 2019). More importantly, beneficial effects of a decrease in glycolytic activity were also observed in the case of HAMLET. Even though HAMLET displays a very satisfactory toxicity in therapy resistant cell lines, a brief glucose starvation period has been shown to reduce ATP levels in A549 lung carcinoma cells, coincidentally also further increasing their sensitivity to HAMLET (Storm *et al.*, 2011).

As seen above, there are many approaches to decreasing the glycolytic activity in cancer cells. Literature reports mentioned above exploited the addition of glycolysis inhibitors to the culture media, such as 2-DG, 3-bromopyruvate, or dichloroacetate (Pradelli *et al.*, 2010; Nakano *et al.*, 2011; Chu *et al.*, 2018; Gründker *et al.*, 2019; Korga *et al.*, 2019). These molecules reduce glycolytic rate by inhibiting glycolytic enzymes, such as hexokinase 2 (Fan *et al.*, 2019) or pyruvate dehydrogenase kinase (Tataranni and Piccoli, 2019). Glucose starvation depletes the cells of the preferred substrate of glycolysis, therefore preventing the glycolytic reactions from happening (Storm *et al.*, 2011). One other method of reducing glycolytic activity in cultured cells is supplementing the culture medium with galactose instead of glucose. Galactose is a hexose abundantly present in milk, dairy, fruits, and vegetables. It differs from glucose solely in the configuration of the hydroxyl group at the carbon-4 position (Coelho *et al.*, 2015). Despite its structural similarity to glucose, galactose metabolism shows some important differences. It can be accommodated by the glycolytic pathway since it can be processed into glucose-1-phosphate (Novelli and Reichardt, 2000); however, while the yield of pyruvate production through the glycolytic metabolism of glucose is 2 net ATP, the glycolytic metabolism of galactose yields 0 net ATP (Aguer *et al.*, 2011). Therefore, cells fed with galactose in the

absence of glucose need to rely on alternative energy pathways to cover their demand for energy. For this reason, supplementing cultured cells with galactose instead of glucose is an interesting way of inhibiting glycolysis – the metabolic machinery remains intact and functional; however, cells choose to resort to other energy producing pathways because glycolysis is not ‘energetically profitable’. This might mean upregulation of OXPHOS, or, for example, fatty acid uptake and β -oxidation (Palm and Thompson, 2017), which is particularly interesting in the context of HAMLET studies. Gradual and long-term galactose adaptation allows for metabolic remodelling without experiencing stress response and cellular activity arrest which could be the case in a sudden change of supplied energy sources.

Since cancer cells largely depend on glycolysis to cover their energy needs, a disruption in the glycolytic activity would cause a significant readjustment of cellular energy metabolism and homeostasis. It is widely reported that cancers upregulate surface transporters for various nutrients to fuel their metabolism, and it correlates with aggressiveness and poor prognosis (Finicle *et al.*, 2018). An acute depletion of glucose introduces a drop in ATP levels; however, as the conditions continue, like in the case of galactose adaptation, cells adapt their metabolism and recover their energetic status (Lang *et al.*, 2014). However, as galactose does not allow for a full compensation of the energy demands of the cancer cells, their ATP levels and proliferation rate might not recover completely. Such deficiency in energy and biomass production might be overcompensated by a further upregulation of nutrient scavenging pathways. Nutrient uptake has been found to be influenced by AMPK and mTORC1 activity in response to metabolic disruptions and nutrient stress (Palm and Thompson, 2017; Finicle *et al.*, 2018).

AMPK is a heterotrimeric enzyme, that binds AMP, ADP and ATP directly, thus detecting their cellular levels (Rahmani *et al.*, 2019). AMPK can be activated by glucose depletion, a drop in ATP levels, or oxidative stress (Finicle *et al.*, 2018). The targets of AMPK include a wide range of proteins involved in a multitude of cellular processes, many of which directly and indirectly participate in nutrient uptake, such as integrins, clathrin heavy chain, dynamin1, and many others (Ducommun *et al.*, 2015; Chen Z. *et al.*, 2019; Rahmani *et al.*, 2019). It has been reported that uptake of glucose through glucose transporters is controlled by AMPK activity (Abbud *et al.*, 2000; Barnes *et al.*, 2002). It is also involved in orchestrating lipid metabolism (Lin and Hardie, 2017). mTORC1 is a protein complex regulating cell growth and function (Valvezan and Manning, 2019). When glucose or cellular ATP levels are low, AMPK

inhibits biosynthesis of molecules partly by blocking mTORC1 activity, in favour of energetically less costly scavenging and catabolic reactions (Palm and Thompson, 2017; Finicle *et al.*, 2018). These reports show an organized cellular response to changes in the extra- and intracellular environment. They also suggest a close connection between the metabolic and energetic state of the cell and alterations to the cellular rate of nutrient uptake.

Cells can acquire nutrients in various ways. Endocytosis is a fundamental property of cells that plays a big role in maintaining homeostasis and cell function. It is particularly important in the case of neurons that rely on endocytosis to sustain neuronal synaptic transmission and in immune cells to perform their functions. However, in the case of cancer cells, various uptake routes are vitally important for cell survival. The cancer cell environment can be very challenging; therefore, cells must utilize a wide range of energy substrates and building blocks to ensure homeostasis and proliferation, such as glucose, amino acids, proteins, fatty acids, various macromolecules, even cell debris, or live cells (Palm and Thompson, 2017; Finicle *et al.*, 2018). There are many routes by which cells can acquire the nutrients and energy substrates necessary for their survival. One important strategy is autophagy, which entails digestion of cellular components to recover molecules needed for energy metabolism or proliferation (Florey and Overholtzer, 2019). Autophagy is often seen in cancer cells, to cope with the difficult environment. A less commonly encountered process is entosis, observed in some cancers, including breast, colon, lung, and cervical tumours, as well as melanomas, and involves absorbing a live, intact, neighbouring cell (Finicle *et al.*, 2018). However, cells rely mostly on the typical endocytic routes (figure 5.1) for scavenging nutrients from the extracellular space or culture medium, and the crosstalk between these processes is tightly controlled by the signalling pathways to orchestrate an adequate response to nutrient starvation.

Phagocytosis is commonly utilised by immune cells to maintain their function. It is a receptor-mediated process of uptake of solid particles or pathogens. It involves F-actin driven engulfment of the particle and creation of a phagosome which further processes the ligand (Garrett and Mellman, 2001).

Clathrin-mediated endocytosis is a common form of fluid-phase receptor-mediated uptake, involving ligand-recognizing, plasma membrane receptors that facilitate internalization of extracellular macromolecules and the direct uptake of ligands for degradation (Palm and Thomp-

son, 2017). The clathrin coats mediating the internalization of the ligand-bound receptor consist of a trimer of heterodimers of clathrin heavy and light chain molecules and an adaptor complex (Garrett and Mellman, 2001). Dynamin is a GTPase that is critical to the clathrin-mediated endocytosis, as it facilitates pinching off of the clathrin coated pits to form vesicles that can be then processed or recycled to the cell surface (Garrett and Mellman, 2001). Clathrin-mediated endocytosis frequently involves scavenging proteins or molecules from the extracellular matrix with the help of heterodimeric surface integrin receptors (Seguin *et al.*, 2015). Integrins regulate cell migration, proliferation, and survival through bridging the intracellular actin cytoskeleton with the extracellular matrix and are regularly found clustered with growth receptors. Integrin levels have been found to be elevated in various cancers (Finicle *et al.*, 2018). There are other specific receptors for various molecules, such as previously mentioned hyaluronic acid receptor, CD44 (see chapter 3), transferrin receptors that facilitate the uptake of transferrin, the carrier of iron (Palm and Thompson, 2017), and albumin receptors, such as megalin or cubilin (Merlot *et al.*, 2014; Bern *et al.*, 2015). To survive the shortage of glucose, cells resort to alternate energy sources and thus increase uptake of alternative nutrients. For instance, albumin, the most abundant plasma protein, is often internalized and recycled by the cells as it is a carrier molecule for other nutrients, such as fatty acids. Nevertheless, in some cases, often when amino acids are scarce, cancer cells take up albumin itself and direct it for degradation in the lysosome to recover its constituents (Merlot *et al.*, 2014; Finicle *et al.*, 2018).

Caveolae-mediated endocytosis is a clathrin-independent uptake pathway. It involves plasma membrane invaginations known as caveolae (little caves) usually coated with a protein named caveolin (Garrett and Mellman, 2001; Parton, 2018). The caveolin-coated pits also require dynamin activity to form vesicles and separate from the plasma membrane before being directed for further processing or recycling (Parton, 2018). Caveolae-mediated endocytosis mainly facilitates the uptake of glycosylphosphatidylinositol-anchored proteins; however, the process seems to have a much lower uptake capacity than clathrin-mediated endocytosis, due to low rate of detachment from the plasma membrane of the caveolae (Parton, 2018). Besides the uptake activity, caveolae are involved in cholesterol and lipid metabolism, and cellular signalling (Garrett and Mellman, 2001; Parton, 2018).

Albumin, as well as other proteins and nutrients, can also be taken up by the cells through a dynamin-independent non-selective mechanism called macropinocytosis. It is utilized by cells to acquire wide range of fluids and macromolecules through large, F-actin-driven vesicles (Kerr and Teasdale, 2009). However, the macropinocytic cargo is not always degraded in the lysosome, as the macropinosomes can be also directed back to the plasma membrane and release their contents into the extracellular space to regulate the nutrient content within the cell (Finicle *et al.*, 2018). Macropinocytosis, though not common in normal cells, has been found to be upregulated in RAS-transformed cancers (Comisso *et al.*, 2013). However, there is evidence that macropinocytosis can also be activated through protein kinase C (Yoshida *et al.*, 2015), stimulation of EGFR by epithelial growth factor (EGF) (Bryant *et al.*, 2007) or similarly, stimulation of platelet-derived growth factor receptor (PDGFR) by platelet-derived growth factor (PDGF) (Schmees *et al.*, 2012). Therefore, it is believed that an active macropinocytosis might be a phenomenon widely present in various cancers.

The increase of scavenging activity and exploitation of alternative survival strategies by cancer cells may be exploitable as a therapeutic intervention. This is especially interesting considering HAMLET studies – as cancer cells are desperately searching for more substrates to internalize to fuel their growth and metabolism. HAMLET, which consists of oleic acid and a partially unfolded α -lactalbumin, could easily pose as a nutrient and enter the cell through macropinocytosis to release its tumourigenic potential. Increased macropinocytosis in cancer cells could be responsible for HAMLET's tumour selectivity and efficacy against therapy resistant cancers. Galactose culture conditions might further upregulate macropinocytosis as well as other endocytic pathways as the cell is desperately trying to find substrates for energy and biomass production in the absence of glucose. Studies on endocytosis and HAMLET toxicity could provide an insight into HAMLET's mode of entry into the cell and its mechanism of action, thus allowing to finally understand the underlying causes of its selectivity against cancer cells.

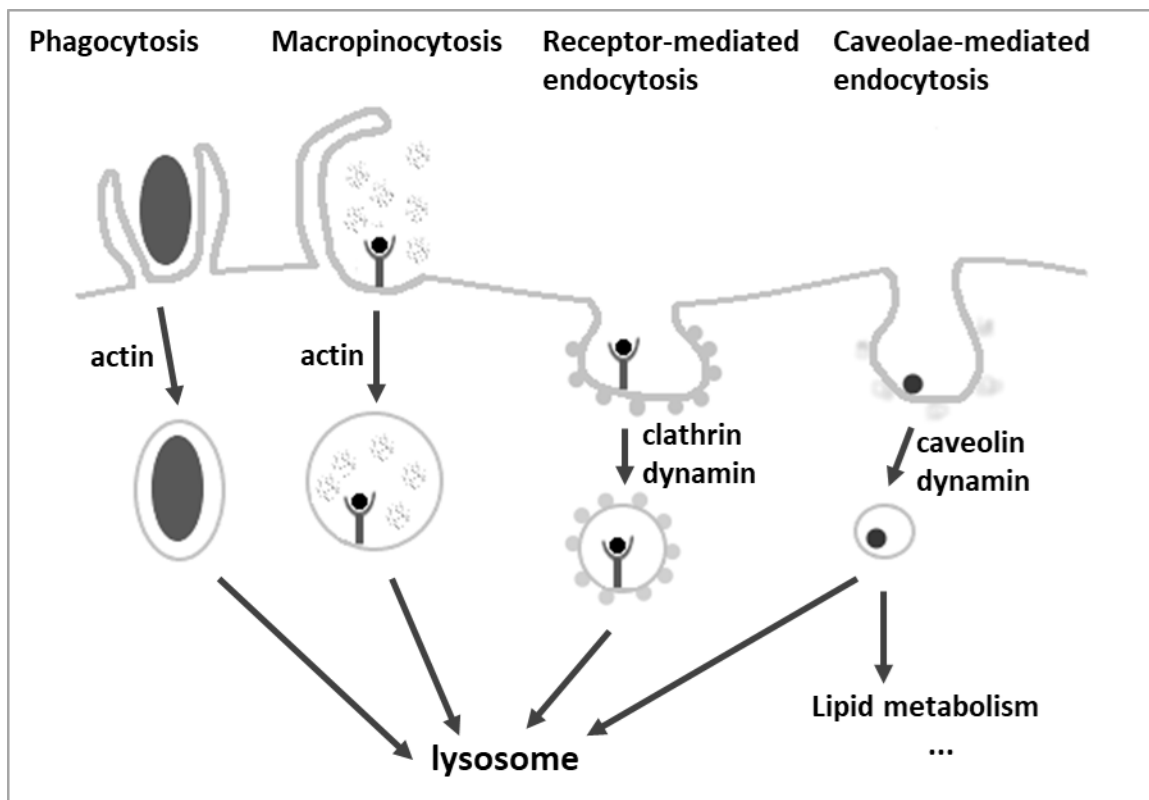


Figure 5.1. Main types of endocytosis in mammalian cells

The four main types of endocytosis utilised by mammalian cells to obtain nutrients and energy substrates for homeostasis. Phagocytosis is a receptor-mediated, actin dependent process of solid particle uptake, usually observed in immune cells. Macropinocytosis is a non-selective mechanism of fluid intake. Macropinosomes are formed with the help of actin and the cargo can be either directed to the lysosome or recycled out of the cell. Receptor-mediated endocytosis requires clathrin-adaptor complex coats and dynamin activity to form the clathrin coated vesicles. Caveolae-mediated endocytosis involves creating caveolae coated vesicles with the help of dynamin. The caveolae cargo can be degraded in the lysosome or partake in cholesterol and lipid metabolism or cell signalling.

5.2. Results

5.2.1. Effect of galactose adaptation on SCC-9, U2OS and KYSE-520 cells' sensitivity to BAMLET

In Chapter 4, galactose adaptation of cells is mentioned as a method to increase their sensitivity to BAMLET. As previously described by Storm *et al.*, (2011) both, a short period of glucose deprivation prior to HAMLET treatment and glycolysis inhibition with 2-DG increased the HAMLET sensitivity of A549 lung carcinoma cell line. In this project, the adopted approach included gradual adaptation of cells to decreasing glucose concentrations with simultaneous increase of galactose concentrations in the culture medium. Ultimately, the culture medium contained 100 % galactose, without any glucose addition. To correct for trace amounts of glucose in the FBS, a dialysed serum was used. As seen in chapter 4.2.5., the initial study of galactose adaptation illustrated about a 4-fold increase in the sensitivity to BAMLET of the SCC-9 cells in comparison to cells grown in glucose containing medium. Based on previous literature reports, a sensitization to BAMLET was predicted; however, the extent was not expected. To understand the underlying causes of the change in sensitivity and investigate if they could be utilised in increasing HAMLET/BAMLET's potential as an anticancer agent, a more comprehensive study of galactose adaptation's effect on BAMLET sensitivity was carried out.

To perform a broad examination of galactose adaptation and its effects on cancer cells, all three initially studied cell lines were employed in the study. U2OS and KYSE-520 cells were also gradually adapted to culture in galactose conditioned medium over several weeks and then exposed to a range of concentrations of BAMLET along with the SCC-9 cells. All three cell lines displayed an increase in sensitivity to BAMLET (figure 5.2) under galactose adaptation conditions. Curiously, all three cell lines of different origin showed a similar range of BAMLET sensitivity after galactose adaptation suggesting involvement of a universal mechanism. Galactose adapted SCC-9 cells showed an IC_{50} value of 0.4 μ M while control SCC-9 cells – 1.2 μ M, galactose adapted U2OS cells – 0.4 μ M while control U2OS cells – 2.5 μ M, galactose adapted KYSE-520 cells – 0.5 μ M while control KYSE-520 cells – 6 μ M. Due to the diversity between the cell lines in their initial sensitivity to BAMLET in control conditions, the levels of sensitization differed, with a 3-fold increase in sensitivity in SCC-9 cells, an over

6-fold rise in U2OS cells, and a dramatic 12-fold enhancement in sensitivity in KYSE-520 cells. It is not clear what caused the initial differences in sensitivity and differing resistance levels between the examined cell lines; however, galactose adaptation has clearly unified the cells and introduced a high level of sensitization to BAMLET. A decrease of IC₅₀ values to similar levels might suggest a certain maximal level of sensitivity in cancer cells that can be reached by diverse cell lines upon successful stimulation.

Storm *et al.*, (2011) reported that addition of glucose reversed the sensitization effect of the short period of glucose starvation. To test if it is true with galactose adaptation, galactose adapted SCC-9, U2OS, and KYSE-520 cells were exposed to various concentrations of BAMLET in glucose containing medium. Upon simultaneous treatment with BAMLET and glucose, the galactose adaptation effect was reversed, and the IC₅₀ values of galactose adapted cells treated with glucose were similar to control cells, that were continually grown in glucose medium (figure 5.3). Galactose adapted SCC-9 cells upon glucose addition displayed an IC₅₀ value of 1 μ M, U2OS cells – 2.6 μ M, and KYSE-520 cells – 5.4 μ M – all showing no significant differences from the IC₅₀ values obtained by the control glucose grown cells of each cell line.

In the inverse of the above study, cells grown in glucose were simultaneously exposed to BAMLET in galactose medium. However, the abrupt deprivation of glucose and exposure to galactose and BAMLET did not introduce any enhanced sensitization (figure 5.4). The IC₅₀ values of glucose grown SCC-9, U2OS, and KYSE-520 cells treated with BAMLET either in glucose or galactose containing media were not significantly different. This result suggests that a starvation/adaptation period prior to BAMLET treatment is necessary to introduce metabolic changes and promote sensitization of the cells. A likely explanation is that the increased BAMLET sensitivity of galactose adapted cells stems from the transcriptional changes that drive the cellular response to new growth conditions. This takes time for the cells to display the effect; and therefore, is only observed in pre-treated cells.

5.2.2. Metabolic characterisation of galactose adapted SCC-9, U2OS, and KYSE-520 cells

To obtain an understanding of the metabolism after galactose adaptation, SCC-9, U2OS, and KYSE-520 cells were studied by Seahorse Metabolic Flux Analyser. Both, the oxygen consumption rate and extracellular acidification rates were examined in control and galactose adapted cells, both in glucose and galactose conditioned medium. Media were changed at the moment of assay execution.

The metabolic studies revealed that glycolysis is downregulated in cells of all three tested cell lines in galactose conditioned medium, both after gradual galactose adaptation, and sudden change of medium (figure 5.5 and figure 5.7). However, upon addition of glucose to the galactose adapted cells, the extracellular acidification rate instantly increases, indicating a rapid initiation of glycolysis. In fact, galactose adapted SCC-9 and U2OS cells show a significantly higher glycolysis rate after addition of glucose than the control cells grown in glucose, suggesting the machinery was intact and functional, despite the galactose conditioning and that the cells are overcompensating for the period of glucose starvation. In the case of KYSE-520 cells, galactose adapted cells do initiate glycolysis upon glucose injection; however, the rate is significantly lower than in control KYSE-520 cells, suggesting adaptation to galactose might have introduced some long-lasting metabolic remodelling involving reliance on alternative energy producing pathways.

On the contrary, the oxygen consumption rate changes did not follow universal patterns in the three examined cell lines (figure 5.6 and figure 5.7). SCC-9 cells displayed expected OCR patterns, with cells cultured in galactose medium during the assay displaying significantly higher OCR in non-stimulated basal respiration state than the control cells injected with glucose. Control U2OS cells showed increased OCR in cells in galactose conditioned medium, while galactose adapted U2OS cells displayed the same elevated level of OCR in both, glucose and galactose conditioned medium. In the case of KYSE-520 cells, the control cells showed no significant difference in OCR in glucose or galactose conditioned media, whereas galactose adapted cells in glucose medium displayed a significant decrease of OCR and a significant increase in galactose containing medium.

All the Seahorse Metabolic Flux assays were normalized for cell number in the three cell lines with the help of Hoechst 33258 double-stranded DNA binding dye to provide an accurate measure of the ECAR and OCR per 10,000 cells from each well of the 96-well plates. A cell number standard curve was first prepared for each cell line (figure 5.8) to establish the difference in fluorescence levels as the number of the cells in the wells rises. Next, after each Seahorse Metabolic Flux assay, the plates were stained with Hoechst 33258 dye to determine the cell number in each well of the plate. Subsequently, the OCR and ECAR data were normalized for cell number in order to make sure the metabolic results are mirroring the situation in cell culture as accurately as possible.

The discrepancies between metabolic behaviour in the various conditions revealed that the reaction to galactose adaptation is not metabolically universal, but the remodelling depends on the cell line and its nutrient and energy substrate preferences. However, despite the different metabolic patterns, all three cell lines displayed similar sensitization to BAMLET suggesting that no matter which actual metabolic compensation mechanisms are chosen by each cell line, the final outcome remains the same. This suggests that lack of glucose in the medium is not easily compensated for with the various energetic pathways utilized by the cells, and that cells might activate other pathways to fulfil their energy demands. The enhancement of macropinocytosis as a secondary response to galactose adaptation would easily explain why the three cell lines, despite showing slightly different metabolic patterns, still showed the same patterns in BAMLET sensitivity.

5.2.3. Endocytosis study in SCC-9 cells

There are multiple literature reports hinting at a close relationship between metabolism and cellular uptake rate. Previous studies show how the metabolic and energetic status of the cell can control the endocytic traffic of nutrients into the cell. Depending on the available nutrient, cells can upregulate different endocytic pathways, with receptor-mediated endocytosis recruiting certain nutrients through their own receptors and transporters, and macropinocytosis being a non-specific general mechanism. Multiple reports mention constitutive activation of macropinocytosis in cancers with certain mutations, such as RAS-transformation (Comisso *et*

al., 2013), EGFR upregulation (Bryant *et al.*, 2007), and multiple others. There is enough evidence to speculate that macropinocytosis is activated in many cancers in general, not only in starvation or during circumstances of nutrient scarcity, when it is acting as a ‘life-saving’ mechanism.

To test the hypothesis that HAMLET/BAMLET molecules are actively recruited into the cell and that upregulated endocytosis under galactose adaptation is responsible for an increased BAMLET toxicity in the tested cell lines, an endocytosis study on SCC-9 cells ensued. With the help of the aforementioned fluorescent uptake markers, the rates of clathrin-mediated endocytosis and macropinocytosis were measured. As described in chapter 3, lucifer yellow is a small anionic molecule, used to track macropinocytosis. The 70-kDa Dextran-Texas Red is a polysaccharide derivative of glucose which, due to its molecular weight, can enter the cell either through macropinocytosis or *via* the mannose receptor to which it has affinity. Therefore, it provides an outlook on both, macropinocytosis and clathrin-mediated endocytosis, while the conjugation with Texas Red fluorophore provides it with a red fluorescence. To obtain a comprehensive look on the cellular uptake, the fluorescent HA polymer derivative, NF2792, synthesised and characterised in chapter 3, was utilised in the study to illustrate clathrin-mediated endocytosis, as it enters SCC-9 cells through the HA receptor, CD44 (Aruffo *et al.*, 1990; Hua *et al.*, 1993; Yu *et al.*, 2016).

SCC-9 cells previously grown in glucose or galactose were treated with the fluorescent endocytosis markers in their respective media. The assay was performed both at 37 °C or 4 °C, to differentiate between active, ATP-dependent endocytic processes and passive diffusion or binding to the plasma membrane. In the case of each marker, 70-Dextran-Texas Red, lucifer yellow, and NF2792, the cell lysates of galactose adapted cells showed higher fluorescence at 37 °C indicating significantly higher active uptake of the molecule (figure 5.9). Glucose grown SCC-9 cells assayed at 37 °C showed a higher fluorescence than the cells treated at 4 °C, suggesting both macropinocytosis and clathrin-mediated endocytosis are active in SCC-9 cells even when cultured in glucose containing medium. Both, galactose adapted and control glucose grown SCC-9 cells showed basic level of fluorescence upon exposure to the endocytic markers at 4 °C, indicating only a low level of non-active binding or diffusion. These observations coupled with the BAMLET toxicity data from figure 5.2 strongly indicate that an up-regulated endocytic traffic in galactose adapted cells is responsible for the increase in BAM-

LET sensitivity in galactose adapted cells. The obtained results support the theory that HAMLET/BAMLET's selectivity against cancer cells might be driven by their active uptake mechanisms even in abundance of nutrients in the medium.

To test if addition of glucose will reverse the uptake rate as it did with BAMLET toxicity (figure 5.3), galactose adapted SCC-9 cells were treated with endocytic markers in glucose containing medium. Correlating with the BAMLET toxicity results, the uptake of 70-kDa Dextran-Texas Red, NF2792, and lucifer yellow was significantly decreased (figure 5.10). This result confirms the theory that cancer cells further upregulate an already active endocytosis in glucose deplete conditions, to secure cell survival and maintain the elevated proliferation rate. Curiously, when the glucose grown control SCC-9 cells were treated with the endocytic markers upon replacement of medium with the galactose conditioned one, no significant difference in uptake was visible in neither of the tested molecules (figure 5.11). This result is also in agreement with previous BAMLET toxicity studies on glucose grown cells treated with galactose conditioned medium (figure 5.4). The observed result suggests that a pre-treatment with glucose deplete conditions might be needed to observe an elevated uptake rate. An acute glucose deprivation might force the cells to undergo a molecular and transcriptional remodelling in response. Therefore, an elevated uptake rate would be observed only after a certain adaptation time necessary for the transcriptional changes to come into effect.

5.2.4. Impact of endocytic inhibitors on BAMLET toxicity in SCC-9 cells

The correspondence between increased endocytic traffic in galactose adapted cells and BAMLET sensitization suggest a close link between metabolism, cellular uptake, and HAMLET/BAMLET toxicity. To examine the connection of BAMLET and endocytosis, an investigation into the uptake patterns upon BAMLET treatment was launched. SCC-9 cells were pre-treated with the endocytosis inhibitors mentioned in chapter 3; amiloride to block macropinocytosis (Koivusalo *et al.*, 2010), and the dynamin inhibitor dynasore (Macia *et al.*, 2006) to limit clathrin-mediated endocytosis. A treatment for 30 min was employed to provide enough time for the suppression of the distinct endocytic pathways. Next, cells were treated with

BAMLET for 6 h and the cell death response was measured in comparison with non-pre-treated cells *via* flow cytometry and Western blotting.

Flow cytometry data obtained from Annexin V/PI double staining revealed that treatment with the endocytic inhibitors alone does not induce apoptosis in SCC-9 cells (figure 5.12). A 6 h exposure to 20 μ M BAMLET induced a significant cell death response in SCC-9 cells. Interestingly, 30 min pre-treatment with 1 mM amiloride protects SCC-9 cells from BAMLET's toxic effect. On the contrary, 100 μ M dynasore pre-treatment prior to exposure to 20 μ M BAMLET does not exhibit the same protective properties, with cells showing signs of induction of apoptosis. The Western blot data on Procaspase 3 levels showed the same patterns of apoptotic induction (figure 5.13). A significant decrease of Procaspase 3 levels was observed in cells treated with 20 μ M BAMLET alone and with 100 μ M dynasore pre-treatment, with a reduced level of Procaspase 3 associated with its activation due to induction of apoptosis. Cells exposed to 1 mM amiloride for 30 min before 20 μ M BAMLET treatment exhibited high levels of Procaspase 3, suggesting no apoptosis induction. These observations strongly suggest that BAMLET is actively recruited into SCC-9 cells through macropinocytosis and that inhibiting this cellular process can protect the cells from BAMLET's toxicity. The inhibition of dynamin activity does not lower BAMLET's toxicity in SCC-9 cells indicating that it is not internalized through clathrin-mediated endocytosis.

5.3. Discussion

It is vital to identify the characteristics that differentiate cancer cells from normal healthy cells in order to develop a highly selective cancer therapy. Cancer cells evolve to grow in very hostile and nutrient poor environments; and therefore, adapt their metabolism to support survival and proliferation in spite of the unfavourable circumstances (DeBerardinis and Chandel, 2016). It is clear that cancer metabolism is very different from what can be observed in normal differentiated cells. One such difference is the upregulation of glycolysis, under aerobic conditions (Warburg, 1956). Multiple studies have targeted glycolysis as a cancer selective therapeutic approach. Glucose starvation and inhibition of glycolysis have been reported to induce cell death through depletion of ATP levels within the cell and thereby acutely disrupting cellular homeostasis (Pradelli *et al.*, 2010; Nakano *et al.*, 2011). Co-treatment of patients with glycolysis inhibitors together with chemotherapeutics was shown to reinstate chemosensitivity (Nakano *et al.*, 2011). Interestingly, periodical glucose starvation and inhibition of glycolysis with 2-DG have been previously reported to increase HAMLET sensitivity *in vitro* (Storm *et al.*, 2011).

This project endeavoured to decipher the mechanism behind sensitization of cells to HAMLET/BAMLET treatment by disruption of glycolysis. The approach adopted in this study was to gradually adapt cells to increasing concentrations of galactose while simultaneously decreasing the content of glucose in the culture medium over several weeks. Eventually, the cells were cultured exclusively in a galactose conditioned medium, without glucose, to stimulate a stable metabolic remodelling. Since the metabolism of galactose by the cell is not energetically profitable, cells developed changes in their metabolic activity to deal with the altered availability of energy substrates in the culture medium.

Galactose adapted SCC-9 squamous cell carcinoma of the tongue cells, U2OS osteosarcoma cells, and KYSE-520 oesophageal squamous cell carcinoma cells showed a dramatic increase in their sensitivities to BAMLET in comparison to cells grown in glucose containing medium. Interestingly, upon simultaneous exposure to BAMLET and glucose, the galactose adapted cells of all three cell lines reverted their sensitivity to the basal level of cells continuously grown in glucose. Conversely, substitution of glucose with galactose upon BAMLET treatment of control glucose grown cells did not introduce a sensitization. Metabolic characteriza-

tion has proven that there is no glycolytic activity in cells assayed in galactose conditioned medium. However, an instant activation of the glycolytic pathway can be observed upon injection of glucose. This demonstrates that glycolytic machinery remains functional in galactose adapted cells. It has been expected that galactose adapted cells would largely switch to oxidative phosphorylation to cover their energetic needs. This was the case for SCC-9 cells, where oxygen consumption rate was higher in cells in galactose medium. However, U2OS and KYSE-520 did not follow the same pattern, indicating dependence on alternative energy producing pathways. In galactose adapted KYSE-520 cells, upon addition of glucose, a significantly lower increase of ECAR was observed than in the case of highly glycolytic control KYSE-520 that were continually cultured in glucose. This observation might suggest a long-term metabolic remodelling to derive energy from alternative sources and not depend largely on glycolysis.

The results suggest a close link between glycolysis rate and BAMLET sensitivity. Previous reports have pointed at the possibility of a decrease in ATP levels in cells with dysfunctional glycolysis which could sensitize the cells to therapy or, in extreme cases, cause the activation of cell death mechanisms. In the case of the BAMLET studies described here, galactose adaptation does sensitize the cells to BAMLET. Moreover, despite the initial differences in IC_{50} values between the three examined cell lines, the sensitivity to BAMLET after galactose adaptation showed sensitivities within a similar range, possibly indicating a maximal sensitization level that cancer cells of different origins could attain. It is interesting that addition of glucose could immediately reverse the sensitization effect and reverted the IC_{50} values to the levels from before the adaptation, suggesting activation of glycolysis has a protective effect. However, the observed sensitization and desensitization patterns point to a necessity of a pre-treatment and adaptation period to new circumstances prior to HAMLET/BAMLET treatment to introduce the increase in sensitivity. It suggests an involvement of a universal mechanism, closely connected to the energy level of the cells and to metabolism, that is activated in glucose- or energy-depleted conditions.

Literature reports suggest a connection between cellular metabolism and endocytic traffic. Cancer cells grow in harsh and often nutrient deplete conditions and need to adapt their metabolism effectively to ensure survival and maintain a high proliferation rate (Vander Heiden and DeBerardis, 2017). Therefore, it is vital for cancer cells to modify their nutrient uptake to

respond to their metabolic and energetic needs. There were reports of upregulated endocytosis in cancer cells exposed to glucose deficient conditions or glycolysis inhibitors (Kim *et al.*, 2018). In light of this, the endocytic traffic in galactose adapted SCC-9 cells has been studied in comparison to control glucose grown SCC-9 cells. It has been observed that galactose adaptation stimulates the uptake of 70-kDa Dextran Texas Red, NF2792, and lucifer yellow in SCC-9 cells, suggesting both the specialised clathrin-mediated endocytosis and non-specialised macropinocytosis uptake pathways are upregulated after galactose adaptation. Simultaneous addition of glucose with the endocytic markers, just like in the case of BAMLET treatment, reverted the uptake rate to the control level, indicating that in the presence of glucose the need for upregulation of endocytic traffic is negated. Similar to the BAMLET toxicity studies, simultaneous treatment with endocytic markers and galactose conditioned medium did not increase uptake in control cells, suggesting an adaptation period is necessary to deplete cellular energy levels before treatment occurs to induce sensitization and uptake increase. Since the results of the endocytosis studies and BAMLET toxicity studies are aligned, a strong connection between the rate of uptake and sensitivity to BAMLET has been drawn. It is important to note that the treatment at 37 °C versus 4 °C revealed that the control cells grown in glucose also show a certain level of clathrin-mediated endocytosis and macropinocytosis activation, albeit significantly lower than galactose adapted cells. This observation draws attention to the fact that despite the presence of glucose in the medium and, presumably, satisfied energy and biomass production of the cells, a considerable level of ATP-related acquisition of nutrients is active in SCC-9 cells, whereas no such phenomenon is noticeable in cells assayed at 4 °C, where no ATP production occurs. This phenomenon, mentioned to occur in cancer cells with certain mutations (Bryant *et al.*, 2007; Schmees *et al.*, 2012; Comisso *et al.*, 2013; Yoshida *et al.*, 2015), might be in fact occurring in a wider group of cancers and perhaps is the origin of HAMLET's cancer selectivity.

To explore this notion further, SCC-9 cells were pre-treated with amiloride, an inhibitor of macropinocytosis and dynasore, an inhibitor of dynamin related to clathrin-mediated endocytosis prior to BAMLET treatment. Interestingly, despite reports of albumin uptake in cancer cells and the existence of albumin receptors, dynasore pre-treatment does not protect SCC-9 cells from BAMLET's toxicity. However, SCC-9 cells pre-treated with amiloride have not displayed induction of apoptosis, indicating BAMLET is internalized into the cells through macropinocytosis and its inhibition has a protective effect. In fact, the protective effect of ami-

loride against HAMLET was mentioned previously in literature, albeit not in connection to macropinocytosis but ion channels inhibition (Storm *et al.*, 2013).

This result confirms that BAMLET's mode of entry into SCC-9 cells is through macropinocytosis. Galactose adaptation disrupts the metabolism and homeostasis in cancer cells as they largely depend on glycolysis for energy production. To compensate for loss of ATP produced through glycolysis, cells resort to other energy producing pathways, largely through catabolic reactions; and therefore, upregulation of nutrient uptake rate is necessary to ensure survival. Due to HAMLET/BAMLET's structure, incorporating oleic acid and α -lactalbumin, it can pose as a nutrient to be taken up through macropinocytosis by the cells in order to be metabolised. The upregulation of macropinocytosis rate results in an increased volume of HAMLET/BAMLET entering the cells, therefore, increasing its toxicity in galactose adapted cells. Inhibition of macropinocytosis in SCC-9 cells showed to protect the cells from HAMLET/BAMLET's toxicity through inhibition of its uptake. Since HAMLET/BAMLET cannot enter the cells, it cannot induce cell death. Moreover, as macropinocytosis is frequently upregulated and constitutively activated in various cancers (Bryant *et al.*, 2007; Schmees *et al.*, 2012; Comisso *et al.*, 2013; Yoshida *et al.*, 2015), and not in normal differentiated cells, we speculate that macropinocytosis is in fact responsible for HAMLET/BAMLET's selectivity against cancer cells.

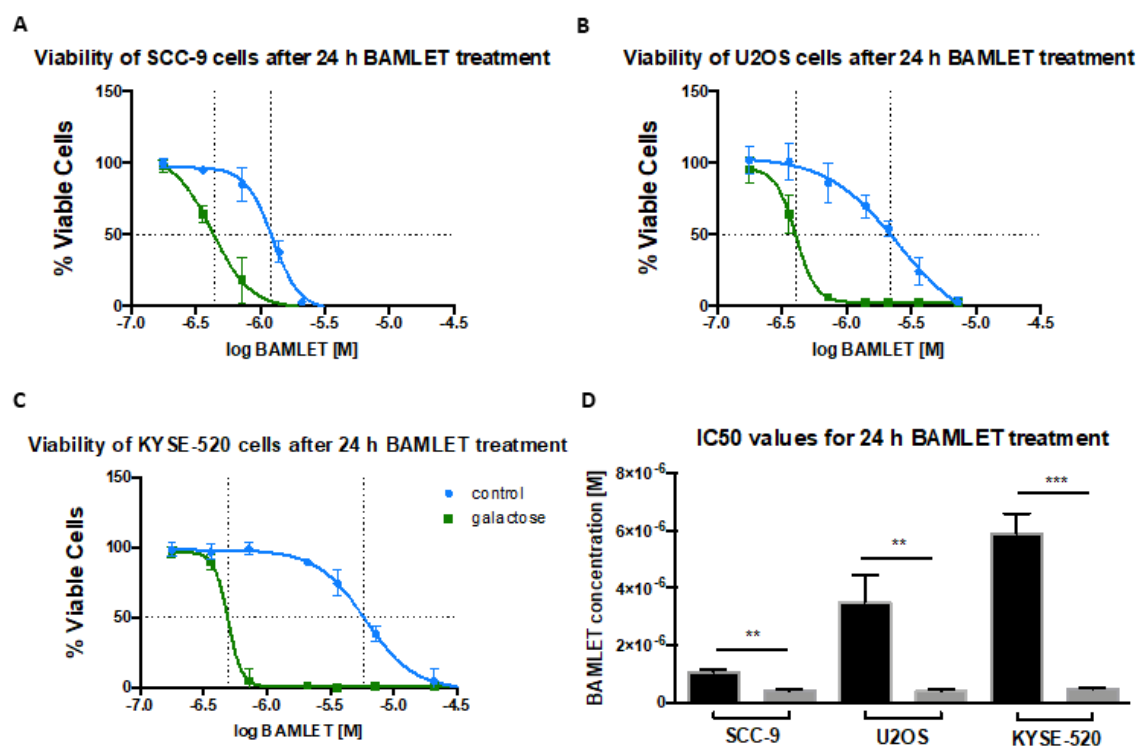


Figure 5.2. Cell viability of galactose adapted cells after 24 h exposure to BAMLET

Cytotoxicity curves for A - SCC-9, B - U2OS, and C - KYSE-520 cells. Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well. All cell lines were serum starved for 16 h prior to treatment with various concentrations of BAMLET in serum-free medium for 24 h. Galactose adapted cells were treated consistently in galactose conditioned medium. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. Graphs represent the mean $\pm$ SD of the values. D – IC₅₀ value comparison for all 3 cell lines in control conditions and after galactose adaptation. IC₅₀ values were as follows: control SCC-9 – 1.2 μ M, galactose adapted SCC-9 – 0.4 μ M, control U2OS – 2.5 μ M, galactose adapted U2OS – 0.4 μ M, control KYSE-520 – 6 μ M, galactose adapted KYSE-520 – 0.5 μ M. Student t-test, *** $p < 0.001$, * $p < 0.05$.

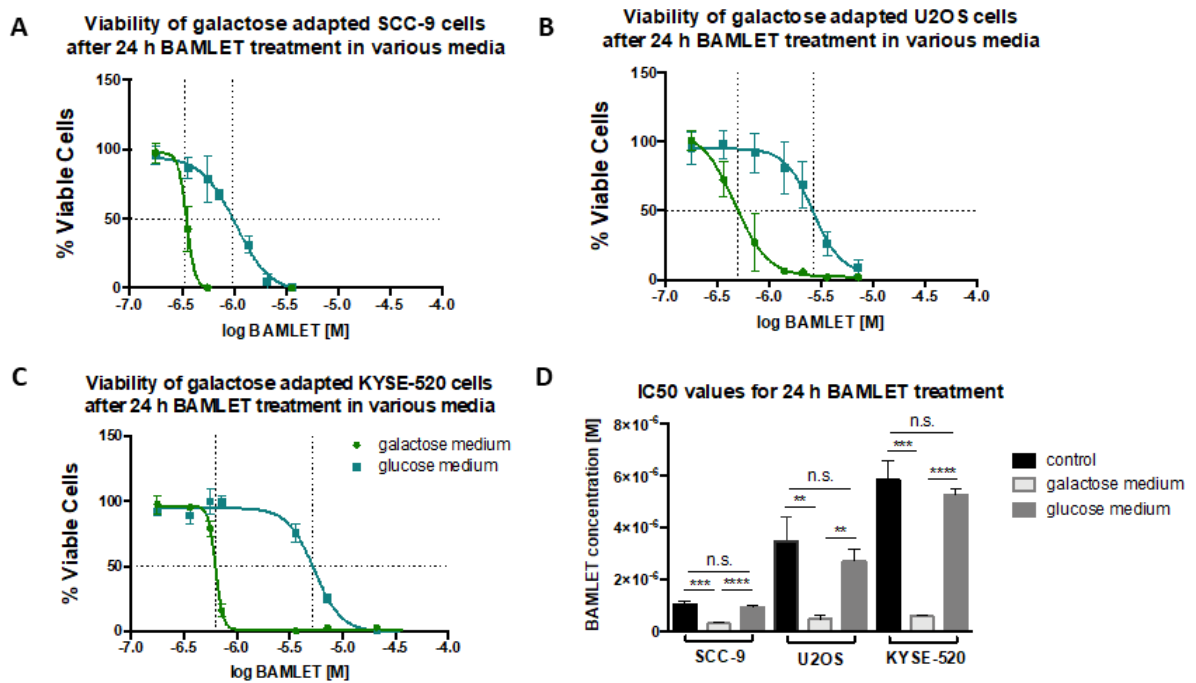


Figure 5.3. Cell viability of galactose adapted cells after 24 h exposure to BAMLET in medium containing galactose or glucose

Cytotoxicity curves for galactose adapted A – SCC-9, B – U2OS, and C – KYSE-520 cells treated in media containing galactose or glucose. Galactose adapted cells were seeded on a 96-well plate at a density of 5×10^3 cells/well. All cell lines were serum starved for 16 h prior to treatment. Various concentrations of BAMLET were administered in serum-free medium for 24 h, either in glucose or galactose conditioned medium. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. D – IC₅₀ value comparison for galactose adapted SCC-9, U2OS, and KYSE-520 cells treated in galactose or glucose containing media and control cells treated in glucose containing medium. Values represent the mean $\pm$ SD of three experiments. IC₅₀ values were as follows: control SCC-9 in glucose medium – 1.243 μ M, galactose adapted SCC-9 in galactose medium – 0.352 μ M, galactose adapted SCC-9 in glucose medium – 1 μ M, control U2OS in glucose medium – 2.5 μ M, galactose adapted U2OS in galactose medium – 0.5 μ M, galactose adapted U2OS in glucose medium – 2.6 μ M, control KYSE-520 in glucose medium – 6 μ M, galactose adapted KYSE-520 in galactose medium – 0.6 μ M, galactose adapted KYSE-520 in glucose medium – 5.4 μ M. Student t-test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. – not significant.

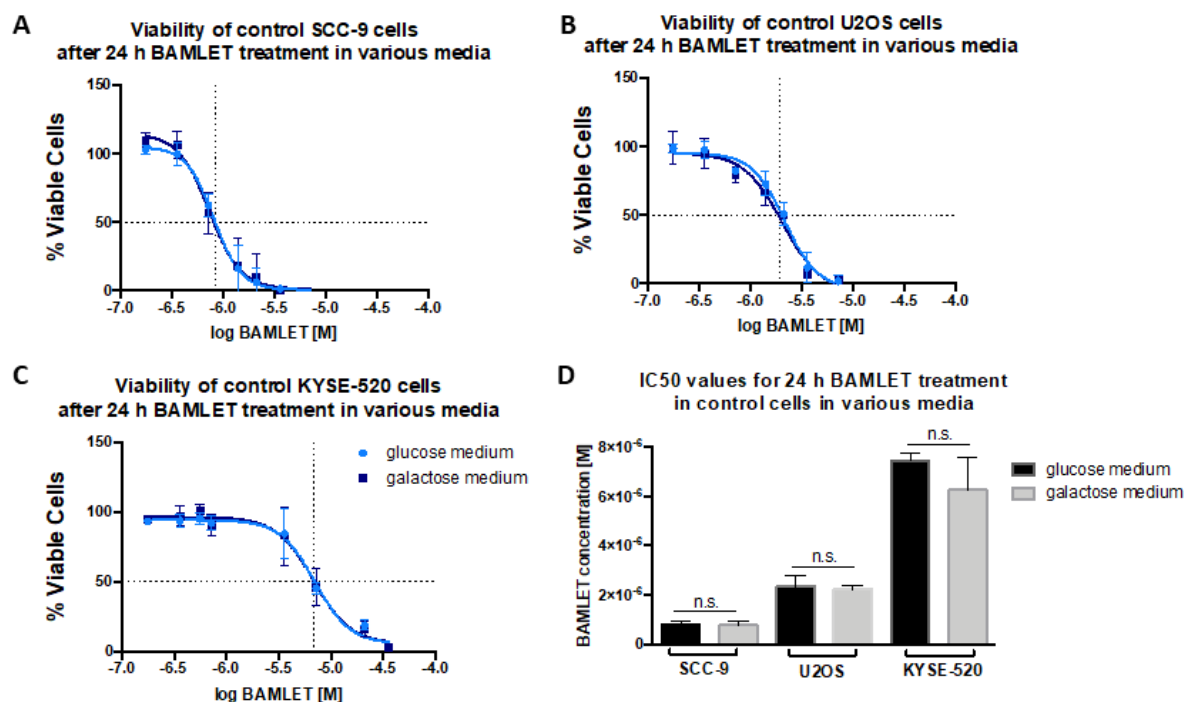


Figure 5.4. Cell viability of control cells after 24 h exposure to different concentrations of BAMLET in medium containing galactose or glucose

Cytotoxicity curves for control A – SCC-9, B – U2OS, and C – KYSE-520 cells treated in media containing galactose or glucose. Cells were seeded on a 96-well plate at a density of 5×10^3 cells/well. All cell lines were serum starved for 16 h prior to treatment. Various concentrations of BAMLET were administered in serum-free medium for 24 h, either in glucose or galactose conditioned medium. The viability of the cells was assessed by Alamar Blue assay. The concentrations are shown in a log scale. D – IC₅₀ value comparison for control SCC-9, U2OS, and KYSE-520 cells treated in galactose or glucose containing media. Values represent the mean $\pm$ SD of three experiments. IC₅₀ values were as follows: control SCC-9 in glucose medium – 0.8 μ M, control SCC-9 in galactose medium – 0.7 μ M, control U2OS in glucose medium – 2.7 μ M, control U2OS in galactose medium – 2.1 μ M, control KYSE-520 in glucose medium – 7 μ M, control KYSE-520 in galactose medium – 6.8 μ M. Student t-test, n.s. – not significant.

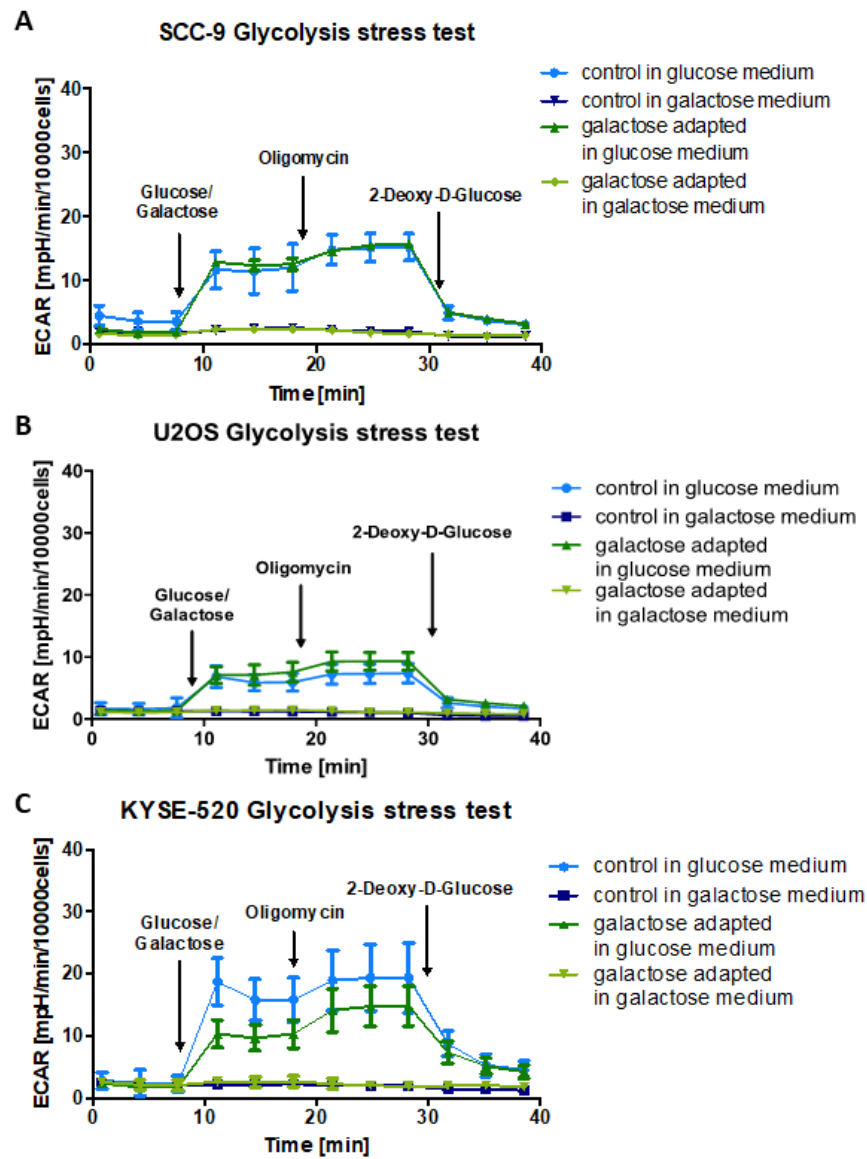


Figure 5.5. Glycolysis stress test in A – SCC-9, B – U2OS, C – KYSE-520 cells after galactose adaptation in various media

ECAR in control and galactose adapted cells in glucose or galactose conditioned media. A – SCC-9 cells, sequential injections of glucose (10 mM), oligomycin (1 μ M), 2-deoxy-D-glucose (30 mM). B – U2OS cells, sequential injections of glucose (10 mM), oligomycin (2 μ M), 2-deoxy-D-glucose (30 mM). C – KYSE-520 cells, sequential injections of glucose (10 mM), oligomycin (2 μ M), 2-deoxy-D-glucose (30 mM). Graphs represent means $\pm$ SD of values obtained in three independent experiments.

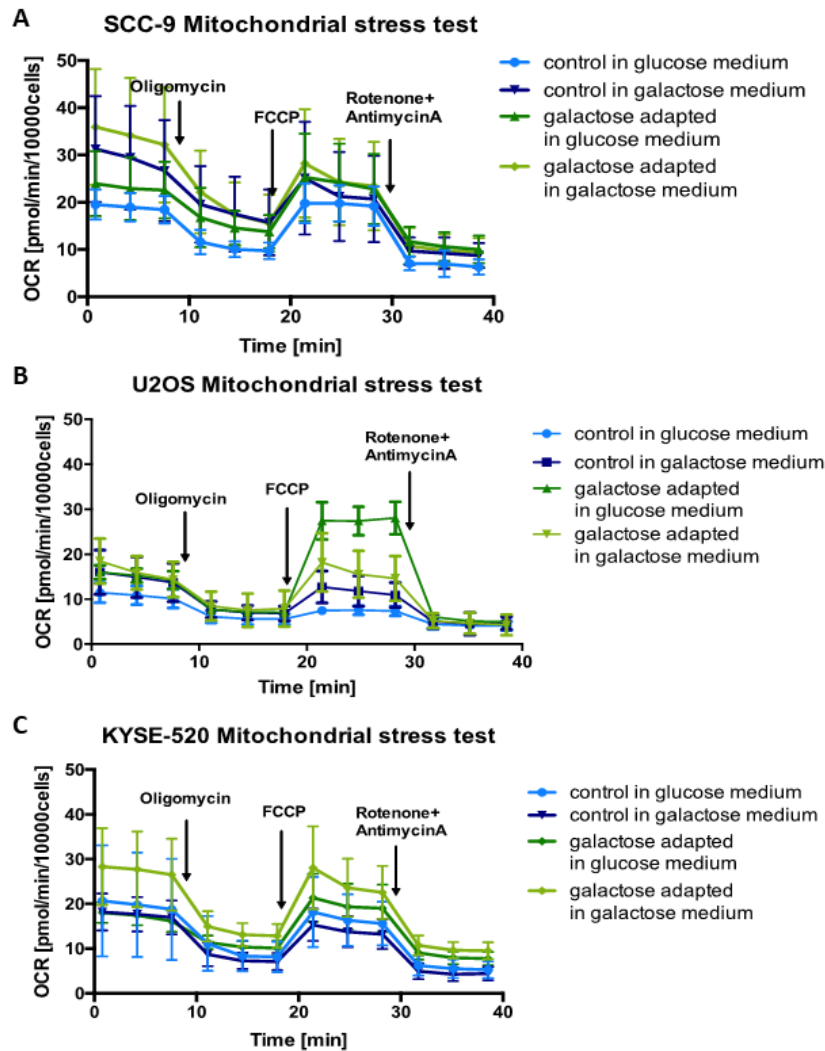


Figure 5.6. Mitochondrial stress test in A – SCC-9, B – U2OS, and C – KYSE-520 cells after galactose adaptation in various media

OCR in control and galactose adapted cells in glucose or galactose conditioned media. A – SCC-9 cells, sequential injections of oligomycin (1 μ M), FCCP (0.125 μ M), rotenone (0.1 μ M) and antimycin A (4 μ M). B – U2OS cells, sequential injections of oligomycin (2 μ M), FCCP (0.5 μ M), rotenone (0.1 μ M) and antimycin A (4 μ M). C – KYSE-520 cells, sequential injections of oligomycin (2 μ M), FCCP (0.125 μ M), rotenone (0.1 μ M) and antimycin A (4 μ M). Graphs represent means $\pm$ SD of values obtained in three independent experiments.

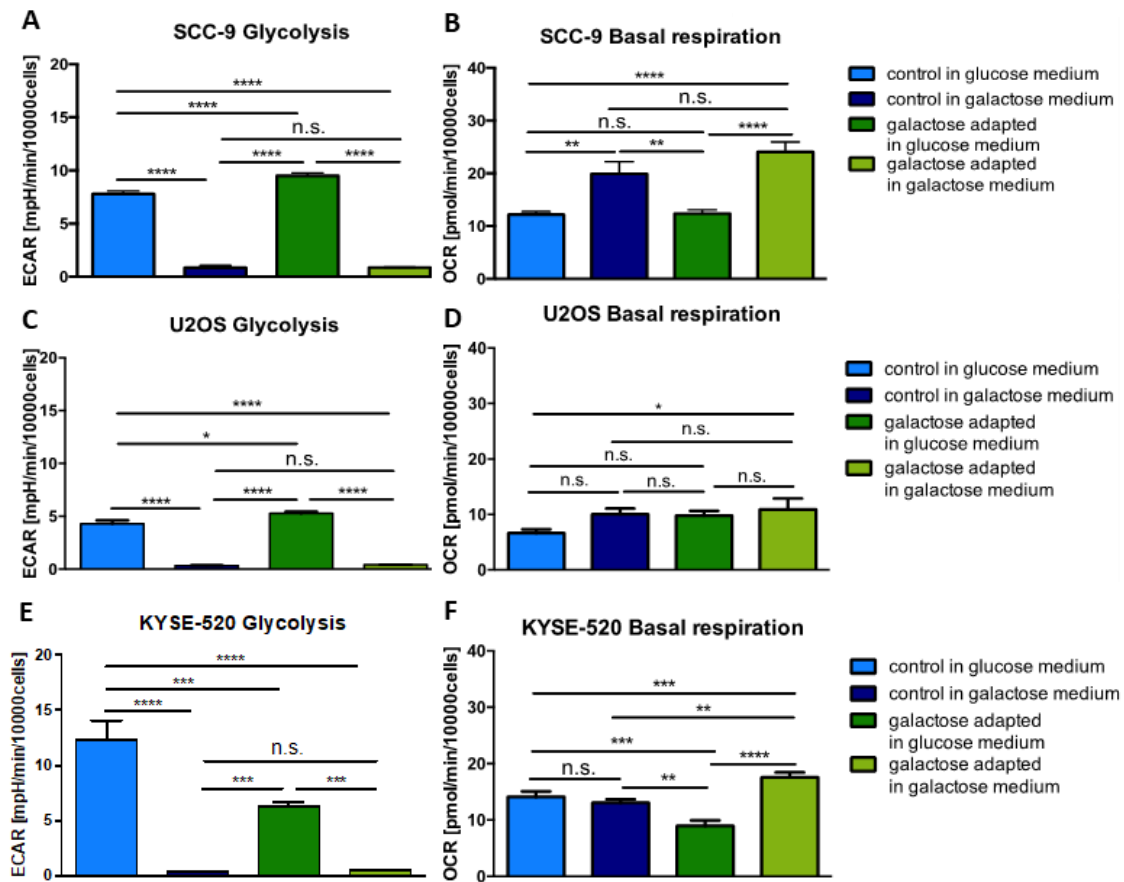


Figure 5.7. Metabolic differences between SCC-9, U2OS, and KYSE-520 cells after galactose adaptation in various media

Glycolysis rate – ECAR in control and galactose adapted cells in glucose or galactose conditioned media after injection of glucose or galactose in A – SCC-9 cells, C – U2OS, E – KYSE-520 cells. Basal respiration – OCR in control and galactose adapted cells in glucose or galactose conditioned media before any injection in B – SCC-9 cells, D – U2OS, F – KYSE-520 cells. Graphs represent means $\pm$ SD of values obtained in three independent experiments. One-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. – not significant.

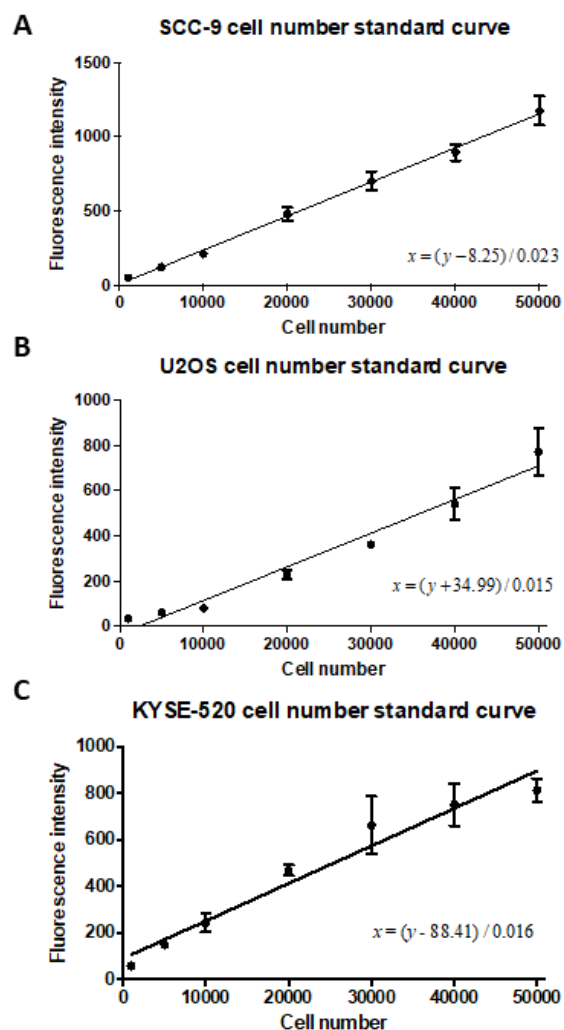


Figure 5.8. Cell number determination by Hoechst 33258 staining for normalization of data from the Seahorse Analyser

Cell number determination curves for A – SCC-9, B – U2OS, and C – KYSE-520 cells. Cells were seeded on a 96-well plate at a density of 1×10^3 , 5×10^3 , 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , and 5×10^4 cells/well. After attachment, medium was removed, and the plates were frozen at -80°C . Plates were subsequently thawed, incubated at 37°C with H_2O , and then stained with Hoechst 33258. Fluorescence was measured at an excitation of 360 nm and emission of 460 nm. Increase in fluorescence was proportional to increase in cell number. The equation for a straight line through the data points for each cell line was used to determine cell number in Seahorse Metabolic Flux Analyser assays.

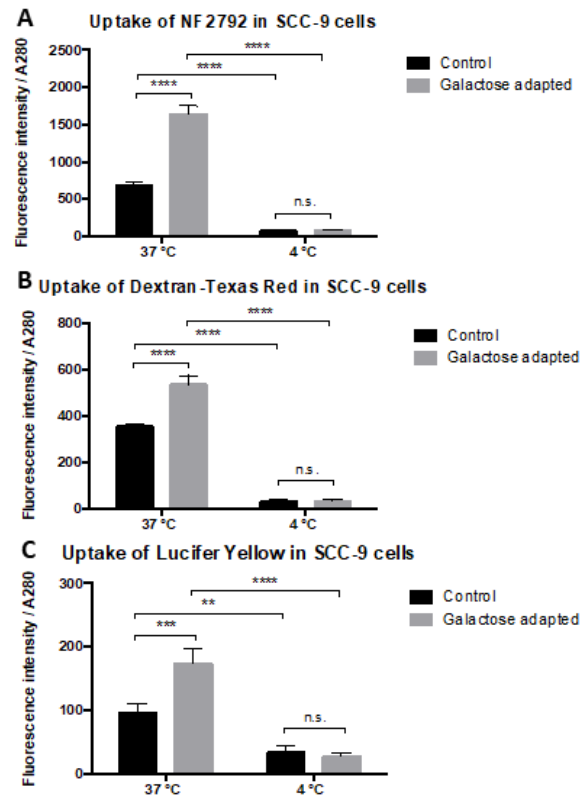


Figure 5.9. Study of endocytosis in SCC-9 cells after galactose adaptation

Uptake patterns of A – NF2792, B – 70 kDa Dextran-Texas Red, and C – Lucifer Yellow in control and galactose adapted SCC-9 cells. Cells were seeded on a 6-well plate at a density of 3×10^5 cells/well and serum starved for 16 h prior to treatment. Then, cells were treated with either 5 μ M of NF2792 (A), 1 μ M of 70 kDa Dextran-Texas Red (B), or 15 μ M of Lucifer Yellow (C) for 1 h in serum-free, either glucose or galactose conditioned medium. Cells were incubated at 37 °C or 4 °C to distinguish between endocytosis and non-specific binding to the plasma membrane. Next, cells were washed twice in PBS and lysed. Fluorescence of the cell lysates was read at an excitation of 335 nm and emission of 435 nm for NF2792, an excitation of 590 nm and emission of 615 nm for 70 kDa Dextran-Texas Red, and an excitation of 425 nm and emission of 540 nm for Lucifer Yellow. Fluorescence values were then normalized for the absorbance at 280 nm. Values represent the mean $\pm$ SD of three independent experiments. Two-way ANOVA, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. – not significant.

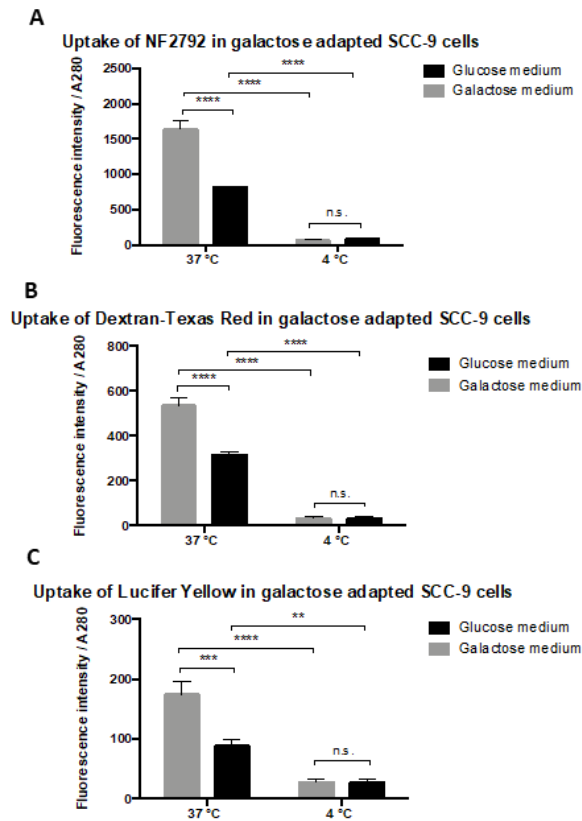


Figure 5.10. Study of endocytosis in galactose adapted SCC-9 cells upon addition of glucose

Uptake patterns of A – NF2792, B – 70 kDa Dextran-Texas Red, and C – Lucifer Yellow in galactose adapted SCC-9 cells incubated in various media. Galactose adapted cells were seeded on a 6-well plate at a density of 3×10^5 cells/well and serum starved for 16 h prior to treatment. Then, cells were treated with either 5 μ M of NF2792 (A), 1 μ M of 70 kDa Dextran-Texas Red (B), or 15 μ M of Lucifer Yellow (C) for 1 h in serum-free, either glucose or galactose conditioned medium. Cells were incubated at 37 °C or 4 °C to distinguish between endocytosis and non-specific binding to the plasma membrane. Then, cells were washed twice in PBS and lysed. Fluorescence of the cell lysates was read at an excitation of 335 nm and emission of 435 nm for NF2792, an excitation of 590 nm and emission of 615 nm for 70 kDa Dextran-Texas Red, and an excitation of 425 nm and emission of 540 nm for Lucifer Yellow. Fluorescence values were then normalized for the absorbance at 280 nm. Values represent the mean $\pm$ SD of three independent experiments. Two-way ANOVA, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. – not significant.

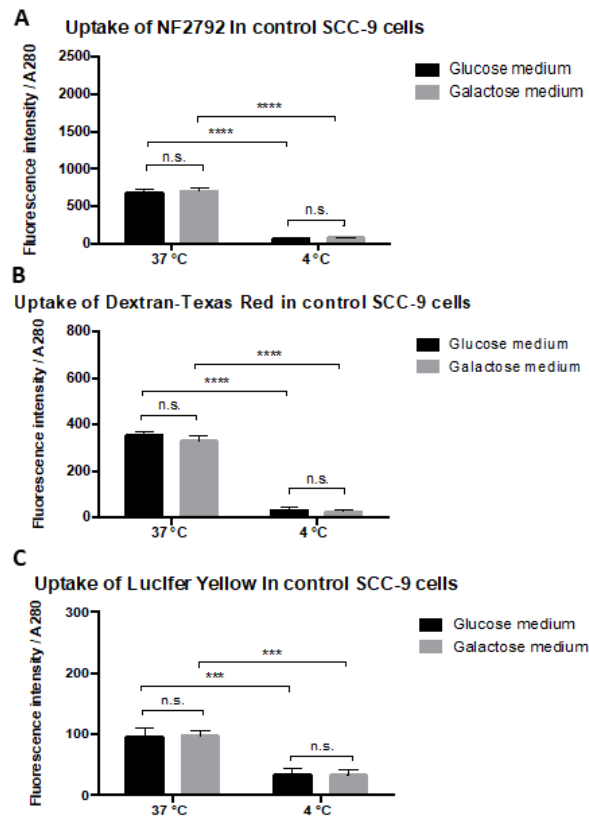


Figure 5.11. Study of endocytosis in control SCC-9 cells incubated in glucose or galactose conditioned medium

Uptake patterns of A – NF2792, B – 70 kDa Dextran-Texas Red, and C – Lucifer Yellow in control SCC-9 cells incubated in various media. Cells were seeded on a 6-well plate at a density of 3×10^5 cells/well and serum starved for 16 h prior to treatment. Then, cells were treated with either 5 μ M of NF2792 (A), 1 μ M of 70 kDa Dextran-Texas Red (B), or 15 μ M of Lucifer Yellow (C) for 1 h in serum-free, either glucose or galactose conditioned medium. Cells were incubated at 37 °C or 4 °C to distinguish between endocytosis and non-specific binding to the plasma membrane. Next, cells were washed twice in PBS and lysed. Fluorescence of the cell lysates was read at an excitation of 335 nm and emission of 435 nm for NF2792, an excitation of 590 nm and emission of 615 nm for 70 kDa Dextran-Texas Red, and excitation of 425 nm and emission of 540 nm for Lucifer Yellow. Fluorescence values were then normalized for the absorbance at 280 nm. Values represent the mean $\pm$ SD of three independent experiments. Two-way ANOVA, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. – not significant.

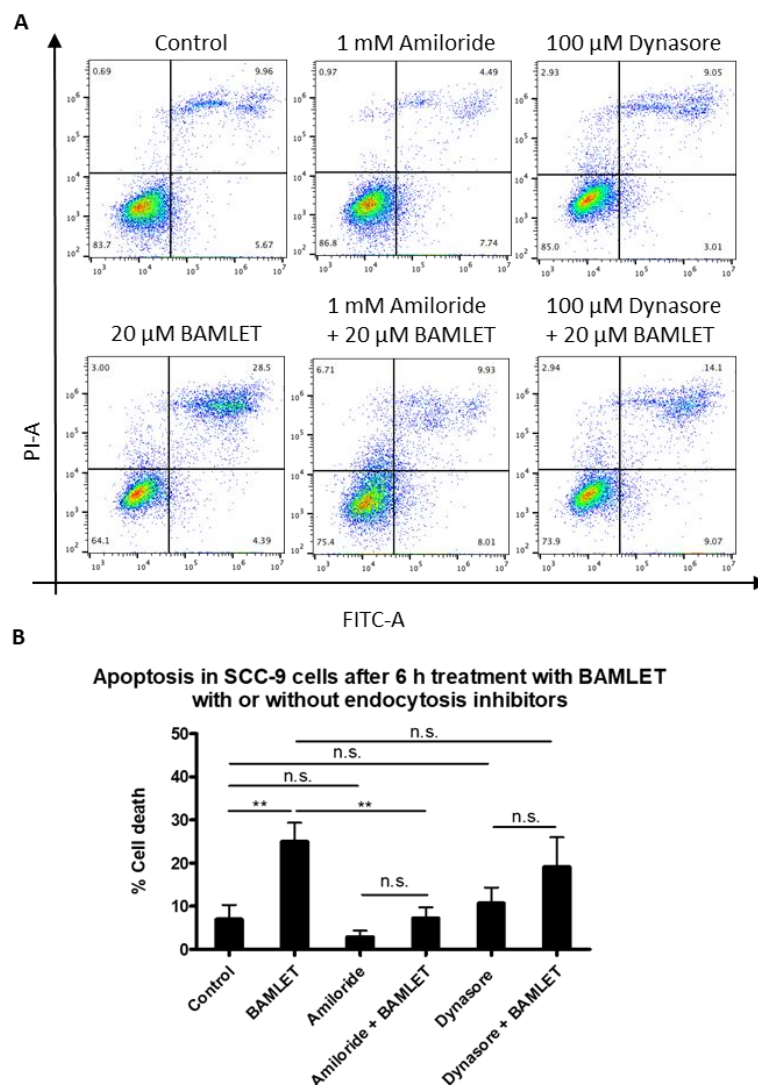


Figure 5.12. Analysis of the effect of Amiloride and Dynasore on BAMLET toxicity in SCC-9 cells – Annexin V/PI staining

A – A representative of three independent flow cytometry experiments. Cells were seeded on a 6-well plate at a density of 2×10^5 cells/well and serum starved for 16 h prior to 30 min treatment with either 0.01% DMSO as vehicle control, 1 mM Amiloride or 100 μ M Dynasore. After that, cells were treated either with 20 μ M BAMLET or equivalent volume of MilliQ H₂O as a vehicle control. After 6 h of treatment, cells were harvested, stained with Annexin V-FITC conjugate and propidium iodide, and analysed by flow cytometry. B - Quantitative apoptosis levels assessment after treatment. Graph represents the means $\pm$ SD of percentages of double stained (Annexin V+/PI+) cells in three independent experiments. Two-way ANOVA, ** $p < 0.01$, n.s. – not significant.

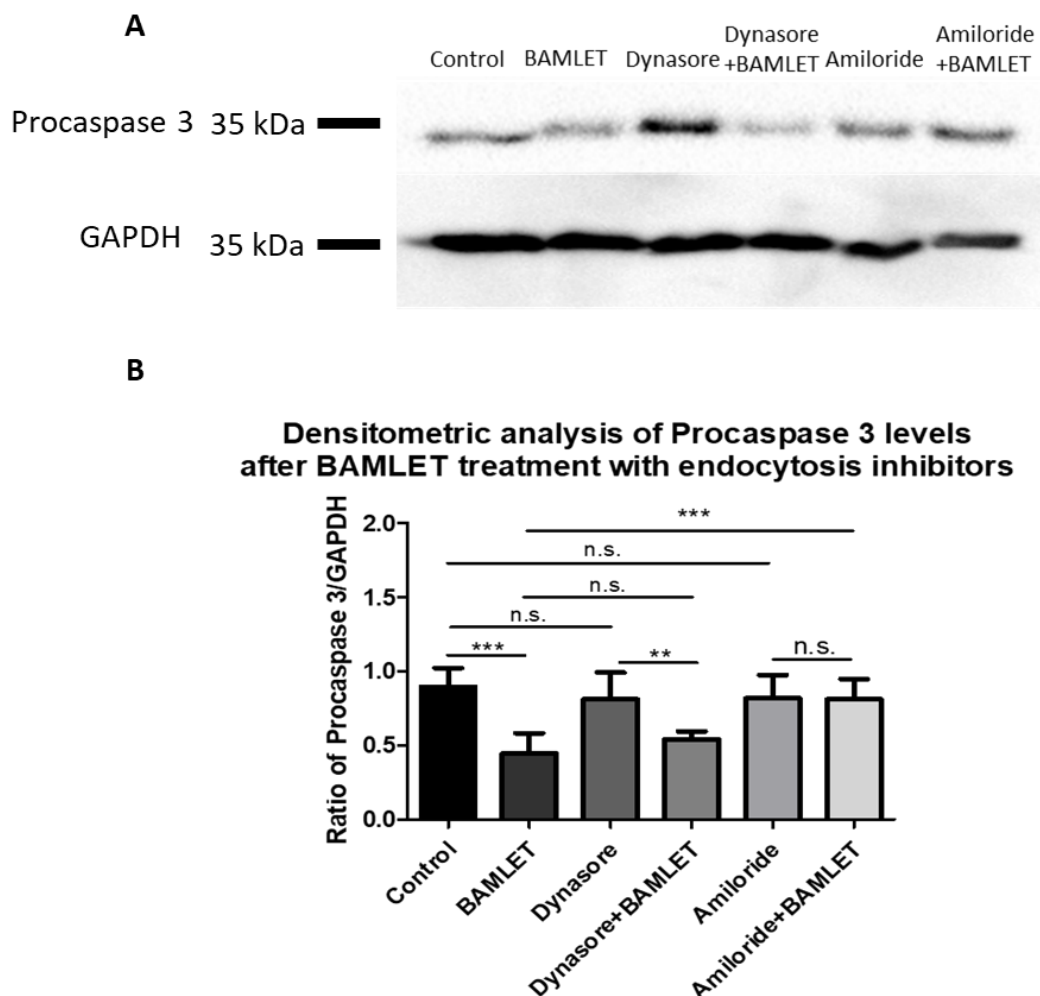


Figure 5.13. Analysis of the effect of Amiloride and Dynasore on BAMLET toxicity in SCC-9 cells – Procaspase 3 levels

A – A representative of three independent Western Blot analyses of Procaspase 3 levels in SCC-9 cells after BAMLET treatment with endocytosis inhibitors. Cells were seeded on a 6-well plate at a density of 2×10^5 cells/well and serum starved for 16 h prior to 30 min treatment with either 0.01% DMSO as vehicle control, 1 mM Amiloride or 100 μ M Dynasore. Subsequently, cells were treated either with 20 μ M BAMLET or equivalent volume of MilliQ H₂O as a vehicle control. After 6 h of treatment, cells were lysed and Procaspase levels were established by Western Blot compared to GAPDH, a housekeeping protein. B – Densitometry analysis of three separate Western Blot experiments. Graph represents the means $\pm$ SD of ratio of Procaspase 3 to GAPDH band intensity. One-Way ANOVA, ** $p < 0.01$, *** $p < 0.001$, n.s. – not significant.

6. Final discussion

Oral and oesophageal cancers are diseases that are challenging to treat. In particular, the difficulties arise from resistance to both radio- and chemotherapy and issues with patient malnutrition due to the localisation of these tumours. Moreover, late diagnosis is a common problem because of a lack of effective biomarkers and diagnostic strategies. Resective surgery is often employed in oral and oesophageal cancer and remains the main treatment strategy. Usually this involves the removal of a large margin of healthy tissue to prevent relapse (Rivera, 2015; Kato and Nakajima, 2013). Surviving patients often have to suffer physical and psychological pain and discomfort caused by severe disfigurement of the face and upper digestive tract (Omura, 2014; Lagergren *et al.*, 2017). Therefore, the field is in a desperate need of novel effective imaging agents that facilitate early diagnosis and therapeutic strategies that would negate the need for invasive surgery.

Despite the need to meet these different objectives, diagnostic and therapeutic molecules adhere to a common principal. They have to be highly selective in their ability to image or kill cancer cells without imparting harmful effects on the healthy adjacent tissue. Developing agents that embody this vital property requires a deep understanding of the cellular characteristics that distinguish cancer and non-cancer cells. One of the most obvious differences between the cancerous and normal tissue is their metabolism. Cancer cells are typically focused on survival in a harsh environment, often without a steady supply of oxygen or nutrients, and yet maintain a high rate of proliferation. Therefore, their metabolism is fundamentally different from normal cells (DeBerardinis and Chandel, 2016; Vander Heiden and DeBerardis, 2017). Since normal cells commonly grow in environments supplied with oxygen and nutrients and a regular blood supply, their energy needs can be largely fulfilled by oxidative phosphorylation (van Horssen *et al.*, 2013). Cancer cells, however, depend largely on glycolysis for ATP supply and biomass production. The ever-increasing energy needs of cancer cells and the unpredictability of the environment they grow in require them to be able to quickly adapt their metabolism to changes in nutrient supply and activate new metabolic pathways to ensure their survival (Lang *et al.*, 2014). This includes activation of endocytosis and increased uptake of nutrients to supply energy substrates and biomass for catabolic reactions requiring less energy than *de novo* production (Palm and Thompson, 2017; Finicle *et al.*, 2018; Rahmani *et al.*, 2019). Our research provides insight on the crosstalk that exists between the metabolic state and rate of endocytosis that could potentially be exploited to develop successful, effective anticancer agents and cancer imaging diagnostics as well as selective therapies.

Our study revealed a pronounced increase in macropinocytosis and receptor-mediated endocytosis through dynamin-dependent processes in SCC-9 squamous cell carcinoma that had been restricted to a galactose medium. Presumably, the observed phenomenon is an adaptive response to a decrease in ATP levels due to lack of glucose, the preferred energy substrate. It was particularly notable that the effects on endocytosis were immediately reversed upon addition of glucose to the medium, revealing that cancer cells have the ability to engage and disengage the process as the need requires. Such a mechanism is likely to be a universal property of cancer, wherein the lack of glucose leads the cells to resort to alternative metabolic routes to fulfil their energy needs. In order to achieve that, cells must increase the uptake of the alternative nutrients and energy sources, hence the need to upregulate the endocytic pathways. This is borne out by the observation that upon addition of glucose to the media, wherein glycolysis is restored, there was an immediate decrease in the rate of endocytosis, presumably due to the fact that other metabolic nutrients were no longer needed.

As mentioned previously, the increased endocytic uptake is likely be a commonly exploited mechanism by cancer cells to cope with the harsh growth conditions that they experience. In fact, activation of macropinocytosis has already been observed in many cancers (Bryant *et al.*, 2007; Schmees *et al.*, 2012; Comisso *et al.*, 2013; Yoshida *et al.*, 2015). Arguably, all cancer cells might exhibit a certain degree of activation of the endocytic traffic to meet their ever-increasing metabolic, energetic, and biomass production needs. Therapeutically, it may be possible to exploit the increase in the endocytic rate in cancer patients through periodical calorie or glucose restriction. This could promote the uptake of imaging molecules or therapeutic agents by the cancer cells. Reports suggest that a ketogenic diet can improve the outcome of cancer treatment and may serve as a neoadjuvant therapy in itself (Zhou *et al.*, 2007; Allen *et al.*, 2013; Allen *et al.*, 2014). While it is not clear if a ketogenic diet in fact could serve as a treatment strategy alone, we suggest that such an approach may be effective in combination with imaging and therapeutic agents, especially agents of natural origin that mimic the nutrients required by the ketotic cancer cells.

We show that a natural fluorophore could effectively exploit uptake through clathrin-mediated endocytosis by means of the CD44 receptor. The compound, NF2792, which is based on a hyaluronan backbone with an oleic acid moiety and a ferulic acid fluorophore, did not display

any toxic effects or apoptosis induction in SCC-9 cells. Moreover, its uptake could be quantified by multiphoton microscopy, allowing for *in vivo* identification of tumours residing deep in a tissue. Its fluorescence in cells gradually decreased over a 24 h span of incubation upon changing the culture medium, indicating that the NF2792 molecule is released from the cells. The ferulic acid moiety has an interesting Stokes shift, which has been further improved upon in the NF2792 molecule, increasing its imaging potential. The fluorescence of the NF2792 molecule is suspected to be inducible upon aggregation of the agent within the cells, thus effectively reducing background noise. The CD44 receptor overexpression has been observed in many cancers and is a marker of cancer stem cell status (Gotoda *et al.*, 2000; Christopoulos *et al.*, 2006; Chen *et al.*, 2014; Yu *et al.*, 2016; Qin *et al.*, 2019), suggesting that the compound would be preferentially taken up by cancer cells. The higher volume of CD44 receptor on the plasma membrane of certain cancer types indicates that the uptake of its ligands is increased in those cells, possibly as it is necessary to fulfil the cell activity or fuel up their metabolism. This phenomenon would facilitate successful imaging with the NF2792 fluorophore, due to its structure. We suspect that in times of nutrient scarcity the uptake of the NF2792 molecule would be dramatically increased as most of its component parts could be utilised by the cells; and therefore, its similarity to a nutrient would stimulate its uptake, thus raising the fluorescence well above background levels and allowing for successful imaging. Most interestingly, the uptake of the NF2792 fluorogen was further increased upon galactose adaptation of the cells, proving that its endocytosis can be additionally increased through metabolic or energetic modulation of the cells.

These observations open a range of possibilities for the development of novel, highly effective cancer imaging agents with potential uses in cancer diagnostics and intraoperative imaging. Natural compounds can be covalently bound together to consolidate their properties and create effective, non-toxic imaging probes. In fact, our research proves that there are natural fluorophores with interesting fluorescence properties and satisfying Stokes shifts, that could be further improved upon through chemical modifications of the covalent bonds between the moieties. The chemical structures and flexibility of the bonds seem crucial – more flexible structures with hydrophilic and hydrophobic residues presumably have better solubility, improved self-assembly properties, or more possibilities to interact with membranes. Such agents, administered as drinks or mouthwashes to increase their exposure to oral or oesophageal tumours, could serve as diagnostic dyes, aiding the diagnostic process. It could be argued that

the nutrient scarcity in a solid tumour appears once the tumour outgrows the blood supply; therefore, the endocytosis rate might not be as rapid as after additional stimulation of nutrient deprivation. However, many cancers show activation of macropinocytosis (Bryant *et al.*, 2007; Schmees *et al.*, 2012; Comisso *et al.*, 2013; Yoshida *et al.*, 2015), and all cancers display an increased metabolic and proliferation rate, indicating it is a strategy worth following. The choice of components of the naturally assembled probes might prove critical. In fact, depending on the cancer type, the expression of CD44 can be elevated, which could allow for a quick and effective imaging in the overexpressing tumours. Interestingly, CD44 expression has been detected in a large portion of oesophageal (Gotoda *et al.*, 2000) as well as oral cancers (Chen *et al.*, 2014) suggesting high practicality and advantage of the structure of NF2792 in imaging of these tumours. In other instances, a diet or periodical dietary restriction could induce an enhancement of nutrient scavenging in the cancer cells and providing with a compound consisting of multiple residues that are normally metabolised by the cells could prove to be a very effective strategy. In the case of cancers not expressing the CD44 receptor, the oleic acid could prove critical – as it is a fatty acid normally metabolised by the cell, it could possibly drive the uptake of the molecule into the cells.

However, natural compounds can also acquire other interesting functions through slight structure and conformation modifications. α -lactalbumin, one of the main proteins of mammal milk, through partial unfolding achieves the ability to form complexes with oleic acid, such as HAMLET, BAMLET, or other HAMLET-like complexes (lipotides which are toxic to cancer cells). Not only do these complexes possess high selectivity against cancer cells, they also do not harm healthy cells and can bypass therapy resistance. Our research shows that BAMLET appears to be entering the cells through macropinocytosis – which means it is effectively recruited into the cell due to its camouflage as a nutrient. Arguably, HAMLET-like complexes' toxicity is actually driven by oleic acid (Wen *et al.*, 2015; Delgado *et al.*, 2015), while α -lactalbumin, or any other partially unfolded protein forming the complex, is just a carrier (Min *et al.*, 2012). Cancers have been observed to take up larger amounts of albumin, particularly misfolded albumin (Merlot *et al.*, 2014; Finicle *et al.*, 2018) to quench the thirst for amino acids and fuel biomass production and proliferation. This phenomenon is more accentuated during low availability of glucose and other nutrients, when the cells are forced to remodel their metabolism in order to survive and continue to proliferate, such as in the case of a solid tumour. The increased uptake of protein to maintain the rate of biomass production might be

the reason for the toxicity and selectivity of liprotides. As the proteins are complexed to multiple, even dozens of molecules of fatty acids depending on the efficacy of the experimental protocol used to obtain them (Wilhelm *et al.*, 2009; Mossberg *et al.*, 2010; Spolaore *et al.*, 2010; Nakamura *et al.*, 2013), each macropinocytosed protein can prove to be a Trojan horse. In fact, the number of fatty acid molecules entering the cells exposed to HAMLET-like complexes might be much greater than in the case of treatment with that fatty acid alone. Therefore, the protein-fatty acid complexes can effectively exploit the cancer cell metabolism, and high rate of biomass production.

Unfortunately, there is a number of non-cancer cell types, like blood cells and embryonic primary cells, that are also vulnerable to HAMLET-like complexes, that need to be addressed in order to understand the basis of their sensitivity. Macropinocytosis has been reported not to be active in normal cells in standard conditions (Comisso *et al.*, 2013; Finicle *et al.*, 2018). Perhaps the cells, due to higher activity, have a higher metabolic rate which could lead to higher nutrient uptake, in turn inducing higher sensitivity to liprotides. However, as oral and oesophageal cancers, due to their localisation, can be easily treated with topical administration of therapeutics in mouthwashes or drinks, the sensitivity of some normal cells to liprotides can be avoided and bypassed. Interestingly, activation of macropinocytosis has previously been linked to EGFR stimulation by EGF (Bryant *et al.*, 2007). In fact, upregulation of EGFR has been observed in both oral and oesophageal cancers, indicating an elevated macropinocytosis rate (Grandis and Tweardy, 1993; Itakura *et al.*, 1994; Hanawa *et al.*, 2005; Hiraishi *et al.*, 2006). This, in turn, suggests a possibly higher susceptibility to liprotides, that could be further exploited through diet or calorie restriction. While the natural origin of HAMLET-like complexes might not in fact be responsible for their cancer selectivity, and the lack of side effect could indeed be traced to the lack of macropinocytosis in normal cells, the notion that liprotides and their constituents can be readily found in food could bring great comfort to the patients. Moreover, approval of drug regulation agencies for therapeutics based on natural, edible compounds might prove easier to achieve, thus making these therapies available for patients sooner.

7. Future directions

Further validation is necessary to confirm that imaging and diagnostic agents based on natural compounds could in fact be successfully used in oncology. The fate of the NF2792 molecule synthesised and characterised in this project after its internalisation into the cell has to be confirmed. Despite establishing that inhibition of the CD44 receptor with a monoclonal antibody reduces the uptake of the molecule, additional corroboration of the endocytic route is needed. Further confirmation on its localisation within the cell and its release patterns is necessary. It is vital to ensure whether the loss of fluorescence after incubation in fresh medium is in fact due to release of the compound from the cells or degradation of the molecule in the lysosomes. Determination of its toxicity and uptake in other cancer cell lines of different origins and normal non-cancer cells is crucial, and upon confirming its non-toxic status, *in vivo* tests in model organisms must be carried out to establish its selectivity.

To further explore the field, a larger study on a wider range of cell lines is necessary in order to confirm the macropinocytosis entry route of HAMLET-like complexes into cancer cells of various origins. A confocal microscopy study of a fluorescently labelled HAMLET/BAMLET to examine its localisation in the cells immediately after internalization to prove that it is accumulated in macropinocytic vesicles would be necessary. An interesting concept would be to examine the link of metabolic processes to endocytosis on CRISPR knockouts of various metabolic pathways. Despite the fact that BAMLET and presumably other HAMLET-like complexes enter the cells through macropinocytosis, the mechanism of action is still not deciphered. Hopefully, establishing the entry route will help to bring about the discovery of HAMLET's mode of action and cellular targets.

References

- Abbud, W., *et al.*, “*Stimulation of AMP-activated protein kinase (AMPK) is associated with enhancement of Glut1-mediated glucose transport*”, 2017
- Abnet, C.C., *et al.*, “*Epidemiology of Esophageal Squamous Cell Carcinoma*”, 2018, *Gastroenterology*, 154, 360–373;
- Aguer, C., *et al.*, “*Galactose Enhances Oxidative Metabolism and Reveals Mitochondrial Dysfunction in Human Primary Muscle Cells*”, 2011, *PLoS one*, 6(12), e28536;
- Aits, S., *et al.*, “*HAMLET (human α -lactalbumin made lethal to tumor cells) triggers autophagic tumor cell death*”, 2009, *International Journal of Cancer*, 124, 1008–1019;
- Alam, H., *et al.*, “*Novel function of keratins 5 and 14 in proliferation and differentiation of stratified epithelial cells*”, 2011, *Molecular Biology of the Cell*, 22, 4068-4078;
- Alasadi, A., *et al.*, “*Effect of mitochondrial uncouplers niclosamide ethanolamine (NEN) and oxyclozanide on hepatic metastasis of colon cancer*”, 2018, *Cell Death and Disease*, 9, 215;
- Allen, B.G., *et al.*, “*Ketogenic Diets Enhance Oxidative Stress and Radio-Chemo-Therapy Responses in Lung Cancer Xenografts*”, 2013, *Clinical Cancer Research*, 19(14), 3905-3913;
- Allen, B.G., *et al.*, “*Ketogenic diets as an adjuvant cancer therapy: History and potential mechanism*”, 2014, *Redox Biology*, 2, 963-970;
- Amash, A., *et al.*, “*CD44 Antibody Inhibition of Macrophage Phagocytosis Targets Fc γ Receptor– and Complement Receptor 3–Dependent Mechanisms*”, 2016, *The Journal of Immunology*, 196 (8), 3331-3340;
- Ampel, N.M., *et al.*, “*The mannose receptor mediates the cellular immune response in human coccidioidomycosis*”, 2005, *Infection and Immunity*, 73(4), 2554-2555;
- Antonescu, C.N., *et al.*, “*Reciprocal Regulation of Endocytosis and Metabolism*”, 2014, *Cold Spring Harbour Perspectives in Biology*, 6(7):a016964,
- Arends, J.J.A., Jacquin, M.F., “*Lucifer yellow staining in fixed brain slices: Optimal methods and compatibility with somatotopic markers in neonatal brain*”, 1993, *Journal of Neuroscience Methods*, 50 (3), 321-339;

- Artym, J., Zimecki, M., “*Milk-derived proteins and peptides in clinical trials*”, 2013, Postępy Higieny i Medycyny Doświadczalnej (Advances in Hygiene and Experimental Medicine), 2013; 67: 800-816;
- Aruffo, A., *et al.*, “*CD44 is the principal cell surface receptor for hyaluronate*”, 1990, Cell, 61(7), 1303-1313;
- Baffy, G., “*Mitochondrial uncoupling in cancer cells: Liabilities and opportunities*”, 2017, Biochimica et Biophysica Acta – Bioenergetics, 1858(8), 655-664;
- Bagan, J., *et al.*, “*Oral cancer: Clinical features*”, 2010, Oral Oncology, 46, 414–417;
- Barnes, K., *et al.* “*Activation of GLUT1 by metabolic and osmotic stress: potential involvement of AMP-activated protein kinase (AMPK)*”, 2002, Journal of Cell Science, 115, 2433–2442;
- Beere, H.M., Hickman, J.A., “*Differentiation: a suitable strategy for cancer chemotherapy?*”, 1993, Anticancer drug design, 8(4), 299-322;
- Bentebibel, A., *et al.*, “*Novel Effect of C75 on Carnitine Palmitoyltransferase I Activity and Palmitate Oxidation*”, 2006, Biochemistry, 45(14), 4339-4350;
- Bern, M., *et al.*, “*The role of albumin receptors in regulation of albumin homeostasis: implications for drug delivery*”, 2015, Journal of Controlled Release, 211, 144-162;
- Boekema, E.J., “*A Passive Function of Mitochondrial ATPSynthase: Target for Tumor Killer HAMLET*”, 2015, Journal of Molecular Biology, 427, 1863–1865;
- Bourguignon, L.Y., *et al.*, “*Hyaluronan-CD44 interaction promotes oncogenic signaling, microRNA functions, chemoresistance, and radiation resistance in cancer stem cells leading to tumor progression*”, 2014, Advanced Cancer Research, 123, 255-275;
- Brest, P., *et al.*, “*Histone Deacetylase Inhibitors Promote the Tumoricidal Effect of HAMLET*”, 2007, Cancer Research, 67(23), 11327-11334;
- Brinkmann, C.R., *et al.*, “*Preparation and comparison of cytotoxic complexes formed between oleic acid and either bovine or human α -lactalbumin*”, 2011, Journal of Dairy Science, 94(5), 2159-2170;
- Bryant, D.M., *et al.*, “*EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin*”, 2007, Journal of Cell Science, 120, 181-1828;

- Busiello, R., *et al.*, “*Mitochondrial uncoupling proteins and energy metabolism*”, 2015, *Frontiers in Physiology*, 6:36, doi: 10.3389/fphys.2015.00036;
- Cadenas, S., “*Mitochondrial uncoupling, ROS generation and cardioprotection*”, 2018, *Biochimica et Biophysica Acta – Bioenergetics*, 1859(9), 940-950;
- Calder, P.C., “*Fatty acids Long-chain fatty acids and inflammation*”, 2012, *Proceedings of the Nutrition Society*, 71, 284–289;
- Cappelli, A., *et al.*, “*Hyaluronan-based graft copolymers bearing aggregation-induced emission fluorogens*”, 2018, *Royal Society of Chemistry Advances*, 8, 5864-5881;
- Caro-Maldonado, A., Muñoz-Pinedo, C., “*Dying for Something to Eat: How Cells Respond to Starvation*”, 2011, *The Open Cell Signaling Journal*, 3, 42-51;
- Casado-Díaz, A., *et al.*, “*Influence of olive oil and its components on mesenchymal stem cell biology*”, 2019, *World Journal of Stem Cells*, 11(12), 1045–1064;
- Castaigne, S., *et al.*, “*All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results*”, 1990, *Blood*, 76(9), 1704-1709;
- Castro, C., *et al.*, “*Modifiable factors and esophageal cancer: a systematic review of published meta-analyses*”, 2018, *Journal of Gastroenterology*, 53, 37–51;
- Chambon, P., “*A decade of molecular biology of retinoic acid receptors*”, 1996, *FASEB Journal*, 10(9), 940-954;
- Chaudhuri, A., Chattopadhyay, A., “*Lipid binding specificity of bovine α -lactalbumin: A multidimensional approach*”, 2014, *Biochimica et Biophysica Acta*, 1838, 2078–2086;
- Chaudhuri, A., *et al.*, “*Protein-dependent Membrane Interaction of A Partially Disordered Protein Complex with oleic acid: Implications for Cancer Lipidomics*”, 2016, *Scientific Reports*, 6, 35015, doi: 10.1038/srep35015;
- Chen, C., *et al.*, “*The biology and role of CD44 in cancer progression: therapeutic implications*”, 2018, *Journal of Hematology and Oncology*, 11, 64;
- Chen, J., *et al.*, “*Significance of CD44 expression in head and neck cancer: a systemic review and meta-analysis*”, 2014, *BMC Cancer*, 14, doi: 10.1186/1471-2407-14-15;

- Chen, M., *et al.*, “*Serum Starvation Induced Cell Cycle Synchronization Facilitates Human Somatic Cells Reprogramming*”, 2012, PLoS One, 7(4), e28203, doi: 10.1371/journal.pone.0028203;
- Chen, Q., Liu, Z., “*Albumin Carriers for Cancer Theranostics: A Conventional Platform with New Promise*”, 2016, Advanced Materials, 28, 10557–10566;
- Chen, Y., *et al.*, “*A novel monoclonal antibody KMP1 has potential antitumor activity of bladder cancer by blocking CD44 in vivo and in vitro*”, 2018, Cancer Medicine, 7(5), 2064–2077;
- Chen, Z., *et al.*, “*Global phosphoproteomic analysis reveals ARMC10 as an AMPK substrate that regulates mitochondrial dynamics*”, 2019, Nature Communications, 10(1), 104;
- Cheng, Z., Ristow, M., “*Mitochondria and Metabolic Homeostasis*”, 2013, Mitochondria and Metabolism, 19(3), 240-242;
- Ching, J.K., *et al.*, “*A role for AMPK in increased insulin action after serum starvation*”, 2010, American Journal of Physiology. Cell Physiology, 299(5), C1171-C1179;
- Cho, Y., *et al.*, “*Cleaved CD44 intracellular domain supports activation of stemness factors and promotes tumorigenesis of breast cancer*”, 2015, Oncotarget, 6(11), 8709-8721;
- Christopoulos, T.A., *et al.*, “*Hyaluronidase and CD44 hyaluronan receptor expression in squamous cell laryngeal carcinoma*”, 2006, 1760(7), 1039-1045;
- Coelho, A.I., *et al.*, “*Galactose metabolism and health*”, 2015, Current Opinion in Clinical Nutrition and Metabolic Care, 18(4), 422–427;
- Cohen, M., *et al.*, “*Spatial and temporal sequence of events in cell adhesion: from molecular recognition to focal adhesion assembly*”, 2004, Chembiochem, 5(10), 1393-1399;
- Comisso, C., *et al.*, “*Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells*”, 2013, Nature, 497, 633-637;
- Corbet, C., Feron, O., “*Cancer cell metabolism and mitochondria: Nutrient plasticity for TCA cycle fueling*”, 2017, Biochimica et Biophysica Acta, 1868, 7-15;
- Costantini, L. *et al.*, “*Retinoic Acids in the Treatment of Most Lethal Solid Cancers*”, 2020, Journal of Clinical Medicine, 9(2), 360;

- Crabtree, H.G., *“Observations on the carbohydrate metabolism of tumours”*, 1929, Biochemical Journal, 23(3), 536–545;
- Cui, J., *et al.*, *“All-trans retinoic acid inhibits proliferation, migration, invasion and induces differentiation of hepa1-6 cells through reversing EMT in vitro”*, 2015, International Journal of Oncology, 48(1), 349-357;
- Dannenberg, A.J., *et al.*, *“Cyclo-oxygenase 2: a pharmacological target for the prevention of cancer”*, 2001, Lancet Oncology, 2, 544 – 551;
- Danuthai, K., *et al.*, *“Oral cancer: A multicenter study”*, 2018, Medicina Oral Patologia Oral y Cirugia Bucal, 23(1), e23–e29;
- Davis, J.T., Okunola, O., Quesada, R., *“Recent advances in the transmembrane transport of anions”*, 2010, Chemical Society Reviews, 39, 3843-3862;
- DeBerardinis, R.J., *et al.*, *“Beyond aerobic glycolysis:transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis”*, 2007, Proceedings of the National Academy of Sciences of the United States of America, 104(49), 19345-19350;
- DeBerardinis, R.J., Chantel, N.S., *“Fundamentals of cancer metabolism”*, 2016, Science Advances, 2, e1600200;
- Defize, I.L., *et al.*, *“Restaging after chemoradiotherapy for locally advanced esophageal cancer”*, 2019, Annals of Translational Medicine, 7(Suppl 8), S288, doi:10.21037/atm.2019.11.57;
- Delgado, Y., *et al.*, *“The cytotoxicity of BAMLET complexes is due to oleic acid and independent of the α -lactalbumin component”*, 2015, FEBS Open Bio, 5, 397–404;
- Deng, W., Ling, S.H., *“Advances in radiotherapy for esophageal cancer”*, 2018, Annals of Translational Medicine, 6(4), 79-88;
- Derdak, Z., *et al.*, *“The Mitochondrial Uncoupling Protein-2 Promotes Chemoresistance in Cancer Cells”*, 2008, Cancer Research, 68(8) 2813-2819;
- Detterbeck, F.C., *et al.*, *“Seeking a home for a PET, part 3: emerging applications of positron emission tomography imaging in the management of patients with lung cancer”*, 2004, Chest, 126, 1656–1666;

- Domper Arnal, M.J., et al., “*Esophageal cancer: Risk factors, screening and endoscopic treatment in Western and Eastern countries*”, 2015, World Journal of Gastroenterology, 21(26), 7933-7943;
- Drage, N., et al., “*Imaging patients with cancer of the oral cavity*”, 2018, British Dental Journal, 225(9), 827-832;
- Ducommun, S., et al., “*Motif affinity and mass spectrometry proteomic approach for the discovery of cellular MAPK targets: identification of mitochondrial fission factor as a new AMPK substrate*”, 2015, Cellular Signalling, 27(5), 978-988;
- Düringer, C., et al., “*HAMLET interacts with histones and chromatin in tumor cell nuclei*”, 2003, Journal of biological Chemistry, 278, 42131-42135;
- Economopoulou, P., et al., “*Diagnostic tumor markers in head and neck squamous cell carcinoma (HNSCC) in the clinical setting*”, 2019, Frontiers in Oncology, 9, 827, doi: 10.3389/fonc.2019.00827;
- Ettinger, K.S., et al., “*Oral cavity cancer*”, 2019, Oral and Maxillofacial Surgery Clinics of North America, 31(1), 13-29;
- Evans, R.M., “*The Steroid and Thyroid Hormone Receptor Superfamily*”, 1988, Science, 240(4854), 889–895;
- Fakunerjad, S., et al., “*Intraoperative molecular imaging for ex vivo assessment of peripheral margins in oral squamous cell carcinoma*”, 2020, Frontiers in Oncology, 9, 1476, doi: 10.3389/fonc.2019.01476;
- Fan, T., et al., “*Tumor Energy Metabolism and Potential of 3-Bromopyruvate as an Inhibitor of Aerobic Glycolysis: Implications in Tumor Treatment*”, 2019, Cancers, 11(3), 317;
- Fang, B., et al., “*Bovine lactoferrin binds oleic acid to form an anti-tumor complex similar to HAMLET*”, 2014, Biochimica et Biophysica Acta, 1841 (2014), 535–543;
- Fast, J., et al., “*Stability of HAMLET—A kinetically trapped α -lactalbumin oleic acid complex*”, 2005, Protein Science, 14(2), 329–340;
- Finicle, B.T., et al., “*Nutrient scavenging in cancer*”, 2018, Nature Reviews – Cancer, 18, 619-633;

- Fischer, W., *et al.*, “*Human α -Lactalbumin Made Lethal to Tumor Cells (HAMLET) Kills Human Glioblastoma Cells in Brain Xenografts by an Apoptosis-Like Mechanism and Prolongs Survival*”, 2004, *Cancer Research*, 64, 2105–2112;
- Florey, O., Overholtzer, M., “*Macropinocytosis and autophagy crosstalk in nutrient scavenging*”, 2019, *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374, 20180154;
- Frislev, H., *et al.*, “*Liprotides kill cancer cells by disrupting the plasma membrane*”, 2017, *Scientific Reports*, 7(1):15129;
- Gama Sosa, M.A., *et al.*, “*Fibroblast growth factor rescues brain endothelial cells lacking presenilin 1 from apoptotic cell death following serum starvation*”, 2016, *Scientific Reports*, 6, 30267, doi: 10.1038/srep30267;
- Garrett, W.S., Mellman, I., “*Studies of endocytosis*”, 2001, *Dendritic Cells: Biology and Clinical Applications*, Chapter 16, Academic Press, ISBN 0-12-455851-8;
- Gigliotti, J., *et al.*, “*Delays in oral cavity cancer*”, 2019, *International Journal of Oral and Maxillofacial Surgery*, 48, 1131-1137;
- Gilroy, D.W., *et al.*, “*COX-2 expression and cell cycle progression in human fibroblasts*”, 2001, *American Journal of Physiology. Cell Physiology*, 281(1), C188-C194;
- Gimeno, R.E., *et al.*, “*Characterization of a Heart-specific Fatty Acid Transport Protein*”, 2003, *The Journal of Biological Chemistry*, 278, 16039-16044;
- Gohil, V.M., *et al.*, “*Nutrient-sensitized screening for drugs that shift energy metabolism from mitochondrial respiration to glycolysis*”, 2010, *Nature Biotechnology*, 28, 249–255;
- Golpour, M., *et al.*, “*Human Fibroblast Switches to Anaerobic Metabolic Pathway in Response to Serum Starvation: A Mimic of Warburg Effect*”, 2014, *International Journal of Molecular and Cellular Medicine*, 3(2), 74-80;
- Goswami, S., Sharma-Walia, N., “*Crosstalk between osteoprotegerin (OPG), fatty acid synthase (FASN) and, cyclooxygenase-2 (COX-2) in breast cancer: implications in carcinogenesis*”, 2016, *Oncotarget*, 7(37), 58953–5897;
- Gotoda, T., *et al.*, “*Expression of CD44 variants and prognosis in oesophageal squamous cell carcinoma*”, 2000, *Gut*, 46(1), 14–19;

- Goyeneche, A.A., *et al.*, “*Cell death induced by serum deprivation in luteal cells involves the intrinsic pathway of apoptosis*”, 2006, *Reproduction*, 131(1), 103-111;
- Grabber, J.H., Ralph, J.H., Hatfield, R.D., “*Cross-linking of maize walls by ferulate dimerization and incorporation into lignin*”, 2000, *Journal of agricultural and food chemistry*, 48(12), 6106-6113;
- Grandis, J.R., Tweardy, D.J., “*Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer*”, 1993, *Cancer Research*, 53, 3579-3584;
- Griesinger, C.B., Richards, C.D., Ashmore, J.F., “*FMI-43 Reveals Membrane Recycling in Adult Inner Hair Cells of the Mammalian Cochlea*”, 2002, *The Journal of Neuroscience*, 22(10): 3939–3952;
- Grubbe, E.H., “*X-rays in the treatment of cancer and other malignant diseases*”, 1902, *Medical Record*, 62(18), 1866-1922;
- Gründker, C., *et al.*, “*Inhibition of aerobic glycolysis enhances the anti-tumor efficacy of Zoptarelin Doxorubicin in triple-negative breast cancer cells*”, 2019, *The Journal of Obstetrics and Gynaecology Research*, 45(7), 1334-1342;
- Gustafsson, L., *et al.*, “*Treatment of skin papillomas with topical alpha-lactalbumin-oleic acid*”, 2004, *The New England Journal of Medicine*, 350(26), 2663-2672;
- Gustafsson, L., *et al.*, “*HAMLET Kills Tumor Cells by Apoptosis: Structure, Cellular Mechanisms, and Therapy*”, 2005, *The Journal of Nutrition*, 135(5), 1299–1303;
- Gustafsson, L., *et al.*, “*Changes in proteasome structure and function caused by HAMLET in tumor cells*”, 2009, *PLOS one*, 4, e5229;
- Hagens, E.R.C., *et al.*, “*Conditional survival after neoadjuvant chemotherapy and surgery for oesophageal cancer*”, 2020, *British Journal of Surgery*, doi:10.1002/bjs.11476;
- Hakansson, A., *et al.*, “*Apoptosis induced by a human milk protein*”, 1995, *Proceedings of the National Academy of Sciences of the United States of America*, 92, 8064-8068;
- Hakansson, A., *et al.*, “*Multimeric α -Lactalbumin from Human Milk Induces Apoptosis through a Direct Effect on Cell Nuclei*”, 1999, *Experimental Cell Research* 246, 451–460;

- Hakansson, A., *et al.*, “Apoptosis-like death in bacteria induced by HAMLET, a human milk lipid-protein complex”, 2011, PLoS one, 6(3), e17717;
- Hallgren, O., *et al.*, “HAMLET triggers apoptosis but tumor cell death is independent of caspases, Bcl-2 and p53”, 2006, Apoptosis, 11(2), 221-233;
- Hallgren, O., *et al.*, “Apoptosis and Tumor Cell Death in Response to HAMLET (Human α -Lactalbumin Made Lethal to Tumor Cells)”, 2008, Advances in Experimental Medicine and Biology, 606, 217-240;
- Hanawa, M., *et al.*, “EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus”, 2005, International Journal of Cancer, 118, 1173-1180;
- Hansen, L.A., *et al.*, “Retinoids in chemoprevention and differentiation therapy”, 2000, Carcinogenesis, 21(7), 1271–1279;
- Harasztosi, C., Gummer, A.W., “Different rates of endocytic activity and vesicle transport from the apical and synaptic poles of the outer hair cell”, 2019, HNO, 67(6), 449–457;
- Harte, N.P., *et al.*, “Amyloid Oligomers and Mature Fibrils Prepared from an Innocuous Protein Cause Diverging Cellular Death Mechanisms”, 2015, Journal of Biological Chemistry, 290, 28343-28352;
- Hartner, L. “Chemotherapy for Oral Cancer”, 2017, Dental Clinics of North America, 62(1), 87-97;
- Hayashi, K., *et al.*, “Overexpression of Retinoic Acid Receptor β Induces Growth Arrest and Apoptosis in Oral Cancer Cell Lines”, 2001, Japanese Journal of Cancer Research, 92(1), 42-50;
- Hein, J.E., Fokin, V.V., “Copper-catalyzed azide–alkyne cycloaddition (CuAAC) and beyond: new reactivity of copper(I) acetylides”, 2010, Chemical Society Reviews, 39(4), 1302–1315;
- Hema, K.N., *et al.*, “Epigenetics in oral squamous carcinoma”, 2017, Journal of Oral and Maxillofacial Pathology, 21(2), 252-259;
- Herrmann, H., Aebi, U., “Intermediate Filaments: Molecular Structure, Assembly Mechanism, and Integration Into Functionally Distinct Intracellular Scaffolds”, 2004, 73, 749-789;

- Hiraishi, Y., *et al.*, “Immunohistochemical expression of EGFR and p-EGFR in oral squamous cell carcinomas”, 2006, Pathology & Oncology Research, 12, 87–91;
- Ho, J.C.S., *et al.*, “HAMLET: functional properties and therapeutic potential”, 2012, Future Oncology, 8(10), 1301-1313;
- Ho, J.C.S., *et al.*, “Lipids as tumoricidal components of human α -lactalbumin made lethal to tumour cells (HAMLET): unique and shared effects on signalling and death”, 2013, The Journal of Biological Chemistry, 288(24), 17460–17471;
- Ho, J.C.S., *et al.*, “The Molecular Motor F-ATP Synthase Is Targeted by the Tumoricidal Protein HAMLET”, 2015, Journal of Molecular Biology, 427, 1866-1874;
- Ho, J.C.S., *et al.*, “Targeting of nucleotide-binding proteins by HAMLET—a conserved tumor cell death mechanism”, 2016, Oncogene, 35, 897–907;
- Ho, J.C.S., *et al.*, “HAMLET - A protein-lipid complex with broad tumoricidal activity”, 2017, Biochemical and Biophysical Research Communications, 482, 454-458;
- Hong, G., *et al.*, “Near-infrared fluorophores for biomedical imaging”, 2017, Nature Biomedical Engineering, 1, 0010, doi: 10.1038/s41551-016-0010;
- Hoque, M., *et al.*, “Oleic Acid May Be the Key Contributor in the BAMLET Induced Erythrocyte Hemolysis and Tumoricidal Action”, 2013, Plos ONE, 8(9), e68390;
- Hoque, M., *et al.*, “Oleic acid complex of bovine α -lactalbumin induces eryptosis in human and other erythrocytes by a Ca^{2+} -independent mechanism” 2015, Biochimica et Biophysica Acta, 1850(9), 1729-1739;
- Hostetler, H.A, *et al.*, “Peroxisome Proliferator-activated Receptor α Interacts with High Affinity and Is Conformationally Responsive to Endogenous Ligands”, 2005, The Journal of Biological Chemistry, 280(19), 18667–18682;
- Hsu, P.P., Sabatini, D.M., “Cancer cell metabolism: Warburg and beyond”, 2008, Cell, 134, 703-707;
- Hu, C., *et al.*, “ATRA induces the differentiation of hepatic progenitor cells by upregulating microRNA-200a”, 2019, In Vitro Cellular & Developmental Biology – Animal, 55, 713–722;

- Hu, Z., *et al.*, “*Hypothalamic malonyl-CoA as a mediator of feeding behaviour*”, 2003, Proceedings of the National Academy of Sciences of the United States of America, 100, 12624-12629;
- Hua, Q., *et al.*, “*Internalization of hyaluronan by chondrocytes occurs via receptor-mediated endocytosis*”, 1993, Journal of Cell Science, 106, 365-375;
- Huang, F-L., Yu, S-I., “*Esophageal cancer: Risk factors, genetic association, and treatment*”, 2018, Asian Journal of Surgery, 210-215;
- Huang, G., Huang, H., “*Application of hyaluronic acid as carriers in drug delivery*”, 2018, Drug Delivery, 25(1), 766-772;
- Huang, M.E., *et al.*, “*Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia*”, 1988, Blood, 72(2), 567-572;
- Huang, S., O’Sullivan, B., “*Oral cancer: Current role of radiotherapy and chemotherapy*”, 2013, Medicina Oral Patologia Oral y Cirugia Bucal, 18(2), 233-240;
- Huang, Y., *et al.*, “*Serum starvation-induces down-regulation of Bcl-2/Bax confers apoptosis in tongue coating-related cells in vitro*”, 2018, Molecular Medicine Reports, 17(4), 5057-5064;
- Inokuchi, R., *et al.*, “*Fluorometric Quantification of Ferulic Acid Concentrations Based on Deconvolution of Intrinsic Fluorescence Spectra*”, 2016, Environmental Control in Biology, 54(1), 57-64;
- Irmie, A.I., *et al.*, “*Current Insights into Oral Cancer Epigenetics*”, 2018, International Journal of Molecular Sciences, 19, 670;
- Itakura, Y., *et al.*, “*Epidermal growth factor receptor overexpression in esophageal carcinoma. An immunohistochemical study correlated with clinicopathologic findings and DNA amplification*”, 1994, Cancer, 74, 795-804;
- Jenkins, R., *et al.*, “*Mini-review: fluorescence imaging in cancer cells using dye-doped nanoparticles*”, 2016, RSC Advances, 6, 65459–65474;
- Jensen, R.G., “*Lipids in human milk*”, 1999, Lipids, 34: 1243-1271;

- Jiang, W., *et al.*, “*¹⁸F-FDG PET-CT metabolic findings can predict the short-term curative effects in esophageal cancer*”, 2019, International Journal of Clinical and Experimental Pathology, 12(11), 4130-4136;
- Jump, D.B., “*Mammalian Fatty Acid Elongases*”, 2009, Methods in Molecular Biology, 579, 375–389;
- Kato, H., Nakajima, M., “*Treatments for esophageal cancer: a review*”, 2013, General Thoracic and Cardiovascular Surgery, 61:330–335;
- Kelly, R., “*Immunotherapy for Esophageal and Gastric Cancer*”, 2017, American Society of Clinical Oncology Educational Book, asco.org/edbook;
- Kerr, M.C., Teasdale, R.D., “*Defining macropinocytosis*”, 2009, Traffic, 10, 364-371;
- Kim, J.H., *et al.*, “*Genome-wide screen identifies novel machineries required for both ciliogenesis and cell cycle arrest upon serum starvation*”, 2016, Biochimica et Biophysica Acta, 1863(6), 1307-18;
- Kim, S.M., *et al.*, “*PTEN deficiency and AMPK activation promote nutrient scavenging and anabolism in prostate cancer cells*”, 2018, Cancer Discovery, 8, 1-18;
- Kirchhausen, T., Macia, E., Pelish, H.E., “*Use of dynasore, the small molecule inhibitor of dynamin, in the regulation of endocytosis*”, 2008, Methods in Enzymology, 438, 77-93;
- Klaus, S., Ost, M., “*Mitochondrial uncoupling and longevity - A role for mitokines?*”, 2020, Experimental gerontology, 130, 110796, doi: 10.1016/j.exger.2019.110796;
- Köhler, C., *et al.*, “*A folding variant of alpha-lactalbumin induces mitochondrial permeability transition in isolated mitochondria*”, 2001, European Journal of Biochemistry, 28, 186-191;
- Koivusalo, M., *et al.*, “*Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling*”, 2010, Journal of Cell Biology, 188(4), 547–563;
- Kolb, H.C., *et al.*, “*Click Chemistry: Diverse Chemical Function from a Few Good Reactions*”, 2001, Angewandte Chemie (International Edition in English), 40(11), 2004-2021;

- Kokatnur, M.G., *et al.*, “*Fatty acid composition of human adipose tissue from two anatomical sites in a biracial community*”, 1979, The American Journal of Clinical Nutrition, 32, 2198-2205;
- Korga, A., *et al.*, “*Inhibition of glycolysis disrupts cellular antioxidant defense and sensitizes HepG2 cells to doxorubicin treatment*”, 2019, FEBS Open Bio, 9, 959-972;
- Kuhajda, F.P., *et al.*, “*Fatty acid synthesis: a potential selective target for antineoplastic therapy*”, 1994, Proceedings of the National Academy of Sciences of the United States of America, 91(14), 6379-6383;
- Kurosu, T., *et al.*, “*Rottlerin synergistically enhances imatinib-induced apoptosis of BCR/ABL-expressing cells through its mitochondrial uncoupling effect independent of protein kinase C-delta*”, 2007, Oncogene, 26, 2975–2987;
- Lagergren, J., *et al.*, “*Oesophageal cancer*”, 2017, Lancet, 390, 2383–2396;
- Lang, M.J., *et al.*, “*Glucose starvation inhibits autophagy via vacuolar hydrolysis and induces plasma membrane internalization by down-regulating recycling*”, 2014, Journal of biological Chemistry, 289(24), 16736-16747;
- Larson, A.M., “*Multiphoton microscopy*”, 2011, Nature Photonics, 5(1);
- Li, J.-N., *et al.*, “*Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53*”, 2001, Cancer Research, 61, 1493-1499;
- Lin, S-C., Hardie, D.G., “*AMPK: sensing glucose as well as cellular energy status*”, 2017, Cell Metabolism
- Lippman, S.M., *et al.*, “*Retinoids as preventive and therapeutic anticancer agents (Part I)*”, 1987, Cancer Treatment Reports, 71(4), 391-405;
- Lišková, K., *et al.*, “*Cytotoxic complexes of sodium oleate with b-lactoglobulin*”, 2011, European Journal of Lipid Science and Technology, 113, 1207–1218;
- Liu, H-W., *et al.*, “*Recent progresses in small-molecule enzymatic fluorescent probes for cancer imaging*”, 2018, Chemical Society Reviews, 47, 7140-7180;
- Liu, P.T., *et al.*, “*Cutting Edge: All-trans Retinoic Acid Down-Regulates TLR2 Expression and Function*”, 2005, The Journal of Immunology, 174(5), 2467-2470;

- Loftus, T. M., *et al.*, “Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors”, 2000, Science 288, 2379-2381;
- Lokshin, A., *et al.*, “Early effects of retinoic acid on proliferation, differentiation and apoptosis in non-small cell lung cancer cell lines”, 1999, Anticancer Research, 19(6B), 5251-5254;
- Lorentz, M., Burant, C.F., Kennedy, R.T., “Reducing time and increasing sensitivity in sample preparation for adherent mammalian cell metabolomics”, 2011, Analytical Chemistry, 83, 3406-3414;
- Lu, J., *et al.*, “ATRA-inhibited proliferation in glioma cells is associated with subcellular redistribution of β -catenin via up-regulation of Axin”, 2008, Journal of Neuro-Oncology, 87, 271–277;
- Luo, J., *et al.*, “Aggregation-induced emission of 1-methyl-1,2,3,4,5-pentaphenylsilole”, 2001, Chemical communications, 18, 1740-1741;
- Luo, S., *et al.*, “A review of NIR dyes in cancer targeting and imaging”, 2011, Biomaterials, 32(29), 7127-7138;
- Macia, E., *et al.*, “Dynasore, a Cell-Permeable Inhibitor of Dynamin”, 2006, Developmental Cell, 10(6), 839-850;
- MacLellan, J.D., *et al.*, “Physiological increases in uncoupling protein 3 augment fatty acid oxidation and decrease reactive oxygen species production without uncoupling respiration in muscle cells”, 2005, Diabetes, 54, 2343-2350;
- Mahoney, J.L., Condon, R.E., “Adenocarcinoma of the Esophagus”, 1987, Annals of Surgery, 205(05), 557-562;
- Malik, U.U., *et al.*, “Oral squamous cell carcinoma: Key clinical questions, biomarker discovery, and the role of proteomics”, 2016, Archives of Oral Biology, 63, 53-65;
- Mannath, J., Ragunath, K., “Role of endoscopy in early oesophageal cancer”, 2016, Nature Reviews Gastroenterology & Hepatology, 13, 720–730;
- Mansson, H.L., “Fatty acids in bovine milk fat”, 2008, Food & Nutrition Research, 52:1, doi: 10.3402/fnr.v52i0.1821;

- Martinez-Outschoorn, U.E., *et al.*, “*Cancer metabolism: a therapeutic perspective*”, 2017, *Clinical Oncology*, 14(1), 11-31;
- Mélida, H., *et al.*, “*Unraveling the Biochemical and Molecular Networks Involved in Maize Cell Habituation to the Cellulose Biosynthesis Inhibitor Dichlobenil*”, 2010, *Molecular Plant*, 3(5), 842-853;
- Mercader, J., *et al.*, “*All-Trans Retinoic Acid Increases Oxidative Metabolism in Mature Adipocytes*”, 2007, *Cell Physiology and Biochemistry*, 20, 1061-1072;
- Mercer, N., *et al.*, “*Applications of Site-Specific Labeling to Study HAMLET, a Tumoricidal Complex of α -Lactalbumin and Oleic Acid*”, 2011, *PLoS One*, 6(10), e26093;
- Merlot, A.M., *et al.*, “*Unraveling the mysteries of serum albumin – more than just a serum protein*”, 2014, *Frontiers in Pysiology*, 5, 299;
- Meyer, S., *et al.*, “*UV-induced blue-green and far-red fluorescence along wheat leaves: a potential signature of leaf ageing*”, 2003, 54(383), 757-769;
- Miles, E.A., Calder, P.C., “*Modulation of immune function by dietary fatty acids*”, 1998, *Proceedings of the Nutrition Society*, 57, 277-292;
- Min, S., *et al.*, “*Alternatively folded proteins with unexpected beneficial functions*”, 2012, *Biochemical Society Transactions*, 40(4), 746-751;
- Ming, X-Y., *et al.*, “*Integrin $\alpha 7$ is a functional cancer stem cell surface marker in oesophageal squamous cell carcinoma*”, 2016, *Nature Communications*, 7:13568;
- Miranda-Filho, A., Bray, F., “*Global patterns and trends in cancers of the lip, tongue and mouth*”, 2020, *Oral Oncology*, 102, 104551, doi: 10.1016/j.oraloncology.2019.104551;
- Mitchell, D.J., *et al.*, “*Simultaneous monitoring of gastric and oesophageal pH reveals limitations of conventional oesophageal pH monitoring in milk fed infants*”, 2001, *Archives of Disease in Childhood*, 84, 273-276;
- Mok, K.H., *et al.*, “*HAMLET, protein folding, and tumor cell death*”, 2007, *Biochemical and Biophysical Research Communications*, 354, 1–7;
- Montero, P.H., Patel, S.G., “*Cancer of the oral cavity*”, 2015, *Surgical Oncology Clinics of North America*, 24(3), 491–508;

- Mossberg, A.K., *et al.*, “*Bladder cancers respond to intravesical instillation of HAMLET (human alpha-lactalbumin made lethal to tumor cells)*”, 2007, International Journal of Cancer, 121(6), 1352-1359;
- Mossberg, A.K., *et al.*, “*Structure and function of human alpha-lactalbumin made lethal to tumour cells (HAMLET)-type complexes*”, 2010, FEBS Journal, 277, 4614-4625;
- Mossberg, A.K., *et al.*, “*HAMLET Interacts with Lipid Membranes and Perturbs Their Structure and Integrity*”, 2010b, PLoS one, 5(2), e9384;
- Mossberg, A.K., *et al.*, “*HAMLET Treatment Delays Bladder Cancer Development*”, 2010c, The Journal of Urology, 183, 1590-1597;
- Nadeem, A., *et al.*, “*Protein receptor-independent plasma membrane remodelling by HAMLET: a tumoricidal protein-lipid complex*”, 2015, Scientific Reports, 5:16432;
- Nadeem, A., *et al.*, “*Beta-sheet-specific interactions with heat shock proteins define a mechanism of delayed tumor cell death in response to HAMLET*”, 2019, Journal of Molecular Biology, 431(14), 2612-2627;
- Naik, P.P., *et al.*, “*Implications of cancer stem cells in developing therapeutic resistance in oral cancer*”, 2016, Oral Oncology, 62, 122-135;
- Nakamura, T., *et al.*, “*Molecular mechanisms of the cytotoxicity of human α -lactalbumin made lethal to tumor cells (HAMLET) and other protein-oleic acid complexes*”, 2013, The Journal of Biological Chemistry, 288(20), 14408–14416;
- Nakano, A., *et al.*, “*Glycolysis Inhibition Inactivates ABC Transporters to Restore Drug Sensitivity in Malignant Cells*”, 2011, PLoS one, 6(11), e27222;
- Nam, S.Y., *et al.*, “*Clinical Factors to Predict the Response to Concurrent Chemoradiotherapy and Survival in Esophageal Cancer Patients*”, 2020, Gut and Liver, doi:10.5009/gnl19165;
- Nelson, W.G., Sun, T.T., “*The 50- and 58-kdalton keratin classes as molecular markers for stratified squamous epithelia: cell culture studies*”, 1983, Journal of Cell biology, 97(1), 244–251;
- Neville, B.W., Day, T.A., “*Oral Cancer and Precancerous Lesions*”, 2002, CA: A Cancer Journal for Clinicians, 52(4), 195-215;

- Newell, K.J., Tannock, I.F., “*Reduction of intracellular pH as a possible mechanism for killing cells in acidic regions of solid tumors: effects of carbonylcyanide-3-chlorophenylhydrazone*”, 1989, *Cancer Research*, 49, 4477–4482;
- Novelli, G., Reichardt, J.K.V., “*Molecular Basis of Disorders of Human Galactose Metabolism: Past, Present, and Future*”, 2000, *Molecular Genetics and Metabolism*, 71, 62–65;
- Omura, K., “*Current status of oral cancer treatment strategies: surgical treatments for oral squamous cell carcinoma*”, 2014, *International Journal of Clinical Oncology*, 19, 423–430;
- Orfali, N., *et al.*, “*All-trans retinoic acid (ATRA)-induced TFEB expression is required for myeloid differentiation in acute promyelocytic leukemia (APL)*”, 2020, *European Journal of Haematology*, 104, 236–250;
- Owen, R.W., *et al.*, “*Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene*”, 2000, *Food and Chemical Toxicology*, 38, 647-659;
- Owens, E.A., *et al.*, “*Tissue-Specific Near-Infrared Fluorescence Imaging*”, 2016, *Accounts of Chemical Research*, 49, 1731–1740;
- Ozer, E., *et al.*, “*The role of angiogenesis, laminin and CD44 expression in metastatic behavior of early-stage low-grade invasive breast carcinomas*”, 1997, *Cancer Letters*, 121(2),119-123;
- Pak, K.C., *et al.*, “*The inhibitory effect of Gleditsia sinensis on cyclooxygenase-2 expression in human esophageal squamous cell carcinoma*”, 2009, *International Journal of Molecular Medicine*, 23,121-129;
- Palm, W., Thompson, C.B., “*Nutrient Acquisition Strategies of Mammalian Cells*”, 2017, *Nature*, 546(7657), 234–242;
- Parton, R.G., “*Caveolae: Structure, Function, and Relationship to Disease*”, 2018, *Annual Review of Cell and Developmental Biology*, 34, 111-136;
- Pathania, D., *et al.*, “*Opportunities in discovery and delivery of anticancer drugs targeting mitochondria and cancer cell metabolism*”, 2009, *Advanced Drug Delivery Reviews*, 61, 1250–1275;

- Pavlova, N.N., Thompson, C.B., *“The emerging hallmarks of cancer metabolism”*, 2016, Cell Metabolism, 23(1), 27–47;
- Pelicano, H., *et al.*, *“Glycolysis inhibition for anticancer treatment”*, 2006, Oncogene, 25, 4633–4646;
- Pettersson-Kastberg, J., *et al.*, *“ α -Lactalbumin, Engineered to be Non-native and Inactive, Kills Tumor Cells when in Complex with Oleic Acid: A new biological function resulting from partial unfolding”*, 2009, Journal of Molecular Biology, 394, 994–1010;
- Pirkmajer, S., Chibalin, A.V., *“Serum starvation: caveat emptor”*, 2011, American Journal of Physiology-Cell Physiology, 301, C272–C279;
- Pitt, M.A., *“Overexpression of uncoupling protein-2 in cancer: metabolic and heat changes, inhibition and effects on drug resistance”*, 2015, Inflammopharmacology, 23, 365–369
- Plötz, T., *et al.*, *“The monounsaturated fatty acid oleate is the major physiological toxic free fatty acid for human beta cells”*, 2017, Nutrition & Diabetes, 7, 305;
- Pradelli, L.A., *et al.*, *“Glycolysis inhibition sensitizes tumor cells to death receptors-induced apoptosis by AMP kinase activation leading to Mcl-1 block in translation”*, 2010, Oncogene, 29, 1641–1652;
- Preta, G., *et al.*, *“Dynasore - not just a dynamin inhibitor”*, 2015, Cell Communication and Signalling, 13(24), doi: 10.1186/s12964-015-0102-1;
- Prince, M.E., *et al.*, *“Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma”*, 2007, Proceedings of the National Academy of Sciences of the USA, 104(3), 973–978;
- Puthia, M., *et al.*, *“Prevention and treatment of colon cancer by peroral administration of HAMLET (human α -lactalbumin made lethal to tumour cells)”*, 2014, Gut, 63, 131–142;
- Qasba, P. K., *et al.*, *“Structure and function of β -1,4-galactosyltransferase”*, 2008, Current Cancer Drug Targets, 9, 292–309;
- Qian, J, Tang, B.Z., *“AIE Luminogens for Bioimaging and Theranostics: From Organelles to Animals”*, 2017, Chem, 3(1), 56–91;
- Qin, Y., *et al.*, *“Evaluation of the correlativity of gender determining region Y-box 4, N-cadherin, CD44 and E-cadherin expression in the prognosis of esophageal squamous*

- cell carcinoma*”, 2019, International Journal of Clinical and Experimental Pathology, 12(5), 1745–1756;
- Rahmani, S., *et al.*, “*Energetic adaptations: Metabolic control of endocytic membrane traffic*”, 2019, Traffic, 20(12), 887-888;
- Ralph, S.J., *et al.*, “*Bioenergetic pathways in tumor mitochondria as targets for cancer therapy and the importance of the ROS-induced apoptotic trigger*”, 2010, Molecular Aspects of Medicine, 31(1), 29-59;
- Rammer, P., *et al.*, “*BAMLET Activates a Lysosomal Cell Death Program in Cancer Cells*”, 2010, Molecular Cancer Therapeutics, 9(1), 24-32;
- Rao, J., *et al.*, “*Fluorescence imaging in vivo: recent advances*”, 2006, Current Opinion in Biotechnology, 18, 17–25;
- Rappaport, J., Garnacho, C., Muro, S., “*Clathrin-Mediated Endocytosis Is Impaired in Type A–B Niemann–Pick Disease Model Cells and Can Be Restored by ICAM-1-Mediated Enzyme Replacement*”, 2014, Molecular Pharmaceutics, 11(8), 2887–2895;
- Rappaport, J., *et al.*, “*A Comparative Study on the Alterations of Endocytic Pathways in Multiple Lysosomal Storage Disorders*”, 2016, Molecular Pharmaceutics, 13(2), 357–368;
- Rath, E.M., *et al.*, “*Structure and Potential Cellular Targets of HAMLET-like Anti-Cancer Compounds made from Milk Components*”, 2015, Journal of Pharmacy & Pharmaceutical Sciences, 18(4) 773 - 824, 2015;
- Rath, E., *et al.*, “*BAMLET kills chemotherapy-resistant mesothelioma cells, holding oleic acid in an activated cytotoxic state*”, 2018, Plos ONE, 13(8): e0203003;
- Ren, T-B., *et al.*, “*A General Method To Increase Stokes Shift by Introducing Alternating Vibronic Structures*”, 2018, Journal of the American Chemical Society, 140(24) 7716-7722;
- Rhodus, N.L., *et al.*, “*Oral cancer: leukoplakia and squamous cell carcinoma*”, 2014, Dental Clinics of North America, 58, 315–340;

- Ribot, J., *et al.*, “*Novel Markers of the Metabolic Impact of Exogenous Retinoic Acid with A Focus on Acylcarnitines and Amino Acids*”, 2019, International Journal of Molecular Sciences, 20(15), 3640;
- Rivera, C., “*Essentials of oral cancer*”, 2015, International Journal of Clinical and Experimental Pathology, 8(9), 11884-11894;
- Rodini, C.O., *et al.*, “*Oral cancer stem cells – properties and consequences*”, 2017, Journal of Applied Oral Science, 25(6), 708-715;
- Rough, S.M., *et al.*, “*Qualitative Analysis of Cancer Patients' Experiences Using Donated Human Milk*”, 2009, Journal of Human Lactation, 25(2), 211–219;
- Rubin, A.L., Rice, R.H., “*differential Regulation by Retinoic Acid and Calcium of Transglutaminases in cultured Neoplastic and Normal Human Keratinocytes*”, 1986, Cancer Research, 46, 2356-2361;
- Sales-Campos, H., *et al.*, “*An Overview of the Modulatory Effects of Oleic Acid in Health and Disease*”, 2013, Mini-Reviews in Medicinal Chemistry, 13, 201-210;
- Sallusto, F., *et al.*, “*Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products*”, 1995, Journal of Experimental Medicine, 182(2): 389–400;
- Santra, S., Malhotra, A., “*Fluorescent nanoparticle probes for imaging of cancer*”, 2011, WIREs Nanomedicine Nanobiotechnology, 3, 501-510;
- Schlick, C.J.R., *et al.*, “*Margin Positivity in Resectable Esophageal Cancer: Are there Modifiable Risk Factors?*”, 2020, Annals of Surgical Oncology, doi:10.1245/s10434-019-08176-z;
- Schmees, C., *et al.*, “*Macropinocytosis of PDGF β -receptor promotes fibroblast transformation by H-RasG12V*”, 2012, Molecular Biology of the Cell, 23, 2571-2582;
- Schweizer, J., *et al.*, “*New consensus nomenclature for mammalian keratins*”, 2006, Journal of Cell Biology, 174(2), 169–174;
- Scully, C., Bagan, J., “*Oral squamous cell carcinoma overview*”, 2009, Oral Oncology, 45, 301–308;

- Sebanjo, L.T., Chellaiah, M.A., “*CD44: A Multifunctional Cell Surface Adhesion Receptor Is a Regulator of Progression and Metastasis of Cancer Cells*”, 2017, *Frontiers in Cell and Developmental Biology*, 5, 18;
- Seguin, L., *et al.*, “*Integrins and cancer: regulators of cancer stemness, metastasis, and drug resistance*”, 2015, *Trends in Cell Biology*, 25(4), 234-240;
- Sharp, J.A., *et al.*, “*Dimeric but not monomeric α -lactalbumin potentiates apoptosis by up regulation of ATF3 and reduction of histone deacetylase activity in primary and immortalised cells*”, 2017, *Cellular Signalling*, 33, 86–97;
- Shurety, W., Stewart, N.L., Stow, J.L., “*Fluid-Phase Markers in the Basolateral Endocytic Pathway Accumulate in Response to the Actin Assembly-promoting Drug Jasplakinolide*”, 1998, *Molecular Biology of the Cell*, 9(4), 957–975;
- Siddikuzzaman, Guruvayoorappan, C., Berlin Grace, V.M., “*All Trans Retinoic Acid and Cancer*”, 2010, *Immunopharmacology and Immunotoxicology*, 33(2), 241-249;
- Sobolewski, C., *et al.*, “*The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies*”, 2010, *International Journal of Cell Biology*, v2010, 215158;
- Spolaore, B., *et al.*, “ *α -Lactalbumin Forms with Oleic Acid a High Molecular Weight Complex Displaying Cytotoxic Activity*” 2010, *Biochemistry*, 49, 8658–8667;
- Storm, P., *et al.*, “*Conserved features of cancer cells define their sensitivity of HAMLET-induced death; c-Myc and glycolysis*”, 2011, *Oncogene*, 30(48), 4765–4779;
- Storm, P., *et al.*, “*A unifying mechanism for cancer cell death through ion channel activation by HAMLET*”, 2013, *PLOS one*, 8(3), e58578;
- Sullivan, L.M., *et al.*, “*The Formation of an Anti-Cancer Complex Under Simulated Gastric Conditions*”, 2013, *Food Digestion*, 4, 7–18;
- Sullivan, L.M., *et al.*, “*Gastric digestion of α -lactalbumin in adult human subjects using capsule endoscopy and nasogastric tube sampling*”, 2014, *British Journal of Nutrition*, 112(4), 638-646;
- Svanborg, C., *et al.*, “*HAMLET Kills Tumor Cells by an Apoptosis-Like Mechanism Cellular, Molecular, and Therapeutic Aspects*”, 2003, *Advances in Cancer Research*, 88, 1-29;

- Svensson, M., *et al.*, “Conversion of α -lactalbumin to a protein inducing apoptosis”, 2000, Proceedings of the National Academy of Sciences of the United States of America, 97(8), 4221–4226;
- Svensson, M., *et al.*, “ α -Lactalbumin unfolding is not sufficient to cause apoptosis, but is required for the conversion to HAMLET (human α -lactalbumin made lethal to tumor cells)”, 2003, Protein Science, 12(12), 2794–2804;
- Svensson, M., *et al.*, “Lipids as cofactors in protein folding: Stereo-specific lipid–protein interactions are required to form HAMLET (human α -lactalbumin made lethal to tumor cells)”, 2003b, Protein Science, 12 2805–2814
- Tahara, E.B., *et al.*, “Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation”, 2009, Free Radical Biology and Medicine, 46, 1283-1297;
- Tataranni, T., Piccoli, C., “Dichloroacetate (DCA) and Cancer: An Overview towards Clinical Applications”, 2019, Oxidative Medicine and Cellular Longevity, 8201079, doi: 10.1155/2019/8201079;
- Taveira Elias, S., *et al.*, “Combined paclitaxel, cisplatin and fluorouracil therapy enhances ionizing radiation effects, inhibits migration and induces G0/G1 cell cycle arrest and apoptosis in oral carcinoma cell lines”, 2015, Oncology Letters, 10, 1721-1727;
- Terao, M., *et al.*, “Role of mitochondria and cardiolipins in growth inhibition of breast cancer cells by retinoic acid”, 2019, Journal of Experimental & Clinical Cancer Research, 38, 436;
- Thomas, R., Kim, M.H., “Targeting the hypoxia inducible factor pathway with mitochondrial uncouplers”, 2007, Molecular and Cellular Biochemistry, 296, 35-44;
- Toime, L.J., Brand, M.D., “Uncoupling protein-3 lowers reactive oxygen species production in isolated mitochondria”, 2010, Free Radical Biology and Medicine, 49, 606-611;
- Tolin, S., *et al.*, “The oleic acid complexes of proteolytic fragments of α -lactalbumin display apoptotic activity”, 2010, FEBS Journal, 277, 163-173;
- Tomo, S., *et al.*, “History and future perspectives for the use of fluorescence visualization to detect oral squamous cell carcinoma and oral potentially malignant disorders”, 2019, Photodiagnosis and Photodynamic Therapy, 28, 308–317;

- Trulsson, M., *et al.*, “*HAMLET Binding to α -Actinin Facilitates Tumor Cell Detachment*”, 2011, PLoS One, 6(3), e17179;
- Tu, C., Hsu, P., “*The frontline of esophageal cancer treatment: questions to be asked and answered*”, 2018, Annals of Translational Medicine, 6(4) 83-91;
- Valvezan, A.J., Manning, B.D., “*Molecular logic of mTORC1 signalling as a metabolic rheostat*”, 2019, Nature Metabolism, 1, 321-333;
- Van Horssen, R. *et al.*, “*Cancer cell metabolism regulates extracellular matrix degradation by invadopodia*”, 2013, European Journal of Cell Biology, 92(3), 113-121;
- Van Rossum, P.S.N., *et al.*, “*Imaging strategies in the management of oesophageal cancer: what’s the role of MRI?*”, 2013, European Radiology, 23:1753–1765;
- Vander Heiden, M.G., “*Targeting cancer metabolism: a therapeutic window opens*”, 2011, Drug Discovery, 10, 671-682;
- Vander Heiden, M.G., DeBerardinis, R.J., “*Understanding the intersections between metabolism and cancer biology*”, 2017, Cell, 168, 657-669;
- Vazquez, A., *et al.*, “*Cancer metabolism at a glance*”, 2016, Journal of Cell Science, 129, 3367-3373;
- Villarreal-Lozoya, J.E., *et al.*, “*Phytochemical constituents and antioxidant capacity of different pecan [*Carya illinoensis* (Wangenh.) K. Koch] cultivars*”, 2007, Food Chemistry, 102(4), 1241-1249;
- Wang, C.H., *et al.*, “*Mitochondrial dysfunction in insulin insensitivity: implication of mitochondrial role in type 2 diabetes*”, 2010, Annals of the New York academy of Sciences, 1201, 157–165;
- Wang, J.T.H., Teasdale, R.D., Liebla, D., “*Macropinosome quantitation assay*”, 2014 MethodsX, 1, 36–41;
- Wang, Q., *et al.*, “*Postoperative Chemotherapy for Thoracic Pathological T3N0M0 Esophageal Squamous Cell Carcinoma*”, 2020, Annals of Surgical Oncology, doi:10.1245/s10434-019-08112-1;
- Wang, S.J., *et al.*, “*CD44 variant isoforms in head and neck squamous cell carcinoma progression*”, 2009, Laryngoscope, 119(8), 1518-30;

- Wang, Y., *et al.*, “*Uncoupling Hepatic Oxidative Phosphorylation Reduces Tumor Growth in Two Murine Models of Colon Cancer*”, 2018, Cell Reports, 24, 47–55;
- Warburg, O., “*On the Origin of Cancer Cells*”, 1956, Science, 123(3191), 309-314;
- Wen, H., *et al.*, “ *α -Lactalbumin:Oleic Acid Complex Spontaneously Delivers Oleic Acid to Artificial and Erythrocyte Membranes*”, 2015, Journal of Molecular Biology, 427, 3177-3187;
- Wilhelm, K., *et al.*, “*Protein oligomerization induced by oleic acid at the solid–liquid interface – equine lysozyme cytotoxic complexes*”, 2009, The FEBS Journal, 276(15), 3975-3989;
- Wise, D.R., Thompson, C.B., “*Glutamine addiction: a new therapeutic target in cancer*”, 2010, Trends in Biochemical Sciences, 35, 427-433;
- Xiao, Z., *et al.*, “*A molecular complex of bovine milk protein and oleic acid selectively kills cancer cells in vitro and inhibits tumour growth in an orthotopic rat bladder tumour model*”, 2013, BJU International, 112, E201–E210;
- Xie, Y., *et al.*, “*Electrostatic interactions play an essential role in the binding of oleic acid with α -lactalbumin in the HAMLET-like complex: A study using charge-specific chemical modifications*”, 2012, Proteins, 81(1), 1-17;
- Yang, W., *et al.*, “*A technique to reduce skin toxicity in radiotherapy treatment planning for esophageal cancer*”, 2020, Journal of Applied Clinical Medical Physics, doi:10.1002/acm2.12812;
- Yi, M., *et al.*, “*Emerging role of lipid metabolism alterations in Cancer stem cells*”, 2018, Journal of Experimental & Clinical Cancer Research, 37:118;
- Yoshida, S., *et al.*, “*Differential signalling during macropinocytosis in response to M-CSF and PMA in macrophages*”, 2015, Frontiers in Physiology, 6(8);
- Yu, C., *et al.*, “*Cryotherapy for oral precancers and cancers*”, 2014, Journal of the Formosan Medical Association, 113, 272-277;
- Yu, K.S., *et al.*, “*Receptor-Meditated Endocytosis by Hyaluronic Acid@Superparamagnetic Nanovector for Targeting of CD44-Overexpressing Tumor Cells*”, 2016, Nanomaterials, 6(8), 149;

- Yu, Q., Stamenkovic, I., “*Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion*”, 1999, *Genes & Deelopment*, 13(1), 35-48;
- Yusoff, AA.M., *et al.*, “*Understanding Mitochondrial DNA in Brain Tumorigenesis*”, IntechOpen, 2015, doi:10.5772/58965. Available from: <https://www.intechopen.com/books/molecular-considerations-and-evolving-surgical-management-issues-in-the-treatment-of-patients-with-a-brain-tumor/understanding-mitochondrial-dna-in-brain-tumorigenesis>;
- Zheng, Z., *et al.*, “*Monoclonal antibodies to CD44 and their influence on hyaluronan recognition*”, 1995, *Journal of Cell Biology*, 130 (2), 485–495;
- Zhou, W., *et al.*, “*The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer*”, 2007, *Nutrition & Metabolism*, 4(5), doi : 10.1186/1743-7075-4-5;
- Zhu, X., *et al.*, “*Il-Trans Retinoic Acid-Induced Deficiency of the Wnt/ β -Catenin Pathway Enhances Hepatic Carcinoma Stem Cell Differentiation*”, 2015, *PLoS One*, 10(11), e0143255;
- Zipfel, W.R., *et al.*, “*Nonlinear magic: multiphoton microscopy in the biosciences*”, 2003, *Nature Biotechnology*, 21, 1369–1377.