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*Development of novel pyrrolo-1,5-benzoxazepines
as anti-cancer agents for the treatment of chronic
myeloid leukaemia*

*Thesis submitted to the
University of Dublin, Trinity College
for the Degree of
Doctor of Philosophy*

By

*Sandra A. Bright, BA(Mod)
School of Biochemistry and Immunology
Trinity College Dublin
Jan 2010*

“To all whose lives have been touched in some way by cancer”



9381

Declaration

I certify that all the work submitted in this thesis is entirely my own and has not previously been submitted as an exercise for a degree at this or any other University. The library at Trinity College, Dublin has my full permission to lend or copy this thesis upon request.



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Abbreviations

ABC	ATP-binding cassette
Abl	Abelson
ADMET	Absorption, digestion, metabolism, elimination and toxicity
AIF	Apoptosis-inducing factor
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
Apaf-1	Apoptotic-protease activating factor-1
ASM	Aggressive systemic mastocytosis
APS	Ammonium persulphate
ATP	Adenosine triphosphate
Bcl-2	B-cell leukaemia/lymphoma 2
Bcr	Breakpoint cluster region
BCRP	Breast cancer resistant protein
BH domains	Bcl-2 homology domains
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CCR	Complete cytogenetic response
CD45	Cluster of differentiation 45
CDK	Cyclin-dependent kinase
CEL	Chronic eosinophilic leukaemia
cFLIP	Cellular FLICE-inhibitory protein
CHR	Complete haematological response
CI	Combination index
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CNS	Central nervous system
CYP	Cytochrome P450
DD	Death domain
DED	Death effector domain
DFSP	Dermatofibrosarcoma protuberans
dH ₂ O	Deionised water

DISC	Death-inducing signalling complex
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
ERK1/2	Extra cellular signal-related kinase 1/2
FACS	Fluorescent activated cell sorting analysis
FADD	Fas associated death domain
FasL	Fas ligand
FBS	Foetal bovine serum
FDA	(U.S.) Food and drug administration
FITC	Fluorescein isothiocyanate
FLICE	FADD-like IL1- β converting enzyme
GAP	Guanine triphosphate-activating protein
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GIST	Gastrointestinal stromal tumour
Grb2	Growth factor receptor-bound protein 2
GTP	Guanine triphosphate
GVHD	Graft-versus-host disease
HES	Hypereosinophilic syndrome
HHT	Homoharringtonine
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
IC ₅₀	50% Inhibitory concentration
IFN	Interferon
i. t.	Intratumoural
i. v.	Intravenous
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase

Mcl-1	Myeloid leukaemia cell differentiation protein-1	DISC
MCR	Major cytogenetic response	DMSO
MDR	Multi-drug resistant	DNA
MDS	Myelodysplastic	DTT
MEK	Mitogen-activated, ERK-activating kinase	ECL
MPD	Myeloproliferative disorder	EGF
MPF	Maturation-promoting factor	ERK1/2
MTA	Microtubule targeting agent	FACS
MTD	Maximum tolerated dose	FADD
NCI	National Cancer Institute	FasL
NT	No (drug) treatment	FBS
NSCLC	Non-small cell lung cancer	PDA
PAGE	Polyacrylamide gel electrophoresis	FTIC
PARP	Poly (ADP-ribose) polymerase	FLICE
PBMC	Peripheral blood mononuclear cell	GAP
PBOX	Pyrrolo-1,5-benzoxazepine	GDP
PBR	Peripheral benzodiazepine receptor	GEP
PBS	Phosphate buffered saline	GFP
PDGF	Platelet-derived growth factor	GIST
P-gp	P-glycoprotein	Gp130
Ph	Philadelphia chromosome	GTP
PI3K	Phosphatidylinositol 3-kinase	GVHD
PKA	Protein kinase A	HES
PKC	Protein kinase C	HHT
PVDF	Polyvinylidene difluoride	HRP
Rb	Retinoblastoma protein	LAP
RBC	Red blood cell	IC ₅₀
RNA	Ribonucleic acid	IFN
SAHA	Suberoylanilide hydroxamic acid	IL
s.c.	Subcutaneous	IV
SCF	Stem cell factor	JNK
SDS	Sodium dodecyl sulphate	KDa
S.E.M.	Standard error of the mean	mAb
SH	Src homology domain	MAPK

SLD	Stathmin-like domain
STAT	Signal transducer and activator of transcription
STI571	Signal transduction inhibitor-571
TBS	Tris buffered saline
TEMED	N,N,N',N',-Tetramethylethylenediamine
TLCK	N α -Tosyl-L-lysine chloromethyl ketone hydrochloride
TNF	Tumour necrosis factor
TPCK	N- <i>p</i> -Tosyl-L-phenylalanine chloromethyl ketone
TRADD	TNF receptor associated death domain
TRAIL	Tumour necrosis factor-related apoptosis inducing ligand
TRIS	Hydroxymethylaminomethane
VEGF	Vascular epithelial growth factor
v/v	Volume/volume
WBC	White blood cell
w/v	Weight/volume
xg	Times gravity
XPB	Xeroderma pigmentosum B

Abstract

Chronic myeloid leukaemia (CML) is characterised by the presence of a constitutively active tyrosine kinase known as Bcr-Abl. The Bcr-Abl kinase inhibitor, STI571, is the first line treatment for CML, but the recent emergence of STI571 resistance has led to the search for alternative drug treatments and the examination of combination therapies for CML. The recently developed novel set of compounds, the pyrrolo-1,5-benzoxazepines, (PBOXs), can be subdivided into two groups with distinct mechanisms of action, G1-arresting agents and apoptosis-inducing compounds. In this report, we sought to analyse the effect of various representative PBOX compounds alone and in combination with anti-cancer agents on CML cells.

The first results chapter of this report describes how the G1-arresting compound, PBOX-21, potentiated the apoptotic ability of STI571 in CML cells. Co-treatment of CML cells with PBOX-21 and STI571 induced more apoptosis than either drug alone in parental and STI571-resistant cell lines. This potentiation of apoptosis was specific to Bcr-Abl-positive leukaemia cells with no effect observed on Bcr-Abl-negative HL-60 acute myeloid leukaemia cells. Apoptosis induced by PBOX-21/STI571 resulted in activation of caspase-8, cleavage of the DNA repair enzyme PARP and the anti-apoptotic protein Bcl-2, upregulation of the pro-apoptotic protein Bim and a downregulation of Bcr-Abl. Repression of proteins involved in Bcr-Abl transformation, the anti-apoptotic proteins Mcl-1 and Bcl-XL was also observed. The combined lack of an early change in mitochondrial membrane potential, release of cytochrome c and cleavage of pro-caspase-9 suggested that this pathway is not involved in the initiation of apoptosis by PBOX-21/STI571. Apoptosis was significantly reduced following pretreatment with either the general caspase inhibitor Boc-D-FMK or the chymotrypsin-like serine protease inhibitor TPCK, but was completely abrogated following pretreatment with a combination of these inhibitors. This demonstrates the important role for each of these protease families in this apoptotic pathway.

The pro-apoptotic PBOX compounds are a novel series of microtubule targeting agents (MTAs) that depolymerise tubulin. Recent papers demonstrating enhanced MTA-induced tumour cell apoptosis when combined with the cyclin-dependent kinase-1 (CDK1) inhibitor flavopiridol prompted us to examine whether this compound could similarly enhance the effect of the pro-apoptotic PBOX compounds. Our results show that sequential treatment with the pro-apoptotic PBOX compounds and flavopiridol synergistically

induced apoptosis in CML cells, we thus characterised part of mechanism underlying this synergism. Flavopiridol reduced the number of polyploid cells formed following PBOX treatment but only to a small extent, suggesting that inhibition of endoreplication was unlikely to play a major role in this combination. However, the addition of flavopiridol following PBOX-6 treatment did result in an accelerated exit from the G2/M transition accompanied by an enhanced downregulation and deactivation of the CDK1/cyclin B1 complex and an enhanced degradation of the inhibitor of apoptosis protein (IAP) survivin, a key target for many anti-cancer therapeutics.

As many CML patients harbour point mutations within the Bcr-Abl gene which exhibit various degrees of resistance to anti-leukaemic therapies, it was important to determine if the PBOX compounds alone and in combination with the anti-cancer agents listed above could similarly induce/enhance apoptosis in CML cells that contained such mutations. Using a series of mutant Bcr-Abl cell lines, results presented herein demonstrated the PBOX compounds to actively induce apoptosis in cells which contain the E225K and H396P mutations along with the highly resistant T315I mutation *in vitro*. The induction of apoptosis was significantly enhanced in native Bcr-Abl Baf/3 cells when combined with STI571 and also in T315I-Bcr-Abl Baf/3 cells when combined with flavopiridol. The PBOX compounds also induced apoptosis in clinical *ex vivo* CML patient samples including those resistant to STI571. We also demonstrated for the first time, the *in vivo* efficacy of the pro-apoptotic PBOX compound, PBOX-6, in a CML mouse model which also harbours the T315I Bcr-Abl mutation.

In conclusion, results from this study highlight the potential of these novel series of PBOX compounds, either alone or in combination with other chemotherapeutic agents, as an effective therapy against CML including STI571-resistant forms of the disease.

Presentations, publications and awards

Presentations:

- November 2009 Biochemical Society - Irish Area Section, Dublin.
- Oral presentation: Development of novel pyrrolo-1,5-benzoxazepines as anti-cancer agents for the treatment of chronic myeloid leukaemia.
- Sept 2007 ECCO14, Barcelona, Spain.
- Poster presentation: PBOX-21 synergistically enhances the apoptotic efficacy of STI571 in human chronic myeloid leukaemia cells.
- March 2007 IACR, Cork.
- Nov 2006 AICC, Belfast.
- Poster presentation: Evaluation of the enhancement of the apoptotic efficacy of STI571 by PBOX-21 in human chronic myeloid leukaemia cell lines.

Publications:

Bright, S.A., Greene, L.M., Greene, T.F., Campiani, G., Butini, S., Brindisi, M., Lawler, M., Meegan, M.J., Williams, D.C., Zisterer, D.M. (2009). The novel pyrrolo-1,5-benzoxazepine, PBOX-21, potentiates the apoptotic efficacy of STI571 (imatinib mesylate) in human chronic myeloid leukaemia cells. *Biochem Pharmacol.* **77**, 310-21.

Bright, S.A., Campiani, G., Deininger, M.W., Lawler, M., Williams, D.C., Zisterer, D.M. (2010). Sequential treatment with flavopiridol synergistically enhances pyrrolo-1,5-benzoxazepine-induced apoptosis in human chronic myeloid leukaemia cells including those resistant to imatinib treatment. *Biochem Pharmacol.* In press.

Awards:

The Oncology Scholars Travel Award from the Irish Cancer Society, 2007.

Chapter 1.
Introduction

1.1 Cancer

Cancer is a group of diseases in which cells acquire mutations causing them to grow and spread abnormally. The most common cancers for men are prostate, colorectal and lung cancer while for women it is breast cancer, followed by colorectal and lung cancer. Lung cancer, with its high mortality rate accounts for the largest percentage of cancer deaths. Tumourigenesis is a multi-step process in which a cell accumulates mutations in specific classes of genes, resulting in a loss of growth control. The accumulation of one or two such mutations results in a 'pre-cancerous' cell which although looks normal, proliferates at an abnormally high rate. Typically, between four and seven of these mutations are required to completely transform a normal cell into a malignant one that proliferates uncontrollably. Clonal expansion of this single progenitor cell produces a tumour mass which has the potential to metastasise or invade other tissues¹. Mutations occur in two types of genes, dominant oncogenes which typically produce upregulated or overactive proteins and tumour suppressor genes which are recessive and require both alleles to be mutated to produce the altered phenotype. There are many widely diverse causative factors of these culminated mutations including chemical toxins such as tobacco smoke, radiation e.g. U.V. light, viruses such as the epstein-barr and human papilloma viruses which cause burkitts lymphoma and cervical cancer, environmental, dietary influences and hereditary factors. Mutations lead to six characteristics commonly found in cancer cells: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis or programmed cell death, limitless replicative potential, angiogenesis, the formation of *de novo* blood vessels and finally the ability to metastasise¹. Two-thirds of secondary tumours will metastasise to locations determined by mechanical factors such as the bloodstream, however the remaining tumours will develop due to the 'seed and soil' hypothesis as first described in 1889. This hypothesis predicts that cells will survive and grow into new tumours depending on growth factors and chemotaxis from surrounding tissues. Metastasis is the cause of most cancer deaths².

1.1.1 Leukaemia

Leukaemia, which literally means 'white blood' in Greek, is a haematological malignancy that begins in the bone marrow when white blood cells (WBCs) or leukocytes

begin to proliferate uncontrollably. These abnormal malignant leukocytes, referred to as blasts, fail to mature properly; they do not die and cannot be used up. Blast cells eventually outnumber and reduce the numbers of mature platelets (causing red skin blotches, bruising and nosebleeds) and decrease the levels of red blood cells (RBCs) (leading to fatigue and anaemia), while a lack of mature WBCs can cause repeated infections³.

There are four main types of leukaemia; these are acute myeloid leukaemia (AML), acute lymphocytic leukaemia (ALL), chronic lymphocytic leukaemia (CLL) and finally chronic myeloid leukaemia (CML). Leukaemia affects about ten times more adults than children and while AML is the most common form of leukaemia, ALL is the most common type seen in children. The average incidence and mortality rates of leukaemia in the U.S.A. in 2006, the latest year for which statistics are available, were 12 and 7 per 100,000 population respectively⁴. It is therefore estimated that there will be almost 45,000 new cases of leukaemia and 22,000 deaths in the U.S.A. in the year 2009.

1.2 Chronic myeloid leukaemia (CML)

CML usually affects the myeloid family which consists of the blood cells granulocytes, monocytes, RBCs and platelets. Symptoms of CML can include fatigue, sweats or weight loss along with an abdominal lump due to an enlarged spleen. Symptoms due to severe anaemia or bleeding are less common and there is not the predisposition to infection that is seen in other forms of leukaemia³.

The clinical course of CML can be divided into three distinct phases - an initial chronic phase, a subsequent accelerated or transformation phase and an acute blastic stage. The chronic phase can last 5 years or more, during which time the patient's symptoms can be controlled quite easily by drugs allowing the patient to lead a relatively normal life. Drugs ensure the WBC count stays near the normal range of 4,000 to 10,000 cells/mm³ with fewer than 6% of peripheral blood and bone marrow cells being blast cells. Eventually the chronic phase of CML will progress, usually unpredictably, to become a much more aggressive disease where effective control of CML is much more difficult to achieve. Blast cells account for between 6 to 30% of blood and bone marrow cells during the accelerated phase⁵. When this figure subsequently rises to over 30%, patients have entered into the invariably fatal acute blastic stage of CML.

The incidence of CML in the U.S.A. in 2006 was 1.55 cases per 100,000 population. The 5 year survival rate is currently ~56%⁴ but is dependent on numerous factors such as a patient's age, general health status, the phase of CML and therefore the amount of blasts in the blood or bone marrow and the size of the spleen at diagnosis. Diagnosis involves a simple blood test where the number of WBCs and platelets in a blood sample are counted. If the WBC count is found to be abnormal a subsequent bone marrow biopsy from the iliac crest would then be performed. Final confirmation would come from cytogenetic analysis of bone marrow testing positive for the Philadelphia (Ph) chromosome. The Ph chromosome is a unique and abnormal chromosome which characterises CML and is caused by the reciprocal translocation between two genes, Bcr and Abl, forming an aberrantly expressed oncogene known as Bcr-Abl⁶. While the development of any blast cell is associated with Bcr-Abl, progression of the disease is associated with mutations in additional oncogenes such as Ras or tumour suppressor genes such as p53 and retinoblastoma (Rb).

1.2.1 The Bcr gene

The Breakpoint cluster region (Bcr) gene is situated on the long arm of chromosome 22 (22q11) (Fig 1.1) in a 5' to 3' orientation with the 5' end closest to the centromere. The gene spans 130 kb, contains 23 exons and is ubiquitously expressed. The gene codes for two major proteins with molecular weights of 130,000 (p130) and 160,000 (p160) kDa⁶. In mouse and human tissues, mRNA levels are found to be highest in brain and haematopoietic cells, while the Bcr protein is primarily expressed in the early stages of myeloid differentiation and progressively decreases as cells mature. The protein is mainly cytoplasmic but can be found in the nucleus associated with condensed DNA or with the xeroderma pigmentosum B (XPB) protein, which plays a role in DNA repair, general transcription initiation and cell cycle regulation⁷.

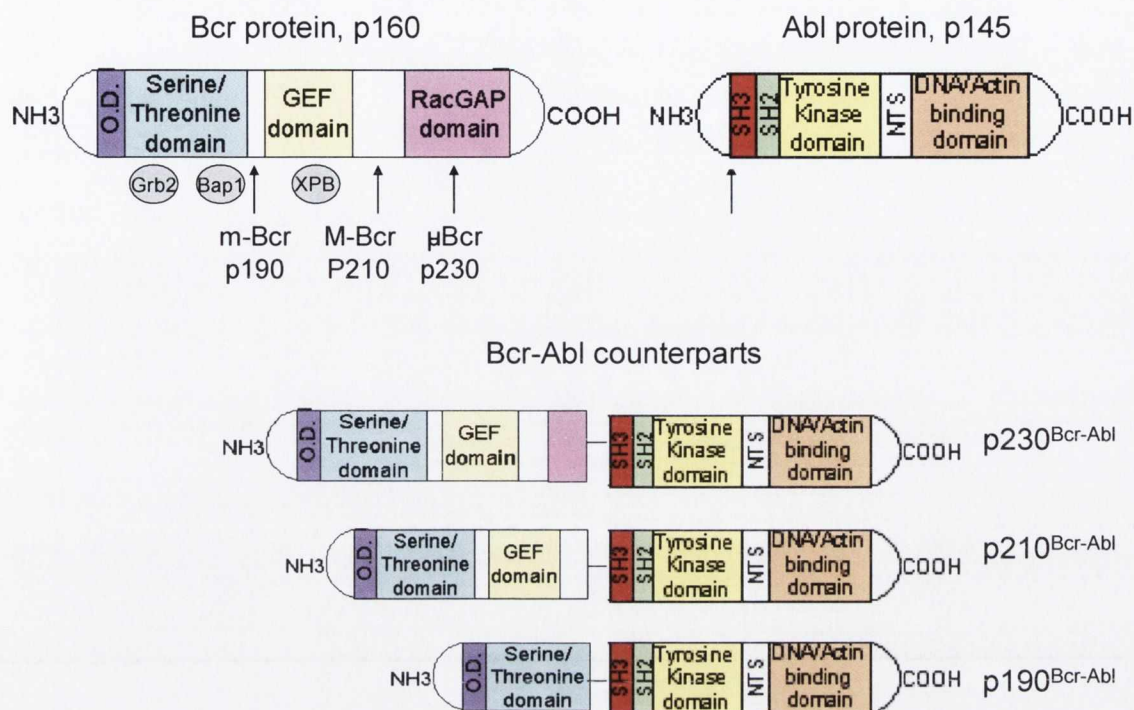


Fig. 1.1 Structure of the Bcr and Abl proteins, and their respective fusion counterparts. O.D. - Oligomerisation domain; GEF – Guanine nucleotide exchange factor; SH - Src homology; NTS - Nuclear translocation signal; Grb2 - Growth factor receptor-bound protein 2; Bap-1 - BRCA1 associated protein-1; XPB - Xeroderma pigmentosum B; Arrowheads = position of breakpoints, figure adapted from Deininger *et al.*, (2000)⁶.

Bcr-related pseudogenes have also been found but they are not translated into proteins, these include Bcr2, Bcr3 and Bcr4 also found on chromosome 22q11. The 3' sequences of these pseudogenes are identical to those comprising the last seven exons of the main Bcr gene (Bcr1)⁸.

The Bcr gene is implicated in two major signalling pathways, phosphorylation and guanine triphosphate (GTP) binding. The first exon of the Bcr gene is crucial to oncogenesis as it is the one exon included in all three Bcr-Abl fusion proteins (Section 1.2.3). It is thought to form a homotetramer allowing intermolecular cross-phosphorylation of the tyrosine kinase and activation of Abl⁹. This exon includes a serine/threonine kinase domain where phosphoserine and phosphothreonine residues interact with Bap-1, a ubiquitin hydrolase also known as 14-3-3, which is involved in cell cycle/DNA damage checkpoint control; two Src homology (SH) binding domains stretching from amino acids

192-242 and 293-413 to which Grb2 a signalling adaptor molecule binds; and an oligomerisation domain (OD).

The central guanine nucleotide exchange factor (GEF) domain which interacts with XPB and the C-terminal GTP-activating protein (GAP) domain both interact with various G proteins, essential players in intracellular signalling, cell growth and normal development. GEFs activate G proteins, in contrast GAPs inactivate G proteins by hydrolysis of GTP; the Bcr gene contains both these functions.

1.2.2 *The Abl gene*

The Abelson (Abl) gene is found on chromosome 9q24 (Fig 1.1), it is at least 230 kb long with 11 exons. The protein has a molecular weight of 145,000 (p145) and mRNA transcripts are either 6 or 7 kb. As with Bcr, Abl is ubiquitously expressed, protein levels decrease with myeloid maturation and it can be localised to both the nucleus and cytoplasm where it can bind to the actin cytoskeleton. Abl is a non-receptor tyrosine kinase, whose phosphorylation capabilities are tightly controlled by motifs in the N-terminal variable domain, these motifs are lost in the formation of the Bcr-Abl gene resulting in a constitutively active enzyme largely responsible for oncogenesis¹⁰. As the gene contains an SH2 and SH3 domain, it can associate with the SH2/SH3 adaptor protein Crkl. The Abl gene also contains a nuclear translocation signal for transportation of the protein to the nucleus where it can interact with DNA and F actin via a DNA/actin binding domain found at the C-terminus. The gene associates with the cell cycle proteins Rb, p53, p73, ATM and cyclin D¹⁰.

1.2.3 *The Philadelphia (Ph) chromosome*

The genetic hallmark of CML is the Bcr-Abl gene or Ph chromosome, this oncogene is formed by a reciprocal t(9;22)(q34;q11) chromosomal translocation between chromosomes 9 and 22 and is only expressed in peripheral blood and bone marrow cells. The Bcr-Abl gene codes for a constitutively active tyrosine kinase whose single oncogene product has been identified as having a central role in CML pathology¹¹. As with the levels of the Bcr and Abl proteins, the levels of Bcr-Abl also decrease with myeloid differentiation.

The breakpoints within the Abl gene can occur over a large area but all yield the same mRNA molecule following splicing. However in contrast to this, the breaks in the Bcr gene are localised to three breakpoint cluster regions. In CML and about a third of Bcr-Abl-positive ALL patients the breakpoint occurs in a region known as the major breakpoint cluster region, M-Bcr, spanning the Bcr exons 12-16, giving rise to a 210 kDa fusion protein (p210^{Bcr-Abl}). In the remaining Bcr-Abl-positive ALL patients the breakpoint occurs upstream of the M-Bcr in a region known as the minor breakpoint cluster region (m-Bcr) coding for a 190 kDa fusion protein (p190^{Bcr-Abl})⁶. A third breakpoint cluster region (μ -Bcr) downstream of exon 19 gives rise to a 230 kDa fusion protein (p230^{Bcr-Abl}) and is associated with the rare Ph-positive chronic neutrophilic leukaemia¹² (Fig. 1.1). Therefore the smallest protein (p190^{Bcr-Abl}) contains the least amount of the Bcr protein, while they all contain the same amount of Abl suggesting that Abl is likely to carry the transforming principle while the different sizes of Bcr may dictate different disease phenotypes.

The majority of proteins phosphorylated by Bcr-Abl are components of signalling pathways activated in normal cells by growth factor signalling or involved in cell adhesion. Exactly which of these pathways are responsible for the anti-apoptotic effects of Bcr-Abl is poorly understood but will be discussed later in relation to novel therapies that target these aberrantly expressed pathways (Section 1.3.6). Bcr-Abl is capable of autophosphorylation, these phosphotyrosine residues create binding sites for many adaptor proteins containing SH2 domains.

1.2.4 *Traditional treatments for CML*

Until the 1950s, total-body or splenic radiation therapy was the mainstay of therapy however it did not affect overall survival rates and is rarely used today, if at all¹³. An alkylating agent known as busulfan was subsequently introduced in the 1950s. While it controls haematological variables, is given orally and is inexpensive, side effects are plenty including severe, prolonged myelosuppression in 10% of patients, marrow, endocardial and pulmonary fibrosis, skin pigmentation and marrow aplasia^{14; 15}. In 1972, a DNA synthesis inhibitor known as hydroxyurea was introduced that specifically targets the myeloid cells neutrophils, eosinophils and basophils. Side effects are rare. Together hydroxyurea and busulfan produce a complete haematological response (CHR) which is defined as the normalisation of blood counts and the WBC differential as well as the disappearance of all

symptoms and signs of disease in 50 to 80% of patients. Unfortunately however, some of the major disadvantages of these two drugs include a lack of major cytogenetic responses (MCRs) which is the presence of less than 35% Bcr-Abl-positive metaphases. When these two agents were compared in a randomised study of patients with early chronic phase CML, median survival rates were significantly longer in patients receiving hydroxyurea (56 mths) when compared to patients who had received busulfan (44 mths)¹⁵. The median survival rate of patients who receive no treatment is 24 to 36 mths.

Interferon- α (IFN- α), is a naturally occurring anti-viral glycoprotein particularly effective in chronic phase CML. Blood counts in 70 to 80% of patients return to normal after treatment with IFN- α while 10 to 20% of CML patients achieve a complete remission (defined as a major reduction or complete disappearance of Bcr-Abl-positive cells). IFN- α causes temporary flu-like symptoms while more persistent side effects include weight loss, depression, alopecia, rashes and autoimmune disorders. High levels of toxicity causes ~20% of patients to discontinue treatment¹⁵. Up until the beginning of the 21st century, IFN- α remained the treatment of choice for CML sufferers; however since then a novel drug, known as STI571 (Section 1.3) has emerged and revolutionised CML treatment.

1.2.5 *Experimental models of CML*

Critical tools in helping to increase our understanding of CML are Bcr-Abl-positive cell lines. There is a wide selection of cell lines available such as the K562, KYO.1, LAMA84 and BV173 cell lines. These cell lines contain genetic alterations in addition to Bcr-Abl and therefore reflect the blast crisis phase. To date, no cell line has been established from the chronic phase. Cell lines are crucial model systems for determining Bcr-Abl transformation and biochemical signalling pathways. Many of the proteins that interact with Bcr-Abl were originally identified in these cell lines as they are more abundantly expressed than in primary cells. Recently, Baf/3 murine cells have been transfected with the Bcr-Abl gene, such genetic manipulation has also allowed for the incorporation of different Bcr-Abl point mutations involved in chemotherapy resistance and the analysis of novel compounds which may over-ride such resistance (Section 1.3.5)¹⁶.

Due to the genetic changes required to produce stable cell lines, they do not as accurately represent CML as primary cells used in *ex vivo* studies do. However, primary cells do not survive for long in culture and there is a large variation between patients.

Access to primary samples is also a major limiting factor, but nonetheless it is vitally important to confirm results obtained from cell lines in these primary samples.

1.2.6 *Animal models of CML*

The main aims of animal models to study CML are to establish a disease phenotype that closely mimics the human disease and to test the efficacy of potentially novel drugs *in vivo*. Early *in vivo* CML mouse models involved the induction of mice with mutagens while more recent approaches have included propagation of Bcr-Abl-positive cell lines, xenotransplantation of primary leukaemic cells into immunodeficient SCID mice, Bcr-Abl transgenic mice and retroviral transduction^{16; 17; 18}.

Most commonly used for the initial *in vivo* testing of new therapeutic approaches to CML are mouse cytokine-dependent haematopoietic cell lines such as 32D or Baf/3, which are transformed to cytokine independence by Bcr-Abl. These cells can be grown in syngeneic hosts (C3H or Balb/c mice respectively) without requiring irradiation. A similar idea is xenotransplantation which involves injecting a human cell line into an immunodeficient mouse, preventing rejection of the foreign tissue. These techniques have been successfully used for the drug testing of numerous anti-cancer agents including the Bcr-Abl inhibitor SGX393¹⁶ and the farnesyltransferase inhibitor SCH66336 (lonafarnib)¹⁹. Recent advances in xenotransplants for solid tumours involve administering cell lines into the site of normal tumour induction in humans. This is known as orthotransplantation and while it is more accurate than straight forward xenotransplants, it is more difficult to perform, requires extensive veterinary training, fluorescently labeled cells and *in vivo* imaging equipment to track the progress of tumours. Another slight modification of this technique involves the transplant of clinical *ex vivo* samples derived from patients. Leukaemic cells from blast crisis patients easily engraft and re-populate bone marrow however cells from chronic phase patients are much more difficult to propagate^{17; 18; 20}.

As the Bcr-Abl gene is so toxic during embryonic stages Bcr-Abl transgenic mice have been difficult to develop. However, successful transgenic systems have been developed using the metallothionien promoter, the haematopoietic-specific *tec* promoter and more recently a tetracycline-regulated system. In this system two transgenic strains of mice are generated, one expressing the tetracycline-responsive transcriptional activator protein tTa and one expressing Bcr-Abl under control of a DNA-response element with

multiple tTa binding sites. The two strains are mated to generate double-transgenic mice where the expression of Bcr-Abl is controlled with tetracycline-treated water¹⁷.

Retroviral transduction models of CML began in 1990 with the studies of Daley *et al.*, and are to date the most accurate animal model of CML²¹. The procedure involves transfecting a packaging cell line with the Bcr-Abl gene, producing retroviral products used to infect stem cells derived from donor mice. These infected stem cells are then re-introduced intravenously (i.v.) into sub-lethally irradiated, genetically identical recipient mice creating models of chronic phase CML. More recently, studies have demonstrated that simultaneous expression with a second mutation, Nup98/HoxA9, known to be affected during the blast crisis phase, leads to an acute form of disease that resembles blast crisis CML^{22; 23}.

1.3 STI571

While most cancers involve a complex disruption of various genes and signalling pathways, CML is one of the few malignancies in which there is a single signalling defect known to cause oncogenesis, the tyrosine kinase Bcr-Abl. This paved the way for the development of a therapy specifically aimed at this singular defect, known as signal transduction inhibitor-571 (STI571, GleevecTM, CGP57148B, imatinib mesylate, Glivec[®]) (Fig. 1.2).

1.3.1 Development of STI571

The development of STI571 began with the identification of a lead compound, a 2-phenylaminopyrimidine derivative identified in a screen of protein kinase C (PKC) inhibitors^{24; 25}. Cellular activity was improved by the addition of a 3'-pyridyl group at the 3'-position of the lead compound (Fig. 1.2A). A benzamide group at the phenyl ring then enhanced activity against tyrosine kinases (Fig. 1.2B). Introduction of a "flag-methyl" group attached to the diaminophenyl ring (Fig. 1.2C) prevented the inhibition of PKC activity leading to a compound that successfully inhibited the Bcr-Abl protein but had poor oral bioavailability with low water solubility. Subsequent attachment of the polar side chain, N-methylpiperazine (Fig. 1.2D), adequately improved these problems thus generating a highly specific, well tolerated tyrosine kinase inhibitor (4-[(4-methyl-1-

piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]phenyl]benzamide methanesulfonate, STI571²⁶.

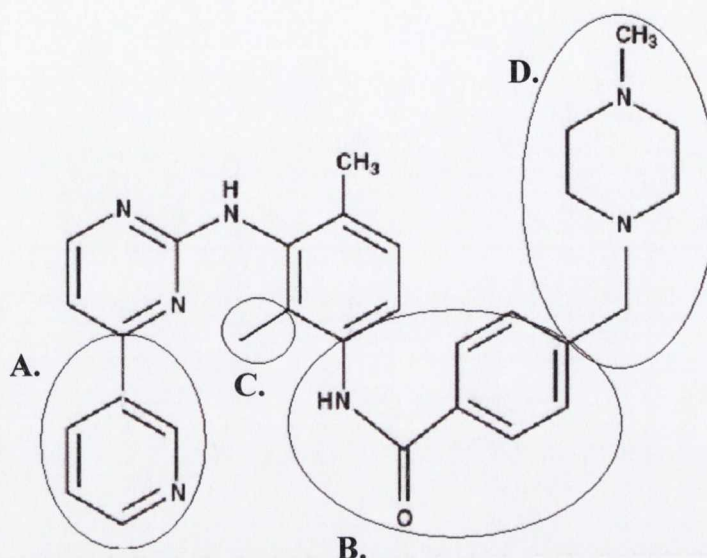


Fig. 1.2 Chemical structure of the tyrosine kinase inhibitor STI571. Diagram adapted from Deininger *et al.*, (2005)²⁶. **A.** Addition of a 3'-pyridyl group for improved cellular activity. **B.** A benzamide group increased activity. **C.** Introduction of a "flag-methyl" group prevented the inhibition of PKC activity. **D.** Attachment of the polar side chain N-methylpiperazine increased bioavailability and water solubility.

1.3.2 Mode of action of STI571

The constitutively active property of Bcr-Abl is a result of adenine triphosphate (ATP) attaching to a specific binding pocket in the kinase domain of the protein; this causes the phosphorylation of selected tyrosine residues on the substrates of Bcr-Abl and on the protein itself. STI571 works by actively competing with ATP for this binding pocket, however while it synthetically mimics the structure of ATP and fits neatly into the same binding pocket, it does not provide the essential phosphate group to be transferred to substrates²⁷. The tyrosine residues on the Bcr-Abl protein also remain unphosphorylated so Bcr-Abl cannot assume the necessary conformation needed to ensure association with its effector molecules. Consequently, the downstream chain of reactions cannot proceed (Fig. 1.3)⁵. STI571 is also an inhibitor of the receptor tyrosine kinases c-KIT and platelet-derived growth factor (PDGF)²⁸.

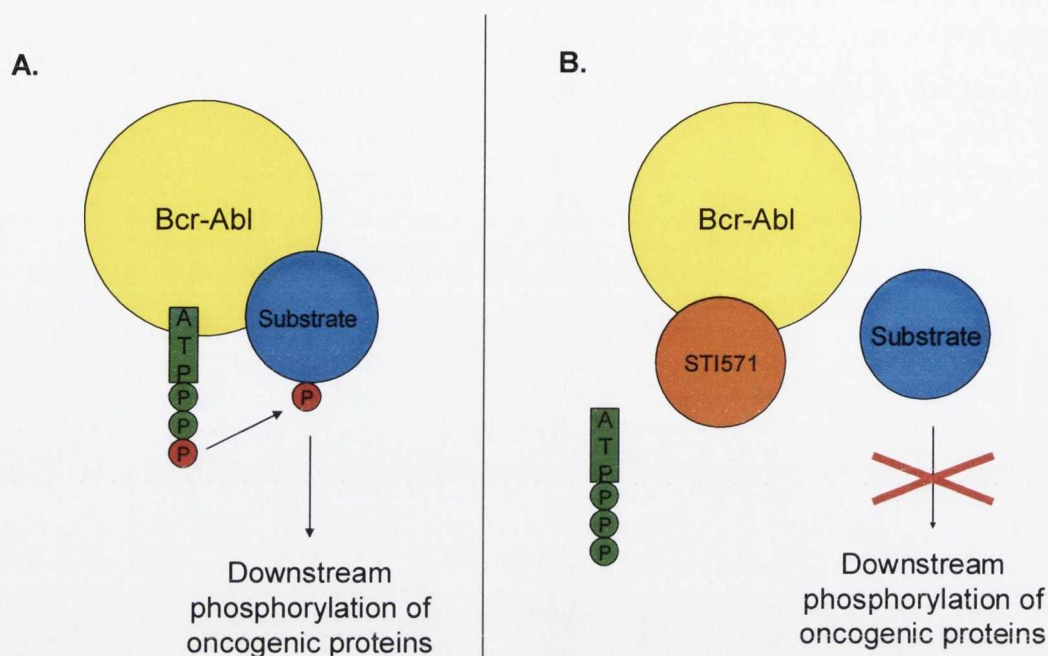


Fig. 1.3 Mechanism of action via which STI571 binds to Bcr-Abl. **A.** ATP binds to the Bcr-Abl protein in the absence of STI571. **B.** STI571 competitively inhibits the binding of ATP preventing the subsequent phosphorylation of substrates and activation of downstream signalling pathways.

1.3.3 Clinical trials with STI571

By 1998 a phase I clinical trial involving 54 chronic phase CML patients had begun. Treatment which was given orally once a day ranging from 300 to 1,000mg was sufficient to provide continuous kinase inhibition²⁹. Following this success, the trial was expanded to a further 58 patients; 38 patients had myeloid blast crisis and 20 had ALL or lymphoid blast crisis. Of the 38 patients with myeloid blast crisis, 11% achieved a CHR but most relapsed within weeks to mths³⁰. Following the success of these phase I clinical trials, three large multinational phase II trials were undertaken, the first involved a total of 532 patients with late chronic phase CML receiving 400mg of oral STI571, after 18 mths of this treatment regime only 11% of patients had progressed to a later stage of the disease³¹. The second of these trials involved 235 patients in the accelerated phase³², while the last trial involved 260 myeloid blast crisis patients³³. A phase III trial conducted on 1106 chronic phase patients comparing the effects of IFN- α in combination with low-dose cytarabine against STI571 conclusively showed STI571 to be far superior at inducing

CHRs and MCRs³⁴. It was also better tolerated and prolonged the time to disease progression when compared to the combined treatment, side effects were minimal.

1.3.4 Current use of STI571

The results of these clinical trials led to STI571 being approved for clinical use in all phases of CML in 2001, less than three years after the start of the first phase I study. As STI571 is taken orally it does not usually require hospital stays as many other chemotherapeutics do. In 2002, STI571 was approved for the treatment of c-KIT-positive gastrointestinal stromal tumours (GISTs), which can not be surgically removed or have metastasised. In October 2006, STI571 was also approved for the treatment of four more blood disorders and another solid tumour in the U.S.A., representing the first time that a regulatory authority has ever simultaneously approved one drug for so many disorders²⁸.

This included approval for use in combination with standard chemotherapy in adult patients with newly diagnosed Bcr-Abl-positive ALL and as a single agent for patients with relapsed Bcr-Abl-positive ALL. Bcr-Abl-positive ALL is a rapidly progressive haematological malignancy also characterised by the presence of the Ph chromosome. The other blood disorders for which STI571 was also approved for are Hypereosinophilic syndrome/chronic eosinophilic leukaemia (HES/CEL), characterised by the persistent overproduction of eosinophils and PDGF gene re-arrangements; Aggressive systemic mastocytosis (ASM), which is marked by the presence of too many mast cells and certain c-KIT mutations and some forms of myelodysplastic/myeloproliferative diseases (MDS/MPD) which involve certain blood cells made in the bone marrow. STI571 was also approved for treatment of adult patients with unresectable, recurrent and/or metastatic dermatofibrosarcoma protuberans (DFSP) who are not eligible for surgery. DFSP is a solid tumour found in the skin of the chest, abdomen or leg and progresses to invade nearby tissues²⁸. It is hoped that STI571 will also be as effective for these diseases as it has been for CML.

1.3.5 Resistance to STI571

While most patients endure a long-lasting CHR and/or MCR with STI571 treatment, residual leukaemia burden always remains. While STI571 has not been available

long enough to definitely say that no patient will ever be completely cured with STI571, it seems unlikely to be true. To date, resistance to STI571 has not been a significant issue in patients with chronic phase disease³⁵, however patients in the accelerated and blastic stages of the disease become increasingly resistant to STI571. There are many different mechanisms through which patients become resistant to STI571 including Bcr-Abl dependent mechanisms such as an increase in the levels of Bcr-Abl mRNA expression and corresponding upregulation of protein levels, amplification of the Bcr-Abl gene and an efflux of STI571 via multi-drug resistant (MDR) proteins such as P-glycoprotein (P-gp, MDR-1)³⁶. Bcr-Abl independent mechanisms include activation of signalling pathways downstream of Bcr-Abl or activation of signalling pathways separate to that of the Bcr-Abl gene³⁷.

However the major factor influencing STI571 resistance is due to mutations at critical points in the Bcr-Abl gene. These mutations can occur in several functionally distinct regions within the kinase domain, including the P-loop, the activation loop and the site of an essential hydrogen bond with STI571, distinct mutations confer various degrees of resistance to STI571³⁸. To date over 60 different amino acid substitutions in the kinase domain have been found, some of these are illustrated below (Fig. 1.4). Of these, T315I, a single amino acid substitution from threonine-315 to isoleucine, is one of the most common Bcr-Abl mutations found in CML patients. Crucially, it is also associated with the highest degree of STI571-resistance, preventing the formation of the critical hydrogen bond and changing the conformation of the Bcr-Abl protein in such a way as to render the protein completely resistant to STI571 binding.

Other commonly found Bcr-Abl mutations occur at locations including E255, M351, Y253, F359, G250, Q252, H396, F317, M244 and E355. Many of these mutations can be found mutated at more than one alternative residue. These mutations raise the possibility that additional drugs that target the Bcr-Abl protein via alternative mechanisms may be potentially successful³⁵. Recent data suggests that the survival rate of patients harbouring a T315I mutation is dependent on disease phase at the time of mutation detection with chronic phase patients responding to some investigational compounds in clinical trials^{39; 40}.

Recent studies have demonstrated the existence of these mutations, particularly the prevalent mutations E255K and T315I, in some Bcr-Abl-positive ALL and CML patients before the onset of STI571 treatment^{41; 42; 43; 44; 45}. These mutations demonstrate enhanced kinase activities and proliferation rates allowing for the clonal selection and multiplication

of STI571-resistant CML cells resulting in subsequent disease progression^{38; 41; 46}. Today, alot of these STI571-resistant mutations are sensitive to second generation tyrosine kinase inhibitors such as dasatinib and nilotinib (Section 1.3.8) with the notable exception of T315I mutation which is resistant to all current treatments^{47; 48}.

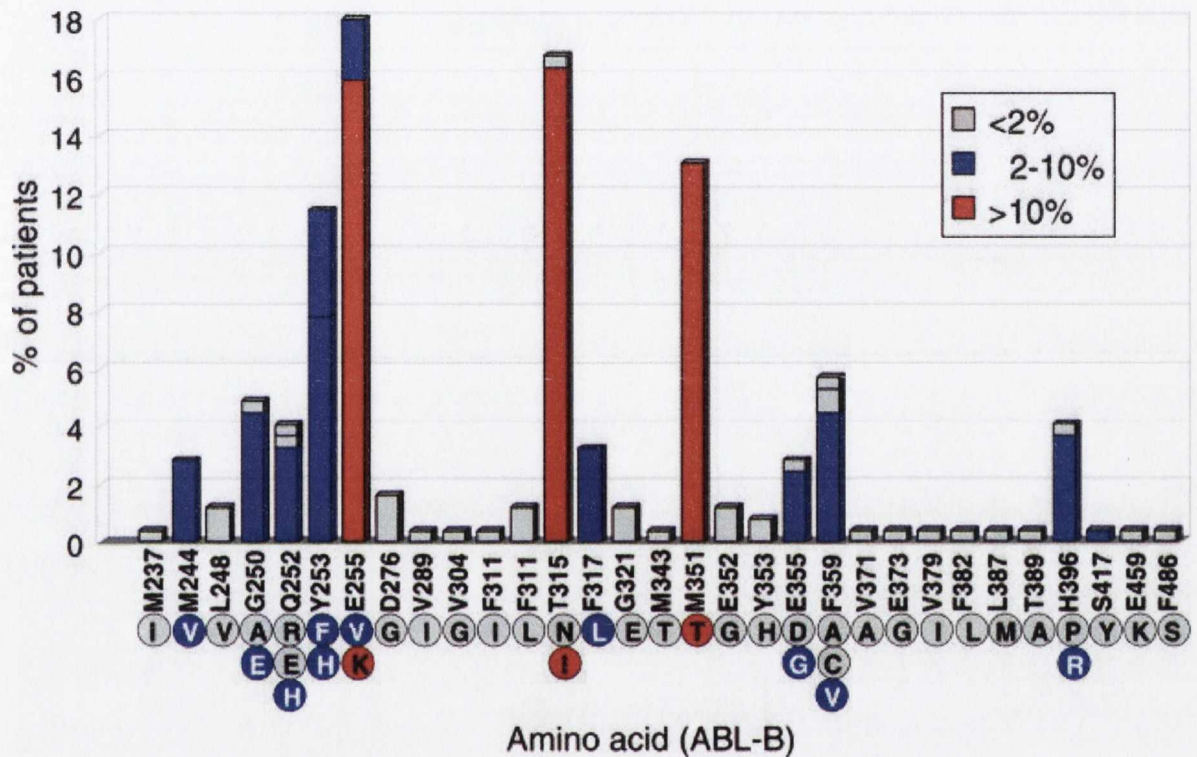


Fig. 1.4 The relative frequency of Bcr-Abl kinase domain mutations. Clinical specimens from 245 patients (219 with CML and 26 with Ph-positive ALL) in whom 31 different mutations were detected. The letters inside the circles represent the mutated amino acids. At some positions two or three possible different amino acids exist. The percentage of patients with each mutation is colour-coded. The most common mutations are E255K and T315I. Data was collected from 20 papers and compiled by Hughes *et al.*, (2006)⁴⁹.

1.3.6 STI571 in combination with other chemotherapeutic drugs

In order to try to overcome the problems of resistance associated with STI571 and to improve its efficacy, a number of studies have been conducted with STI571 in combination with other anti-cancer therapeutics. Those agents with established activity in

CML and with least overlapping toxicity and low cross-resistance were considered first. STI571 produced synergistic effects with IFN- α and additive effects with other anti-leukaemic drugs such as hydroxyurea, cytarabine, homoharringtonine (HHT), doxorubicin and etoposide *in vitro*⁵⁰. However, *in vivo*, the general theme is that a complete cytogenetic response (CCR) tends to occur earlier, but at the expense of more toxicity, particularly myelosuppression. For example, while a phase II trial with STI571/cytarabine showed a moderate increase of MCRs when compared to STI571 monotherapy, the rate of haematological toxicities (grade 3/4) had more than doubled (53% compared to ~20% with STI571 alone)⁵¹. While a more recent study involving STI571/cytarabine showed more positive results with 46% of patients experiencing a MCR after 1 year of treatment; however dose-limiting toxicities, mainly streptococcal infections, still proved problematic⁵². Similar observations were made with a combination of STI571 and IFN- α ; a 5 year phase II study of STI571 in combination with IFN- α revealed 87% of patients had achieved a MCR, however as only 13% of patients were continuing with the treatment it was hard to determine the true benefits of the combination treatment^{53; 54}. Due to the limited success of traditional CML treatments in combination with STI571, a more recent approach has focused on the combination of STI571 with inhibitors of signalling molecules downstream of Bcr-Abl.

1.3.7 STI571 in combination with inhibitors of Bcr-Abl activated signalling pathways

Bcr-Abl has been linked to many downstream signalling pathways through which it elicits its oncogenic effect including the signal transducer and activator of transcription (JAK/STAT) pathway, the phosphatidylinositol 3-kinase/Akt (PI3K) pathway and the SH2-containing inositol-5-phosphatase (SHIP) proteins that downregulate the pathway, the oncogenic Ras and mitogen-activated protein kinase (MAPK) pathways and the tyrosine phosphoprotein Crkl, an SH2/SH3 adaptor protein. Therefore combinations of known signalling inhibitors with STI571 have yielded some promising results. Some of these are outlined below (Fig. 1.5).

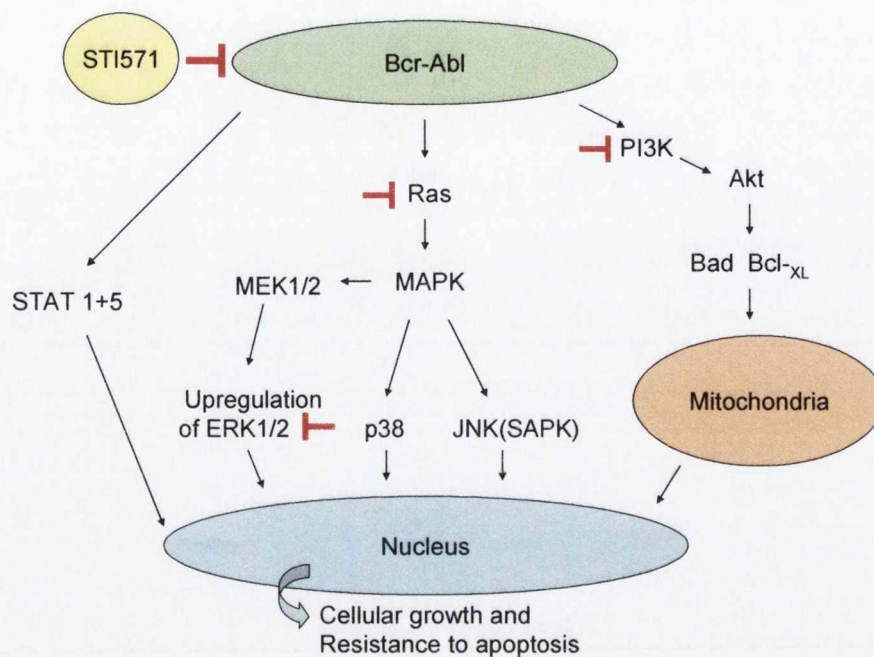


Fig. 1.5 Signalling pathways with mitogenic potential in Bcr-Abl transformed cells. Some of the main signalling pathways activated by Bcr-Abl include the STAT, PI3K and MAPK pathways. These pathways can be inhibited at various different points as indicated by the symbol **—|**. Activation of the MAPK p38 has been demonstrated in v-Abl transformed cells but not Bcr-Abl expressing cells (adapted from McGee, M.M., 2001)⁵⁵.

Farnesyltransferase inhibitors: Ras is a small GTP-binding molecule; mutations in this oncogene have been identified in ~30% of cancers. It is thought to play a central role in the pathogenesis of CML by transmitting signals to downstream molecules such as Raf and MAPKs. Bcr-Abl autophosphorylation of Tyr177 provides a docking site for the adapter molecule Grb2 which stabilises Ras in its active GTP-bound form⁵⁶. In order to elicit their full activity, Ras proteins require several modifications including transfer to the plasma membrane. The enzyme farnesyltransferase is required for this transportation. Farnesylation is one of the most critical steps in the post-translational modification of Ras proteins, therefore inhibition of farnesylation is a rational strategy for suppressing the downstream function of Ras proteins. Studies conducted with the farnesyltransferase inhibitor SCH66336 in combination with STI571 have shown a reduction in the proliferation of CML cell lines⁵⁷, inhibition of *ex vivo* CML bone marrow samples and impressive disease free survival rates of over a year in a CML mouse model¹⁹. A recent clinical study on CML patients with failed or intolerable STI571 or IFN- α therapy (n=13)

who were treated with SCH66336 alone showed responses in only a minority of patients (n=2) and dose-limiting side-effects¹³. SCH66336 was also recently assessed in combination with STI571 in a phase I trial with 23 CML patients who failed STI571 therapy, 2/9 and 1/9 chronic phase patients achieved a CHR and CCR, 2/11 accelerated phase patients achieved a CHR, while 2/3 blast crisis patients demonstrated a haematological improvement⁵⁸.

MAPK inhibitors: The Ras pathway leads to the downstream upregulation of the MAPK family which consists of three major serine-threonine kinases: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase. Upon activation by various stimuli, JNK and p38 induce apoptosis whereas ERK1/2 elicits cytoprotective effects. In all stages of CML, ERK1/2 and its substrate MEK1/2 are constitutively activated, however this enhanced activity is inhibited by STI571 in chronic and accelerated phases of CML⁵⁹. The MEK1/2 inhibitor PD184352 has been shown to synergistically potentiate STI571-induced lethality in CML cells with minimal toxicity to normal peripheral blood cells. Similar results were obtained using other pharmacological MEK1/2 inhibitors PD184352, PD98059 and U0126⁶⁰. The PD184352 inhibitor has also been successfully combined with other anti-leukaemic agents highlighting the important role this pathway plays in Bcr-Abl induced leukogenesis⁶¹.

PI3K/Akt inhibitors: PI3K is thought to associate with and become activated by Bcr-Abl, following which, it can transduce survival signals to Akt kinase protecting cells from apoptosis by inhibiting the pro-apoptotic protein Bad and blocking the caspase-dependent apoptotic pathway. PI3K has also been implicated in resistance to apoptosis induced by some growth factors. However, studies have shown that CML cells treated with wortmannin, a potent inhibitor of PI3K, eliminated PI3K activity but did not interfere with the resistance of these cells to apoptosis suggesting that PI3K seems to be important for the proliferation of CML cells, but it does not play a role in Bcr-Abl-mediated resistance to apoptosis^{59; 62}. Thus, the PI3K/Akt pathway may play a critical role in the leukogenesis of CML cells. Recently, a novel PI3K inhibitor BAG956, enhanced the inhibitory effects of STI571 in CML cells both *in vitro* and *in vivo*⁶³.

Histone deacetylase inhibitors: Histone deacetylase inhibitors are molecules that promote histone acetylation, resulting in the relaxation of chromatin structure, examples include trichostatin A, short chain fatty acids such as sodium and phenylbutyrate, depsipeptide,

MS-275 and suberoylanilide hydroxamic acid (SAHA) among others⁶⁴. Results of a phase I clinical trial with SAHA showed no dose-limiting toxicities and good anti-tumour activity⁶⁵. SAHA also showed synergistic potentiation of dasatinib, a recently developed second generation tyrosine inhibitor, in STI571-resistant CML cell lines⁶⁶. The novel histone deacetylase inhibitor valproic acid was recently shown to enhance the activity of STI571, amongst other anti-leukaemic agents, in Bcr-Abl-positive ALL and CML cells *in vitro*⁶⁷.

1.3.8 *The second generation tyrosine kinase inhibitors*

The need for alternative or additional treatments for STI571-resistant CML has led to the development of a second generation of Bcr-Abl targeted therapies, resulting in the design of novel small-molecule inhibitors such as nilotinib, dasatinib, NS-187 and ON012380⁶⁸. Of these dasatinib (BMS-354825, Sprycel™) (Fig. 1.6A) has proved the most successful and was approved by the FDA for treatment of all phases of STI571-resistant CML and Bcr-Abl-positive ALL in June 2006⁶⁹. Dasatinib is an orally active, small molecule that works by inhibiting Bcr-Abl, the Src family, c-KIT, EPHA2 and PDGFR- β , thus inhibiting blast cells. In clinical trials, a CHR was achieved in 37/40 chronic phase CML patients and 31/44 accelerated phase patients. In the same groups, a MCR was achieved in 45% and 25% of patients who could not tolerate or were resistant to STI571. Phase II trials showed dasatinib to be effective in accelerated phase CML patients who were either resistant or intolerant to STI571, 76% of patients were estimated to be alive and progression-free after 10 mths⁷⁰. However, while this new drug does offer some hope to patients who relapse while on STI571 treatment, it is also possible that patients may gain resistance to this drug in the future.

Nilotinib (AMN107, Tassigna®, Novartis) is an analogue of STI571 and was similarly approved by the FDA for treatment of STI571-resistant CML patients (Fig. 1.6B). In one phase II trial a CHR was observed in 18/23 chronic phase patients (78%). In acute phase patients, 6/16 (38%) demonstrated partial haematological responses while 2 of these (13%) achieved a MCR. Of 25 blast crisis patients, partial haematological responses were achieved in 2 (8%) and MCR in 3 (12%)⁷¹. Dasatinib and nilotinib are effective in most mutant Bcr-Abl cells with the notable exception of the T315I mutation which confers resistance to both drugs⁷².

important factor in the development of cancer. The eukaryotic cell cycle consists of four distinct phases: the M phase where mitosis or cell division takes place; the S phase during which a second identical strand of DNA is duplicated or synthesised; the G1 phase, the gap phase between M and S; and finally the G2 phase, the gap phase between S and M (Fig. 1.7)^{76, 77}.

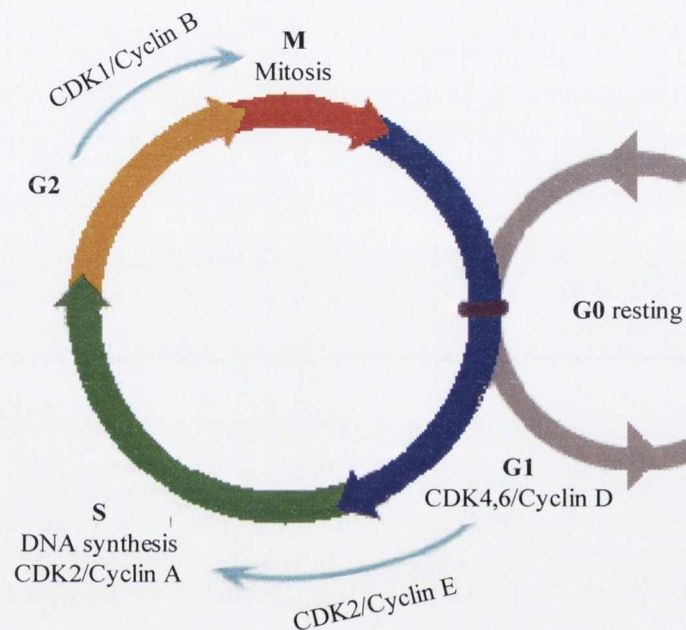


Fig. 1.7 Diagrammatic representation of the eukaryotic cell cycle. The four phases of the cell cycle include M, the mitotic phase where cell division takes place, S, the synthesis phase where duplicate strands of DNA are synthesised and the gap phases between these, G1 and G2. Entry into each phase is tightly regulated and dependent upon the expression levels of different members of the CDK and cyclin families.

Cells spend the majority of their time in the G1 phase where they monitor their internal and external environments to determine whether to divide or remain in a state of quiescence (G0). When a cell reaches a certain threshold size and specific proteins have been activated, the cell progresses from the G1 to S phase, after DNA has been replicated, cells progress through a much shorter growth phase, G2, and finally undergo mitosis and meiosis, starting the cycle over again^{76; 77}. In 1989, Hartwell and Weinert described how later events were dependent on the completion of earlier events through regulatory mechanisms known as ‘checkpoints’ or by the product of an early event being a substrate for a later event (substrate-product relationship)⁷⁸. Core machinery is used to control these

checkpoints and the progression through each phase of the cell cycle. This machinery includes maturation-promoting factor (MPF), originally found in *Xenopus* oocytes which was later found to be made up of two components, a cyclin and a cyclin-dependent kinase (CDK)⁷⁹.

1.5.1 Cyclin and CDKs

CDKs are serine/threonine kinases. To date, nine human and four mammalian CDKs have been found, most but not all are used to control the cell cycle. There are two different types of cyclins, cell cycle related (A, B, D and E) and non-cell cycle related (H and E). Each phase of the cell cycle is associated with different cyclins and CDKs that bind together to form what is known as a CDK/cyclin complex; these active complexes can phosphorylate specific substrates, one of the main targets being the Rb protein, transcribed and translated from a tumour suppressor gene. Rb is an important component in the G1/S transition. In an unphosphorylated form, Rb suppresses cellular growth through the suppression of transcription factors, phosphorylated Rb activates DNA transcription and cell cycle progression. The CDK/cyclin complex is held in an inactive state by phosphorylation of the CDK component, dephosphorylation is required for activation. After exerting its activity, the CDK/cyclin complex needs to be inactivated or replaced with machinery required for the next cell cycle phase, this is achieved through rapid degradation of the cyclin followed by the synthesis of a different cyclin to ensure progression into the subsequent cell cycle phase⁷⁷.

Specifically, CDK4 and CDK6, when complexed with their respective D-type cyclin partners are responsible for a cells progression through the G1 phase. A complex of CDK2/cyclin E is responsible for a cells progression from the G1 to S phase. Therefore, the G1/S transition is mainly regulated by cyclin D and E. CDK2/cyclin A is required for the cells progression through the S phase and a complex of CDK1 and cyclin B1 is required for mitosis and the regulation of the G2/M transition (Fig. 1.7). These complexes are in turn regulated by a stoichiometric combination with small inhibitory proteins called endogenous CDK inhibitors. The inhibitor of CDK4 (INK4) family includes p16^{ink4a}, p15^{ink4b}, p18^{ink4c}, and p19^{ink4d}, and its members specifically inhibit cyclin D-associated kinases. Members of the kinase inhibitor protein family p21^{waf1}, p27^{kip1} and p57^{kip2} bind and inhibit the activity of complexes of cyclin E and CDK2 and complexes of cyclin A and CDK2⁸⁰.

1.5.2 Mitotic checkpoint spindle and proteins

The role of the mitotic checkpoint spindle is to prevent entry into anaphase unless all mitotic daughter cells have received equal chromatids during mitosis. This is achieved with associated proteins including Mad1, Mad2, BubR1, Bub1 and Bub3. Evidence suggests that the checkpoint prevents cell cycle progression by inhibiting the activity of the anaphase-promoting complex^{81; 82} suggested that checkpoint proteins sense distinct aspects of kinetochore interaction with the spindle, with Mad2 and Bub1 monitoring microtubule occupancy, while BubR1 and Bub3 monitor tension across attached kinetochores. Deficiencies in the BubR1 gene have also recently been associated with premature aging and infertility⁸³.

1.5.3 Survivin

Survivin is a bifunctional protein that acts as a mitotic regulator and an inhibitor of apoptosis protein (IAP). It was discovered in 1997 and at 16.5 kDa is the smallest member of the IAP family but differs from others in that it forms a stable homodimer when in solution. Survivin suppresses apoptosis by targeting caspases, cysteine proteases and downstream executioners of apoptosis (Section 1.7.4). Studies highlighting the critical role of this protein have shown that deleting the survivin gene in mice results in embryonic lethality and immediate loss of tissue/organ viability⁸⁴.

During mitosis survivin is localised to and associates with mitotic spindle microtubules (Section 1.8)⁸⁵. It is cell cycle-dependent and crucial for cell cycle progression through mitosis. Survivin is activated by phosphorylation on Thr34 by the cell cycle complex CDK1/cyclin B1⁸⁶. Another role for survivin is in response to cellular stress during which survivin binds to the molecular chaperone Hsp90, helping cells to cope with unfavourable environments and preserving cell viability⁸⁷.

While survivin is expressed during development and is undetectable in most adult cells except during mitosis, it is overexpressed in most cancer cells. Its selective expression in cancer is contributed to by oncogenes including STAT3⁸⁸, E2F which is associated with the Rb pathway⁸⁹, mutated Ras⁹⁰ or by loss of tumour suppressor genes like p53⁹¹ which cause the survivin gene to become deregulated. High levels of survivin expression have been shown to be associated with a poor prognosis, tumour progression and drug

resistance⁹². This together with its role in multiple signalling pathways makes survivin an important target in the fight against cancer and has led to the development of molecular antagonists, antisense nucleotides, gene therapy, the advent of DNA vaccines and CDK inhibitors all targeted towards survivin⁸⁴. Of these, small chemical inhibitors have proved the most successful with a number of inhibitors currently in clinical trials. For example, YM155 has shown modest activity in non-small-cell lung cancer (NSCLC) in phase I and II trials^{93; 94} and EM-1421 (Terameprocol) has demonstrated potency in phase I/II cervical neoplasia trials⁹⁵. Both compounds are transcriptional repressors of survivin. Results have been positive but modest and these studies indicate survivin inhibitors in combination with other pro-apoptotic agents may be a more attractive option with greater clinical potential.

1.5.4 CDK inhibitors as potential chemotherapeutic targets

Abnormalities in the Rb pathway are found in the majority of cancers, these can be due to hyperactivation of CDKs resulting from the overexpression of positive cofactors (CDKs/cyclins), a decrease in negative factors (endogenous CDK inhibitors) or Rb gene mutations. For cancer patients, the loss of endogenous CDK inhibitors confers poor prognosis. For example, loss of the p27^{kip1} protein predicts a poor outcome in patients with breast, prostate, lung, colon or gastric carcinoma⁹⁶. Loss of p16^{ink4a} is clearly associated with poor prognosis in patients with NSCLC or melanoma. Inhibition of CDK activity therefore represents a novel mechanism for targeting cancer cells. CDK inhibitors act by arresting cells in the G1, S or the G2/M phase of the cell cycle depending on cell type, resulting in cell death⁸⁰.

CDK inhibition has been achieved by overexpressing CDK inhibitors, using small peptides to mimic the effects of CDK inhibitors or peptides that directly bind to and inhibit CDK molecules themselves and using antisense technology like siRNA to deplete CDK mRNA. The activation of CDKs by modulation of upstream phosphatases and/or kinases is also possible through dephosphorylation⁹⁷ or by manipulating the proteolytic machinery that regulates the catabolism of CDK/cyclin complexes or their regulators. Among the better known groups of direct CDK inhibitors are the flavopiridols (flavopiridol and deschloroflavopiridol), the staurosporines (staurosporine and UCN-01), olomucine and the purvalanols.

Newer CDK inhibitors include BAI, which has shown strong anti-cancer activities in head and neck cancers⁹⁸, xylocidine which has demonstrated activity against

hepatocellular carcinoma xenografts *in vivo*⁹⁹, SNS-032 which inhibits transcription and induces cell death in CLL cells¹⁰⁰ and an imidazole pyrimidine amide known as AZD5597 which has displayed activity against a range of cancer cells¹⁰¹.

1.6 Flavopiridol

Of all the CDK inhibitors, flavopiridol (L86-8275 or HMR 1275) [(-)-*cis*-2-(2-chlorophenyl)-5,7-dihydroxy-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4*H*-1-benzopyran-4-one hydrochloride], has proven to be the most successful and has advanced through many clinical trials. It is a semisynthetic flavonoid derived from an alkaloid, rohitukine, found in a plant indigenous to India (Fig. 1.8).

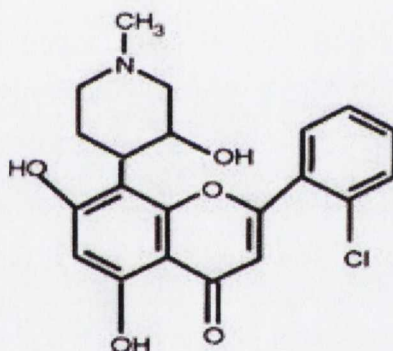


Fig. 1.8 Chemical structure of the CDK inhibitor flavopiridol.

1.6.1 Development of flavopiridol

Flavopiridol was initially found to inhibit the tyrosine kinase domain of protein kinase A (PKA) and the epidermal growth factor (EGF) receptor *in vitro*^{102; 103}. However, an anti-cancer drug screen panel resulted in an anti-proliferative IC₅₀ value of 66nM, ~1,000 times lower than the concentration required to inhibit PKA and the EGF receptor. Flavopiridol was shown to arrest cells in the G1 and G2/M phases of the cell cycle which raised the possibility that flavopiridol may inhibit CDK1 and CDK2⁹⁷. It is currently being developed by Aventis Oncology in collaboration with the National Cancer Institute (NCI) for a variety of solid tumours as well as haematological cancers.

1.6.2 Mode of action of flavopiridol

Studies using purified CDKs showed that flavopiridol inhibited the activities of CDK1, CDK2 and CDK4; this inhibition is competitively blocked by ATP. The crystal structure of flavopiridol and CDK2 showed that flavopiridol binds to the ATP binding pocket of CDK2, with the benzopyran ring occupying the same region as the purine ring of ATP¹⁰⁴. Further studies showed that flavopiridol inhibits all other examined CDKs ($IC_{50} \sim 100\text{nM}$) and slightly less potently, CDK7 ($IC_{50} \sim 300\text{nM}$)¹⁰³. Inhibition of CDK1 resulted in a G2/M arrest while inhibition of CDK2 prevented progression into and through the S phase thus resulting in a G1 arrest.

When arresting cells in the G1 phase of the cell cycle, flavopiridol treatment is also associated with a decrease in the levels of cyclin D1, cofactor for CDK4 and CDK6 activation, which is overexpressed in many human neoplasias and is associated with a poor prognosis¹⁰⁵. When MCF-7 human breast carcinoma cells were incubated with flavopiridol, levels of cyclin D1 decreased within 3 h due to mRNA depletion, followed by a decline in the levels of cyclin D3 and finally loss of CDK4 activity, while no alteration in the levels of cyclin D2, cyclin E or the remaining G1 cyclins was observed¹⁰³.

While it is clear that flavopiridol blocks cells from proliferating by subverting the cell cycle checkpoints it is not clear how flavopiridol actually kills cells. Flavopiridol selectively induced apoptosis in a wide range of haematopoietic cancers including SUDHL4, SUDHL6 (B-cell), Jurkat, MOLT4 (T-cell), HL-60 (myeloid) cell lines¹⁰⁶, *in vivo* CLL samples¹⁰⁷ and *in vivo*¹⁰⁸, however the mechanism/s by which flavopiridol induces apoptosis have not yet been elucidated. Depending on the system involved, flavopiridol may or may not involve Bcl-2 or p53^{107; 109}. Whether flavopiridol-induced inhibition of CDK activity is required for induction of apoptosis is not clear. Studies have also shown flavopiridol to display anti-angiogenic properties *in vitro* by decreasing vascular endothelial growth factor (VEGF) mRNA expression by hypoxia¹¹⁰. Thus, the anti-tumour activity of flavopiridol may be supplemented by anti-angiogenic effects. Whether the various anti-angiogenic actions of flavopiridol result from its interaction with CDK targets or other targets requires further study. Flavopiridol has also been linked to the inhibition of transcription¹¹¹.

1.6.3 *Clinical trials with flavopiridol*

Encouraging *in vivo* results eventually led to a NCI phase I clinical trial of flavopiridol administered as a 72 h continuous infusion in patients with progressive refractory neoplasms¹¹² and in lymphoma, colon and renal cancers^{113; 114; 115}. Plasma concentrations of nM levels of drug thought to be capable of achieving CDK inhibition based on results in tumour cell lines were easily achieved. However in phase II trials, although there were subsets of patients with prolonged stable disease, few responses were observed. Flavopiridol did not exhibit activity as a single agent against advanced colorectal cancer¹¹⁶, renal cancer¹¹⁴, malignant melanoma¹¹⁷, hormone-refractory prostate cancer¹¹⁸, NSCLC¹¹⁹ or gastric cancer¹¹⁵. Dose-limiting toxicities including vascular thrombosis, fatigue¹¹⁵ and diarrhea^{112; 120} and finding an optimal dosing schedule were also problematic. These initial trials involved a continuous infusion schedule.

It was recently found that greater than 90% of flavopiridol is bound to plasma proteins so it is now likely that higher concentrations in the μM range are required to ensure that the necessary levels of free drug required for tumour targeting activity are achieved. Pharmacokinetic models now indicate that this is possible by administering flavopiridol as a 30 min bolus infusion followed by a 4 h infusion¹²¹. This approach has led to considerable improvements of flavopiridol as a monotherapy, particularly in CLL. A 50% response rate was detected with prolonged survival in fludarabine refractory CLL due to significant tumour lysis syndrome¹²¹. In more recent clinical trials, flavopiridol showed significant clinical activity in 19/42 (45%)¹²² and 21/52 (40%)¹²³ of patients with highly refractory late stage and/or poor prognosis CLL achieving a partial response with a median response duration that exceeded 12 mths. In other malignancies, flavopiridol administration has largely benefitted from combination with other other chemotherapeutic agents.

1.6.4 Flavopiridol in combination with other chemotherapeutic drugs

In vitro, various combinations of chemotherapeutics such as cytarabine, topotecan, doxorubicin and etoposide when followed by CDK-inhibiting concentrations of flavopiridol, resulted in sequence-dependent cytotoxic synergy in A549 lung carcinoma cells due to the arrest of cells in the G1 or G2/M phases. Cisplatin was the only agent that resulted in sequence-independent synergy when combined with flavopiridol. In contrast, a synergistic effect was observed when cells were treated with flavopiridol for 24 h before the addition of 5-fluorouracil, which arrests cells in the S phase¹²⁴. As flavopiridol by itself induces a G2/M arrest preventing entry into mitosis, its administration before or concomitantly with taxanes is antagonistic^{124; 125}. However, Chien *et al.*, (1999)¹²⁶ failed to demonstrate a synergistic effect between flavopiridol and cisplatin and/or γ -irradiation in bladder carcinoma models. It has recently been shown that flavopiridol synergistically enhances the apoptotic effect of STI571 in CML cell lines by targeting the mitochondrial apoptotic pathway¹²⁷. It has also been shown to induce apoptosis in GIST tumours that over-express the c-KIT protein suggesting a similar mechanism of action to STI571¹²⁸. Many other CDK inhibitors have also shown similar effects.

As with flavopiridol administration alone, continuous infusion of the drug combined with docetaxel in a phase I study in metastatic breast cancer patients was disappointing due to dose-limiting neutropenia and hypotension¹²⁹. Similarly an early clinical trial examining the sequential combination of paclitaxel followed by 24 h flavopiridol infusions also showed dose-limiting neutropenia¹³⁰. However, in the same study, dose-limiting toxicities were not a problem when a 3 h flavopiridol infusion was used¹³⁰. Similarly, in a study with a 1 h flavopiridol infusion 4 h after the administration of docetaxel, five partial responses were observed in patients within pancreatic, breast and ovarian cancers¹³¹. Other more recent clinical trials involving flavopiridol activity in phase I trials include flavopiridol in combination with paclitaxel and carboplatin in NSCLC¹³² and a phase II trial of sequential flavopiridol, cytarabine and mitoxantrone in AML¹³³. A complete remission was achieved in 12/15 (75%) newly diagnosed secondary AML patients, 18/24 (75%) patients during their first relapse after a short complete remission and 2/13 (15%) primary refractory AML patients.

1.7 Cell death

Cell death was traditionally classified as either apoptosis (Section 1.7.1 to 1.7.7), also known as programmed cell death or necrosis. Necrotic cell death occurs as a result of injury, such as a physical insult or by oxygen deprivation. Increased calcium concentrations result in activation of calcium-dependent DNases, phospholipases and proteases. In contrast to apoptosis which is highly regulated, necrosis is largely uncontrolled. It causes internal organelles, in particular mitochondria, to swell, eventually causing the entire cell to lyse and empty its contents into the surrounding intercellular space. Such an event evokes an inflammatory response, circulating macrophages and other WBCs of the immune system converge on the necrotic cell and digest it. This can in turn damage normal cells in the vicinity.

Recent findings have expanded the classification of cell death to include autophagy, paraptosis, mitotic castrophy and other forms of necrosis-like apoptosis. Autophagy is a catabolic process through which damaged or long-lived proteins, macromolecules and organelles are sequestered in double or multimembrane autophagic vesicles, delivered to and subsequently degraded by the cells own lysosomal degradative machinery. Autophagy is often initiated in response to starvation, when the cell can self-digest and recycle some non-essential components. It has an important role in cellular remodeling due to differentiation, stress or damage induced by cytokines. Although autophagy is non-selective, under oxidative conditions it effectively removes oxidatively damaged mitochondria, peroxisomes and the endoplasmic reticulum. Thus, autophagy can protect the cells from apoptosis and other major injuries and it is considered to be in the cross-road between cell death and survival. However, excess autophagy can destroy essential cellular components and lead to cell death. Breakdown of autophagy has been linked to the development of cancer^{134; 135; 136}.

Paraptosis has recently been characterised by cytoplasmic vacuolation that begins with progressive swelling of mitochondria and the endoplasmic reticulum. It typically does not involve activation of caspases or the formation of apoptotic bodies, both linked with apoptosis. Instead it is thought to be mediated by MAPKs, however reports on paraptosis are limited¹³⁶.

Defective cell cycle checkpoints can lead to mitotic catastrophe and the development of polyploid cells. These cells fail to undergo cytokinesis after cell division,

instead forming large multi-nucleated cells. Mitotic catastrophe can, in particular, be triggered by compounds that target tubulin (Section 1.8.1) and DNA damaging agents ultimately leading to cell death. This death pathway kills the cell independent of p53¹³⁶. It is difficult to conclusively distinguish between different types of cell death as they can overlap and share some signalling pathways. More than one cell death program may also be activated at the same time. However the most sought after form of cell death against cancer cells is apoptosis as it does not lead to damage to surrounding cells. Apoptosis has been intensely studied and well characterised.

1.7.1 Apoptosis

Apoptosis is a specialised and genetically programmed form of cell death. Unlike necrosis however, apoptosis does not evoke an inflammatory response and is therefore less harmful to a tissue, it is essential for normal cellular and embryonic development and tissue homeostasis. Today apoptosis is characterised by specific biochemical and morphological features which include shrinkage of the cell membrane, the formation of apoptotic bodies which are subsequently phagocytosed by neighbouring macrophages, a decrease in mitochondrial membrane potential, reversal of phosphatidyl serine to the outer side of the plasma membrane, DNA degradation or laddering and chromatin condensation¹³⁷. Defects in apoptosis underlie many medical disorders such as autoimmune diseases, neurodegenerative disorders and cancer.

The genetic basis of a signalling pathway leading to apoptosis was initially identified in the nematode *C. elegans*¹³⁸ as apoptotic machinery has been well conserved throughout evolution. Since then two interconnected but yet distinct pathways have been identified. They are known as the extrinsic and the intrinsic pathways.

1.7.2 Caspases

Caspases are a series of intracellular cysteine proteases, many of which play an important role in dismantling the cell during apoptosis. The first caspase to be identified was caspase-1 or interleukin-1 β converting enzyme (ICE). It was identified as the enzyme that converts the precursor of interleukin-1 β (IL-1 β) into its mature form that is a potent mediator of inflammation¹³⁹. In order to be activated, caspases must be cleaved from an

inactive procaspase form to that of an active caspase form. All procaspases contain a highly homologous protease domain which is divided into two subunits, a large subunit of ~20 kDa (p20) and a smaller subunit of ~10 kDa (p10), which may be connected by a short linker (10 amino acids long)¹⁴⁰.

Caspases can be divided into two groups; these are the upstream initiator caspases and the downstream effector caspases. The initiator caspases such as caspase-2, -8, -9 and -10 play a role as upstream components in a proteolytic cascade that amplifies the death signal¹⁴¹. These inflammatory caspases contain prodomains or N-terminal peptides of over 100 amino acids long which contain death effector domains (DEDs) and caspase recruitment domains (CARDs). The downstream effector caspases-3, -6 and -7 function to incapacitate essential homeostatic pathways during the effector phase of apoptosis and in contrast to the prodomains of initiator caspases, the prodomains of effector domains are usually less than 30 amino acids in length¹⁴⁰. Once activated, caspases can then in turn activate downstream substrates including cytoskeletal components of the cell such as lamin and fodrin amongst many others. Caspases are also known to cleave the DNA repair enzyme PARP (Section 1.7.6).

While the 3-D structure of a procaspase has yet to be determined, structures of active caspases have been determined by x-ray diffraction revealing a tetramer (homodimer of the p20 and p10 heterodimers arranged in twofold rotational symmetry), the two adjacent small subunits are surrounded by the two larger subunits. Each p20-p10 heterodimer forms a single globular domain, the core of which is a six-stranded β -sheet flanked by α -helices. These two heterodimers associate with each other primarily through the interaction between the p10 subunits¹⁴².

1.7.3 *The extrinsic pathway*

The extrinsic or receptor-mediated pathway is initiated by the tumour necrosis factor (TNF) receptor superfamily. Members of this family all contain death domains (DD) and include the Fas (CD95) and TNF receptors. Activation of these receptors by their respective ligands, Fas ligand (FasL) and TNF results in the rapid recruitment of adaptor proteins FADD or TRADD which also contain DD domains. When the necessary ligand binds, these receptors undergo a 3-D conformational change forming trimers. Trimerisation brings together the DD domains allowing signalling pathways and further downstream activation to occur¹⁴³. FADD/TRADD also contain a DED domain that interacts with a

homologous DED domain in procaspase-8 resulting in their recruitment¹⁴⁴. The receptor, adaptor proteins and procaspase-8 come together to form a death-inducing signalling complex (DISC) which allows procaspase-8 to oligomerise and activate itself by autocleavage¹⁴³. Caspase-8 can then go on to activate multiple downstream effector molecules (Fig. 1.9A).

The apoptotic pathway must be tightly regulated, if not the consequences would be drastic, this is partly achieved by cellular FLICE-inhibitory protein (cFLIP), an inhibitory component of the DISC complex. cFLIP contains a caspase-like domain and two DED domains which enable the molecule to bind to the DED domain in FADD. Thus, the recruitment of procaspase-8 is blocked and apoptosis is inhibited.

1.7.4 *The intrinsic pathway*

The intrinsic or mitochondrial pathway is activated through internal stimuli such as stress factors, p53, DNA damage and cytotoxic agents. Once triggered, the internal stimuli are relayed to the mitochondria via pro-apoptotic members of the Bcl-2 family (Section 1.7.5). These members then heterodimerise with the outer mitochondrial membrane proteins Bax or Bak resulting in the release of cytochrome c^{145; 146}. Under normal physiological conditions, anti-apoptotic Bcl-2 heterodimerises with pro-apoptotic Bax in healthy cells, inhibiting Bax and preventing the release of cytochrome c from the mitochondria. During apoptosis, Bcl-2 is often phosphorylated and inactivated resulting in the release of cytochrome c, leading into apoptosis (Fig. 1.9B).

Cytochrome c is originally encoded by a nuclear gene and translated by cytosolic ribosomes as apocytochrome c. Apocytochrome c is then subsequently transported into the mitochondria where it is covalently modified by the addition of a heme group catalysed by cytochrome c heme lyase¹⁴⁷. The release of cytochrome c enables the formation of the apoptosome or mitochondrial initiating complex which consists of cytochrome c, dATP and apoptotic protease-activating factor-1 (apaf-1). Apaf-1 is a 130 kDa protein comprising of an N-terminal CARD domain, a CED-4 homology domain and a C-terminal repeat domain¹⁴⁸. Oligomerisation of apaf-1 in the apoptosome triggers the interaction between the CARD domain of apaf-1 and the CARD domain of procaspase-9 resulting in its autocatalysis.

Another mechanism for regulating the apoptotic pathway lies with activation of IAPs such as XIAP, cIAP-1, cIAP-2 and survivin by apoptotic stimuli. IAPs inhibit

caspases through a baculovirus IAP repeat (BIR) domain and a RING finger which contains an ubiquitin protein ligase activity, targeting caspases for degradation by the proteasome¹⁴⁹. The 29 kDa mitochondrial protein Smac/DIABLO relieves the inhibition evoked by IAPs by being processed into a mature protein and translocated to the cytosol following an apoptotic trigger¹⁵⁰. Cross-talk between the two pathways occurs via the cleavage of the BH3-only protein Bid by caspase-8.

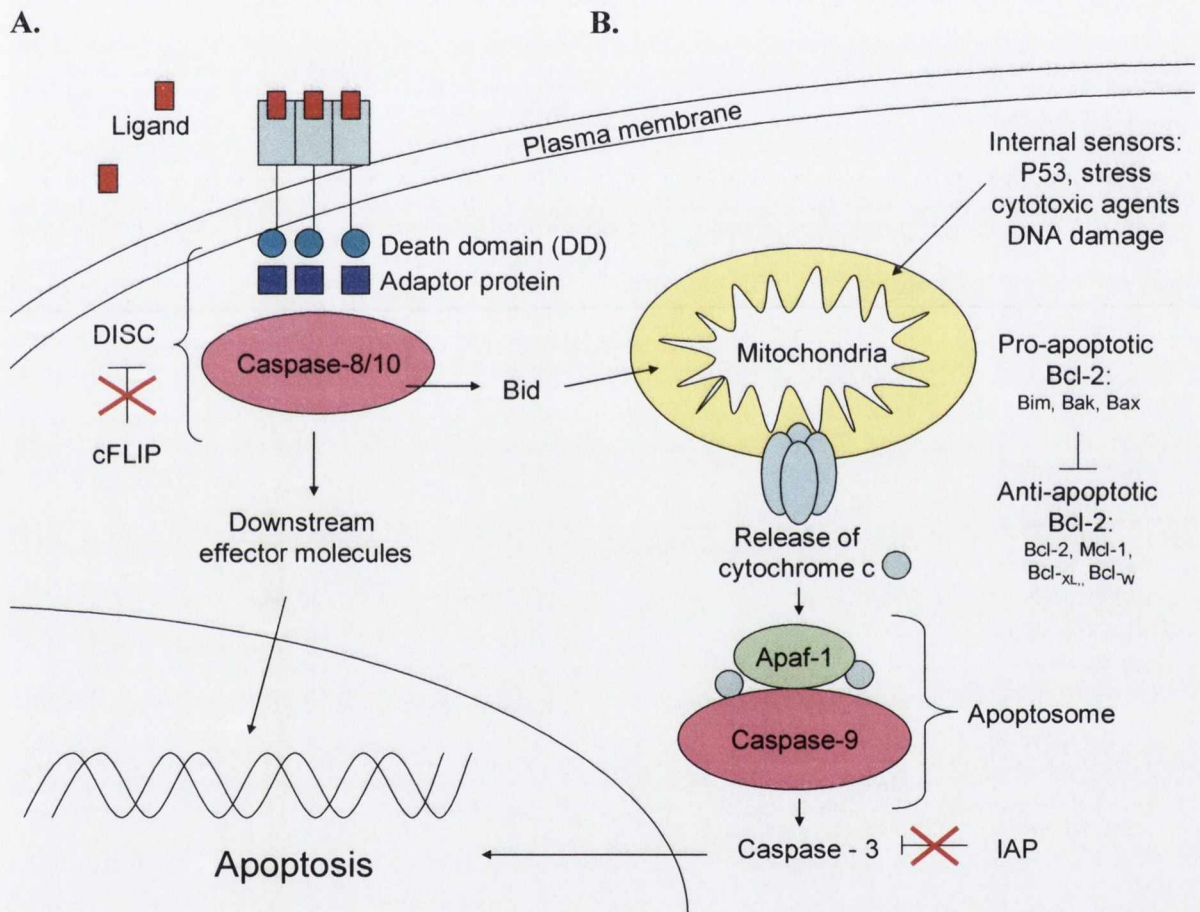


Fig. 1.9 The extrinsic and intrinsic pathways of apoptosis. **A.** The extrinsic apoptotic pathway. Death ligands such as FasL or TNF bind to their respective receptors resulting in the formation of a DISC complex (which can be inhibited by cFLIP) which activates the upstream caspases, caspase-8 and/or caspase-10. Active caspase-8 directly cleaves the effector procaspases, thus activating them, or cytosolic Bid, a pro-apoptotic Bcl-2 protein which can translocate to the mitochondria, initiating the intrinsic pathway **B.** The intrinsic or mitochondrial pathway can also be initiated via internal stimuli resulting in the release of cytochrome c into the cytosol, the subsequent formation of the apoptosome allows auto-activation of procaspase-9 and activation of downstream caspases, figure adapted from Benedict *et al.*, (2002)¹⁴⁵.

1.7.5 The *Bcl-2* family

The Bcl-2 family plays a fundamental role in caspase activation and in the regulation of apoptosis. The Bcl-2 gene was first identified in human B-cell lymphomas where it is involved in a chromosomal translocation¹⁵¹. All proteins in the family contain at least one Bcl-2 homology (BH) domain, a stretch of homologous sequence peptide found uniquely in the Bcl-2 family.

The family can be subdivided into pro-apoptotic and anti-apoptotic proteins. The pro-apoptotic proteins are the most structurally diverse. They include Bax and Bak, which contain three BH domains BH1, BH2 and BH3, and Bid, Bim, Bad, Bik and Bmf which contain only the BH3 domain¹⁵². The anti-apoptotic members include Bcl-2, Bcl-X_L, Bcl-w and Mcl-1, these members contain four BH domains, BH1 to 4; they promote cell survival by binding pro-apoptotic Bcl-2 proteins and thus inhibiting their function.

Bcl-2 can be post-translationally modified and inactivated by phosphorylation at Ser70 within a flexible loop domain in murine IL-3-dependent myeloid cell lines¹⁵³ or it can be cleaved into a shorter inactive fragment. Mcl-1 was originally identified in 1993 due to its increased expression in myeloid cell leukaemias¹⁵⁴. It displays all four BH domains and has been localised to intracellular membranes, particularly the mitochondrial membrane. While they both prolong cellular viability, the effects of Mcl-1 are much less prominent than those produced by Bcl-2¹⁵⁵. Mcl-1 and Bcl-2 are expressed at different stages of differentiation in many normal tissues (e.g. in lymphoid tissues and complex epithelia), although their expression can overlap in some cases¹⁵⁶. Bcl-X_L is known to preferentially bind with Bad and prevents the extrinsic apoptotic pathway through the Fas receptor. Bcr-Abl is known to upregulate the levels of Bcl-X_L through activation of the STAT family, particularly STAT5^{157; 158}.

Three forms of the pro-apoptotic Bcl-2 protein Bim exist due to alternative splicing; these are BimEL, BimL and BimS, each containing a BH3 domain. Protein and mRNA levels are downregulated by cytokines through either the MAPK or PI3K signalling pathways in cell lines. The level of Bim expression is a major determinant of cell fate regulated by cytokines. Bim-deficient mice have increased numbers of mature WBCs but not erythrocytes in peripheral blood. Bim is also likely to be a downstream target of Bcr-Abl¹⁵⁹.

1.7.6 PARP

Poly(ADP-ribose) polymerase (PARP) is a multifunctional enzymatic protein that participates in genome repair, DNA replication and the regulation of transcription. PARP binds to strand breaks in DNA that regularly occur during DNA replication, with the aid of the cofactor NAD, PARP is also accountable for the elongation and branching of the negatively charged poly(ADP-ribose) polymer¹⁶⁰. Cleavage of PARP from a 115 kDa molecule into 85 and 30 kDa fragments by caspase-3 is considered a classical hallmark feature of cells undergoing apoptosis¹⁶¹.

There are ~1 million molecules of the enzyme PARP in the mammalian cell nucleus, most of which are tightly bound to chromatin or the nuclear matrix. The enzyme is found in most eukaryotes, with the exception of yeast¹⁶². In humans the gene encoding PARP has been mapped to chromosome 1q41-q42.

The intact active 115 kDa molecule contains three main domains: the N-terminal 42 kDa domain which contains the signal sequence, two zinc finger structures and a DNA binding domain; the 16 kDa mid-portion of the enzyme which contains automodification sites with 15 conserved glutamate residues as presumed targets for auto-poly(ADP-ribosylation)¹⁶²; and the C-terminal 55 kDa fragment which binds NAD and mediates the enzymatic activity of PARP¹⁶³.

1.7.7 Serine proteases and caspase-independent cell death

Serine proteases are a large and varied group of protein degraders that have been classified into three main groups based on their substrate specificities, with chymotrypsin, trypsin and elastase being proto-typical of the three subgroups. The biological role of chymotrypsin is to catalyse the hydrolysis of proteins in the small intestine, it is synthesised as a single-chain inactive precursor called chymotrypsinogen. It is more commonly known for cleaving peptide bonds on the carboxyl side of the aromatic side chains of tyrosine, tryptophan and phenylalanine and of large hydrophobic residues such as methionine, but it also hydrolyses ester bonds. Trypsin cleaves peptide bonds on the C-terminal side of the basic residues arginine and lysine while elastase prefers small uncharged residues such as glycine. The active sites of serine proteases contain the critical residues Ser195, His57 and Asp102 which form a catalytic triad¹⁶⁴.

However, it is becoming increasingly clear that serine proteases also play a critical role in caspase-independent cell death. Where caspases once seemed to be a defining consequence of apoptosis, there are now numerous accounts of caspase-independent cell deaths which exhibit the traditional features of apoptosis such as chromatin condensation and DNA fragmentation. It is thought that in such cases, serine proteases may play a role, although knowledge of the exact mechanism through which they induce apoptosis is limited^{165; 166}. Cathepsin B (a cysteine protease) and D (an aspartyl protease), lysosomal enzymes known as the calpains and apoptosis-inducing factor (AIF) have also been implicated in some caspase-independent apoptotic pathways triggered by cytotoxic agents^{136; 167}.

1.8 Microtubules

The cytoskeleton is composed of three types of protein filaments: actin filaments, microtubules and intermediate filaments. Microtubules are key components of the cytoskeleton and are extremely important in the processes of mitosis, cell division and signalling¹⁶⁸. Their hollow cylindrical structure consists of heterodimers of two closely related protein subunits, α -tubulin and β -tubulin, bound tightly by noncovalent bonds. Tubulin heterodimers can undergo rapid cycles of assembly and disassembly by polymerising, giving rise to a fast-growing plus end capable of growth, and depolymerising, resulting in a slow-growing minus end. There is a binding site for GTP on α monomers while β monomers can bind either GTP or guanine diphosphate (GDP). GTP hydrolysis to GDP weakens the binding affinity of tubulin heterodimers for adjacent molecules, favouring depolymerisation. During slow microtubule growth, also known as catastrophe, the GTP bound to tubulin is hydrolysed to GDP causing depolymerisation. However, if new GTP-tubulin molecules are added more rapidly than GTP is hydrolysed, the microtubule retains a GTP cap at its plus end and growth is resumed, also known as rescue. Therefore, whether a microtubule grows or shrinks is determined by the rate of GTP hydrolysis relative to the addition of tubulin heterodimers (Fig. 1.10). This dynamic instability of microtubules allows for the reorganisation of the cytoskeleton and formation of mitotic spindles during mitosis^{169; 170}.

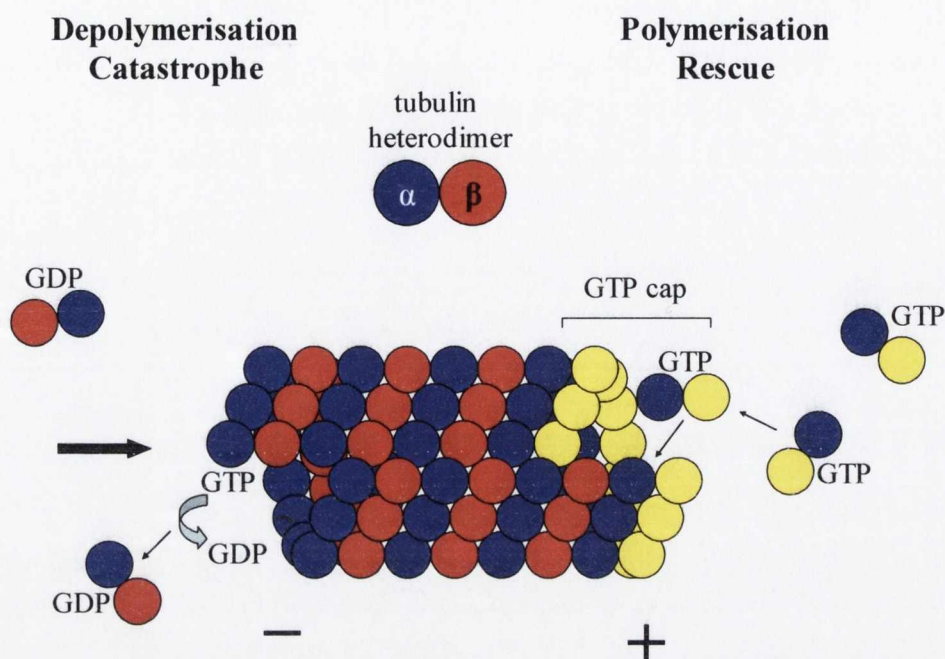


Fig. 1.10 Structure of microtubules. Microtubules are dynamic molecules that can undergo rapid rounds of assembly and disassembly. During polymerisation, new GTP-tubulin heterodimers are added to the fast growing, plus end of microtubules. Alternatively GTP hydrolysis causes tubulin to lose affinity for other tubulin molecules resulting in depolymerisation.

1.8.1 Microtubule targeting agents

A chemically diverse group of anti-cancer agents target microtubules, they are broadly referred to as microtubule targeting agents (MTAs) and have been used with great success in the treatment of cancer¹⁷⁰. Microtubule dynamics can be altered by drugs that target tubulin and affect its dynamic instability. MTAs are classified accordingly as either tubulin polymerisers (taxanes) or depolymerisers (vinca alkaloids and colchicines). Both events can be fatal to the cell as disruption leads to the failure of mitotic spindle formation required for mitosis and proliferation. MTAs therefore affect all rapidly dividing cell types including cancer cells, but also intestinal epithelium and bone marrow. Cells treated with MTAs undergo cell cycle arrest and eventually apoptotic cell death. They all bind β -tubulin but at different locations^{170; 171}.

The discovery of the first set of MTAs, the colchicines, in the 1960s highlighted their potential use as chemotherapeutic agents¹⁷². These agents bind irreversibly at the

interface between α -tubulin and β -tubulin heterodimers, known as the colchicine binding site. They cause depolymerisation of tubulin in the cellular cytoskeleton, preventing polymerisation and arresting cells in mitosis. One well characterised example is colchicine, an alkaloid of the Meadow saffron plant *Colchicum autumnale*, which has been shown to induce apoptosis in *ex vivo* CLL cells^{142; 173} however its main limitation is its toxicity against normal cells. The British drug development company Angiogene is developing a prodrug of colchicine, ZD6126 (ANG453) as a treatment for cancer¹⁷⁴. Other drugs that bind to the colchicine binding site include the combretastatins, e.g. CA4, which is currently undergoing clinical evaluation. The combretastatins are isolated from the African willow tree *Combretum caffrum*¹⁷⁵.

The vinca alkaloids represent another class of MTAs that also cause tubulin depolymerisation by binding to β -tubulin and arresting cells at prometaphase. Vinca alkaloids bind to the polar, opposite end of β -tubulin at the plus end interface and include agents such as vincristine, vinblastine and nocodazole. Vinblastine was first isolated by Robert Noble and Charles Thomas Beer from the Madagascar periwinkle plant *Catharanthus roseus*¹⁷⁶ and has been used to treat various types of cancer including Hodgkin's lymphoma, NSCLC, breast, head and neck and testicular cancer. It is a chemical analogue of vincristine. Vincristine, approved by the FDA in 1963 as Oncovin, is mainly used in non-Hodgkin's lymphoma, nephroblastoma (a common kidney tumour in children) and in combination with prednisone to treat ALL. Vincristine is occasionally used as an immunosuppressant. Both vincristine and nocodazole have shown high cytotoxicity levels and induce apoptosis in CLL cells^{177; 178}.

The third class of MTAs, known as the taxanes, is a highly important and successful group of chemotherapeutic agents including paclitaxel and docetaxel¹⁷⁹. Paclitaxel, commonly referred to as taxol, which is isolated from the bark of the Pacific yew tree *Taxus brevifolia* was discovered in 1967 by the NCI¹⁸⁰. It increases microtubule polymerisation by promoting nucleation and elongation of the polymers, over-stabilising the microtubule lattice and destroying the cells ability to use its cytoskeleton in a flexible manner as a mechanism to transport cellular components. It binds the so called taxane site of β -tubulin within the lumen of the microtubule. This binding even stabilises lateral interactions between the adjacent protofilaments of microtubules. At low concentrations, the taxanes efficiently suppress the dynamic stability of microtubules resulting in a failure of chromosome alignment causing G2/M arrest that leads to apoptosis¹⁷¹. Paclitaxel is now

used to treat patients with lung, ovarian, breast cancer, head and neck cancer and advanced forms of Kaposi's sarcoma.

A novel set of MTAs that act similarly to taxanes known as the epothilones have recently been shown to demonstrate anti-tumour activity and are currently undergoing clinical evaluation¹⁸¹.

While it is widely accepted that MTAs main function is to disrupt microtubule dynamics during mitosis and subsequently activate the mitotic spindle checkpoint leading to mitotic cell cycle arrest, exposure to MTAs can induce differential responses in different cell types. Some cells exhibit a sustained mitotic arrest, whereas others only arrest transiently in mitosis and proceed to undergo apoptosis. It has recently been reported that cells which express high levels of BubR1 are associated with prolonged cell cycle arrest, whereas cells with lower BubR1 levels tend to undergo apoptosis more rapidly¹⁸².

1.9 *PBR* receptor

The peripheral benzodiazepine receptor (PBR) was originally identified in the peripheral system¹⁸³ but was later found also in the central nervous system (CNS). Benzodiazepines such as valium bind to the PBR and to neuronal GABA-gated chloride channels known as the central-type benzodiazepine receptors¹⁸⁴. PBRs are composed of at least three subunits, a binding site for isoquinolines, a voltage-dependent anion channel and an adenine nucleotide carrier. This complex is located on the outer and inner mitochondrial membrane contact sites¹⁸⁵. A number of compounds have been developed for studying the PBR, the best characterised being PK11195 and Ro5-4864¹⁸⁶, however while the isoquinoline drug binding site has been cloned for the receptor, little is known about the exact molecular structure and the true physiological role of the PBR¹⁸⁴. The role of the PBR has been hypothesised to include involvement in the immune system, apoptosis, mitochondrial respiration, steroidogenesis¹⁸⁷ and more recently proliferation¹⁸⁸. Recently a novel series of pyrrolo-1,5-benzoxazepines, more commonly referred to as the PBOX compounds, have been synthesised as high-affinity ligands for the PBR (Fig. 1.11)¹⁸⁹.

1.9.1 *Pyrrolo-1,5-benzoxazepines compounds*

The PBOX compounds can be subdivided into two groups. The first group of compounds arrest cells in the G1 phase of the cell cycle for example PBOX-1, -2 and -21. The second group induces apoptosis in a wide range of cell lines from haematological malignancies and solid tumours; these include PBOX-6, -15 and -16. The pro-apoptotic PBOX compounds have recently been patented as potential chemotherapeutic agents. While the structure-activity relationship has yet to be determined for the compounds, it is likely that the naphthyl ring is responsible for the apoptotic activity of the pro-apoptotic compounds as the G1-arresting and non-apoptotic PBOXs do not contain this ring structure.

Importantly, no cytotoxic effects were observed in normal peripheral blood mononuclear cells (PBMCs) treated with the PBOX compounds^{190, 191} and *in vivo* administration of a representative member, PBOX-6, in unchallenged mice did not result in toxicity (unpublished observation). The mechanism through which the PBOX compounds induce cytostatic and cytotoxic effects has been shown to not involve the PBR receptor. The pro-apoptotic PBOX compounds still exhibit apoptotic activity in PBR-deficient cells such as Jurkat ALL cells whilst also inducing apoptosis in PBR-rich rat Leydig cells¹⁹². However, much of the mechanism through which these compounds elicit their effects has been resolved.

A representative of the G1-arresting PBOX compounds, PBOX-21, has been shown to arrest astrocytoma cells in the G1 phase of the cell cycle; this was preceded by a decrease in CDK2 kinase activity and a downregulation of cyclin D3. This resulted in a reduction in the levels of CDK4, CDK6 and hypophosphorylation of Rb¹⁹³. PBOX-21 inhibited cellular proliferation but did not induce apoptosis in these cells. These results indicate the potential of the PBOX compounds as anti-cancer therapeutics in the treatment of solid tumours and indicate that PBOX-21 exerts its growth inhibitory effects through modulation of the expression and/or activity of several key G1 regulatory proteins. The G1-arresting PBOX-21 compound had no effect on the microtubule network or on the assembly of purified tubulin, a molecular target of the pro-apoptotic PBOX compounds.

The second group of PBOX compounds has been shown to induce apoptosis in a wide range of malignant cells. In CML cells, PBOX-6 has been shown to induce apoptosis by bypassing the Bcr-Abl fusion protein¹⁹⁴ resulting in the release of cytochrome c and the subsequent induction of caspase-3. The MAPK family is activated in response to a range of

various stimuli which include U.V. light and chemotherapeutic drugs. Previous studies have demonstrated that the activation of the JNK signalling pathway is critical for PBOX-6-induced apoptosis in CML cells¹⁹⁵. PBOX-6 has also been shown to induce apoptosis in MCF-7 human breast carcinoma cells which lack caspase-3, by activating caspase-7 instead, indicating that the involvement of caspase-3 which is normally considered a hallmark feature of apoptosis, is not always critical for DNA fragmentation and apoptosis¹⁹⁶. More recent efforts to try and solve the questions surrounding the molecular mechanisms by which these PBOX compounds induce apoptosis have shown that PBOX-6 overcomes Bcl-2-mediated resistance to apoptosis in K562 cells and wild-type T leukaemia CEM cells by the dose-dependent phosphorylation and thus inactivation of the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-x_L¹⁹⁷. Additionally, PBOX-6 induces apoptosis in the prostate carcinoma LnCAP and cervical cancer HeLa cells (unpublished results).

P-gp, along with ABCC1 and breast cancer resistant protein (BCRP) are members of the ABC superfamily of proteins that transport a wide variety of substrates across the plasma membrane, including anti-cancer compounds resulting in MDR. A recent study has determined the pro-apoptotic PBOX compounds are not substrates for ABC drug efflux pumps and are equipotent at inducing apoptosis in parental and P-gp and BCRP expressing MDR cells. A preliminary molecular docking study also showed the PBOXs are unlikely to form favourable interactions with the P-gp substrate binding site¹⁹⁸.

A recent study suggests that the mitotic spindle checkpoint protein BubR1 contributes to the G2/M arrest induced by the PBOX compounds. The phosphorylation of BubR1 correlated with PBOX-induced G2/M arrest in CML cells while a downregulation of BubR1 was associated with the onset of apoptosis. The ability of the PBOX compounds to induce apoptosis in other cell lines was determined to be associated with low levels of endogenous BubR1. Gene silencing of BubR1 by siRNA reduced PBOX-induced G2/M arrest without enhancing the levels of apoptosis¹⁸².

More recently, tubulin has been identified as a molecular target for the pro-apoptotic PBOX compounds¹⁹⁹. PBOX-6 was found to cause depolymerisation of the microtubule network in MCF-7 cells and inhibit the assembly of purified tubulin *in vitro*. This was in a similar manner to other depolymerisers, vinblastine and colchicine. Through competitive binding studies, it was demonstrated that PBOX-6 did not bind to either the vinblastine or the colchicine binding site on tubulin, suggesting that it binds to an as yet

unidentified novel binding site¹⁹⁹. Further work is needed to characterise this novel binding site.

The pro-apoptotic PBOX compounds have been shown to not only induce apoptosis in cancer cells but to also have anti-angiogenic properties. While the formation of new blood vessels from endothelial cells is not initially required for sustaining small solid tumours it is required for the progression and growth of much larger tumours. Our laboratory has shown that PBOX-16 effectively inhibits the proliferation and differentiation of endothelial HUVEC cells into capillary-like tubules when grown on basement membrane matrix preparations (Matrigel). Migration of endothelial cells to tumour sites can be controlled by chemotaxis, VEGF-stimulated HUVEC migration was also inhibited by the PBOX compounds²⁰⁰.

In an attempt to develop these compounds as anti-cancer agents it is necessary to determine their effects on *ex vivo* patient samples and *in vivo* mouse models. PBOX-15 which has recently been shown to be more potent than PBOX-6, induced apoptosis ($IC_{50} = 0.55\mu M$) in *ex vivo* CLL samples (n=55). This is the first time the PBOX compounds have demonstrated efficacy in *ex vivo* samples. PBOX-15-induced apoptosis in CLL cells occurs via partial caspase-8 and JNK activation dependent mechanisms and independent of Bcl-2 phosphorylation. PBOX-15 also induced apoptosis in CLL cells with poor prognostic markers ($CD38^+$, $ZAP-70^+$, unmutated IgV_H genes), including fludarabine resistant cells with chromosomal deletions in 17p, further demonstrating the relevance of PBOX-15 as a potential anti-cancer agent in CLL¹⁹¹.

In a mouse mammary carcinoma model, intratumoural (i.t.) administration of PBOX-6 significantly inhibited tumour growth while also effectively inducing apoptosis in two estrogen-positive and two estrogen-negative breast cancer cell lines highlighting PBOX-6 as a promising anti-cancer agent for both hormone-dependent and -independent breast tumours²⁰¹.

Recently we have reported that STI571 potentiated the apoptotic efficacy of PBOX-6 and other MTAs such as paclitaxel and nocodazole in CML cells. Results showed STI571 reduced PBOX-6-induced G2/M arrest and polyploid formation, leading to a significant increase in apoptosis in CML K562 cells. This increase in apoptosis was associated with the downregulation of Bcr-Abl and repression of the anti-apoptotic proteins Bcl-X_L and Mcl-1¹⁹⁰.

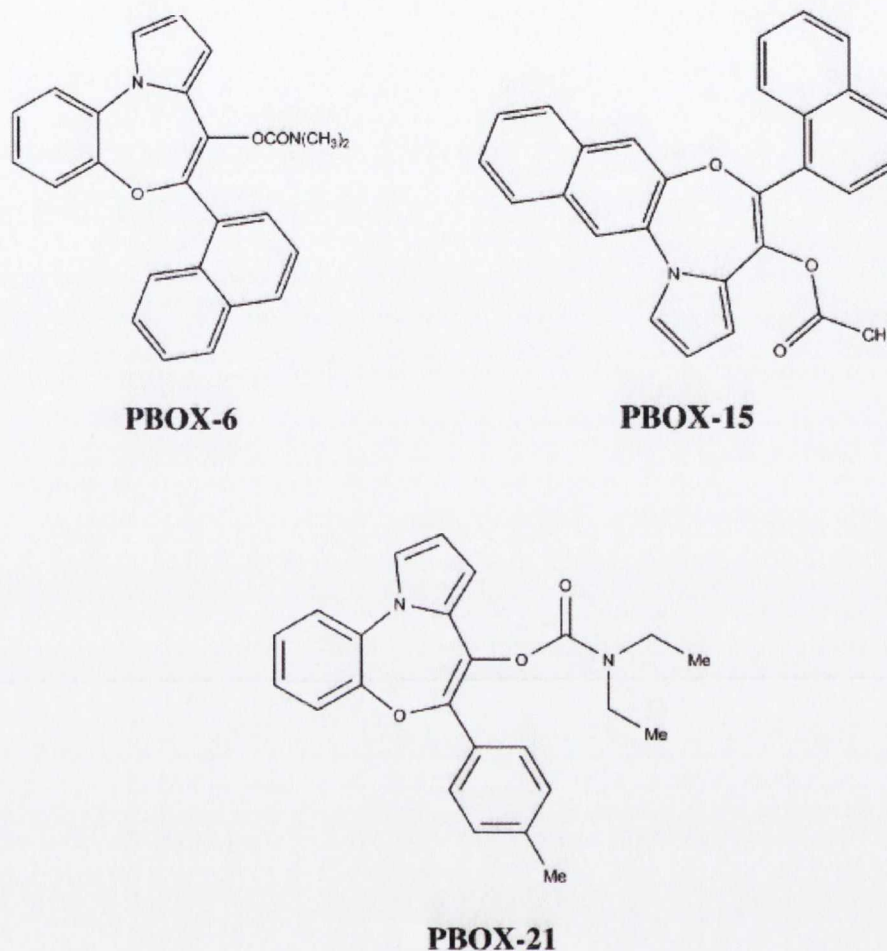


Fig. 1.11 Chemical structures of some members of the PBOX family. These include the pro-apoptotic PBOX compounds PBOX-6, -15 and the G1-arresting PBOX-21.

1.10 Aims of the project

The cytostatic and cytotoxic effects of the PBOX compounds in human tumour cells demonstrate their potential as novel anti-cancer agents. The Bcr-Abl kinase inhibitor STI571 is the first line treatment for CML, but the recent emergence of resistance to STI571 has led to the examination of combination therapies and the search for novel drugs which can bypass this resistance. To further determine the potential of the PBOXs in the treatment of CML, further study is required into the effects of these drugs in *ex vivo* CML patient samples and in an *in vivo* tumour model of CML. In addition, a deeper understanding of the mechanism of the effects of the PBOXs on inducing apoptosis in CML cells and the discovery of new ways to enhance the effect of the PBOXs will be critical to improving the potential therapeutic efficiency of these compounds.

Aims of the study:

- 1) To examine whether a representative member of the G1-arresting PBOXs, PBOX-21, enhances the apoptotic ability of STI571 in CML cells, including those resistant to STI571. To determine the molecular mechanisms underlying apoptosis induced by this combination treatment.
- 2) To determine whether the CDK inhibitor, flavopiridol, significantly enhances PBOX-6-induced apoptosis in CML cells and to gain an understanding of the mechanism underlying apoptosis induced by this treatment.
- 3) To assess the apoptotic potential of the PBOXs on *ex vivo* patient samples.
- 4) To examine the ability of the PBOXs to induce apoptosis in resistant CML cells expressing mutations within the Bcr-Abl kinase domain, such as the T315I mutation and to determine the effect of the PBOXs in an *in vivo* tumour model.

Chapter 2.
Materials and methods

2.1 *Materials and suppliers*

The full names and addresses of suppliers can be found at the end of this list.

21G needles	BD Biosciences
$\gamma^{32}\text{P}$ -ATP	Perkin Elmer
Acrylamide mix	National Diagnostics
Alamar blue	BioSource
Alexa 488	Invitrogen
Annexin V	IQ Products
Annexin V binding buffer	BioSource
Anti-Actin monoclonal antibody (mAb)	Calbiochem
Anti-Bcl-2 mAb	Calbiochem
Anti-Bcl-XL mAb	Calbiochem
Anti-Bcr-Abl mAb	Calbiochem
Anti-Bim mAb	Calbiochem
Anti-caspase-8 mAb	Calbiochem
Anti-caspase-9 mAb	Calbiochem
Anti-CDK1 mAb	Calbiochem
Anti-CD45 mAb	BD Biosciences
Anti-cyclin B1 mAb	Santa Cruz
Anti-Mcl-1 mAb	Calbiochem
Anti-mouse IgG secondary Ab	Promega
Anti-PARP mAb	Calbiochem
Anti-phosphotyrosine mAb	Calbiochem
Anti-rabbit secondary Ab	Cell Signaling Technology
Anti-survivin mAb	R&D Systems
Anti- α -tubulin mAb	Calbiochem
APS	Sigma
Apo-ONE kit	Promega
ATP	Sigma
BCA protein assay reagents A and B	Pierce
Boc-D-FMK	Calbiochem
BSA powder	Sigma

BSA protein standards	Pierce
Calculusyn	Biosoft
Caspase-2 inhibitor Z-VDVAD-FMK	Calbiochem
Caspase-8 inhibitor Z-IETD-FMK	Calbiochem
Caspase-9 inhibitor Z-LEHD-FMK	Calbiochem
Caspase-10 inhibitor Z-AEVD-FMK	R&D Systems
Cremophor EL	Sigma
Cryogenic tubes	Nalgene
DiOC ₆	Sigma
DMSO	Sigma
DTT	Sigma
ECL detection reagents A and B	Pierce
EDTA	Sigma
EGTA	Sigma
Ethanol	Lennox
FACSflow	BD Biosciences
FACS tubes	BD Biosciences
FBS	Invitrogen
Filter paper	Whatman
Flavopiridol	Sigma
Glycerol	Sigma
β -Glycerophosphate	Sigma
Glycine	Sigma
HEPES	Sigma
Histone H1	New England Biolabs
IL-3	Peprotech
Isopropanol	Lennox
Kodak X-omat LS film	Sigma
Methanol	Lennox
MgCl ₂	Sigma
NaCl	Sigma
NaF	Sigma
Nucleofector kit	Lonza
Paclitaxel	Sigma

PBS	Oxoid Ltd
Penicillin	Sigma
PMSF	Roche
Ponceau S	Sigma
Prism GraphPad	Graphpad Software, Inc.
Propidium iodide	Sigma
Protease inhibitor tablets	Roche
Protein A sepharose	Sigma
Protein molecular weight markers	Amersham Biosciences
PVDF	Millipore
RNase A	Sigma
RPMI 1640 medium	Biosciences
scRNA	Cell Signaling Technology
SDS	Sigma
siRNA-survivin	Cell Signaling Technology
Streptomycin	Sigma
Stripping buffer	Pierce
TEMED	Sigma
TLCK	Sigma
TPCK	Sigma
Tris	Sigma
Triton X-100	Sigma
Tween 20	Sigma

All sterile tissue culture flasks, pipettes, pipette tips, plates and tubes were from Greiner Bio-One Ltd. PBOX-6 and PBOX-15 were synthesised by Prof. Guiseppe Campiani, University of Siena, Italy and PBOX-21 by Dr. Tom Greene, School of Pharmacy, Trinity College, Dublin 2, Ireland. STI571 was a generous gift from Dr. Elisabeth Buchdunger, Novartis, Switzerland. Prof. Stephen Grant, Division of Hematology/Oncology, Virginia Commonwealth University, Richmond, VA, U.S.A, kindly provided the STI-resistant K562 cell line. Baf/3 cells were received with thanks from Prof. Deininger, Division of Hematology and Medical Oncology, Oregon Health and Science University, Portland, OR, U.S.A. The LAMA84 and parental K562 cells were received from Prof. Mark Lawler, Institute of Molecular Medicine, St. James's Hospital and Trinity College, Dublin 2,

Ireland, as was the lymphoprep. HL-60 cells were originally sourced from the ECACC. All animals were sourced from Harlan, U.K.

2.2 Postal and e-mail addresses of suppliers/distributors.

Amersham Biosciences: Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, U.K. orders.gb@amersham.com

ATCC: LGC Standards, Queens Road, Teddington, Middlesex TW11 0LY, U.K. atcc@lgcpromochem.com

BD Biosciences: 21 Between Towns Road, Cowley, OX4 3LY Oxford, U.K. BDUK_customerservice@europe.bd.com

Biosciences: 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Co. Dublin, Ireland. orders@biosciences.ie

Biosoft: 20 Dolphin Way, Stapleford, Cambridge CB2 5DW, U.K. info@biosoft.com

BioSource Europe S.A: rue de l'Industrie, 8- B 1400, Belgium. orders@biosciences.ie

Calbiochem: P.O. Box 12087, La Jolla, California 92039-2087, U.S.A. customer.service@merckbiosciences.co.uk

Cell Signaling Technology: 3 Trask Lane, Danvers, Massachusetts 01923, U.S.A. enquiries@isisco.ie

ECACC: Health Protection Agency, Salisbury SP4 0JG, U.K. eiorders@europe.sial.com

GraphPad Software, Inc: 2236 Avenida de la Playa, La Jolla, California 92037, U.S.A. sales@graphpad.com

Greiner Bio-One Ltd: Brunel Way, Stroudwater Business Park, Stonehouse, Gloucestershire GL10 3SX, U.K. orders@cruinn.ie

Harlan Laboratories U.K. Ltd: Blackthorn, Oxon OX25 1TP, U.K. orders@harlan.com

Invitrogen Ltd: 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, U.K. orders@biosciences.ie

IQ Products: Rozenburglaan 13a, 972 DL Groningen, The Netherlands. info@accuscience.ie

Lennox Laboratory Supplies Ltd: John F. Kennedy Drive, Naas Road, Dublin 12, Ireland. sales@lennox.ie

Lonza Cologne AG: Nattermannallee 1, 50829 Cologne, Germany. sales.uk@lonza.com

Millipore Ireland B. V.: Tullagreen, Carrigtwohill, Co. Cork, Ireland. eiorders@europe.sial.com

Nalgene (Europe) Ltd: Unit 1a, Thorn Business Park, Hereford HR2 6JT, U.K.
eiorders@europe.sial.com

National Diagnostics U.S.A: 305 Patton Drive, Atlanta, Georgia 30336, U.S.A.
orders@biosciences.ie

New England Biolabs (UK) Ltd: 75/77 Knowl Piece, Wilbury Way Hitchin, Herts SG4 0TY, U.K. enquiries@isisco.ie

Oxoid Ireland Ltd: Blackthorn Road, Sandyford Industrial, Estate, Foxrock, Dublin 18, Ireland. orders@fanninhealthcare.com

Peptrotech: PeproTech House, 29 Margravine Road, London W6 8LL, U.K.
info@peptrotech.co.uk

Perkin Elmer: Unit G13, Calmount Park, Ballymount, Dublin 12, Ireland.
cc.ie@perkinelmer.com

Pierce Biotechnology, Inc: P.O. Box 117, Rockford, Illinois 61105, U.S.A.
info@medical-supply.ie

Promega U.K: Delta House, Southampton Science Park, Southampton SO16 7NS, U.K.
info@medical-supply.ie

R&D Systems Europe Ltd: 19 Barton Lane, Abingdon Science Park, Abingdon, Oxon OX14 3NB, U.K. info@RnDSystems.co.uk

Roche Diagnostics Ltd: Charles Avenue, Burgess Hill, West Sussex RH159RY, U.K.
uk.biochem@roche.com

Sigma-Aldrich Ltd: Airton Road, Tallaght, Dublin 24, Ireland. eiorders@europe.sial.com

Whatman International Ltd: Springfield Mill, James Whatman Way, Maidstone, Kent ME14 2LE, U.K. information@whatman.com

2.3 Cell culture

2.3.1 Cell lines

The LAMA84 cell line was previously established from the peripheral blood of a 29 year old female with CML (treated for 5 years with busulfan from 1979-1984) 1 mth after the onset of myeloid-megakaryocytic blast crisis²⁰².

The parental K562 cell line (K562S) was originally derived from a 53 year old female CML patient in terminal blast crisis and can spontaneously differentiate into recognisable progenitors of the erythrocyte, granulocyte and monocytic series²⁰³. The STI571-resistant K562 cell line (K562R) obtained from Prof. S. Grant was generated by growing parental K562S cells in progressively higher concentrations of STI571. They were cultured in the absence of STI571 before all experimental procedures²⁰⁴.

The HL-60 cell line was originally obtained by leukopheresis from a 36 year old caucasian female with promyelocytic leukaemia. About 10% of these peripheral blood lymphocytes can spontaneously differentiate.

The Baf/3 cell lines are of murine descent. The parental cell line is a pro-B cell line that is IL-3 dependent. Prof. Deininger and colleagues transfected this parental cell line with the native p210 version of Bcr-Abl, mutant Baf/3 cells contain different Bcr-Abl point mutations which exhibit various degrees of chemotherapeutic resistance¹⁶.

2.3.2 Growth and maintenance of cell lines

The K562S, K562R, LAMA84 and mutant Bcr-Abl Baf/3 cell lines were grown in T75cm² flasks in 20ml RPMI 1640 (Glutamax) medium supplemented with 10% (v/v) foetal bovine serum (FBS) and 50µg/ml penicillin/streptomycin. Parental Baf/3 cells also required 10ng/ml IL-3. HL-60 cells were grown in RPMI medium supplemented with 20% (v/v) FBS and 50µg/ml penicillin/streptomycin. Cells were grown in a humidified environment maintained at 95% O₂ and 5% CO₂ and passaged at least twice weekly depending on their levels of confluency. When required for sub-culturing, growth medium was heated to 37°C, during which time cells were transferred to a sterile 20ml tube and centrifuged at 500xg for 5 min. The supernatant was discarded and the pellet was resuspended in 5ml of medium, cells were then counted using a haemocytometer and

seeded at the optimised density of 2×10^5 cells/ml for HL-60, K562S, K562R and Baf/3 cells and 3×10^5 cells/ml for LAMA84 cells.

2.3.3 Use of a haemocytometer

A haemocytometer is a modified microscope slide containing an accurately subdivided grid that enables the counting of cells in suspension. Each grid (of which there are two) consists of nine primary or large squares, each measuring an area of 1mm^2 . Each of these primary squares contains 25 medium squares, each measuring an area of 0.04mm^2 . When a particular seeding density of cells was required, a clean cover slip was placed on the haemocytometer; $10\mu\text{l}$ of the cell suspension was then pipetted into the groove at the end of the plane. The cell suspension was then drawn across the grid by capillary action. The number of cells within the four outer primary squares was counted; those that touched the lower or right hand side of the grid were not counted. As each medium square is 0.04mm^2 , the total volume per primary square is $1 \times 10^{-4}\text{ml}$. This implies that the total cell concentration in the original suspension (cells/ml) is the average cell count per primary square multiplied by 10^4 .

2.3.4 Cell storage/cryopreservation

Cells were first sub-cultured as previously described (Section 2.3.2), the supernatant was discarded and the pellet was dissolved in 1ml of freezing medium (10% (v/v) DMSO, 30% (v/v) FBS in RPMI medium) and transferred to sterile labeled cryotubes. In an effort to minimise damage caused during cryopreservation, cryotubes were incubated on ice for 15 min and subsequently placed in a freezer at -70°C overnight. Samples could then be stored in liquid nitrogen at -180°C indefinitely. When an aliquot of cells was required, the cells were quickly removed from the liquid nitrogen and thawed at 37°C for 2 min. Just before the samples had fully thawed, their contents were gently pipetted into a sterile 20ml tube to prevent damage to the cells that can be very fragile after cryopreservation. The cells were then centrifuged at $500\times g$ for 5 min. The supernatant was discarded and the pellet was resuspended in 1ml of medium. This solution was then added to a small flask containing a further 6ml of medium. Following 48 h, cells were confluent enough to be passaged as previously described (Section 2.3.2). All cells had been sub-

cultured at least once and analysed by flow cytometry to ensure background levels of apoptotic cells were sufficiently low before any experiments were performed.

2.4 Preparation of stock solutions

A 10mM stock solution of PBOX-21 and 5mM stock solutions of PBOX-6 and PBOX-15 were made up in 100% (v/v) ethanol (for *in vitro* experiments) and maintained at -20°C. Working stock solutions were then made up in ethanol by serial dilutions of the primary stock solution.

STI571 was initially made up as a 10mM stock solution in 100% (v/v) DMSO and subsequently aliquoted out into 10µl samples to prevent repeated freeze-thawing, aliquots were kept for up to 6 mths at -20°C. Immediately before any experiments, STI571 was diluted to a concentration of 100µM in RPMI medium, the required volume of which was added to cells.

Caspase inhibitors, Boc-D-FMK and etoposide were dissolved in 100% (v/v) DMSO. TPCK was reconstituted in 100% (v/v) ethanol, while TLCK was stored in 5ml aliquots in dH₂O. All inhibitors were stored at -20°C.

The CDK inhibitor flavopiridol was reconstituted in 100% (v/v) DMSO to a 10mM stock solution and aliquoted into 5µl samples to prevent repeated freeze-thawing. Prior to experimentation, flavopiridol was diluted in fresh medium to a working stock solution of 25µM and added to cells as required.

All four novel hybrid compounds NF1522-25 were stored as a 10mM stock solution in 100% (v/v) DMSO at -20°C. The compounds contain the basic pyrrolo 7 ring structure characteristic of the PBOX compounds.

2.5 Treatment of cells

CML cells were treated with the indicated concentrations of PBOX-21 and STI571 at the same time for the required incubation period. Controls corresponding to the treatment of cells with each agent alone were also set up as were vehicle samples treated with ethanol, DMSO and single treatments combined with the vehicle of the second treatment. For experiments involving co-treatment with PBOX-6 (P6) and flavopiridol (flavo) at the same time, the same method was followed, however after the initial 18 h treatment, cells were centrifuged for 5 min at 500xg and resuspended in the equivalent

volume of fresh drug-free medium (no treatment, NT) and incubated for a further 24 h, this treatment protocol was designated (P6+flavo)₁₈,NT₂₄. Cells treated sequentially with PBOX-6/15 for X h followed by removal of the compounds and addition of either drug-free medium or medium containing flavopiridol for 24 h were designated P_X,NT₂₄ or P_X,F₂₄.

2.6 Alamar blue viability assay

CML cells were seeded at the relevant density in 200µl medium in a 96-well plate. Cells were treated and incubated as required. Alamar blue (20µl) was then added to each well and incubated at 37°C in the dark for between 3 and 4 h (depending on the cell type). Plates were then read on a fluorescence plate reader (SpectraMax Gemini, Molecular Devices) with excitement and emission wavelengths of 544nm and 590nm respectively. Experiments were performed in triplicate. A sample containing only reagent and medium (without any cells) was used as a blank. Vehicle samples were set as 100% viability from which any decrease in viability was calculated.

2.7 Flow cytometry

2.7.1 Fixing of cells

CML cells were seeded at the relevant density in 1ml of supplemented medium in 12-well plates and treated with the designated compound/s or corresponding vehicle. After the required treatment time, the medium was transferred to a 20ml tube and centrifuged at 650xg for 5 min. The supernatant was removed and the cells were resuspended in 100µl ice-cold PBS. Ice-cold 70% (v/v) ethanol (1ml) was then added to fix the samples which were then stored at 4°C until required.

2.7.2 Propidium iodide staining

Samples were centrifuged at 800xg for 10 min; the supernatant was then carefully discarded so as not to dislodge the pellet. The pellets were resuspended in 200µl FACSflow sheath fluidTM and transferred to FACS tubes. RNase A (12.5µl of 10mg/ml)

and propidium iodide (37.5µl of 1mg/ml) was added to each sample. The samples were wrapped in tin foil and incubated for 30 min at 37°C.

2.7.3 *FACS analysis*

Cell cycle analysis was performed at 488nm using a Becton Dickinson FACS Calibur flow cytometer. The Macintosh-based application CellQuest was then used to analyse the data of 10,000 gated cells once cell debris had been excluded. The data was stored as frequency histograms²⁰⁵.

2.8 *Analysis of drug combination interactions*

Interactions between various drug combinations were investigated using median dose analysis via the computer software Calcosyn. This software analysed the degree of synergism and/or antagonism between compounds generating dose-effect curves, median effect plots and combination index (CI) values. The dose-effect curves show the effect of increasing concentrations of each drug alone and in combination with each other. The median effect plots are a logarithmic version of the dose-effect curves. The CI values are based on the multiple drug-effect equations of Chou-Talalay derived from enzyme kinetic models. CI values of >1 , $=1$ and <1 indicate antagonism, an additive effect and synergism respectively²⁰⁶. The lower the CI value, the stronger the synergism between two compounds as indicated by Table 2.1.

Range of CI	Symbol	Description
<0.1	+++++	Very strong synergism
0.1-0.3	++++	Strong synergism
0.3-0.7	+++	Synergism
0.7-0.85	++	Moderate synergism
0.85-0.90	+	Slight synergism
0.90-1.10	±	Nearly additive
1.10-1.20	—	Slight antagonism
1.20-1.45	— —	Moderate antagonism
1.45-3.3	— — —	Antagonism
3.3-10	— — — —	Strong antagonism
>10	— — — — —	Very strong antagonism

Table 2.1 Recommended symbols for describing synergism or antagonism in drug combination studies analysed with the CI method. (Calculusyn manual, 2005).

2.9 Caspase and serine protease inhibitors

CML cells were seeded at the relevant density in 1ml of medium in 12-well plates. Cells were then pretreated with the required caspase and/or serine protease inhibitors. After 3 h, the cells were then treated with the relevant compound/s. Cells were then fixed, stained with propidium iodide and analysed by flow cytometry (Section 2.7) or Western blotting (Section 2.10).

2.10 Western blotting

2.10.1 Whole cell lysate preparation

Logarithmically growing CML cells were seeded at the relevant density and treated with the required compound/s. Following treatment, cells were centrifuged at 650xg for 5 min. The supernatant was removed and the pellet was resuspended in 1ml PBS. The samples were then transferred to eppendorf tubes and stored at -70°C.

When required, the cell pellets were resuspended in 2X Laemmli buffer (1X = 30mM Tris-base pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol in dH₂O) to lyse the cells (~50-100µl depending on the number of cells and size of pellet) and added to an equal volume of PBS. The samples were then sonicated for 10 s to degrade the DNA. An aliquot (8µl) of each sample was removed for subsequent protein concentration determination via the BCA assay (Section 2.10.3). After this, the samples were boiled for 3 min in a heating block at 100°C and DTT was added (final concentration 50mM). The lysates were stored at -70°C for up to 2 weeks.

2.10.2 Production of cytosolic fractions

Treated cells were harvested by centrifugation at 650xg for 5 min, washed once in ice-cold PBS and resuspended in 100µl of isotonic buffer (200mM mannitol, 70mM sucrose, 1mM EGTA, 10mM HEPES pH 6.9, 1mM DTT in dH₂O). Cells were incubated on ice for 10 min, drawn through a 21G needle ~30 times followed by incubation on ice for a further 15 min. Unbroken cells, nuclei and heavy membranes were pelleted at 1,000xg and discarded. The mitochondrial enriched fraction was produced by pelleting cells in minicentrifuge tubes at 12,000xg for 20 min at 4°C with the recovered supernatant representing the cytosolic fraction. The pellet was washed once with 150µl isotonic buffer and centrifuged at 12,000xg for 5 min, followed by a final resuspension in 40µl hypotonic buffer (10mM Tris pH 7.5, 5mM MgCl₂, 1mM EGTA, 1mM DTT and a 1X protease inhibitor solution containing aprotinin, leupeptin, pepstatin and PMSF in dH₂O).

2.10.3 BCA protein concentration assay

In order to determine the protein concentration of cell pellets made as described above, a bicinchoninic acid (BCA) protein concentration assay was performed. BCA is a stable, water-soluble compound that forms a purple complex with the cuprous ion (Cu⁺¹). Cu⁺¹ is reduced from Cu⁺² by protein in an alkaline medium (biuret reaction). The colourimetric reaction product of this assay increases proportionally over a broad range of increasing protein concentrations and is formed by the chelation of two molecules of BCA with one Cu⁺¹ ion²⁰⁷.

The required samples (8µl) were pipetted into minicentrifuge tubes. These were then diluted to a factor of 1:10 with dH₂O, 20µl of which was then added in triplicate to a

96-well plate. A BSA protein standard (2mg/ml) was serial diluted giving six BSA standards in the range of 2mg/ml to 0.0625mg/ml. Again 20 μ l of these standards were added to the 96-well plates in triplicate, dH₂O was used as a blank. The plates were incubated at room temperature for 30 min in the dark following the addition to each well of 200 μ l of BCA protein reagent B (4% CuSO₄) diluted 1:50 in BCA reagent A (Na₂CO₃, NaHCO₃, BCA, NaC₄H₄O₆ in 0.1M NaOH). The absorbance at 650nm of each unknown sample was then read using an automated microplate reader (Softmax, Molecular devices). A standard curve was then generated using Prism GraphPad software (such as the one illustrated in Fig. 2.1), from which the concentration of unknown samples could be determined. Each sample was then diluted to give a final protein concentration of 40 μ g/ μ l.

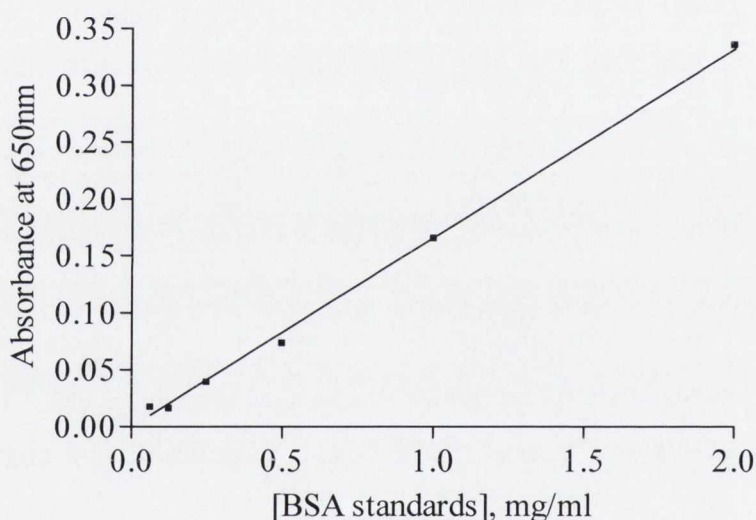


Fig. 2.1 An example of a BSA standard curve used to determine protein concentration.

2.10.4 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an 8, 12 or 15% resolving gel and a 5% stacking gel (Table 2.2). The volumes stated are those required to make up one gel. The resolving gel was poured between two glass plates; methanol (200 μ l) was added to the top of the gel to even it out. The gel was then left to polymerise for 30 min. The solvent was then washed out and any remaining traces of the solvent were removed using filter paper.

The stacking gel was poured on top of the resolving gel and a comb was added before the gel had polymerised. The apparatus was assembled and filled with 1X running buffer (5X = 125mM Tris, 1M Glycine, 0.5% (v/v) SDS in dH₂O). A protein marker and the required samples (10µl) were loaded onto the gel and run at 80 volts through the stacking gel (20 min) and 120 volts until the samples reached the end of the gel.

	8% resolving gel	12% resolving gel	15% resolving gel	5% stacking gel
dH₂O	4.6ml	3.3ml	2.3ml	3.4ml
Acrylamide mix	2.7ml	4.0ml	5.0ml	830µl
1.5M Tris pH 8.8	2.5ml	2.5ml	2.5ml	-
1M Tris pH 6.8	-	-	-	630µl
10% (w/v) SDS	100µl	100µl	100µl	50µl
10% (w/v) APS	100µl	100µl	100µl	50µl
TEMED	6µl	4µl	4µl	5µl

Table 2.2 Constituents required for 10ml of an 8, 12 and 15% resolving gel and 5ml of a 5% stacking gel.

2.10.5 Western blotting

The proteins on the gel were then transferred to a 0.2µM polyvinylidene difluoride (PVDF) membrane pre-soaked in methanol and transfer buffer. The PVDF membrane was placed on top of the gel in a 'transfer sandwich' which consisted of four layers of filter paper and two sponge layers and placed in a transfer cassette. The cassette was then placed in the transfer apparatus along with a cooling block and transfer buffer (1X = 25mM Tris, 200mM glycine, 10% (v/v) methanol in dH₂O). The gel was transferred using a wet transfer apparatus system (BioRad mini trans blot) at a constant current of 150mAmps. The PVDF membrane was then removed and immersed for 1 min in Ponceau S and rinsed in TBS-Tween 0.5% (v/v) Tween 20; (10X TBS = 100mM Tris, 1.5M NaCl in dH₂O, pH 7.6). Non-specific sites on the PVDF membrane were blocked by a 1 h incubation with gentle agitation in 5% (w/v) marvel milk made up in TBS-Tween. After blocking, the membrane was transferred to a 50ml tube containing the necessary primary antibody and placed on a roller at 4°C overnight.

The following morning the membrane was rinsed in TBS-Tween 3 times for 10 min each. The membrane was then placed in a 50ml tube containing the required secondary antibody and placed on a roller for 1 h. The secondary antibody was bound to horseradish peroxidase (HRP) which then binds to the primary antibody, after which the membrane was again washed 3 times for 10 min each. The membrane was then placed in enhanced chemiluminescence (ECL) reagents for 5 min and transferred to a developing cassette. The light emission was detected by exposing the membrane to Kodak X-Omat LS film and developed using an automated developer (Fuji x-ray film processor). Individual experiments required slight modifications to this general protocol depending on the protein being analysed. Further details are provided in Table 2.3.

2.10.6 Stripping and re-probing of blots

PVDF membranes were placed in a 50ml tube containing 5ml of stripping buffer for at least 30 min at 37°C. The membranes were then washed with TBS-Tween for 30 min (3 x 10 min each). Finally, the membranes were incubated with the desired primary and secondary antibodies (Section 2.10.5).

2.10.7 Western blot analysis for phosphotyrosine residues and the Bcr-Abl protein

Protein phosphorylation was examined by SDS-PAGE and Western blotting using the same protocols as outlined above with a few minor modifications (Section 2.10.4 & 2.10.5). Proteins were transferred for 1 h 30 min to a PVDF membrane, which was then blocked with 5% (w/v) BSA for 1 h at room temperature. The anti-phosphotyrosine mAb was made up in 5% BSA as was the HRP conjugated secondary antibody. The Bcr-Abl protein was also separated by SDS-PAGE but was transferred overnight at 4°C and incubated for 1 h at room temperature with the primary antibody.

Protein	Lysate	Mr (kDa)	Gel	Transfer	1°Ab	2°Ab	Host	Detergent
Actin	Whole cell	42	12%	30 min	1:6000	1:6000	Mouse	5% Marvel
Bcr-Abl	Whole cell	210	8%	Overnight	1:1000	1:2000	Mouse	5% Marvel
Bcl-2	Whole cell	24	12%	30 min	1:500	1:2000	Mouse	5% Marvel
Bcl-xL	Whole cell	34	12%	30 min	1:500	1:2000	Mouse	5% Marvel
Bim	Whole cell	24, 15, 12	12%	30 min	1:500	1:1000	Rabbit	5% Marvel
Mcl-1	Whole cell	42	12%	30 min	1:2000	1:2000	Mouse	5% Marvel
PARP	Whole cell	115 + 85	12%	1 h	1:1000	1:2000	Mouse	5% Marvel
Phosphotyrosine	Whole cell	All bands	12%	1 h 30 min	1:500	1:2000	Mouse	5% BCA
Caspase-8	Whole cell	57, 28	12%	30 min	1:1000	1:2000	Mouse	5% Marvel
Caspase-9	Whole cell	46, 35	12%	30 min	1:1000	1:2000	Mouse	5% Marvel
Cytochrome c	Cytosolic	15	15%	30 min	1:1000	1:2000	Mouse	5% Marvel
Cyclin B1	Whole cell	60	12%	1 h	1:1000	1:2000	Mouse	5% Marvel
CDK1	Whole cell	34	12%	1 h	1:1000	1:2000	Mouse	5% Marvel
Survivin	Whole cell	17	12%	30 min	1:1000	1:2000	Rabbit	2.5% Marvel

Table 2.3. Western blotting details for specific proteins.

2.10.8 Immunoprecipitation

K562 cells were seeded at the relevant density and treated as required. Following the treatment regimens, cells were centrifuged for 650xg and resuspended in 500µl of lysis buffer (50mM HEPES pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 1mM DTT, 1mM NaF, 10mM β-glycerophosphate and a 1X protease inhibitor solution containing aprotinin, leupeptin, pepstatin and PMSF). The cell suspension was vortexed vigorously for 10 s and incubated on ice for 30 min. The samples were then sonicated and centrifuged at 12,000xg for 20 min. A BCA assay was performed on the resulting supernatants (Section 2.10.3)

An aliquot of 1µg of CDK1 antibody was used per sample. The volume of the incubation buffer was then made up to 500µl with lysis buffer. The samples were then incubated on a roller at 4°C for 3 h. A 50% slurry of protein A sepharose beads in PBS (50µl) was then added to the samples and incubated overnight on a roller at 4°C. The samples were then centrifuged for 2 min at 500xg to pellet the beads. The supernatant was carefully removed using gel loading tips making sure not to disturb the beads. The beads were then washed 2 times in 1ml of ice-cold lysis buffer and subjected to a CDK kinase activity assay.

2.10.9 CDK kinase activity assay

Following the washing of immunoprecipitated beads, the beads were washed once with ice-cold kinase buffer (50mM HEPES pH 7.5, 10mM MgCl₂, 2.5mM EGTA, 1mM DTT, 1mM NaF, 10mM β-glycerophosphate and 0.1mM Na₃VO₄). The lysates were then resuspended in 30µl kinase buffer supplemented with unlabeled ATP, 2µCi [γ ³²P]-ATP and 10µg Histone H1 (CDK1 substrate). Samples were incubated at 30°C for 30 min, following which samples were boiled at 100°C for 3 min and added to 3X Laemmli buffer (20µl). Samples were then run on a 12% gel (Section 2.10.4), exposed to Kodak X-Omat film at -70°C overnight and analysed for Histone H1 by autoradiography.

2.10.10 Densitometry

Band intensities were normalised by densitometry (ImageJ software, NIH website) with respect to actin and expressed as fold change over control. Representative blots of three independent experiments were analysed.

2.11 Measurement of mitochondrial membrane potential

LAMA84 cells were treated for up to 24 h and subsequently incubated with 3,3'-dihexyloxacarbocyanine iodide (DiOC₆, final concentration 150nM) for 20 min at 37°C in the dark. Cells were then centrifuged for 5 min, washed and resuspended in PBS (200µl) and analysed immediately by flow cytometry using the FL-1 channel (Section 2.7.3).

2.12 Immunofluorescence

Following treatment, cells (1ml) were cytocentrifuged for 2 min at 200xg onto a glass slide and air dried. Cells were then fixed in 100% ice-cold methanol, washed in PBS-T (0.1% (v/v) Triton X-100 in PBS) and blocked for 30 min in 5% (w/v) BSA in PBS-T. Cells were then incubated with a mouse anti-tubulin antibody (diluted 1:20 in blocking buffer) for 1 h, washed 3 times with PBS-T and incubated for a further 1 h in the secondary antibody Alexa 488 (diluted 1:2000 in blocking buffer). Cells were again washed 3 times in PBS-T, after which the DNA was stained with 0.2µg/ml propidium iodide in blocking buffer for 2 min. Slides were mounted with phenylene diamine (2µl/ml) in 50:50 PBS/glycerol. Projected images from a z-series of images were captured using the OLYMPUS 1X81 microscope coupled with Olympus Fluoview Ver 1.5 software. All images were collected on the same day using identical parameters.

2.13 Caspase-3/7 activity assay

The Apo-ONE caspase-3/7 activity assay includes a profluorescent caspase-3/7 consensus substrate, (Z-DEVD-Rhodamine-110) and an optimised bifunctional cell lysis/activity buffer. The buffer efficiently lyses cultured mammalian cells and supports optimal caspase-3/7 enzymatic activity. Upon cleavage on the C-terminal side of the

aspartate residue in the DEVD peptide substrate sequence by activated caspase-3/7 enzymes, the rhodamine 110 becomes fluorescent when excited at a wavelength of 499nm. Thus the amount of fluorescence released is indicative of the amount of active caspase-3/7 present in the sample.

Cells were seeded at the relevant density in a black 96-well plate (interfering fluorescence reducing plate) (100µl/well), pretreated with a general caspase inhibitor for 3 h followed by the required treatment regime. After the required incubation time, an Apo-ONE kit containing the substrate (10µl) and buffer (990µl), was thawed and thoroughly mixed according to the manufacturer's instructions. This reagent was then added to each well in a 1:1 ratio with cells. The contents were gently mixed on an orbital plate shaker for 1 - 2 h in the dark at room temperature. The fluorescence was then read on a plate reader (SpectraMax Gemini, Molecular Devices) with excitement and emission wavelengths of 499nm and 521nm respectively. A well containing a 1:1 ratio of reagent and medium (without any cells) was used as a blank. Readings were corrected for cell viability (Section 2.6). Vehicle samples were set as a base-line factor for 100% caspase activity from which any increases/decreases were calculated.

2.14 *siRNA of survivin*

K562 cells in the log phase of growth were transfected with 100ng of a validated siRNA duplex to target survivin or a fluorescently labeled control scrambled (sc) RNA which does not bind to any known sequence. Cells were transfected for 24 h with a nucleofector solution (100µl) and device from Lonza (Programme T-016) according to the manufacturer's instructions. Cells were then examined by confocal microscopy to ensure efficient transfection or Western blotting (Section 2.10) to ensure satisfactory protein knock-down. Transfected cells were then treated as required and analysed for apoptosis by flow cytometry (Section 2.7).

2.15 *Ex vivo* CML patient samples

2.15.1 Patients

Peripheral blood (10ml) was collected with informed consent from newly diagnosed treatment naïve (n = 4) or blast crisis STI571-resistant CML patients (n = 2) in EDTA-anticoagulant tubes. Ethical approval for all work carried out on *ex vivo* CML patient samples was obtained from the St. James's Hospital and Adelaide and Meath incorporating the National Children's Hospital Ethics Committee. The diagnosis of CML was clinically established on the basis of morphological examination and the presence of the Ph chromosome, the mutational status of Bcr-Abl was detected by RT-PCR.

2.15.2 Isolation of white blood cells/PBMCs

Fresh peripheral blood was diluted 1:3 with RPMI medium. This was then carefully added drop by drop to half the equivalent volume of lymphoprep, ensuring both layers did not mix. Samples were then centrifuged at 750xg for 30 min to form a Ficoll gradient (Fig. 2.2), following which, the top layer of platelets was removed. The white buffy layer at the interface of the platelet and lymphoprep layers containing the WBCs or peripheral blood mononuclear cells (PBMCs) was carefully removed to a sterile 50ml tube. The WBCs were diluted to a volume of 50ml with RPMI medium and centrifuged again for 10 min at 650xg. The supernatant was then removed and the white pellet resuspended in 1ml RPMI medium supplemented with 10% (v/v) FBS, 50µg/ml penicillin/streptomycin and 2mM L-glutamine.

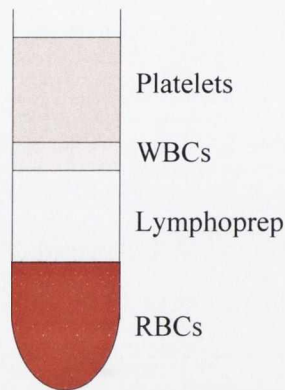


Fig. 2.2 Separation of WBCs from fresh peripheral blood using a Ficoll gradient.

Peripheral blood (10ml) was diluted 1:3 with non-supplemented medium, gently layered over lymphoprep and centrifuged at 750 $\times$ g for 30 min. Separated WBCs were then removed with a pasteur pipette.

2.15.3 *Annexin V staining*

WBCs were seeded at a density of 1×10^6 cells/1ml medium in a 24-well plate and treated with the required agents. After a 72 h incubation the cells were transferred to FACS tubes and centrifuged at 650 $\times$ g for 5min. The supernatant was discarded and the pellet was resuspended in 100 μ l anti-CD45 antibody (diluted 1:50 in medium). Samples were then left in the dark at room temperature for 10 min. Samples were centrifuged at 650 $\times$ g for 5 min and washed in 1ml annexin V binding buffer (diluted 1:10 in PBS) and recentrifuged at 650 $\times$ g for 5 min. Following this, samples were resuspended in 100 μ l annexin V (diluted 1:50 in binding buffer) and left to incubate on ice for 15 min in the dark. Binding buffer (1ml) was then added followed by a recentrifugation. Pellets were finally resuspended in 500 μ l annexin V binding buffer and analysed by flow cytometry. Leukaemic cells were identified and gated based on their low to medium side scatter and low CD45 expression and then analysed for annexin V staining²⁰⁸.

2.16 *In vivo* tumour model of CML

2.16.1 *Mice*

Specific pathogen free six to eight week old adult female BALB/c-nude (nu/nu) mice were purchased from Harlan laboratories (U.K.). Mice were housed in sterile individually ventilated cages, food and water were provided *ad libitum*, in accordance with the standard operating procedures set down in SI 17/94 of the European Union. Mice were acclimatised for 1 week prior to the initiation of any *in vivo* experiments. Animals were euthanised by CO₂ if found to be in distress (hunching, failure to groom etc.) or if tumour volume exceeded 10% of mouse bodyweight. Ethical approval was obtained by the BioResources Committee, Trinity College, Dublin.

2.16.2 *Tumour inoculation*

The *in vivo* anti-tumour efficacy of PBOX-6 was examined using a Baf/3 xenograft containing the T315I mutant version of Bcr-Abl in a BALB/c mouse model. Female BALB/c-nude mice were inoculated subcutaneously (s.c.) into the right flank with 3×10^6 Baf/3 cells in the log phase of growth. After 8 days, tumours had reached approximately 4mm in diameter.

2.16.3 *Administration of PBOX compounds in vivo*

Mice were randomly divided into two groups of six mice per cage following tumour induction. A stock solution of PBOX-6 was prepared at a concentration of 15mg/ml in 1:1 ethanol:cremophor EL. When required, this was then diluted 1:5 in sterile PBS to give a final concentration of 7.5mg/kg or 0.15mg/mouse. The treatment group (n = 6) received an i.t. injection of PBOX-6 (50µl) once daily for the duration of the experiment starting on day 8. Control mice (n = 6) received a single daily i.t. injection of 50µl of the equivalent vehicle also starting on day 8. Tumour growth was measured every second day with a sterile vernier callipers. The long (L) and short (S) axes were recorded and tumour volume (V) was calculated using the following equation as previously described by Beck *et al.*, (2003)²⁰⁹.

$$V = \frac{S^2 \times L}{2}$$

Mice were sacrificed by CO₂ asphyxiation at the experimental end-point or if found to be under any excess duress.

2.17 Statistical analysis

The software Prism GraphPad was used to carry out statistical analysis when comparing two samples using the Student's paired (or unpaired for *in vivo* studies) t-test or a one-way ANOVA where more than two groups were being analysed. The p values stated indicate the probability of obtaining a more extreme value than the one observed if the values obtained were due to random chance variation, therefore the lower the p value the more significant the result. For illustrative purposes the p values are presented as *, p < 0.05 and **, p < 0.01 respectively.

Chapter 3.

*Evaluation of the apoptotic enhancement of STI571
by PBOX-21 in human chronic myeloid leukaemia cell lines.*

3.1 Introduction

CML is a haematological stem cell disorder characterised by the presence of a Ph chromosome. This unique and abnormal chromosome is caused by the reciprocal t9:22 translocation between two genes, Bcr and Abl coding for a constitutively active tyrosine kinase Bcr-Abl^{6; 35}. The Bcr-Abl protein has been identified as having a central role in CML pathology and is responsible for the continuous downstream phosphorylation of many signalling pathways routinely activated in normal cells or involved in cell adhesion¹⁴. Exactly which of these pathways are responsible for the effects of Bcr-Abl is poorly understood however the Ras, JAK/STAT, MAPK and PI3K pathways are all thought to play key roles in rendering leukaemic blast cells resistant to apoptosis.

In 2001 the release of a novel tyrosine kinase inhibitor, known as STI571, quickly became the frontline treatment for CML²⁶. STI571 binds to the ATP binding site of Bcr-Abl, inhibiting downstream phosphorylation. Despite the high rates of haematological and cytogenetic responses³² to STI571 monotherapy, the emergence of resistance to STI571^{43; 210} has led to the evaluation of combination therapies. Many studies have been conducted to date demonstrating the therapeutic efficacy of STI571 in combination with a range of anti-cancer agents including DNA-damaging agents⁵⁰, farnesyltransferase inhibitors like SCH66336¹³, the CDK inhibitor flavopiridol¹²⁷, histone deacetylase inhibitors such as SAHA^{64; 211} and MAPK inhibitors PD98059 and U0126^{60; 61} amongst others.

Apoptosis is a cell suicide mechanism invoked in disparate situations to remove redundant, damaged or infected cells^{137; 138}. One of the best characterised mediators of apoptosis is a family of cysteine proteases known as caspases¹⁴⁰. These proteases are involved in two distinct apoptotic pathways, the extrinsic and intrinsic pathways. The extrinsic pathway is triggered by death receptors binding to their cognate ligands, whilst the intrinsic pathway is initiated in response to situations such as hypoxia or DNA-damage. This pathway is often called the mitochondrial pathway as it involves release of cytochrome c from the mitochondria and apoptosome formation, a complex consisting of cytochrome c, apaf-1 and procaspase-9. Both pathways converge at the level of the effector caspases that leads to the typical biochemical and morphological changes of the apoptotic cell. However, caspase inhibition does not always prevent cell death and their targeted disruption does not always impair apoptosis and therefore roles for non-caspase proteases such as serine proteases have recently attracted more interest¹⁶⁶.

Recently we have reported how a novel series of PBOX compounds have potential as anti-cancer agents. These compounds can be divided into two groups with distinct mechanisms of action; those that exert anti-proliferative effects by arresting cancerous cells in the G1 phase of the cell cycle without eliciting cytotoxic effects e.g. PBOX-21¹⁹³ and those that exhibit pro-apoptotic ability by binding to tubulin and acting as MTAs e.g. PBOX-6. This second group of MTAs have been shown to cause cell cycle arrest in the G2/M phase and potentially induce apoptosis in a wide range of solid tumours and cell lines derived from the haematopoietic system and also *in vivo*^{190; 192; 194; 201}. More recently we reported that STI571 potentiated the apoptotic efficacy of a representative pro-apoptotic PBOX compound, PBOX-6, and other MTAs such as paclitaxel and nocodazole, by inhibiting endoreplication¹⁹⁰.

In the present study, we sought to determine the effects of STI571 which has previously been shown to cause a G1-arrest, with a PBOX compound that acts in the same stage of the cell cycle. Hence, we examined a representative member (PBOX-21) of the first group of PBOX compounds, the non-cytotoxic G1-arresting compounds, and its ability to lower the apoptotic threshold of STI571. Herein we demonstrate that PBOX-21 synergistically enhanced the apoptotic ability of STI571 in CML cells including those resistant to STI571 through activation of both caspases and serine proteases. Consequently, a series of four novel PBOX and STI571 hybrid compounds were synthesised by a collaborating partner. These compounds were evaluated *in vitro* for their ability to induce apoptosis in CML cell lines. Collectively these findings highlight the potential therapeutic efficacy in combining STI571 with PBOX-21 as a novel anti-leukaemic strategy.

3.2 Results

3.2.1 *The effect of STI571 on CML cell lines.*

STI571 is a commercially available tyrosine kinase inhibitor that has quickly become the frontline treatment for all phases of CML. In order to confirm its effects on the CML cells, LAMA84 and K562 cells lines were treated with a range of concentrations of STI571 (50nM to 5 μ M), fixed in ethanol and analysed by flow cytometry (Section 2.7). Results showed a dose-dependent increase in the number of apoptotic cells with increasing concentrations of STI571. This reached a plateau of $88.0 \pm 1.0\%$ apoptosis when LAMA84 cells were treated with 1 μ M STI571 for 48 h (Fig. 3.1). The IC₅₀ value for LAMA84 cells was determined to be 355nM.

The STI571-resistant K562 (K562R) cell line has recently been determined to over-express the Bcr-Abl protein when compared to parental cells (K562S)¹⁹⁰. K562R cells were determined to be ~7-fold more resistant to STI571 than K562S cells (IC₅₀ values 2.9 μ M and 420nM respectively) although both cell lines did undergo a maximum of 71.4 ± 2.7 and $63.0 \pm 7.1\%$ apoptosis when treated with 5 μ M STI571 (Fig. 3.1).

A concentration of 250nM STI571 was chosen for all subsequent experiments as it is a pharmacologically achievable plasma concentration that is routinely used in research and it is the concentration that has been used in previous STI571 drug combination studies^{64; 190}. In addition, in the present study this concentration of STI571 only induced a small amount of apoptosis.

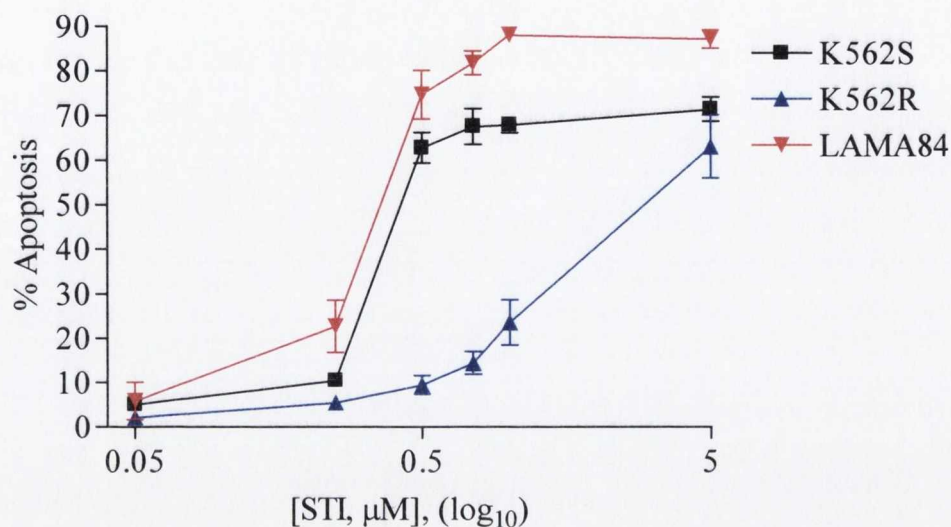


Fig. 3.1 The effect of STI571 on CML cell lines.

CML cells were seeded during the log phase of growth and treated for 48 h (K562S and K562R) or 72 h (LAMA84) with a vehicle (Veh) (0.05% (v/v) DMSO) or a range of STI571 (STI) concentrations. After the required incubation period, cells were fixed in 1ml ethanol and 100 μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments.

3.2.2 PBOX-21 does not augment the G1-arresting effects of STI571.

It has previously been reported that PBOX-21 exerts anti-proliferative effects on 1231N1 human astrocytoma cells by arresting them in the G1 phase of the cell cycle^{193; 212}, therefore it was of interest to see if PBOX-21 could evoke similar effects on the LAMA84 cell line. A concentration of 25µM was chosen for this experiment as this was the concentration used in previous studies with this drug¹⁹³. After 8 h of treatment there were no observable changes in this population of cells regardless of the treatment they received (Fig. 3.2A).

However, results showed that PBOX-21, STI571 and PBOX-21 in combination with STI571 significantly increased the percentage of cells in the G1 phase of the cell cycle after 16 h treatment when compared to control samples (Fig. 3.2B). Although there was a significant increase in the number of cells in the G1 phase compared to control samples following both 16 and 24 h with the combination treatment, there was no significant increase when compared to STI571 treatment alone (Fig. 3.2C).

Cells were subsequently treated with a range of PBOX-21 concentrations (10µM to 40µM) or the corresponding control for up to 72 h. Solubility limited the highest concentration of PBOX-21 tested to 40µM. Samples were then prepared for flow cytometric analysis (Section 2.7). Quantification of the pre-G1 peak which represents the apoptotic cell population was performed. PBOX-21 did not induce apoptosis in these cells. Following treatment with the highest concentration of PBOX-21, 40µM for 72h, 7.7 ± 0.5 and $3.9 \pm 1.0\%$ of K562S and K562R cells had undergone apoptosis, compared to 3.3 ± 0.4 and $3.2 \pm 0.7\%$ apoptosis for their respective controls. Similar results were also obtained for LAMA84 cells (Fig. 3.3).

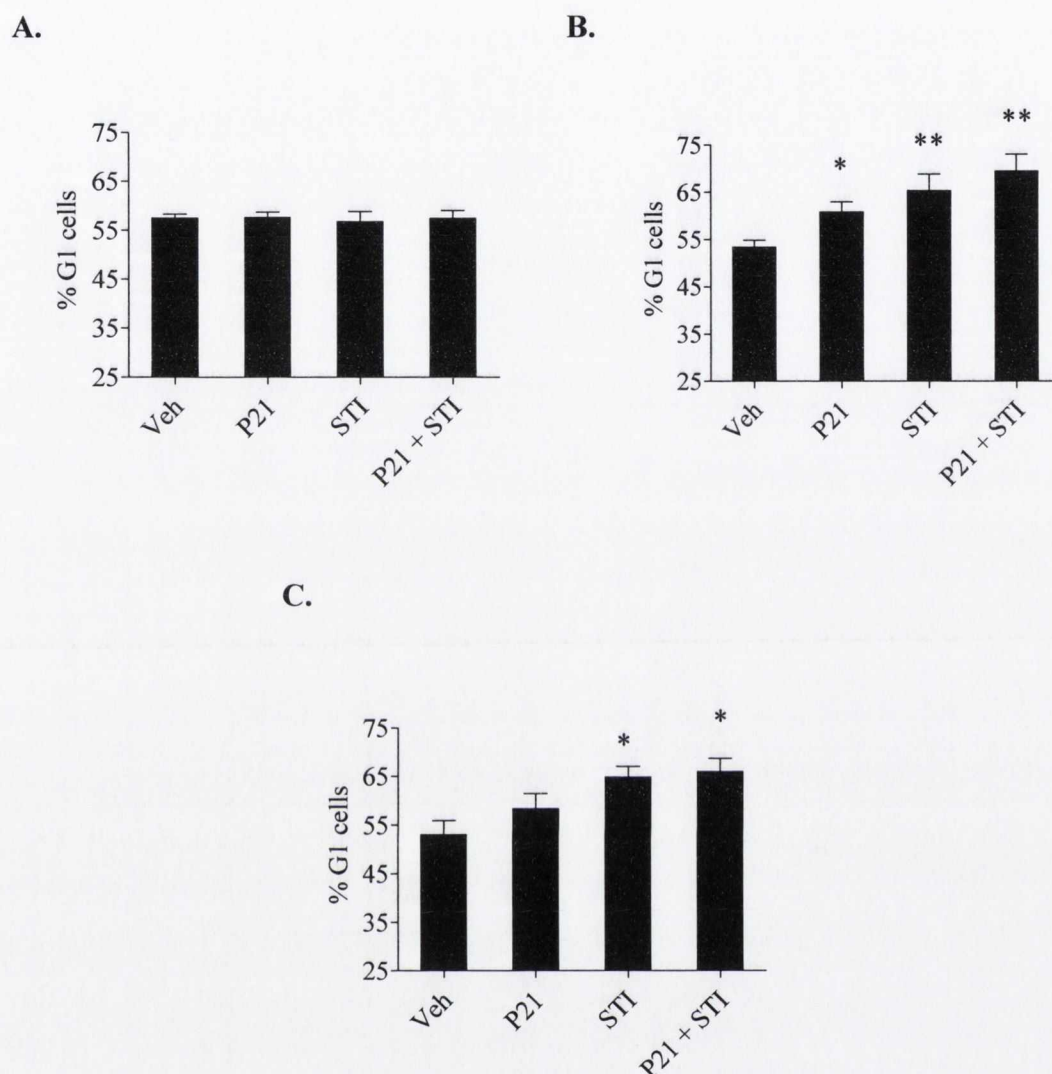


Fig. 3.2 The effect of PBOX-21, alone and in combination with STI571, on the G1 population of CML cells.

LAMA84 cells were seeded during the log phase of growth and treated for up to 24 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 25 μ M PBOX-21 (P21), 250nM STI571 (STI) or a combination of both. After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry for the percentage of cells in the G1 phase of the cell cycle. Cells were treated with PBOX-21 and/or STI for **A.** 8 h, **B.** 16 h and **C.** 24 h. Values represent the mean $\pm$ S.E.M. of three independent experiments. Statistical analysis was carried out using a one-way ANOVA, *, $p < 0.05$; **, $p < 0.01$.

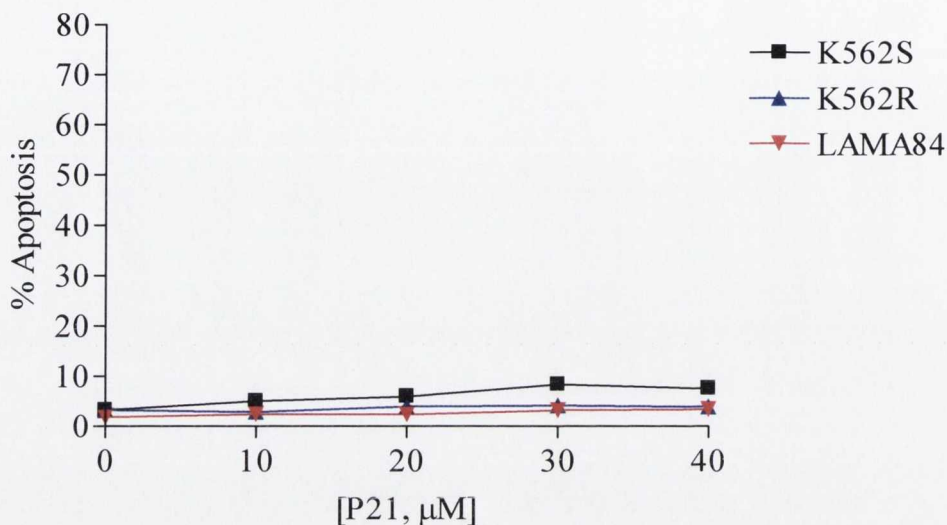


Fig. 3.3 PBOX-21 alone does not induce apoptosis in CML cells.

CML cells were seeded during the log phase of growth and treated for up to 72 h with a vehicle (Veh) (0.4% (v/v) ethanol) or a range of PBOX-21 (P21) concentrations (10μM to 40μM). After the required incubation period, cells were resuspended in 1ml ethanol and 100μl PBS and stained with propidium iodide. Samples were then analysed by flow cytometry, the percentage of apoptosis was measured by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments. Absence of error bars indicates the error was smaller than the size of the symbol.

3.2.3 PBOX-21 augments the apoptotic effect of STI571 in CML cells.

Various researchers have previously demonstrated an enhancement of the apoptotic ability of STI571 when combined with other chemotherapeutic drugs and potentially therapeutic agents^{57, 60}. It has also been shown that when a representative member of the pro-apoptotic PBOX compounds, PBOX-6, was added to CML cells in combination with STI571, there was a significant increase in the percentage of cells undergoing apoptosis when compared to the each compound alone¹⁹⁰. This prompted us to examine if PBOX-21 could also elicit a similar effect.

Results showed that 25 μ M PBOX-21 in combination with 250nM STI571 did increase the percentage of cells undergoing apoptosis when compared to either drug alone as determined by flow cytometry, a representative histogram plot of LAMA84 cells is shown for illustrative purposes (Fig. 3.4A). In more detail, for LAMA84 cells the average percentage of cells in the pre-G1 phase increased from $24.3 \pm 5.3\%$ when treated for 48 h with STI571 alone to $60.6 \pm 3.2\%$ (p value: 0.0083,**) when treated with PBOX-21 in combination with STI571. Similarly, $39.8 \pm 5.1\%$ (p value: 0.0142,*) of K562S and $36.3 \pm 3.3\%$ (p value: 0.0028,**) of K562R cells respectively had undergone apoptosis following combination treatment, this compared to $10.5 \pm 1.6\%$ (K562S) and $5.5 \pm 1.4\%$ (K562R) apoptosis for STI571 alone after 72 h (Fig. 3.4B). This increase in apoptosis corresponded to a decrease in the percentage of cells in the G1, S and G2/M phases of the cell cycle (Table 3.1).

Further experiments in the LAMA84 cell line showed this novel co-treatment to induce a significant time and dose-dependent enhancement of apoptosis. Following treatment with PBOX-21 and STI571 for 8 h $8.7 \pm 2.5\%$ of cells had undergone apoptosis, this rose to $16.5 \pm 6.4\%$ after 24 h and to $60.6 \pm 3.2\%$ after 48 h treatment (Fig. 3.5A).

At the highest concentration of PBOX-21 tested, 40 μ M, the increase in apoptosis when combined with STI571 appeared to have already plateaued with a maximum of $66.5 \pm 4.8\%$ of cells undergoing apoptosis (Fig. 3.5B). Concentrations of less than 10 μ M PBOX-21 in combination with STI571 did not significantly increase the number of cells undergoing apoptosis.

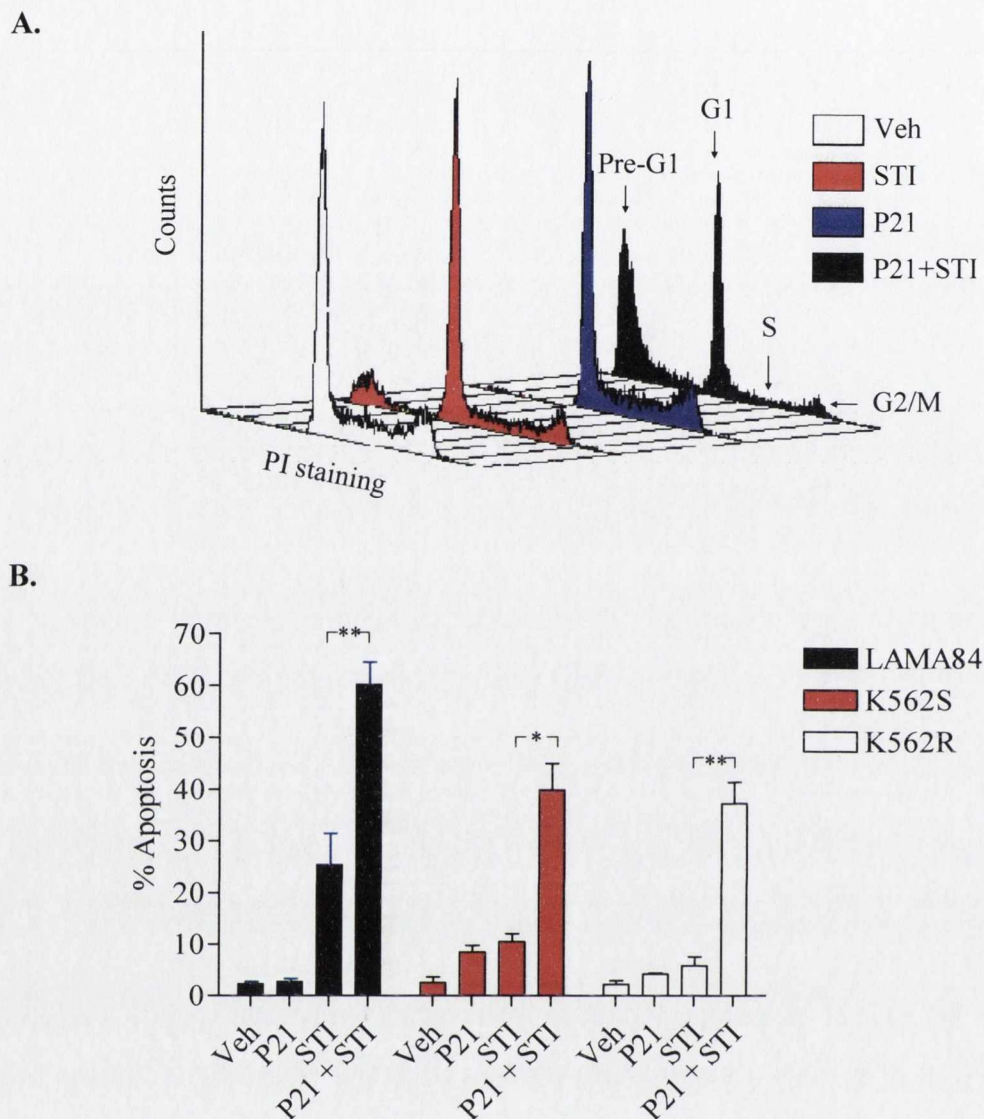


Fig. 3.4 PBOX-21 enhances the apoptotic effect of STI571 in LAMA84 and parental and STI571-resistant K562 cells.

CML cells were seeded during the log phase of growth and treated for up to 72 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 25 μ M PBOX-21 (P21), 250nM STI571 (STI) or a combination of PBOX-21 and STI571. After the required incubation period, cells were resuspended in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Samples were then analysed by flow cytometry, the percentage of apoptosis was measured by quantification of the pre-G1 peak. **A.** A representative histogram plot of LAMA84 cells. **B.** Apoptotic levels in all three cell lines where values represent the mean $\pm$ S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired t-test, *, $p < 0.05$; **, $p < 0.01$.

		Pre-G1	G1	S	G2/M
LAMA84 48 h	Veh	2.5 ± 0.4	57.9 ± 1.5	21.6 ± 0.6	17.6 ± 1.2
	STI	24.3 ± 5.3	51.9 ± 2.7	12.6 ± 2.3	10.5 ± 1.9
	P21	2.5 ± 0.6	54.2 ± 1.5	22.3 ± 1.3	20.0 ± 0.8
	P21 + STI	60.6 ± 3.2	28.3 ± 2.2	7.2 ± 0.9	3.0 ± 0.3
K562S 72 h	Veh	2.9 ± 0.6	52.0 ± 3.2	26.8 ± 1.8	16.6 ± 5.0
	STI	10.5 ± 1.6	43.2 ± 0.8	28.6 ± 1.5	15.7 ± 1.1
	P21	8.4 ± 1.4	58.2 ± 6.9	20.5 ± 1.3	11.5 ± 4.3
	P21 + STI	39.8 ± 5.1	34.4 ± 3.3	16.4 ± 2.0	7.6 ± 1.6
K562R 72 h	Veh	2.8 ± 0.6	49.3 ± 1.2	24.6 ± 1.2	21.0 ± 1.2
	STI	5.5 ± 1.4	51.9 ± 1.6	21.0 ± 0.8	19.5 ± 1.8
	P21	4.2 ± 0.2	49.6 ± 1.8	25.5 ± 0.5	18.4 ± 1.6
	P21 + STI	36.3 ± 3.3	35.6 ± 2.3	17.3 ± 2.2	9.7 ± 0.6

Table 3.1 PBOX-21 enhances the apoptotic effect of STI571 in LAMA84 and parental and STI571-resistant K562 cells.

CML cells were seeded during the log phase of growth and treated for up to 72 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 25µM PBOX-21 (P21), 250nM STI571 (STI) or a combination of PBOX-21 and STI571. After the required incubation period, cells were resuspended in 1ml ethanol and 100µl PBS and stained with propidium iodide. Samples were then analysed by flow cytometry for cell cycle distribution, the percentage of apoptosis was measured by quantification of the pre-G1 peak. Values represent the mean ± S.E.M. of three independent experiments.

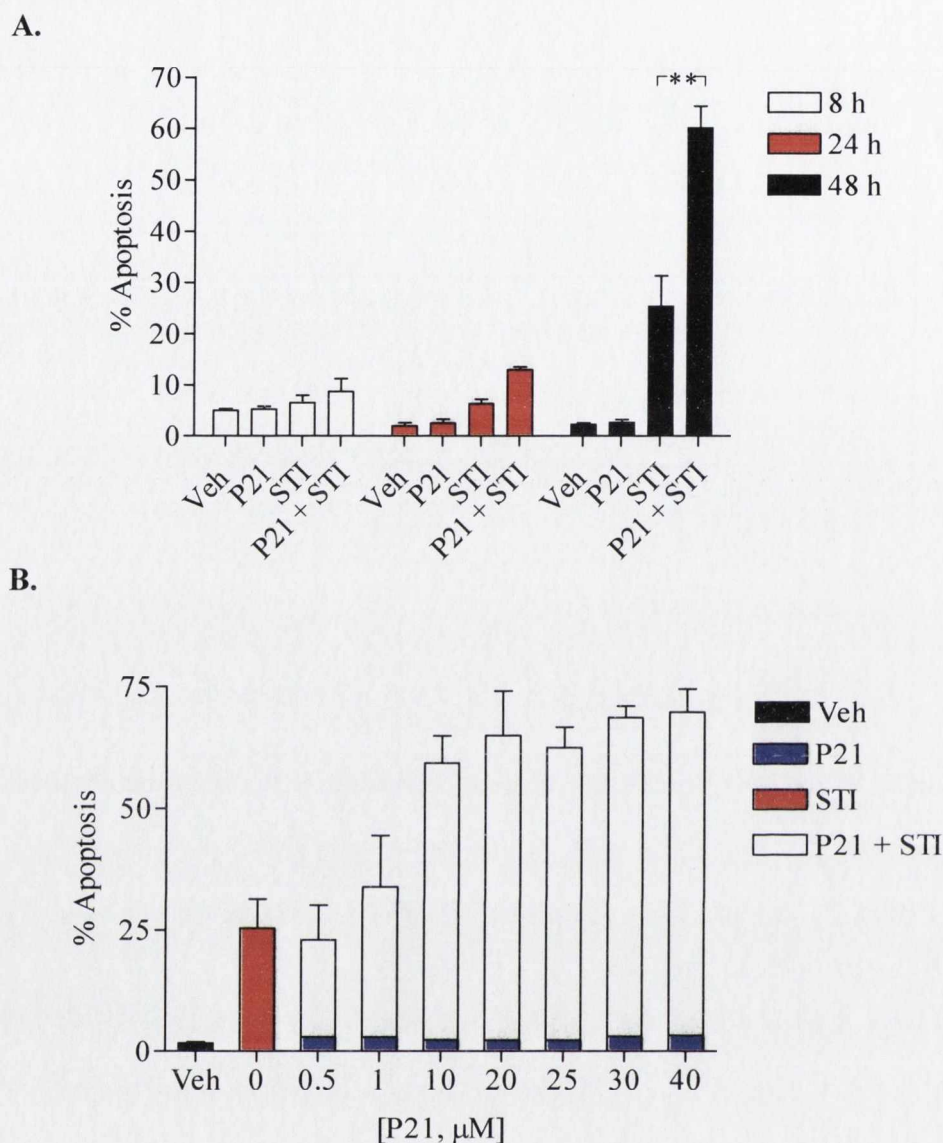


Fig. 3.5 PBOX-21 enhances the apoptotic effect of STI571 in the LAMA84 cell line in a time and dose-dependent manner.

LAMA84 cells were seeded during the log phase of growth and treated for up to 48 h with a vehicle (Veh) (0.4% (v/v) ethanol, 0.0025% (v/v) DMSO), PBOX-21 (P21), 250nM STI571 (STI) or a combination of both. After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. **A.** Cells were treated with 25 μ M PBOX-21 and/or STI571 for either 8, 24 or 48 h **B.** Cells were treated with the indicated concentrations of PBOX-21 in combination with STI571 for 48 h. Values represent the mean $\pm$ S.E.M. of at least three independent experiments. Statistical analysis was performed using the Student's paired t-test, **, $p < 0.01$.

3.2.4 PBOX-21 synergistically enhances the apoptotic effect of STI571 in parental and STI-resistant CML cell lines.

In order to determine how substantial an effect PBOX-21 had on the apoptotic potential of STI571 in CML cells, cells were treated with STI571 in combination with a range of PBOX-21 concentrations (10 μ M to 40 μ M) and analysed by flow cytometry. Data from these experiments was then evaluated by the computer software Calcsyn. This software utilises the median-effect equation of Chou and the CI equation of Chou-Talalay to quantify the amount of synergism between two or more mutually exclusive drugs²⁰⁶. The dose-effect curve shows the effect of increasing concentrations of PBOX-21 and STI571 alone and in combination with each other. The median effect plot is a logarithmic version of the dose-effect curve. The CI versus fractional affect graph represents the CI values obtained for each data plotted against the fraction of cells undergoing apoptosis. These results were determined for all three CML cell lines, LAMA84 (Fig. 3.6), K562S (Fig. 3.7) and K562R (Fig. 3.8).

The CI values for all three cell lines tested were all less than 1 indicating synergism (Table 3.2). The CI values for LAMA84 cells ranged from 0.669 to 0.523 while the CI values ranged from 0.982 to 0.398 for the K562S cell line. All data points for these two parental cell lines were evaluated as being synergistic (+++) (as defined by Table 2.1) with the exception of K562S cells treated with 10 μ M PBOX-21 and 250nM STI571 which fell into a lower category of nearly additive ($\pm$). The STI571-resistant cell line K562R produced CI values of between 0.398 and 0.101, most of the data points for this cell line fell into a strongly synergistic bracket (++++).

This shows there was a synergistic increase in the number of cells undergoing apoptosis when PBOX-21 was combined with STI571 when compared to the addition of STI571 alone in both parental and STI571-resistant CML cell lines.

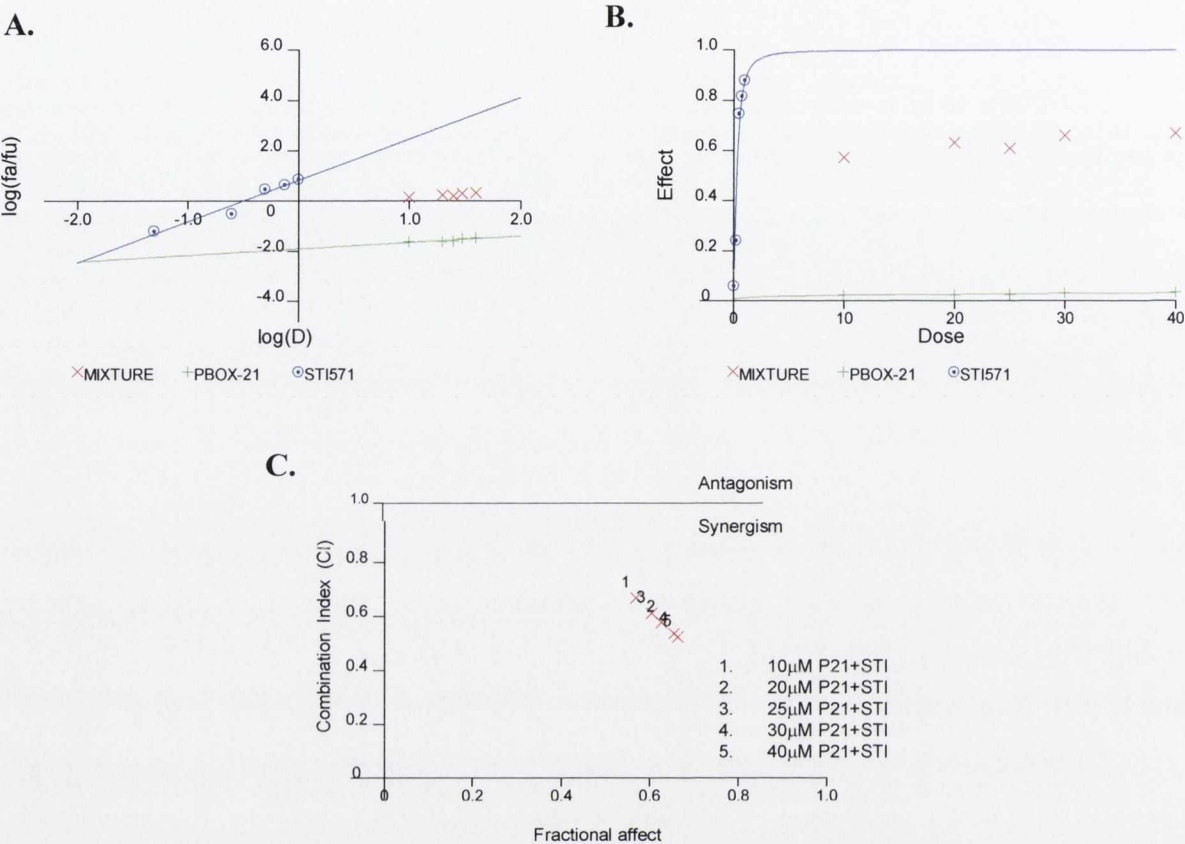


Fig. 3.6 PBOX-21 synergistically enhances the effect of STI571 in LAMA84 cells.

Median dose effect analysis was carried out on LAMA84 cells using the software Calculusyn. Cells were treated with PBOX-21 (P21) (10 μ M to 40 μ M) and/or STI571 (STI) for 48 h. **A.** Median dose plot, **B.** dose-effect curve and **C.** CI versus fraction of cells affected. A CI value of >1, =1 and <1 indicates antagonism, an additive effect and synergism respectively.

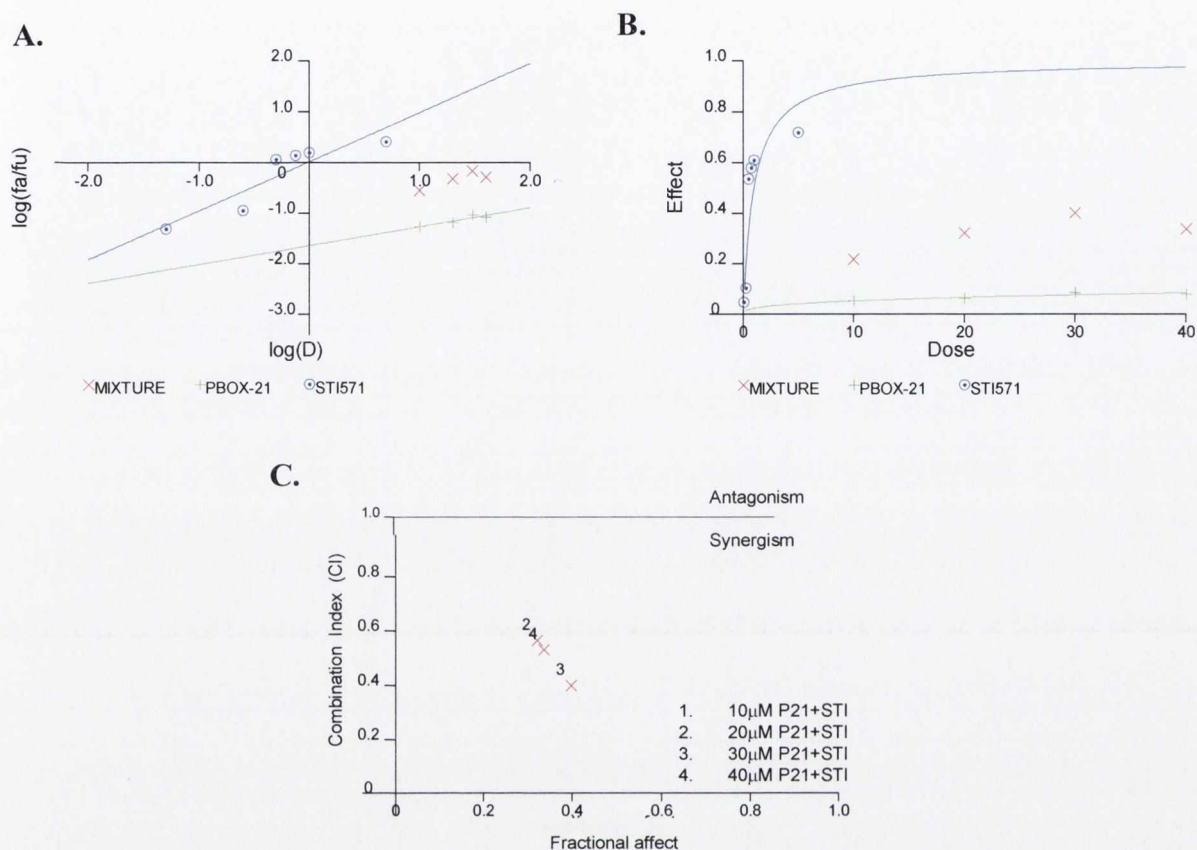


Fig. 3.7 PBOX-21 synergistically enhances the effect of STI571 in K562S cells.

Median dose effect analysis was carried out on K562S cells using the software CalcuSyn. Cells were treated with PBOX-21 (P21) (10 μ M to 40 μ M) and/or STI571 (STI) for 72 h. **A.** Median dose plot, **B.** dose-effect curve and **C.** CI versus fraction of cells affected. A CI value of >1 , $=1$ and <1 indicates antagonism, an additive effect and synergism respectively.

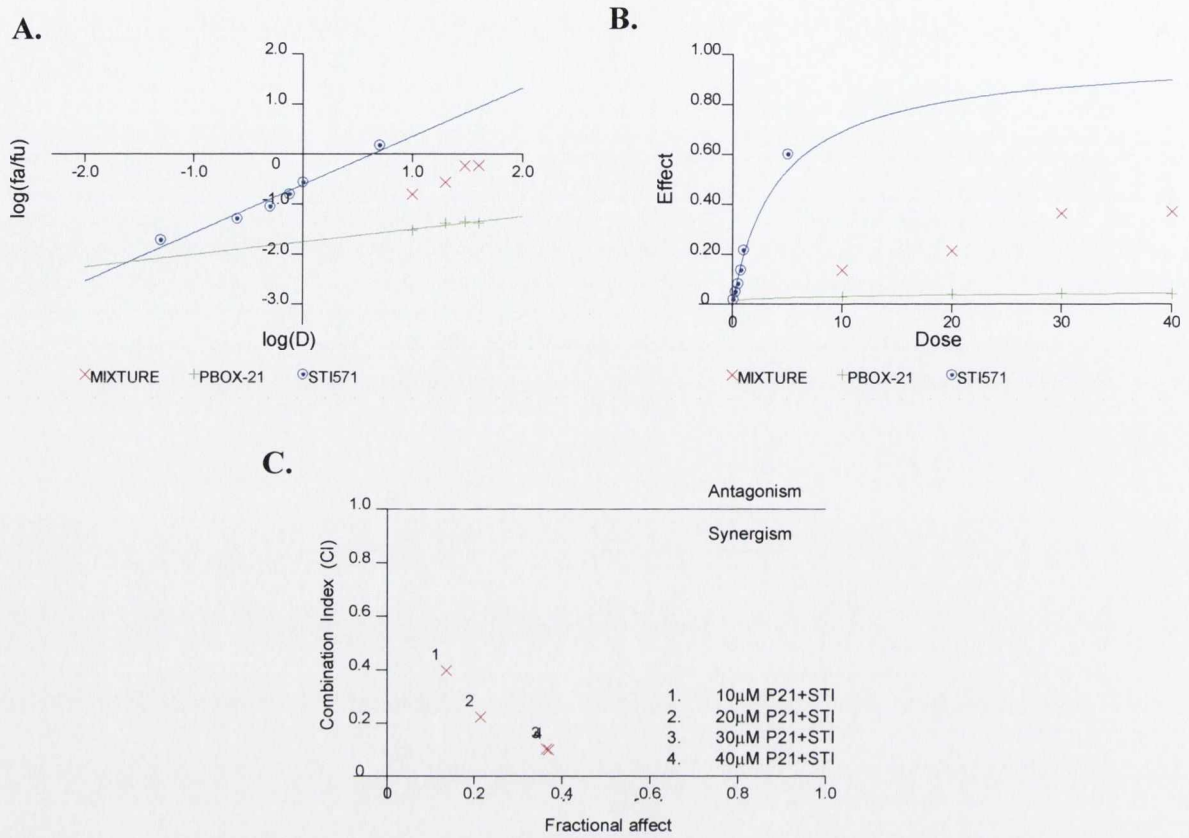


Fig. 3.8 PBOX-21 synergistically enhances the effect of STI571 in K562R cells.

Median dose effect analysis was carried out on K562R cells using the software Calcsyn. Cells were treated with PBOX-21 (P21) (10 μ M to 40 μ M) and/or STI571 (STI) for 72 h. **A.** Median dose plot, **B.** dose-effect curve and **C.** CI versus fraction of cells affected. A CI value of >1, =1 and <1 indicates antagonism, an additive effect and synergism respectively.

	[P21, μM]	[STI, μM]	Fa	CI Value	Rating
LAMA84	10	0.25	0.570	0.669	+++
	20	0.25	0.627	0.578	+++
	25	0.25	0.605	0.611	+++
	30	0.25	0.656	0.536	+++
	40	0.25	0.665	0.523	+++
K562S	10	0.25	0.217	0.982	$\pm$
	20	0.25	0.321	0.564	+++
	30	0.25	0.398	0.397	+++
	40	0.25	0.335	0.531	+++
K562R	10	0.25	0.135	0.398	+++
	20	0.25	0.213	0.224	++++
	30	0.25	0.364	0.103	++++
	40	0.25	0.369	0.101	++++

Table 3.2 CI values of PBOX-21 in combination with STI571 on CML cell lines.

Synergistic analysis was carried out using the software Calcsyn (Biosoft, U.K.). Data from flow cytometry experiments was used to analyse the interaction between PBOX-21 (P21) and STI571 (STI) using an isobologram method described by Chou and Talalay. A CI value of >1 , $=1$ and <1 indicates antagonism (-), an additive effect ($\pm$) and synergism (+) respectively (Section 2.8). Fa - Fraction of cells affected by apoptosis.

3.2.5 STI571 in combination with PBOX-21 does not induce apoptosis in Bcr-Abl-negative cells.

Due to the specific targeting of STI571 to Bcr-Abl, this novel combination was unlikely to be active in Bcr-Abl-negative cell lines. Consequently, it was likely that PBOX-21 in combination with STI571 would only also be specific for Bcr-Abl-positive cells. In order to prove this hypothesis, we tested the ability of this novel combination to induce apoptosis on a Bcr-Abl-negative cell line, HL-60.

HL-60 cells were treated for 72 h with 250nM STI571 and/or PBOX-21 (25µM or 40µM) and analysed by the alamar blue viability assay (Section 2.6) and flow cytometry (Section 2.7). The combination treatment had only a minimal effect on cell viability (Fig. 3.9A) and cells did not undergo a significant change in the percentage of cells in any phase of the cell cycle (Fig. 3.9B). Cells treated with a vehicle for 72 h resulted in a pre-G1 peak of $1.7 \pm 0.7\%$; following treatment with the highest concentration of PBOX-21 tested, 40µM, this apoptotic figure rose non-significantly to only $5.6 \pm 1.2\%$ (Fig. 3.9C). These results support the theory that the pro-apoptotic effect of STI571 in combination with PBOX-21 is specific for Bcr-Abl-positive cells and does not target Bcr-Abl-negative cells.

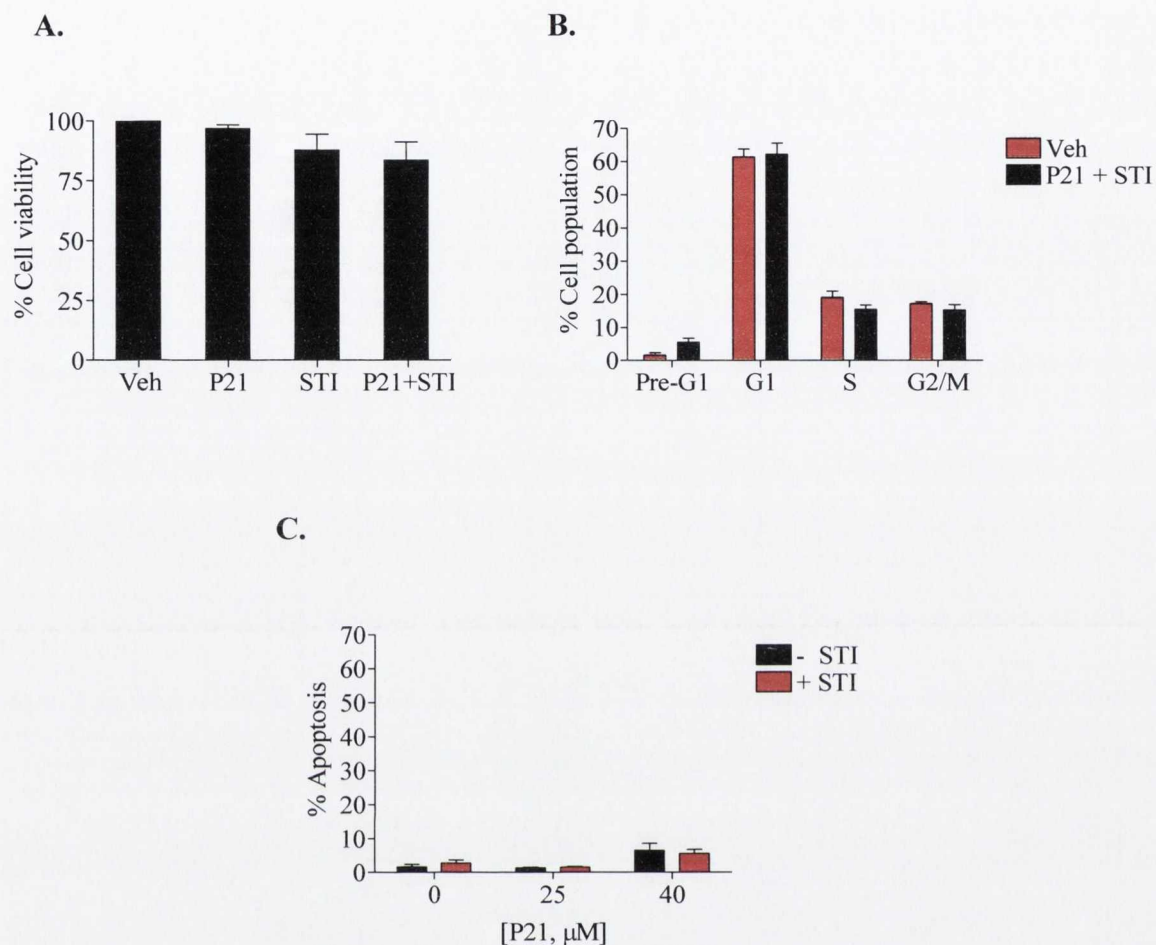


Fig. 3.9 The effect of PBOX-21 in combination with STI571 on Bcr-Abl-negative HL-60 cells.

Bcr-Abl-negative HL-60 cells were seeded during the log phase of growth and treated for 72 h with a vehicle (0.4% (v/v) ethanol, 0.0025% (v/v) DMSO) (Veh), 250nM STI571 (STI), PBOX-21 (P21) or a combination of both compounds. Cells were analysed by **A.** the alamar blue viability assay or flow cytometry for **B.** analysis of cell cycle distribution and **C.** apoptosis by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments.

3.2.6 Time-dependent cleavage of PARP is observed in LAMA84 cells following PBOX-21/STI571 treatment.

Intracellular cysteine proteases, known as caspases, must be cleaved in order to become activated. It has previously been demonstrated that PBOX-6 causes cleavage and thus activation of caspases in the breast cancer cell line MCF-7¹⁹⁶. Once activated, caspases can then in turn cleave numerous substrates including the DNA repair enzyme PARP. Cleavage of PARP has long been considered a classical hallmark of apoptosis. To determine the effect of PBOX-21/STI571 on the levels of cleaved PARP, Western blot analysis was performed on treated CML cells (Section 2.8).

Results showed that PARP was cleaved in LAMA84 cells 24 and 48 h post 25 μ M PBOX-21/250nM STI571 treatment (Fig. 3.10), but not at the earlier time-point of 8 h. Control samples showed no observable PARP cleavage, neither did samples treated with PBOX-21. There was a small amount of PARP cleavage found in STI571-treated samples at 48 h.

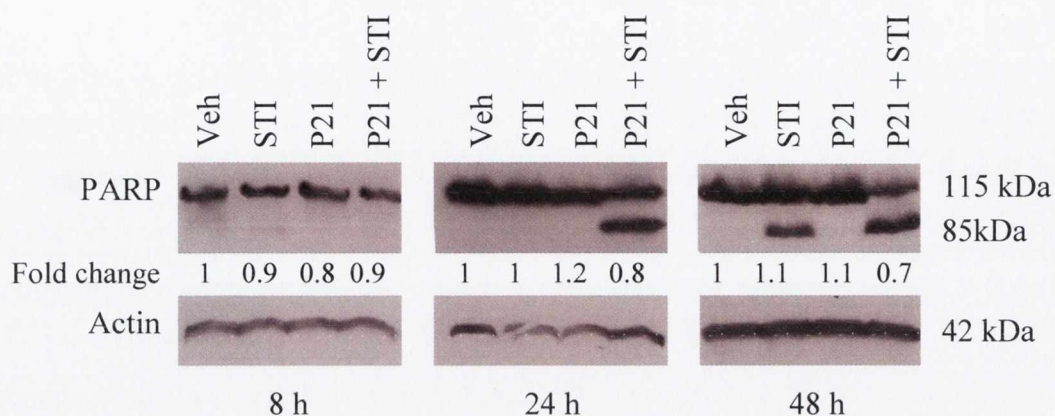


Fig. 3.10 Co-treatment with PBOX-21 and STI571 results in enhanced cleavage of the enzyme PARP.

LAMA84 cells were seeded during the log phase of growth and treated for up to 48 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 250nM STI571 (STI), 25 μ M PBOX-21 (P21) or a combination of both compounds. Proteins (20 μ g) from whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with an anti-PARP mAb (Section 2.10). Actin is shown as a loading control. Band intensities were normalised by densitometry with respect to actin and expressed as fold change over control. Results are representative of three independent experiments.

3.2.7 The effect of PBOX-21/STI571 on the expression levels of Bcr-Abl and phosphorylated Bcr-Abl substrates.

CML is one of the few malignancies where the pathogenesis of the disease is mainly due to one aberrantly expressed protein, p210^{Bcr-Abl}. Bcr-Abl elicits this leukaemic effect by the constant phosphorylation of substrates, that in turn upregulate various downstream signalling pathways (Section 1.2.3 and 1.3.7). STI571 is known to downregulate both the expression levels of the Bcr-Abl and its ability to phosphorylate substrates by competitively binding to its ATP binding site.

Results in Section 3.2.4 have shown that PBOX-21 synergistically enhances the apoptotic effect of STI571; therefore PBOX-21 in combination with STI571 could potentially reduce the protein levels of Bcr-Abl and its phosphorylating capabilities even further. To examine whether this was the case or not, cells were treated for up to 48 h with a vehicle, 25µM PBOX-21, 250nM STI571 or a combination of both compounds. Cell lysates were prepared and run on 8% or 12% gels, proteins were transferred to PVDF membranes and incubated with an antibody against p210^{Bcr-Abl} or an antibody directed against all the general tyrosine phosphorylated proteins in a whole cell lysate extract (Section 2.10).

Results showed that Bcr-Abl was downregulated in LAMA84 cells 24 and 48 h post treatment with STI571 in combination with PBOX-21 (Fig. 3.11), but not at the earlier time-point of 8 h. Cells treated with STI571 alone for 48 h also marginally decreased the levels of Bcr-Abl.

Similarly, the amount of tyrosine phosphorylated proteins were downregulated when STI571-treated cells were compared to vehicle-treated cells, however PBOX-21/STI571 reduced the levels of tyrosine phosphorylated proteins even further (Fig. 3.12).

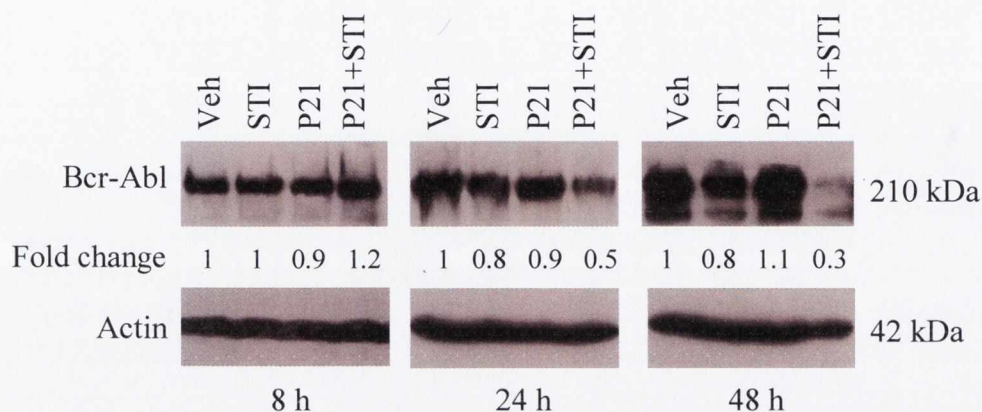


Fig. 3.11 STI571 in combination with PBOX-21 decreases the levels of Bcr-Abl.

LAMA84 cells were seeded during the log phase of growth and treated for up to 48 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 250nM STI571 (STI), 25 μ M PBOX-21 (P21) or a combination of both compounds. Proteins (20 μ g) from whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with an anti-Bcr-Abl mAb (Section 2.10). Actin is shown as a loading control. Band intensities were normalised by densitometry with respect to actin and expressed as fold change over control. Results are representative of three independent experiments.

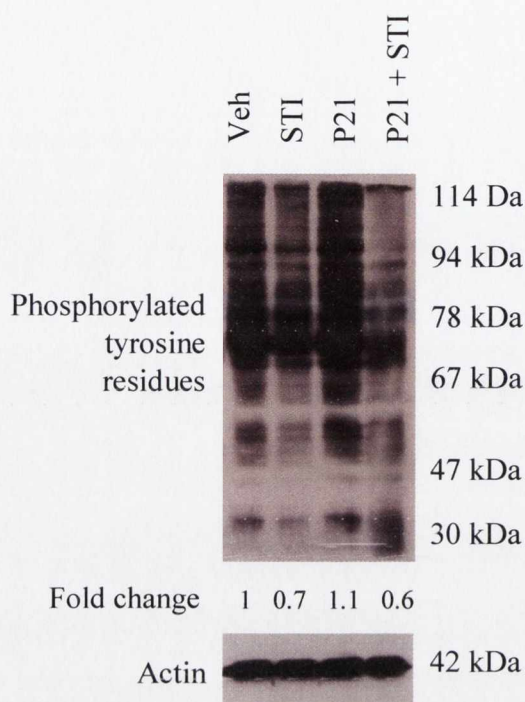


Fig. 3.12 Tyrosine phosphorylated protein levels are reduced following PBOX-21/STI571 treatment for 48 h.

LAMA84 cells were seeded during the log phase of growth and treated for 48 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 250nM STI571 (STI), 25 μ M PBOX-21 (P21) or a combination of both compounds. Proteins (20 μ g) from whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for phosphorylated tyrosine residues (Section 2.10). PVDF membranes were then stripped and re-probed with anti-actin mAb to show equal loading. Band intensities were normalised by densitometry with respect to actin and expressed as fold change over control. Results are representative of three independent experiments.

3.2.8 *The effect of PBOX-21/STI571 on the expression levels of Bcl-2 family members.*

The anti-apoptotic members of the Bcl-2 family (Mcl-1, Bcl-XL, Bcl-2) dominate over the pro-apoptotic members (Bim) in a healthy cell. However the pro-apoptotic members have been extensively researched and are repeatedly shown to be downstream executioners of apoptosis. During apoptosis they are activated and freed from the inhibitory effects of the anti-apoptotic members, therefore identification of the status of different members of the Bcl-2 family is crucial in helping to determine part of the signalling pathway evoked after chemotherapeutic intervention.

Cells were treated with a vehicle, 250nM STI571, 25μM PBOX-21 or a combination of both compounds for 8, 24 and 48 h. Cell lysates were then prepared and run on 12% gels (Section 2.10).

Following treatment with PBOX-21/STI571 for 8 and 24 h there was no detectable change in the protein levels of Bcl-2, however after treatment for 48 h Bcl-2 had been cleaved into a smaller fragment. This cleaved fragment represents an inactive form of the protein (Fig. 3.13)²¹³.

Bcr-Abl-mediated cell survival has been linked to the increased expression of various anti-apoptotic proteins, particularly Bcl-XL. We next sought to examine the effect of the PBOX-21/STI571 combination on Bcl-XL. Similarly to Bcl-2, there was no change in the levels of this protein at the early time-points of 8 and 24 h. However, after 48 h combination treatment, the expression levels had been reduced. Similar results were also observed with Mcl-1. β-actin is shown as a loading control (Fig. 3.13).

The protein Bim was then examined as a representative of a pro-apoptotic Bcl-2 family member. An increase in the expression levels of BimEL, one of three alternative splicing isoforms of Bim (antibody detects all three isoforms), was evident after 48 h of treatment, but not at the earlier time-points of 8 and 24 h (Fig. 3.14), suggesting that regulation of the Bcl-2 family members is a downstream consequence of STI571 added in combination with PBOX-21.

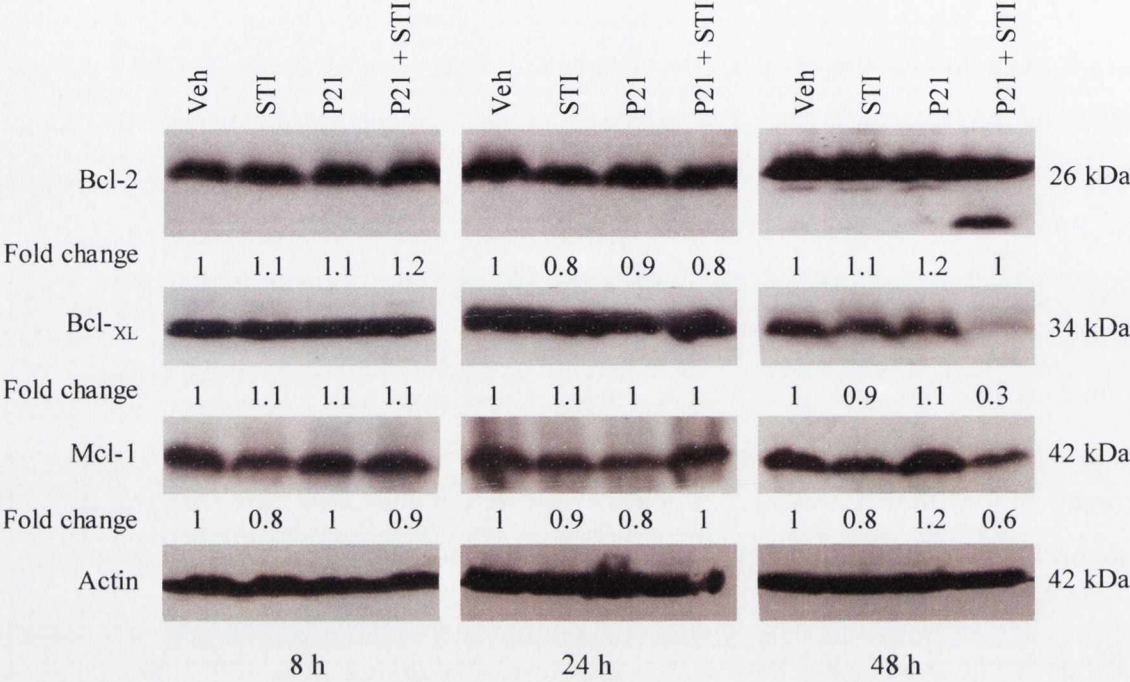


Fig. 3.13 PBOX-21/STI571 treatment and the Bcl-2 family.

LAMA84 cells were seeded during the log phase of growth and treated for up to 48 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 250nM STI571 (STI), 25μM PBOX-21 (P21) or a combination of both compounds. Proteins (20μg) from whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with an anti-Bcl-2, Bcl-XL or Mcl-1 mAb (Section 2.10). Actin is shown as a loading control. Band intensities were normalised by densitometry with respect to actin and expressed as fold change over control. Results are representative of three independent experiments.

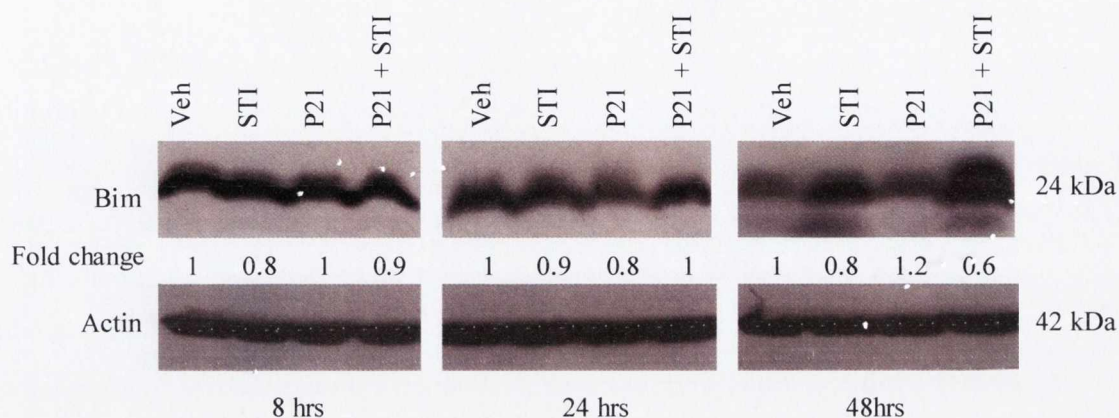


Fig. 3.14 STI571 in combination with PBOX-21 upregulates the expression of the pro-apoptotic Bcl-2 family member BimEL.

LAMA84 cells were seeded during the log phase of growth and treated for up to 48 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 250nM STI571 (STI), 25 μ M PBOX-21 (P21) or a combination of both compounds. Proteins (20 μ g) from whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with an anti-Bim mAb (Section 2.10). Actin is shown as a loading control. Band intensities were normalised by densitometry with respect to actin and expressed as fold change over control. Results are representative of three independent experiments.

3.2.9 PBOX-21/STI571-induced apoptosis does not appear to involve the mitochondrial pathway.

We next sought to examine the involvement of the mitochondria in the PBOX-21/STI571-mediated apoptotic pathway. Normally, following a decrease in mitochondrial membrane potential, an early indicator of the intrinsic apoptosis, cytochrome c would be released from the mitochondria into the cytosol, forming an apoptosome with procaspase-9 molecules, key events associated with the intrinsic apoptotic pathway. A small shift in mitochondrial membrane potential was observed 24 h after treatment with STI571, while the combination treatment resulted in a more pronounced shift. PBOX-21 alone did not have any effect on mitochondrial membrane potential nor did any treatment at the earlier time-points of 8 and 16 h (Fig. 3.15). Following the PBOX-21/STI571 treatment, cytochrome c was not released into the cytosol, although staurosporine-treated LAMA84 cells, as a positive control, showed release of this protein demonstrating the effectiveness of this assay (Fig. 3.16). The initiator caspase involved in the intrinsic pathway, procaspase-9, was not cleaved during PBOX-21/STI571 apoptosis even after 48 h, consistent with a lack of cytochrome c release into the cytosol. Again, staurosporine treatment of LAMA84 cells did however result in procaspase-9 cleavage. Collectively these results suggest a late involvement of the mitochondrial pathway in PBOX-21/STI571-induced apoptosis.

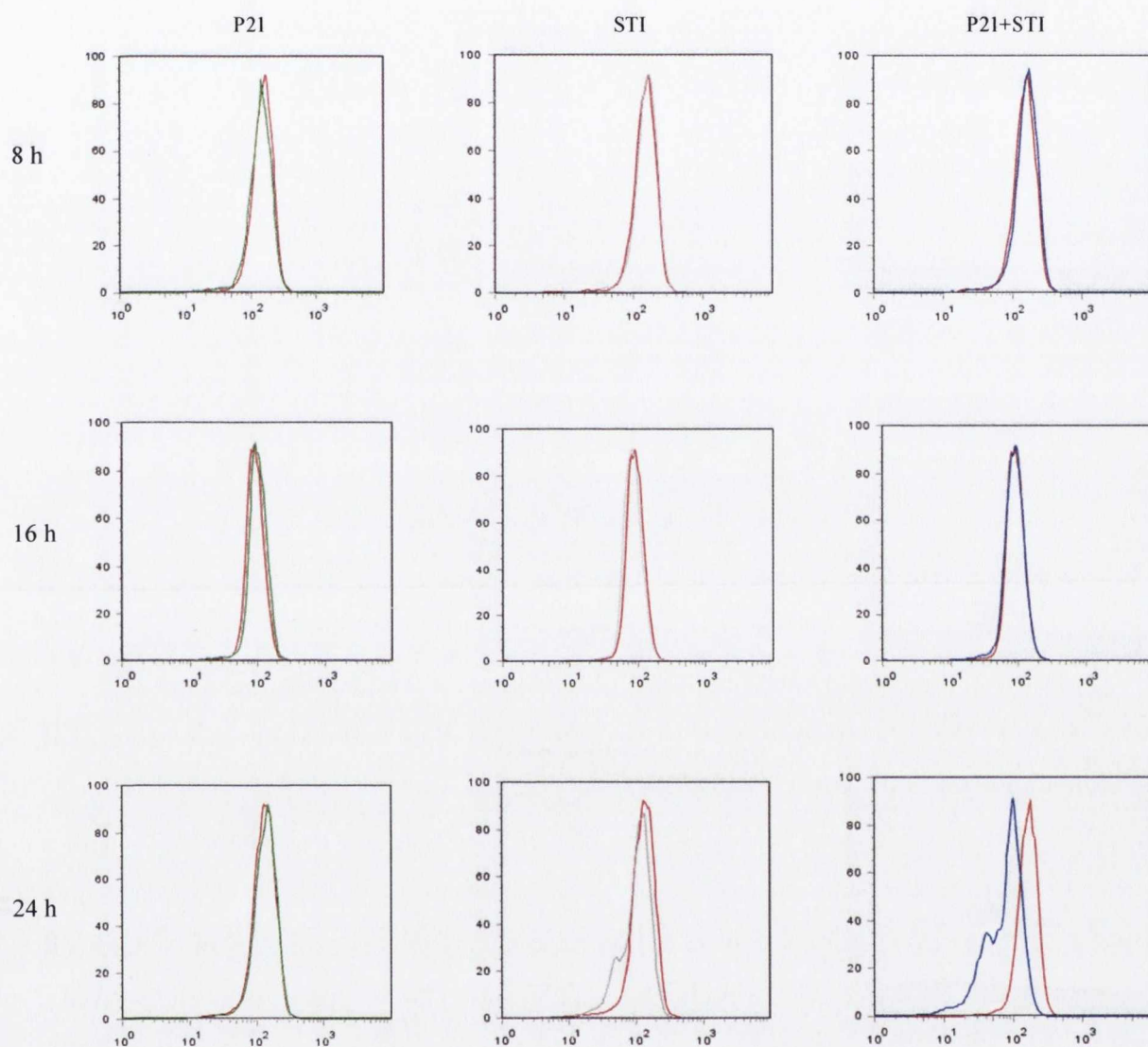


Fig. 3.15 The role of the mitochondria in PBOX-21/STI571-induced apoptosis.

LAMA84 cells were seeded during the log phase of growth and treated for up to 24 h with a vehicle (Veh, -) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 25 μ M PBOX-21 (P21, -), 250 nM STI571 (STI, -) or a combination of both compounds (-). After the required incubation period, treated cells were incubated for 20 min with 150 nM DiOC₆ and analysed by flow cytometry for changes in the mitochondrial membrane potential. Results are representative of three independent experiments.

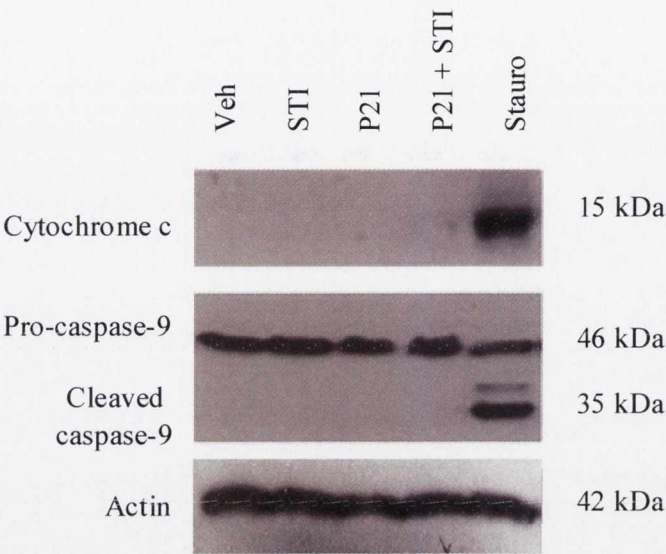


Fig. 3.16 STI571 in combination with PBOX-21 does not evoke the release of cytochrome c from the cytosol or cleavage of procaspase-9.

LAMA84 cells were seeded during the log phase of growth and treated for 48 h with a vehicle (Veh) (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO), 250nM STI571 (STI), 25μM PBOX-21 (P21) or a combination of both compounds. Proteins (20μg) from whole cell lysates (caspase-9) and cytosolic proteins (cytochrome c) were separated by SDS-PAGE, transferred to PVDF membranes and probed with the relevant antibodies (Section 2.10). Actin is shown as a loading control. Cells treated for 16 h with 1μM staurosporine (stauro) were used as a positive control. Results are representative of three independent experiments.

3.2.10 Caspase-8 is a primary caspase involved in PBOX-21/STI571-induced apoptosis.

As we had ruled out the involvement of caspase-9 in the apoptotic pathway, we next sought to determine the role of other caspases in PBOX-21/STI571-induced apoptosis. The broad spectrum caspase inhibitor Boc-D-FMK was utilised first. Pretreatment of cells for 3 h significantly reduced the levels of apoptosis induced by a 48 h treatment with PBOX-21/STI571 from 62.5 ± 11.9 to $44.1 \pm 2.4\%$ (p value: 0.0251,*) (Fig. 3.17).

Caspases can be subdivided into initiator upstream and executioner downstream caspases and caspase-2, -8, -9 and -10 have previously been reported to act as initiators¹⁴¹. We next sought to determine which initiator caspases were involved in PBOX-21/STI571-induced apoptosis. Pretreatment of cells with specific caspase-2, -9 and -10 inhibitors had no effect on the level of apoptosis induced (Fig. 3.18), this is in agreement with a lack of procaspase-9 cleavage (Fig. 3.16). In contrast, cells pretreated under the same conditions with the caspase-8 inhibitor, IETD-FMK, followed by treatment for a further 48 h with PBOX-21/STI571 were assessed for apoptosis and results showed apoptosis levels were significantly reduced from 66.2 ± 7.2 to $36.5 \pm 4.9\%$ (Fig. 3.18), (p value: 0.0102,*).

Western blot analysis showed this novel combination of STI571 and PBOX-21 cleaved procaspase-8 into its active form (Fig. 3.19). This cleavage was prevented by pretreatment with the caspase-8 inhibitor. This would suggest an important role for caspase-8 in this apoptotic pathway.

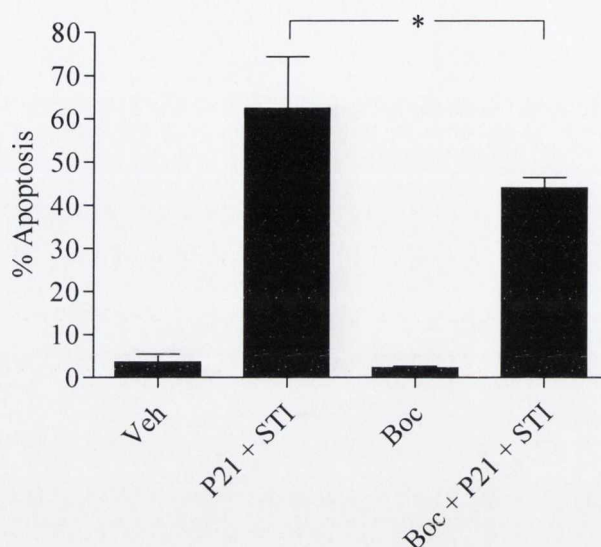


Fig. 3.17 A general caspase inhibitor partially blocks apoptosis.

LAMA84 cells were pretreated for 3 h with a vehicle (Veh) (1% (v/v) DMSO) or 100 μ M Boc-D-FMK followed by treatment for a further 48 h with a vehicle (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO) or a combination of 250nM STI571 (STI) and 25 μ M PBOX-21 (P21). After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments. Statistical analysis was performed using the Student's t-test, *, $p < 0.05$.

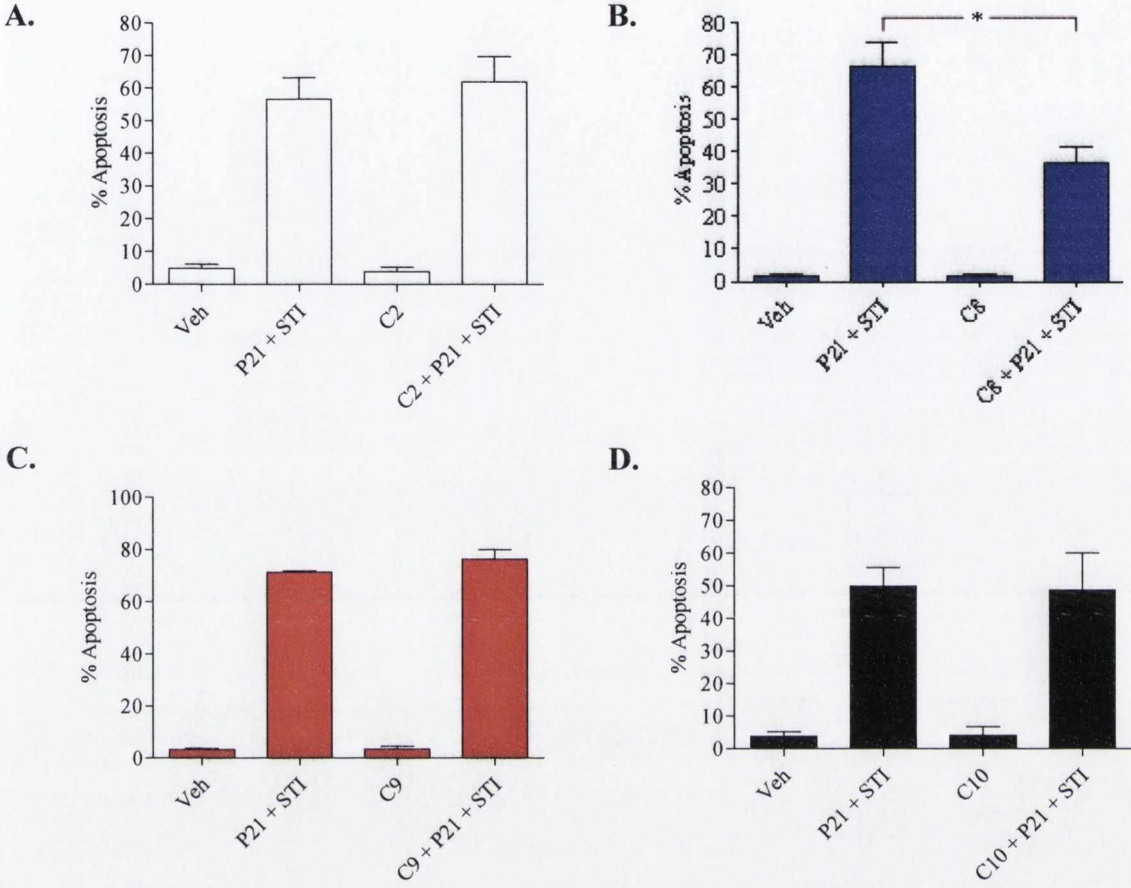


Fig. 3.18 Caspase-8 is required for PBOX-21 and STI571-induced apoptosis.

LAMA84 cells were pretreated for 3 h with a vehicle (Veh) (1% (v/v) DMSO) or a specific caspase inhibitor followed by treatment for a further 48 h with a vehicle (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO) or a combination of 250nM STI571 (STI) and 25μM PBOX-21 (P21). After the required incubation period, cells were fixed in 1ml ethanol and 100μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. Cells were pretreated with **A.** 50μM caspase-2 inhibitor (C2), **B.** 40μM caspase-8 inhibitor (C8), **C.** 40μM caspase-9 inhibitor (C9) or **D.** 40μM caspase-10 inhibitor (C10). Values represent the mean ± S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired t-test, *, $p < 0.05$.

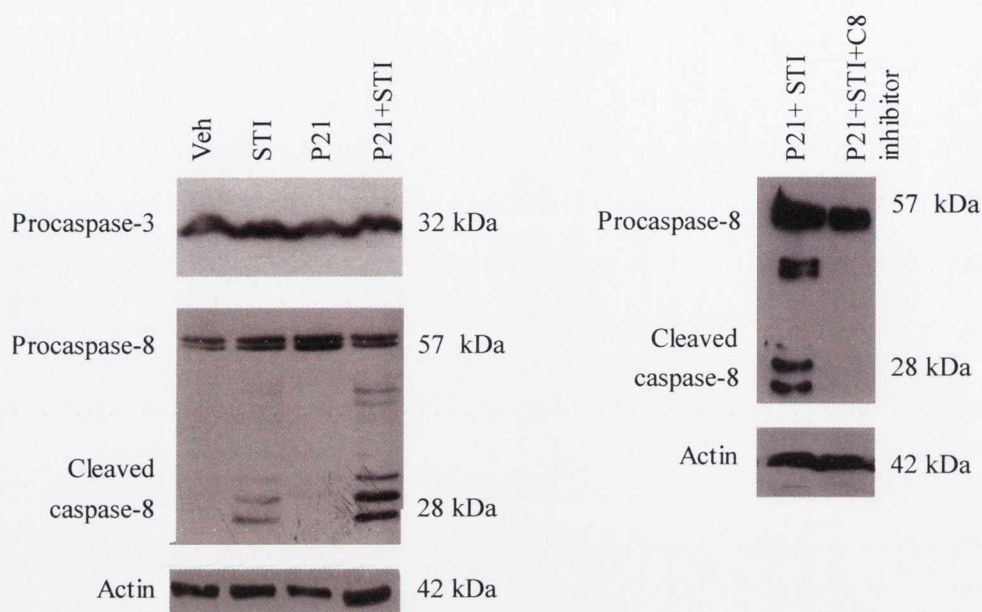


Fig. 3.19 Involvement of caspase-8 in PBOX-21 and STI571-induced apoptosis.

LAMA84 cells were pretreated for 3 h with a vehicle (Veh) (1% (v/v) DMSO) or a specific caspase-8 inhibitor followed by treatment for a further 48 h with a vehicle (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO) or a combination of 250nM STI571 (STI) and 25μM PBOX-21 (P21). Proteins (20μg) from whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with an anti-caspase-8 mAb (Section 2.10). PVDF membranes were then stripped and re-probed with an anti-actin mAb to show equal loading. Results are representative of three independent experiments.

3.2.11 Serine proteases are a critical component of the PBOX-21/STI571 apoptotic pathway.

The general caspase inhibitor could not completely abrogate apoptosis induced by the combined PBOX-21/STI571 treatment suggesting that other proteases may be involved in this apoptotic mechanism. Serine proteases have previously been shown to be involved in apoptosis, however the exact mechanisms through which they elicit their effects are still ill-defined. A recent publication has shown that the pro-apoptotic PBOX-6 compound induces apoptosis in CML cell lines through the involvement of the trypsin-like serine proteases²¹⁴. This prompted us to look at the serine proteases. The inhibitors used were TLCK a specific inhibitor that irreversibly inactivates trypsin-like serine proteases and TPCK a specific inhibitor that irreversibly inactivates chymotrypsin-like serine proteases.

Boc-D-FMK, the general caspase inhibitor was non-toxic to cells. TLCK and TPCK were marginally toxic. CML cells were pretreated for 3 h with the required inhibitor/s and then treated for a further 48 h with 25 μ M PBOX-21/250nM STI571 (Section 2.9). When cells were pretreated with TLCK and subsequently with PBOX-21/STI571, there was no significant change in the levels of apoptosis when compared to cells pretreated with a vehicle, 61.2 ± 7.6 versus $59.3 \pm 18.4\%$ respectively. When this was repeated with Boc-D-FMK in combination with TLCK apoptosis was significantly reduced to $30.7 \pm 16.1\%$ (p value: 0.0346,*), although this was to a similar extent as the reduction observed in cells pretreated with Boc-D-FMK alone.

When cells were pretreated with the chymotrypsin-like serine protease inhibitor TPCK and subsequently with PBOX-21/STI571, the levels of apoptosis were significantly reduced from 58.8 ± 6.2 to $37.9 \pm 9.3\%$ (p value: 0.0360,*). However this reduction in apoptosis was even more pronounced when cells were pretreated with a combination of both TPCK and Boc-D-FMK reducing levels of apoptosis to $8.0 \pm 0.4\%$, comparable to vehicle-treated cells (p value: 0.0034,**) (Fig. 3.20).

As the previous set of results show a combination of a general caspase inhibitor and TPCK prevent PBOX-21/STI571-induced apoptosis it was of interest to see if these inhibitors also prevented Bcr-Abl downregulation. This was examined in LAMA84 cells after 48 h treatment by Western blot analysis. Results showed that these inhibitors did prevent the downregulation of Bcr-Abl as predicted (Fig. 3.21), therefore chymotrypsin-like serine proteases and caspases are likely to be critically important in the PBOX-21/STI571-induced apoptotic pathway.

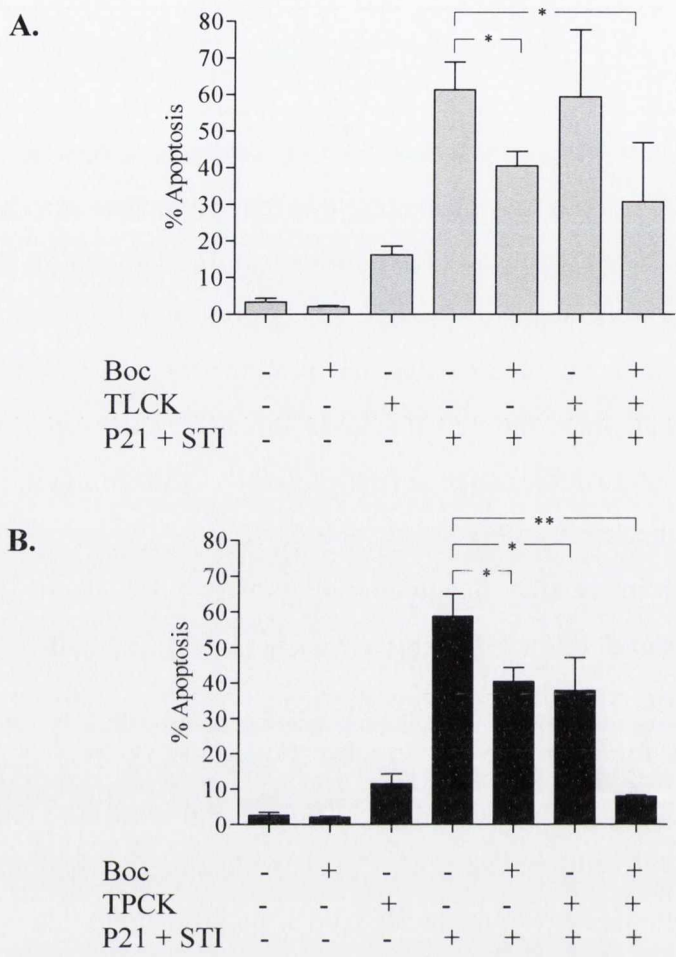


Fig. 3.20 The serine protease inhibitor TPCK protects against PBOX-21 and STI571 induced-apoptosis when combined with the general caspase inhibitor Boc-D-FMK.

LAMA84 cells were pretreated for 3 h with a vehicle (Veh) (1% (v/v) DMSO), 100µM Boc-D-FMK (Boc), a serine protease inhibitor (TLCK or TPCK) or a combination of both followed by treatment for a further 48 h with a vehicle (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO) or a combination of 250nM STI571 (STI) and 25µM PBOX-21 (P21). After the required incubation period, cells were fixed in 1ml ethanol and 100µl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. **A.** Cells were treated with 100µM TLCK and/or Boc. **B.** Cells were treated with 20µM TPCK and/or Boc. Values represent the mean ± S.E.M. of three independent experiments. Statistical analysis was performed using the Student’s unpaired t-test, *, $p < 0.5$; **, $p < 0.01$.



Fig. 3.21 A combination of the general caspase inhibitor, Boc-D-FMK, and the serine-like protease inhibitor, TPCK, prevent PBOX-21/STI571-induced downregulation of Bcr-Abl.

LAMA84 cells were pretreated for 3 h with a vehicle (Veh) (1% (v/v) DMSO), 100μM Boc-D-FMK (Boc), the serine protease inhibitor TPCK or a combination of both followed by treatment for a further 48 h with a vehicle (0.25% (v/v) ethanol, 0.0025% (v/v) DMSO) or a combination of 250nM STI571 (STI) and 25μM PBOX-21. Proteins (20μg) from whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with an anti-Bcr-Abl mAb (Section 2.10). Actin is shown act as a loading control. Results are representative of three independent experiments.

3.2.12 Evaluation of the ability of novel PBOX-STI571 hybrids to induce apoptosis in CML cells.

Due to our previous results demonstrating an enhancement of apoptosis upon co-treatment of CML cells with PBOX-21/STI571, it was decided to design and synthesise a short series of PBOX-21 and STI571 hybrid structures for analysis. These compounds, NF1522-NF1525, were synthesised by a collaborator, Prof. Campiani, Universita d'egli, Siena, Italy and his team and the structures are shown in Fig. 3.22. These compounds are completely novel, no biological information of these hybrid compounds exists thus far.

Results showed that two of the four compounds tested demonstrated activity. Of these, NF1523 and NF1525 were the most potent inducing apoptosis at $10\mu\text{M}$ from 24 h onwards in K562 cells (Fig. 3.23A), these compounds were therefore subjected to a further dose-response study. A range of concentrations, from 500nM to $25\mu\text{M}$, were analysed. Results showed apoptosis peaked following treatment with $2.5\mu\text{M}$ NF1523 which induced $49.7 \pm 3.7\%$ apoptosis while $25\mu\text{M}$ NF1525 induced $52.4 \pm 4.9\%$ apoptosis after a 48 h treatment (Fig. 3.23B). These results are similar to those obtained by PBOX-21 and STI571 in combination.

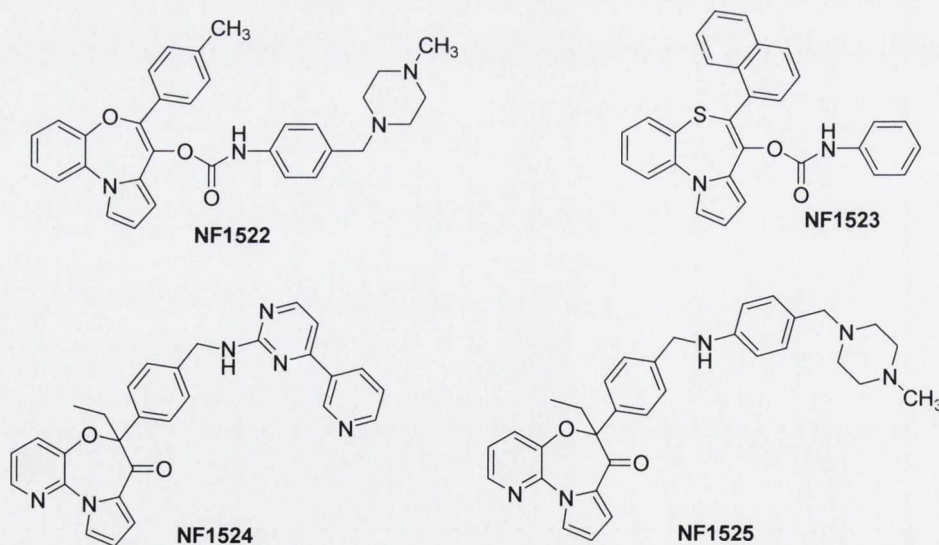
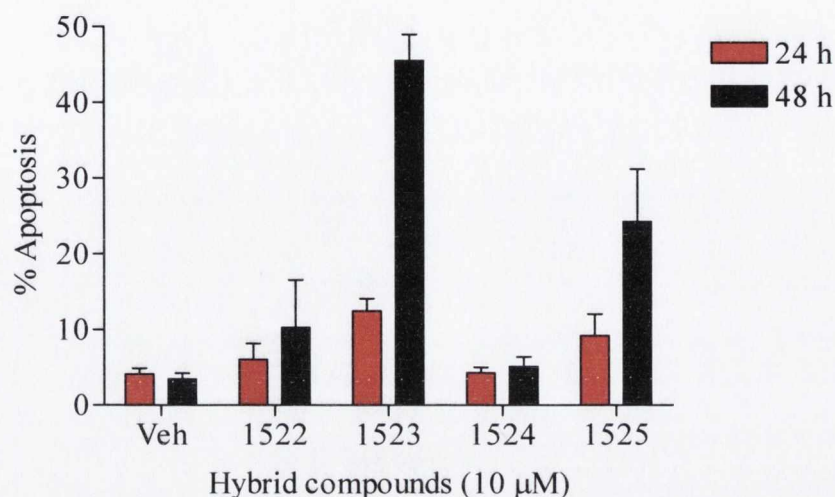


Fig. 3.22 Structures of novel PBOX hybrid compounds.

Chemical structures of STI571 and PBOX hybrid compounds NF1522, NF1523, NF1524 and NF1525.

A.



B.

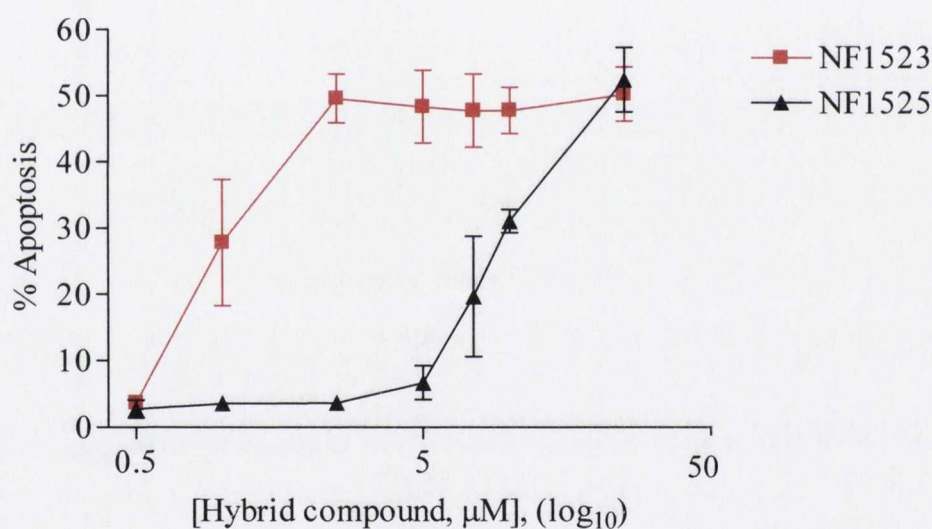


Fig. 3.23 Some PBOX hybrid compounds induce apoptosis in CML cells.

K562 cells were seeded during the log phase of growth and treated for up to 48 h with a vehicle (Veh) (0.1% (v/v) DMSO) or a novel PBOX hybrid compound. After the required incubation period, cells were resuspended in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Samples were then analysed by flow cytometry, the percentage of apoptosis was measured by quantification of the pre-G1 peak. Cells were treated with **A.** 10 μ M of each compound for 24 and 48 h and **B.** a concentration range (0.5 μ M to 25 μ M) of NF1523 and NF1525 for 48 h. Values represent the mean $\pm$ S.E.M. of three independent experiments.

3.3 Discussion

The tyrosine kinase inhibitor STI571 has rapidly become frontline treatment for CML patients since its release on the market in 2001. The emergence of STI571 resistance, intolerance and dose-limiting toxicity has led to the need to develop novel therapeutic strategies for CML patients. One approach to overcome these problems is to combine STI571 with other anti-cancer agents^{50; 13; 60; 61; 65; 66}. We have recently shown that STI571 enhances the apoptotic efficacy of the representative pro-apoptotic PBOX compound, PBOX-6, and other MTAs by inhibiting endoreplication¹⁹⁰. We have previously reported that the functionally distinct non-cytotoxic G1-arresting compound known as PBOX-21 affects the cell cycle by arresting cells in the G1 phase of the cell cycle in both human 132INI astrocytoma and MCF-7 breast carcinoma cells¹⁹³. Herein, in agreement with these reports, we demonstrate that PBOX-21 significantly increased the percentage of LAMA84 cells in the G1 phase. STI571 alone also arrested the number of cells in the G1 phase. When these drugs were combined there was no further increase in the population of cells in the G1 phase indicating that G1 arrest may not be the major mechanism underlying the combination effect of PBOX-21/STI571 on apoptosis.

It is crucially important for novel therapeutic combinations to not only render 'normal' or early stage CML cells susceptible to apoptosis, but to also do so in highly resistant cells and in a synergistic manner. We utilised a K562 cell line that was up to seven times more resistant to STI571 (K562R) than the parental form (K562S) due to the over-expression of Bcr-Abl²⁰⁴. While each compound alone at specific concentrations had little or no effect on either cell line, co-administration of STI571 with PBOX-21 induced a synergistic effect with a CI value of less than 1. Of the many compounds which have emerged showing synergistic potentiation of the lethal effects of STI571 our results show co-treatment of CML cell lines with PBOX-21 and STI571 to be as potent as many of these.

As shown here and elsewhere, STI571 (250nM) alone resulted in a small alteration in Bcr-Abl protein levels in LAMA84 cells^{190; 211}. The co-administration to cells of PBOX-21/STI571 for 24 h, resulted in a marked reduction in Bcr-Abl levels and the expression of Bcr-Abl was almost undetectable by 48 h. PBOX-21/STI571 administration, as expected, did not exhibit activity in Bcr-Abl-negative cells. This novel combination is also likely to reduce the kinase activity of Bcr-Abl as the number of downstream phosphorylated proteins had also significantly decreased following co-treatment for 48 h. The

downregulation of Bcr-Abl at 24 h was concomitant with the onset of apoptosis and the cleavage of PARP. Bcr-Abl-mediated cell survival has been linked to the increased expression of various anti-apoptotic proteins. Co-administration of LAMA84 cells with PBOX-21/STI571 resulted in the downregulation of Bcl-XL and Mcl-1. It has been reported that Bcr-Abl lies upstream of the STAT family, in particular STAT5 which has been implicated in the regulation of Bcl-XL in leukaemia cells^{158; 215}. It is therefore likely that the observed downregulation of Bcl-XL in PBOX-21/STI571-treated cells was a direct consequence of Bcr-Abl downregulation and this may serve to amplify the apoptotic response.

Another anti-apoptotic protein, Bcl-2, was found to be cleaved 48 h after co-administration with PBOX-21/STI571. While Bcl-2 is traditionally inactivated by phosphorylation, there have been numerous studies reporting cleavage of Bcl-2 in response to various apoptotic stimuli^{216; 217}. It is thought that this shorter cleaved fragment of Bcl-2 is not functional and is not capable of dimerisation with other anti-apoptotic proteins thus diminishing its protective effect²¹³. The pro-apoptotic Bcl-2 protein Bim was found to be upregulated following treatment with STI571. This is in agreement with previously published reports^{218; 219} demonstrating that Bim is a critical protein mediating STI571-induced killing of leukaemic cells. In the present study, when LAMA84 cells were treated with a combination of PBOX-21 and STI571, Bim was further upregulated which correlates with our findings that PBOX-21 synergistically enhances the apoptotic effect of STI571.

Despite the central role played by caspases during apoptosis, evidence of the existence of caspase-independent apoptosis suggests that caspases are dispensable for some apoptotic cell death pathways^{165; 166}. In the present study, when cells were pretreated with either the general caspase inhibitor, Boc-D-FMK, or the caspase-8 specific inhibitor, prior to treatment with the PBOX-21/STI571 combination, there was a significant reduction in the number of cells undergoing apoptosis. Treatment of cells with PBOX-21/STI571 resulted in cleavage of procaspase-8 to its active form, these results suggest the importance of caspases, in particular the initiator caspase, caspase-8, in this pathway. This finding is in agreement with previous reports which demonstrated that the combinations of STI571 with either flavopiridol or a histone deacetylase inhibitor resulted in a pronounced increase in procaspase-8 activation^{64; 127}. In the present study, while the pan-caspase inhibitor significantly reduced apoptotic cell death, there were still a large proportion of cells undergoing apoptosis suggesting the involvement of additional caspase-independent

pathways. Pretreatment with the chymotrypsin-like serine protease inhibitor TPCK significantly reduced the extent of apoptosis observed. Apoptosis was, however, completely abrogated by pretreating CML cells with both the general caspase inhibitor and TPCK. While it was believed an inhibition of caspases prevented STI571-induced apoptosis, it is now known that cells actually undergo a delayed caspase-independent necrosis-like form of programmed cell death²²⁰. Such cell death is thought to be elicited through trypsin-like serine proteases which were not found to be of importance in the PBOX-21/STI571-induced apoptotic pathway.

It is hypothesised that there are two main apoptotic pathways induced following co-treatment with STI571 and PBOX-21, a caspase-dependent pathway and a separate caspase-independent pathway that utilises chymotrypsin-like serine proteases. The involvement of two complimentary pathways has also been shown in other types of leukaemia²²¹ and Jurkat T lymphoma cells²²². Interestingly, in each of these studies, the caspase-dependent mechanism also involved caspase-8. Western blot analysis confirmed that pretreatment with caspase and chymotrypsin-like serine protease inhibitors also prevented downregulation of Bcr-Abl.

As caspase-8 is usually associated with the extrinsic apoptotic pathway we sought to delineate the involvement of the other classical apoptotic pathway involving the mitochondria, the intrinsic pathway. A decrease in the mitochondrial membrane potential, an early indicator of the intrinsic apoptotic pathway, was not observed until 24 h after treatment by which stage apoptosis had already been detected. The combined lack of release of cytochrome c into the cytosol, cleavage of procaspase-9 and an early change in mitochondrial membrane potential suggests this pathway is not involved in the initiation of apoptosis by PBOX-21/STI571. This conclusion is further supported by the fact that the caspase-9 inhibitor had no effect on the level of apoptosis induced.

Although the events directly responsible for PBOX-21 mediated potentiation of STI571-induced lethality remain to be determined the possibility exists that interactions between PBOX-21 and STI571 may involve dysregulation of various cell-cycle related proteins. We have previously shown the PBOX-21 causes a downregulation in cyclin D2 and D3 expression and a decrease in CDK2, 4 and 6 activity¹⁹³. The possibility that enhanced apoptosis in PBOX-21/STI571-treated CML cells reflect perturbations in cyclin D expression/activity appear plausible and studies to test this hypothesis are planned. A number of studies have recently demonstrated that combinations of STI571 with

farnesyltransferase inhibitors displayed synergism in CML cell lines^{13; 223; 224}. The possibility that PBOX-21 targets farnesyltransferases should also be tested.

Recent studies have proven STI571 to be effective at treating many other diseases like Bcr-Abl-positive ALL, DFSP, MDS/MPD, HES/CEL and ASM. Most of the work in this report has been carried out on CML cells, but it is also possible that co-administration of PBOX-21 and STI571 is capable of inducing apoptosis in these diseases although further study would be required to confirm this theory.

The results outlined above paved the way for the development of four novel compounds in which components of STI571 were hybridised to the PBOX compounds. Of these, two compounds successfully induced apoptosis in CML cell lines, however, neither compound was more potent than the PBOX-21/STI571 combination. Interestingly, the two compounds that do show activity are the two in the series that show least structural similarities suggesting that the synthesis and testing of further hybrids is required in order to determine any structure-activity relationship. Therefore while these results are encouraging it was decided to wait until a much larger series of hybrid compounds was developed before looking at such compounds in any further detail.

In conclusion, this chapter looked at the combination of a pharmacologically achievable concentration of STI571 with a novel compound, PBOX-21, in the induction of apoptosis in CML cells. PBOX-21 synergistically enhanced the apoptotic effect of STI571 in three CML cell lines, including an STI571-resistant cell line, through the activation of procaspase-8 and chymotrypsin-like serine proteases and the downregulation of Bcr-Abl. Finally, the non-cytotoxic effects of STI571/PBOX-21 combinations on Bcr-Abl-negative HL-60 leukaemia cells highlights its selectivity for Bcr-Abl-positive CML cells. Therefore, combination therapy with PBOX-21 and STI571 represents a potentially novel and effective treatment strategy for the treatment of CML patients, including those that have become resistant to current treatments.

Chapter 4.

*Flavopiridol synergistically enhances PBOX-induced apoptosis
in human chronic myeloid leukaemia cells in a sequence
dependent manner*

4.1 Introduction

CML represents a clonal disorder of haematological stem cells containing a constitutively active tyrosine kinase known as Bcr-Abl. This oncogenic fusion protein is a hallmark of CML and is also found in 10 to 15% of ALL patients^{10; 35}. Bcr-Abl confers cells with a survival advantage due to the continuous activation of many downstream signalling pathways including the Ras/MAPK, JAK/STAT and PI3K pathways rendering cells insensitive to apoptosis⁶. The advent of the novel tyrosine kinase inhibitor, STI571, has revolutionised CML treatment with the majority of newly diagnosed chronic phase patients achieving durable CCRs^{5; 26}. However advanced stages of the disease, the accelerated phase and the fatal blast crisis phase, are commonly associated with the development of mutant Bcr-Abl clones. While STI571 competitively binds to and inhibits the ATP binding pocket of unmutated Bcr-Abl, mutant clones display various degrees of resistance to STI571^{41; 47}. The fact that a substantial subset of CML patients fail STI571 treatment has stimulated the development of novel agents and drug combinations for the treatment of CML²²⁵.

A novel set of MTAs, the pro-apoptotic PBOX compounds, have recently been shown by our group to induce apoptosis in a wide range of solid tumours and haematological malignancies by causing a depolymerisation of the microtubule network^{192; 194}. Mechanistic studies have indicated that the PBOXs induce apoptosis in CML cells by bypassing Bcr-Abl¹⁹⁴ and that activation of JNK signalling pathways¹⁹⁵ and inactivation of the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL¹⁹⁷ are essential during PBOX-induced apoptosis. The PBOX compounds have also demonstrated efficacy in *ex vivo* CLL samples¹⁹¹ and in an *in vivo* mouse mammary carcinoma model²⁰¹. We have recently demonstrated that the PBOXs induce the formation of polyploid cells in CML cells¹⁹⁰, a characteristic shared with other MTAs. Generally in response to MTAs, cells enter mitosis, transiently arrest and then undergo either repair or apoptosis. However, cells with a defective G1 checkpoint e.g. cells lacking p53 or Rb, will continue to replicate without undergoing cell division, a process known as endoreplication, resulting in the accumulation of cells with a >4N DNA content (polyploidy)²²⁶. We have also previously shown that, in a similar manner to other MTAs, a significant proportion of CML cells (approximately 60%) remained resistant to our pro-apoptotic PBOXs. We therefore sought to determine whether a combination treatment with another drug could potentiate PBOX-induced apoptosis.

Flavopiridol is a potent CDK1 inhibitor which has shown activity in a wide variety of malignancies both *in vitro* and in *in vivo* animal models^{121; 227; 228}. Concentrations required to inhibit CDKs were easily achieved in phase I clinical trials. However, the results of many early phase II trials using continuous infusion schedules were disappointing^{116; 114; 118; 115}, dose-limiting toxicities such as neutropenia were also evident^{114; 115; 122}. Recently trials involving shorter infusions and higher plasma concentrations have now seen flavopiridol demonstrate strong anti-cancer activity, particularly in CLL^{122; 123}. The combination of this schedule of flavopiridol with other pro-apoptotic agents has also proved successful, including flavopiridol in combination with cytarabine and mitoxantrone¹³³, and the MTAs docetaxel¹³¹ and paclitaxel (together with carboplatin)¹³². Flavopiridol has also been shown to synergistically enhance the apoptotic effect of MTAs *in vitro*, however the exact mechanism through which this synergism occurs remains unclear¹²⁴.

Survivin is a member of the IAP family that functions to prevent apoptosis by inhibiting caspases, downstream executioners of apoptosis. It is also a cell cycle related protein, with activation occurring during mitosis via phosphorylation of Thr34 by the cell cycle complex CDK1/cyclin B1⁸⁶. Survivin has been associated with cytoprotection and preservation of microtubule integrity. It is expressed during development but is undetectable in most adult tissues except during mitosis. Importantly however, it is overexpressed in the majority of cancers including CML cells. Survivin expression in malignant cells may facilitate evasion from apoptosis and promote aberrant mitotic progression suggesting it may be a key target for anti-cancer agents⁸⁴. The possibility of exploiting the survivin pathway for cancer therapy has been the study of intense investigations. Inhibition of survivin expression and/or function in tumour cells by employment of survivin antisense oligodeoxynucleotides, dominant negative mutants or by siRNA has been shown to trigger apoptosis⁸⁴. Some reports suggest that flavopiridol suppresses CDK1-mediated survivin phosphorylation, accelerating protein destruction through the ubiquitin-dependent proteasome and thus enhancing tumour cell apoptosis²²⁹ whilst others suggest that flavopiridol prevents MTA-induced endoreplication leading to an enhancement of MTA-induced cell death²²⁶.

Studies demonstrating flavopiridol enhanced MTA-induced tumour cell apoptosis prompted us to examine whether this CDK1 inhibitor can similarly enhance PBOX-induced apoptosis in CML cells. In this study, we have characterised the apoptotic and cell cycle events associated with combination therapy of the pro-apoptotic PBOX compounds

and flavopiridol in CML cell lines. Our results show that sequential addition of flavopiridol to PBOX-treated cells results in an accelerated exit from the G2/M transition accompanied by an enhanced downregulation of CDK1/cyclin B1 and survivin. We conclude that this novel combination of PBOX compounds and flavopiridol may have the potential to be effective in the treatment of CML patients.

4.2 Results

4.2.1 Flavopiridol, PBOX-6 and PBOX-15 induce apoptosis in CML cells.

The novel CDK inhibitor flavopiridol has been shown to enhance the apoptotic effect of numerous MTAs including paclitaxel^{124; 125}. As the PBOXs have recently been identified as MTAs¹⁹⁹ it was hypothesised that flavopiridol could also augment PBOX-induced apoptosis. Previous combination studies involving flavopiridol and other chemotherapeutics such as MTAs have identified treatment order as being crucially important for apoptotic synergy. For example, Motwani *et al.*, (1999)¹²⁵ demonstrated that flavopiridol enhances paclitaxel-induced apoptosis in a sequence-dependent manner, with paclitaxel treatment for 18 h followed by flavopiridol treatment for 24 h resulting in optimal induction of apoptosis. It was therefore decided to use a similar sequence dependent treatment regimen to assess the combination of the PBOX compounds and flavopiridol. However, it was first necessary to establish the effects of each compound alone.

Results showed that PBOX-6 and PBOX-15 caused a dose-dependent increase in the number of CML cells undergoing apoptosis as determined by flow cytometry (Section 2.7). Specifically, after 18 h, 5 μ M PBOX-6-treated K562 cells had undergone a small amount of apoptosis, $11.3 \pm 1.5\%$, while LAMA84 cells had undergone $12.5 \pm 4.9\%$ apoptosis. Apoptosis increased significantly to 26.3 ± 3.0 and $17.8 \pm 3.3\%$ following PBOX-6 treatment for 18 h followed by a further 24 h without any treatment (P₆₁₈NT₂₄) in K562 and LAMA84 cells respectively (Fig. 4.1A). Similar results were also seen with PBOX-15 (Fig. 4.1B). As the PBOX compounds were washed away following an 18 h treatment and cells were left to grow in drug free medium for a further 24 h this induction of apoptosis was considered to be irreversible.

Flavopiridol arrested K562 and LAMA84 cells in the G2/M phase of the cell cycle after 24 h (Fig. 4.2A). For K562 cells, a significant dose-dependent increase in the number of cells in the G2/M phase was observed increasing from $20.0 \pm 2.8\%$ for vehicle-treated cells to $30.7 \pm 1.1\%$ for cells treated with 500nM flavopiridol. However, for LAMA84 cells, the number of cells in the G2/M phase rose from $20.5 \pm 2.0\%$ for vehicle-treated cells to a maximum of $30.2 \pm 2.6\%$ following treatment with 200nM flavopiridol before dropping back down to $20.4 \pm 2.0\%$ with 500nM flavopiridol treatment (Fig. 4.2A). This

subsequent reduction in the G2/M phase back down to levels more associated with vehicle-treated samples corresponded to an increase in the number of cells undergoing apoptosis.

Treatment for 24 h with flavopiridol resulted in a dose-dependent increase in apoptosis in LAMA84 cells from 3.1 ± 1.0 to $17.9 \pm 2.3\%$ for cells treated with a vehicle compared to those treated with 500nM flavopiridol. K562 cells appeared less susceptible to flavopiridol-induced apoptosis and while flavopiridol did induce a small amount of apoptosis in these cells, it was negligible (Fig. 4.2B).

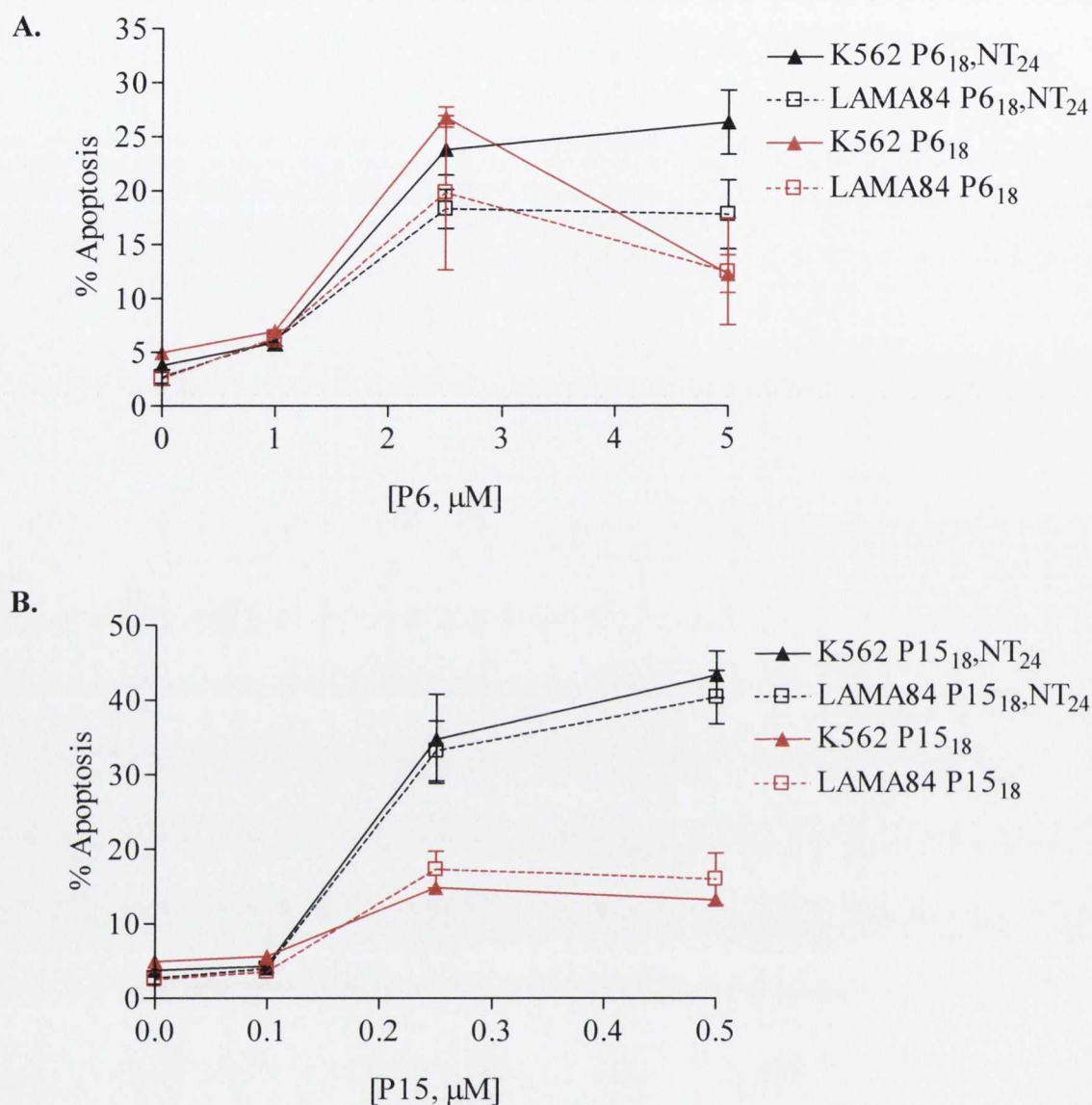


Fig. 4.1 The pro-apoptotic PBOX compounds irreversibly induce apoptosis in CML cells.

CML cells were seeded during the log phase of growth and treated for 18 h with a vehicle (0.1% (v/v) ethanol) or PBOX-6/-15 (P6, P15) and either analysed immediately or centrifuged and resuspended in drug free medium followed by a further 24 h without treatment (NT). After the required incubation period, cells were fixed in 1ml ethanol and 100 μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak.

A. PBOX-6 and **B.** PBOX-15. Values represent the mean $\pm$ S.E.M. of three independent experiments.

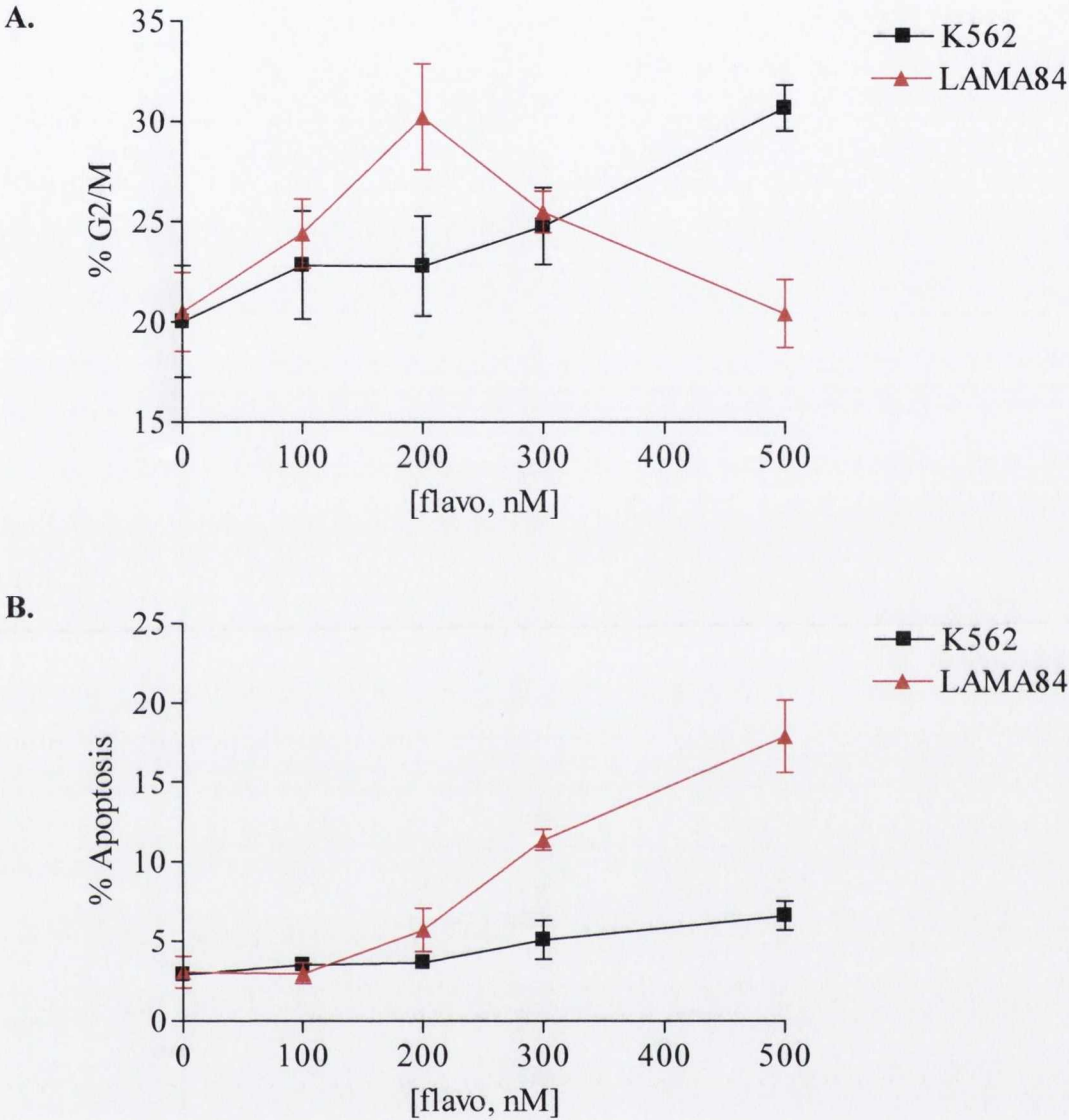


Fig. 4.2 Flavopiridol induces a G2/M arrest and apoptosis in CML cells.

CML cells were seeded during the log phase of growth and treated for 24 h with a vehicle (0.005% (v/v) DMSO) or flavopiridol (flavo). After the required incubation period, cells were fixed in 1ml ethanol and 100µl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry for **A.** the percentage of cells in the G2/M phase of the cell cycle and **B.** apoptosis as determined by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments.

4.2.2 Flavopiridol augments the apoptotic effect of the pro-apoptotic PBOX compounds PBOX-6 and PBOX-15 in CML cells.

Flow cytometry demonstrated that CML cells underwent a dose-dependent increase in apoptosis when treated for 18 h with PBOX-6 or PBOX-15 followed by a further 24 h with flavopiridol treatment when compared to either agent alone. Specifically, the number of K562 cells undergoing apoptosis increased from 6.5 ± 0.4 to $17.4 \pm 6.4\%$ when treated with $1\mu\text{M}$ PBOX-6, from 18.1 ± 1.3 to $41.9 \pm 8.4\%$ with $2.5\mu\text{M}$ PBOX-6 and from 16.6 ± 1.5 to $53.1 \pm 2.7\%$ with $5\mu\text{M}$ PBOX followed by flavopiridol (500nM) treatment (Fig. 4.3A).

Similar enhancements in apoptosis were also seen in PBOX-15-treated K562 cells (Fig. 4.3B). As this increase in apoptosis was strongest with PBOX-6, it was decided to focus on PBOX-6 as a representative compound for further analysis. LAMA84 cells were therefore also treated with PBOX-6 followed by flavopiridol and results also showed a significant enhancement of apoptosis following the sequential addition of flavopiridol when compared to cells treated with PBOX-6 alone (Fig. 4.4).

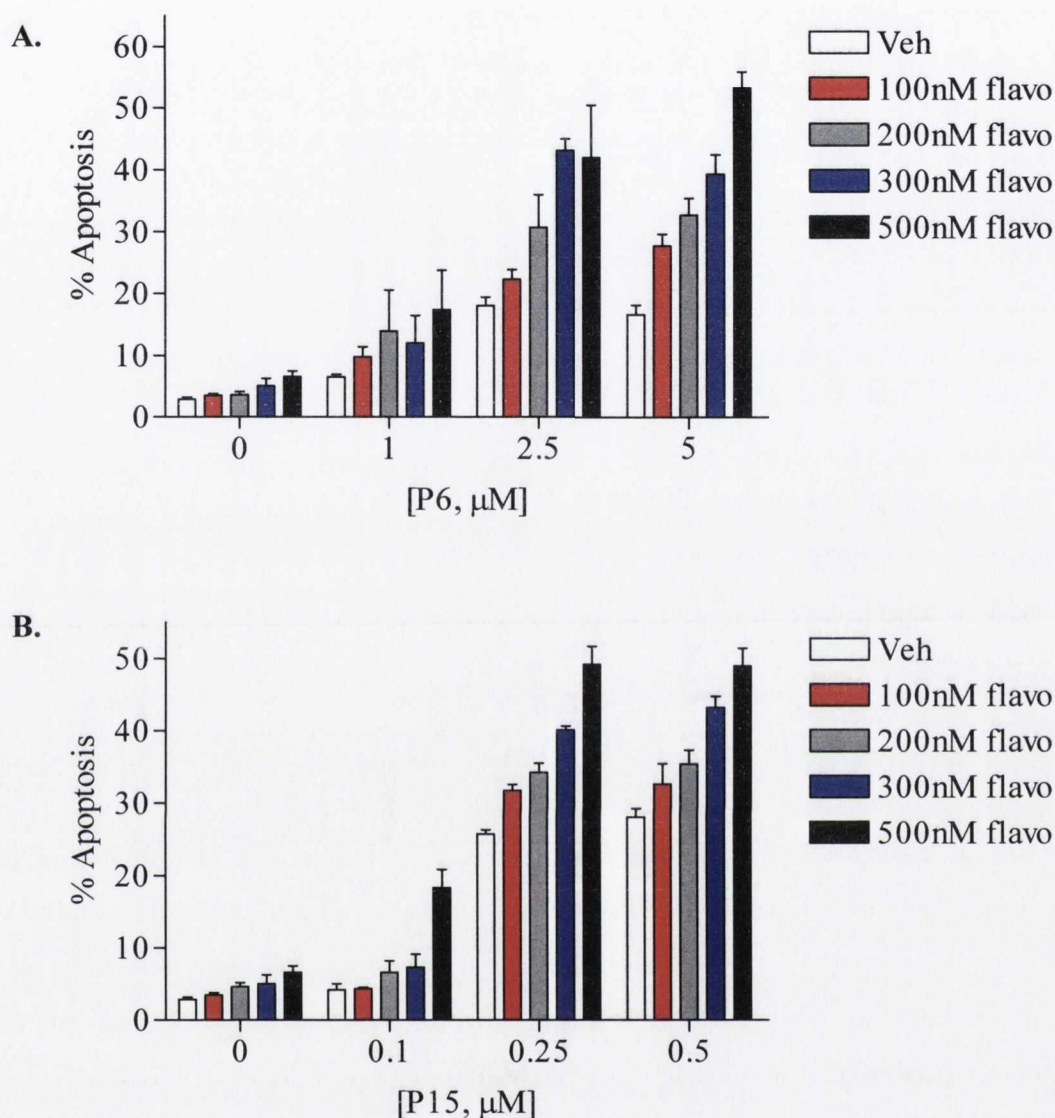


Fig. 4.3 Sequential treatment with PBOX-6/PBOX-15 and flavopiridol enhances apoptosis compared to either agent alone in K562 cells.

K562 cells were treated for 18 h with PBOX-6 (P6) (1 μ M to 5 μ M) or PBOX-15 (P15) (0.1 μ M to 0.5 μ M), centrifuged and resuspended in fresh medium containing flavopiridol (flavo) (100nM to 500nM) for a further 24 h or treated with the relevant controls. After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. **A.** PBOX-6 and **B.** PBOX-15. Values represent the mean $\pm$ S.E.M. of three independent experiments.

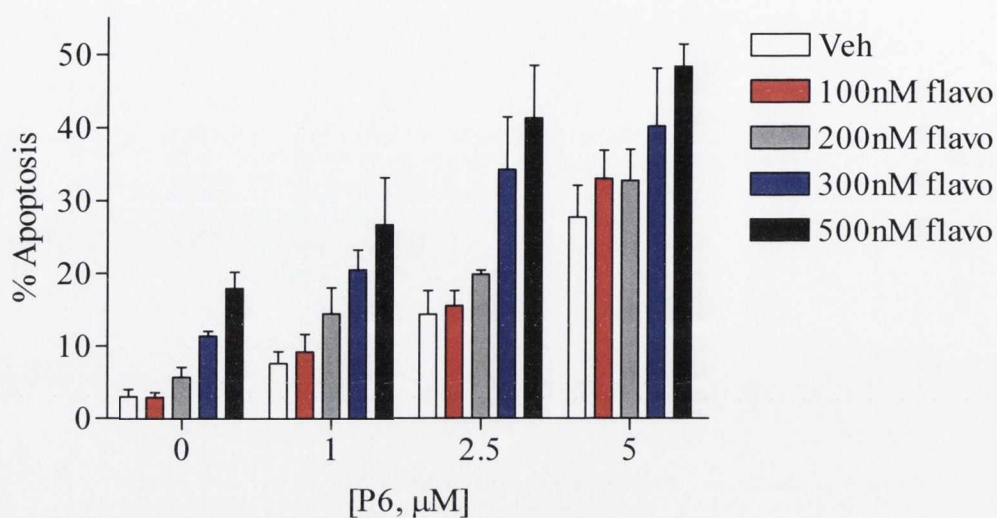


Fig. 4.4 Sequential treatment with PBOX-6 and flavopiridol enhances apoptosis compared to either agent alone in LAMA84 cells.

LAMA84 cells were treated for 18 h with PBOX-6 (P6) (1μM to 5μM), centrifuged and resuspended in fresh medium containing flavopiridol (flavo) (100nM to 500nM) for a further 24 h or treated with the relevant controls. After the required incubation period, cells were fixed in 1ml ethanol and 100μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments.

4.2.3 *Flavopiridol synergistically enhances PBOX-induced apoptosis.*

To evaluate whether the novel combination of PBOX compounds together with flavopiridol resulted in a synergistic increase in apoptosis, CI values were determined. Median dose analysis showed that at all concentrations of PBOX-6 (1 μ M to 5 μ M) and PBOX-15 (100nM to 500nM) tested in K562 cells, a synergistic increase in apoptosis was observed following sequential treatment with flavopiridol (500nM) (Fig. 4.5-6, Table 4.1).

In K562 cells treated with PBOX-6 and flavopiridol, CI values ranged from 0.375 to 0.109 indicating synergism (+++) to strong synergism (++++). Similar results were obtained in K562 cells treated with PBOX-15/flavopiridol with CI values of 0.659 to 0.328 indicating synergism (+++) (Fig. 4.6).

These results were confirmed in LAMA84 cells. LAMA84 cells treated with PBOX-6 and flavopiridol showed CI values ranging from 0.865 to 0.677 indicating slight synergism (+) to synergism (+++) (Fig. 4.7, Table 4.2).

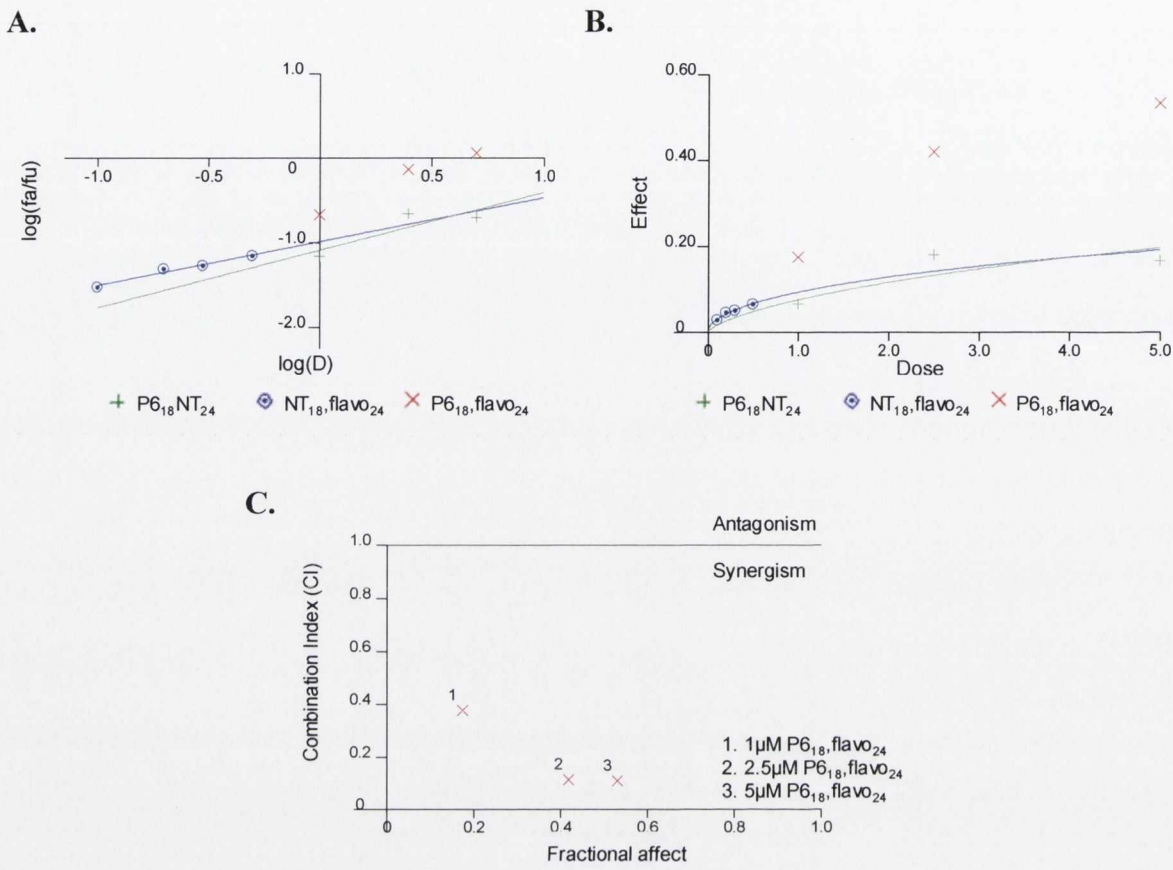


Fig. 4.5 Flavopiridol synergistically enhances PBOX-6-induced apoptosis in K562 cells.

Median dose effect analysis was carried out on K562 cells using the software Calcsyn. Cells were treated with PBOX-6 (P6) ($1\mu M$ to $5\mu M$) for 18 h, centrifuged and resuspended in fresh medium containing flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). **A.** Median dose plot, **B.** dose-effect curve and **C.** CI versus fraction affected. A CI value of >1 , $=1$ and <1 indicates antagonism, an additive effect and synergism respectively.

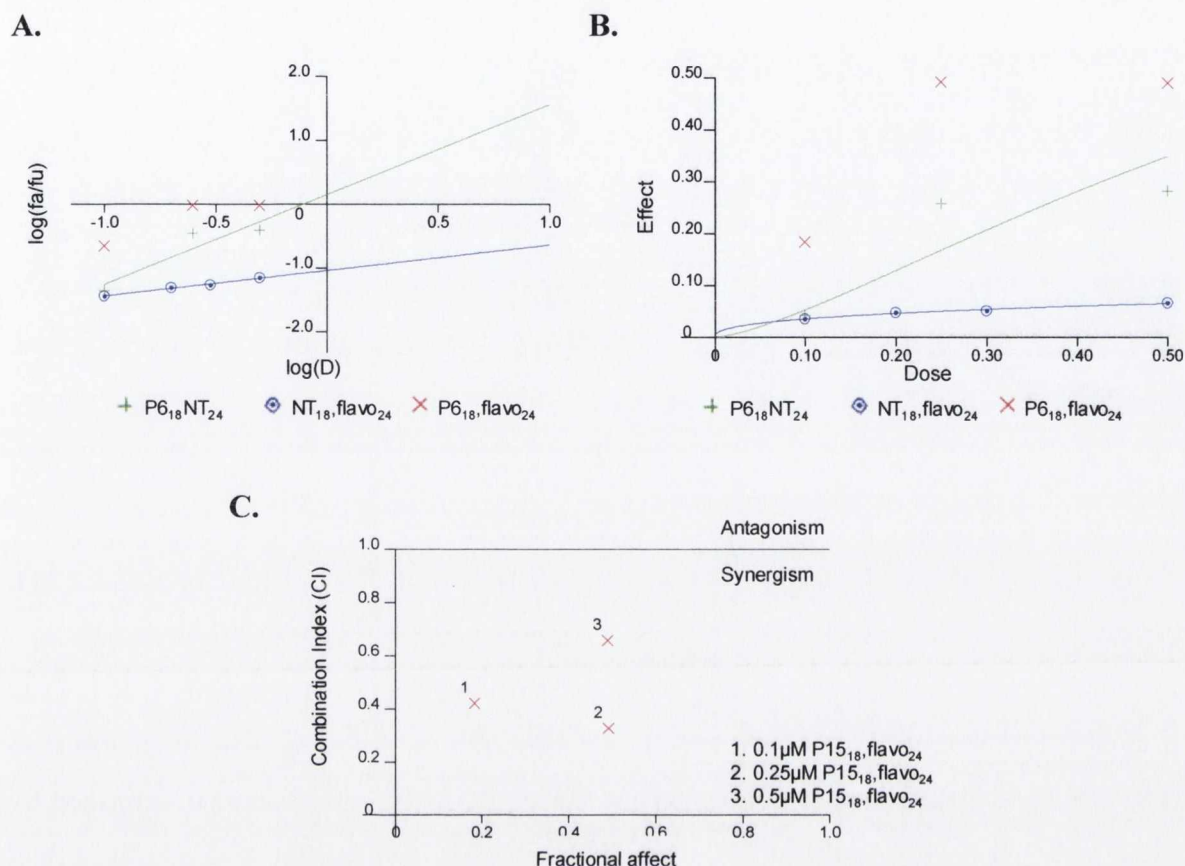


Fig. 4.6 Flavopiridol synergistically enhances PBOX-15-induced apoptosis in K562 cells.

Median dose effect analysis was carried out on K562 cells using the software Calcsyn. Cells were treated with PBOX-15 (P15) (100nM to 500nM) for 18 h, centrifuged and resuspended in fresh medium containing flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). **A.** Median dose plot, **B.** dose-effect curve and **C.** CI versus fraction affected. A CI value of >1 , $=1$ and <1 indicates antagonism, an additive effect and synergism respectively.

	[PBOX, μ M]	[flavo, μ M]	Fa	CI Value	Rating
K562 P6	1	0.5	0.174	0.375	+++
	2.5	0.5	0.419	0.114	++++
	5	0.5	0.531	0.109	++++
K562 P15	0.1	0.5	0.184	0.421	+++
	0.25	0.5	0.492	0.328	+++
	0.5	0.5	0.490	0.659	+++

Table 4.1 CI values of PBOX-6/-15 in combination with flavopiridol in K562 CML cells.

Synergistic analysis was carried out using the software Calcsyn (Biosoft, U.K.). Data from flow cytometry experiments was used to analyse the interaction between PBOX-6 (P6)/PBOX-15 (P15) and flavopiridol (flavo) using an isobologram method described by Chou and Talalay. A CI value of >1, =1 and <1 indicates antagonism (-), an additive effect ($\pm$) and synergism (+) respectively (Section 2.6). Fa - fraction of cells affected by apoptosis.

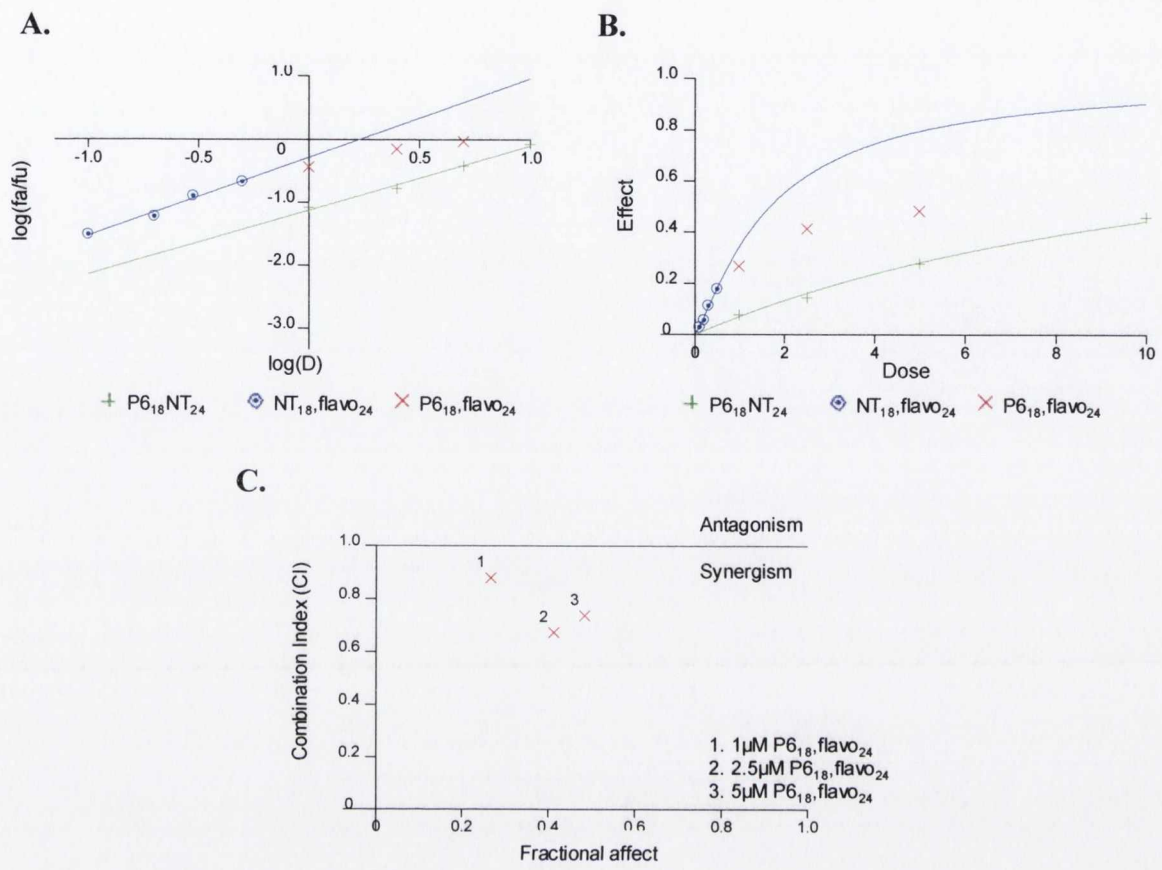


Fig. 4.7 Flavopiridol synergistically enhances PBOX-6-induced apoptosis in LAMA84 cells.

Median dose effect analysis was carried out on LAMA84 cells using the software Calcsyn. Cells were treated with PBOX-6 (P6) (100nM to 500nM) for 18 h, centrifuged and resuspended in fresh medium containing flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). **A.** Median dose plot, **B.** dose-effect curve and **C.** CI versus fraction affected. A CI value of >1, =1 and <1 indicates antagonism, an additive effect and synergism respectively.

	[PBOX, μM]	[flavo, μM]	Fa	CI Value	Rating
LAMA84 P6	1	0.5	0.267	0.865	+
	2.5	0.5	0.413	0.64	+++
	5	0.5	0.484	0.677	+++

Table 4.2 CI values of PBOX-6 in combination with flavopiridol in LAMA84 CML cells.

Synergistic analysis was carried out using the software Calcosyn (Biosoft, U.K.). Data from flow cytometry experiments was used to analyse the interaction between PBOX-6 (P6) and flavopiridol (flavo) using an isobologram method described by Chou and Talalay. A CI value of >1 , $=1$ and <1 indicates antagonism (-), an additive effect ($\pm$) and synergism (+) respectively (Section 2.6). Fa - fraction of cells affected by apoptosis.

4.2.4 *Flavopiridol enhances PBOX-6-induced apoptosis only when administered after PBOX-6 treatment.*

Previous combination studies involving flavopiridol and other chemotherapeutics have identified treatment order as being crucially important for apoptotic synergy^{124; 125}. To determine if this was also the case with our novel combination treatment we decided to analyse the extent of apoptosis following combination treatment with both compounds administered concurrently and following treatment in the reverse order; flavopiridol followed by PBOX-6. Results showed that cells treated with the combined compounds (P6+flavo)₁₈ followed by no treatment (NT₂₄) underwent a lower percentage of apoptosis than those cells treated with 5 μ M PBOX-6 followed by 500nM flavopiridol (P₁₈,F₂₄), $18.7 \pm 7.7\%$ versus $54.8 \pm 1.1\%$ respectively. Alone, PBOX-6 caused $29.2 \pm 7.1\%$ of cells to undergo apoptosis (Fig. 4.8).

In the same experiments repeated with the compounds given in reverse order (i.e. flavopiridol first for 18 h followed by PBOX-6 for 24 h), cells had undergone $13.8 \pm 2.3\%$ apoptosis compared to 13.7 ± 3.9 and $3.0 \pm 0.0\%$ apoptosis for cells treated with PBOX-6 and flavopiridol alone (Fig. 4.9). Sequential treatment with flavopiridol followed by PBOX-6 therefore appeared to induce apoptosis levels similar to that observed when cells were treated with PBOX-6 alone.

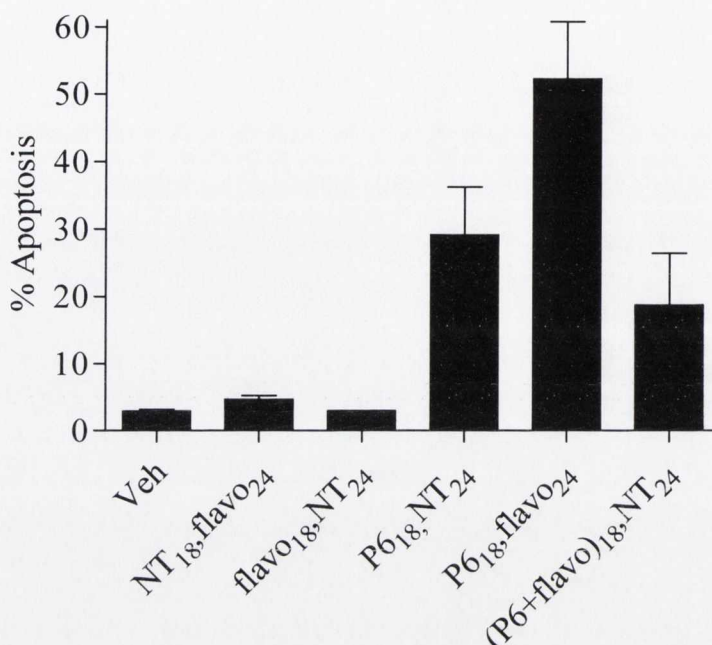


Fig. 4.8 Treatment of K562 CML cells with PBOX-6 and flavopiridol concurrently does not enhance apoptosis compared to either agent alone.

K562 cells were treated for 18 h, followed by a further 24 h with the indicated treatments: 5 μ M PBOX-6 (P6), 500nM flavopiridol (flavo) or the relevant controls (NT). After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments.

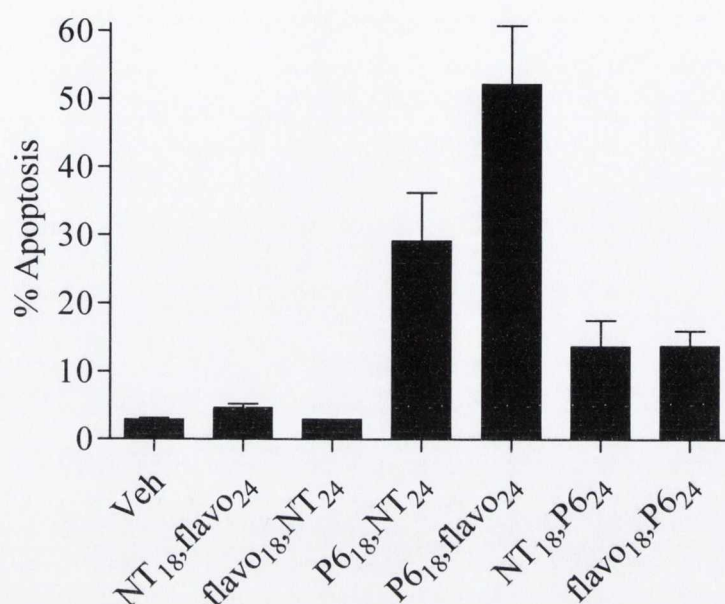


Fig. 4.9 Sequential treatment of K562 CML cells with flavopiridol followed by PBOX-6 does not enhance apoptosis compared to either agent alone.

K562 cells were treated for 18 h, followed by a further 24 h with the indicated treatments: 5 μ M PBOX-6 (P6), 500nM flavopiridol (flavo) or the relevant controls (NT). After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments.

4.2.5 *Flavopiridol reduces the number of polyploid cells associated with PBOX-6 treatment.*

MTAs function via the alteration of microtubule dynamics during mitosis and activation of the mitotic spindle checkpoint. K562 cells which are p53-deficient have been shown to undergo endoreplication in response to MTAs cells (polyploidy)^{226, 230}. Previously, CDK inhibitors such as flavopiridol have been shown to prevent MTA-induced endoreplication in human cancer cells²²⁶. This prompted us to examine whether flavopiridol affects the number of polyploid cells formed following PBOX-6 treatment in K562 cells and whether this contributes to the synergistic enhancement of PBOX-6-induced apoptosis by flavopiridol.

K562 cells were treated with 5 μ M PBOX-6 for 18 h followed by sequential treatment with 500nM flavopiridol for a further 24 h. PBOX-6 caused $5.3 \pm 1.4\%$ of cells to undergo polyploidy, this was reduced, although not significantly, to $1.3 \pm 0.1\%$ following the addition of flavopiridol (p value: 0.1026, ns) (Fig. 4.10A). Similar results were also observed in LAMA84 cells (Fig. 4.10B). While PBOX-6 has been shown to induce up to 40% polyploidy in K562 following a 48 h treatment¹⁹⁰ the lower number of polyploid cells observed after 18 h PBOX treatment made it difficult to determine the true significance of the effect of flavopiridol on this cell population.

For these reasons, results were confirmed by indirect immunofluorescence where cells were stained green (tubulin) and red (nucleus). Control samples showed tubulin evenly spread throughout the cytoplasm, intact singular nuclei and cells displaying a round morphology. In PBOX-6-treated samples, some cells contained up to three or four nuclei, indicative of polyploidy. The number of these cells appeared to be reduced in PBOX-6 and flavopiridol-treated samples. Other morphological changes observed during immunofluorescence showed blebbing of the membrane following flavopiridol treatment whilst combination samples showed partial degradation of the nucleus. In comparison, paclitaxel, a tubulin polymeriser caused tubulin bundling (Fig. 4.11).

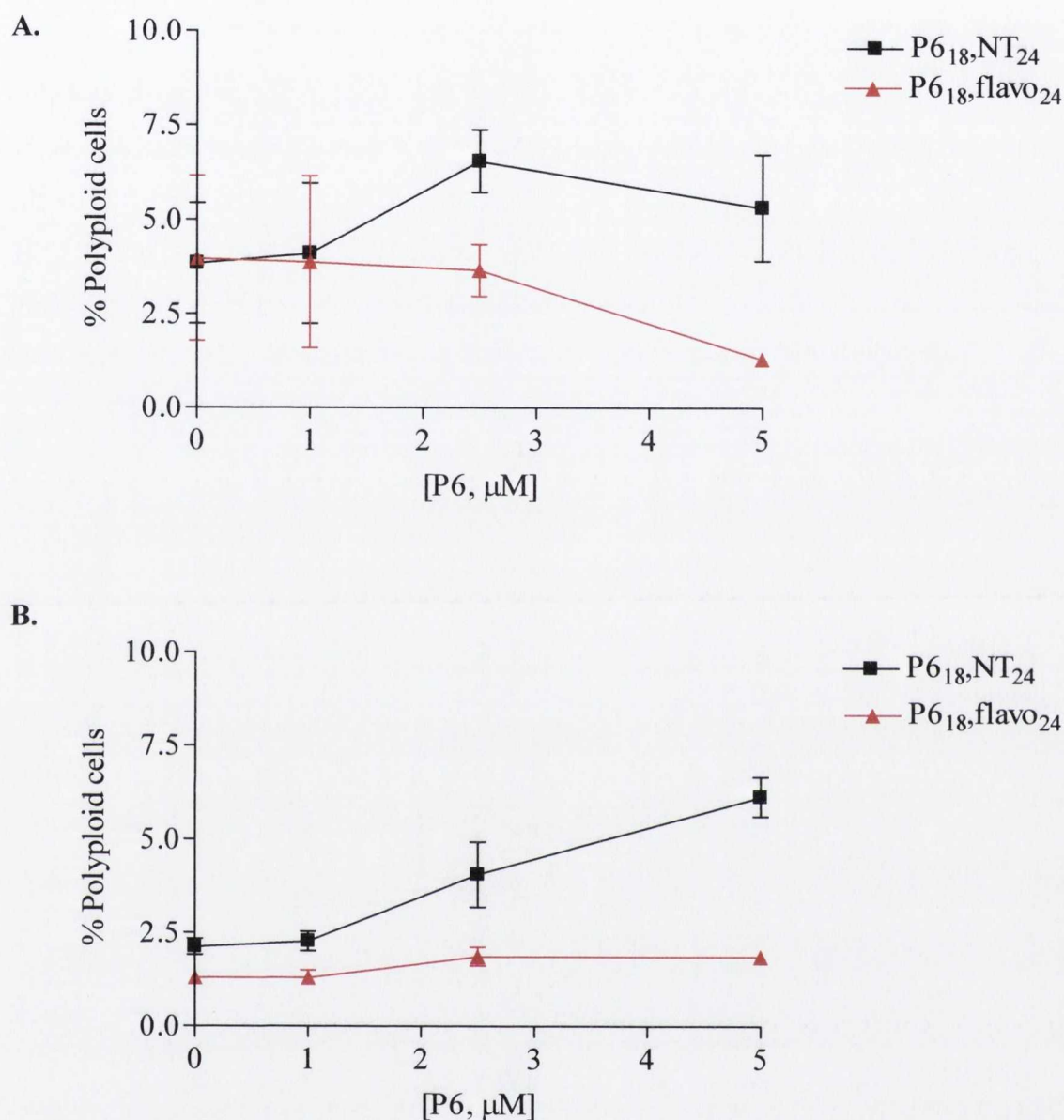
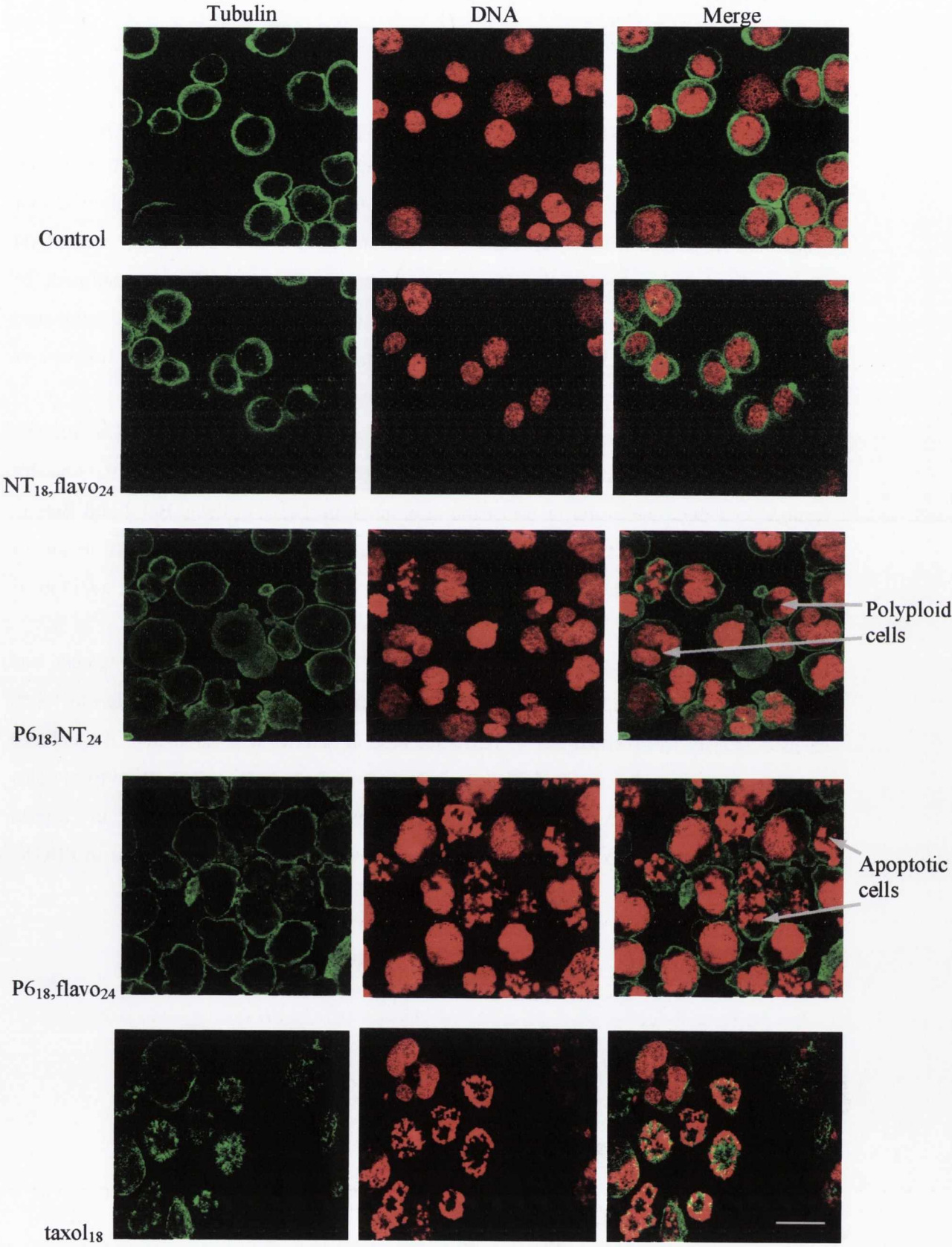


Fig. 4.10 Flavopiridol reduces polyploidy induced by PBOX compounds in CML cells.

CML cells were treated for 18 h with PBOX-6 (P6) (1 μM to 5 μM), centrifuged and resuspended in fresh medium containing 500 nM flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). After the required incubation period, cells were fixed in 1 ml ethanol and 100 μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry; polyploidy was defined as cells containing $>4\text{N}$ DNA content. **A.** K562 and **B.** LAMA84 cells. Values represent the mean $\pm$ S.E.M. of three independent experiments.

Fig. 4.11 The effect of sequential treatment with PBOX-6 and flavopiridol on polyploid cells.

K562 cells were treated for 18 h with 5 μ M PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500 nM flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). Paclitaxel (taxol) (1 μ M) samples were treated for 18 h. After the required incubation period, samples were prepared for immunofluorescence (Section 2.12). Cells were cytocentrifuged, fixed, permeabilised and incubated with a primary anti- α -tubulin and secondary Alexa 488 mAb. DNA (red) and microtubules (green) were then visualised by confocal microscopy. Bar represents 20 μ m. Results are representative of three independent experiments.



4.2.6 Uncovering part of the apoptotic signalling pathway induced by PBOX-6/flavopiridol treatment.

As our previous results had determined flavopiridol to successfully enhance PBOX-6-induced apoptosis, it was next important to ascertain some of the apoptotic signalling pathways involved with this novel combination treatment. Western blotting showed that sequential treatment with 5 μ M PBOX-6 followed by 500nM flavopiridol caused an increase in the cleavage of the DNA repair enzyme PARP, a classical hallmark of apoptosis, when compared to each agent alone. This together with the flow cytometric results confirmed the cells were undergoing apoptosis as opposed to other forms of cell death such as necrosis or apoptosis-like necrosis (Fig. 4.12).

Members of the Bcl-2 family of proteins were found to be involved in PBOX-6/flavopiridol-induced apoptosis. Treatment of K562 cells with PBOX-6 and flavopiridol resulted in a decrease in the levels of the anti-apoptotic Bcl-2 proteins Bcl-2 and Bcl-X_L. The anti-apoptotic Bcl-2 protein, Mcl-1, was downregulated following PBOX-6 treatment alone however, no further downregulation was observed with the subsequent addition of flavopiridol (Fig. 4.13).

The involvement of caspase-3/7 in PBOX-6/flavopiridol-induced apoptosis was next examined by the Apo-ONE caspase activity assay. Vehicle samples were set to 100% caspase activity from which any increase/decrease in activity was calculated. An increase in caspase activity was observed for the combination treatment when compared to samples treated with PBOX-6 and flavopiridol alone, however while this increase in caspase activity was not significant these results may still indicate a role for caspases in PBOX-6/flavopiridol treatment of CML cells (Fig. 4.14).

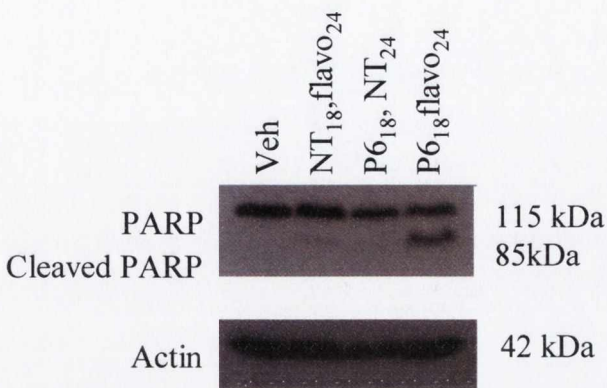


Fig. 4.12 Sequential treatment with PBOX-6 and flavopiridol increases cleavage of the enzyme PARP.

Exponentially growing K562 cells were treated for 18 h with 5µM PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500nM flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). After the required incubation period, samples were prepared for Western blotting (Section 2.10). Samples (20µg of protein) were run on a 12% gel and transferred for 1 h. PVDF membranes were incubated with an anti-PARP mAb overnight and enhanced with ECL. Actin is shown as a loading control. Results are representative of three independent experiments.

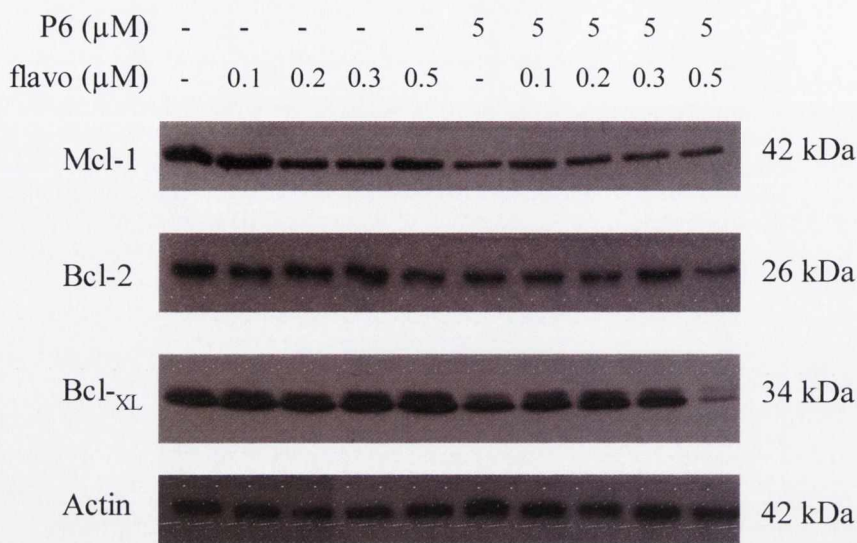


Fig. 4.13 The effect of sequential treatment with PBOX-6 and flavopiridol on members of the Bcl-2 family.

Exponentially growing K562 cells were treated for 18 h with 5μM PBOX-6 (P6), centrifuged and resuspended in fresh medium containing a range of concentrations of flavopiridol (flavo) (100nM to 500nM) for a further 24 h or treated with the relevant controls. After the required incubation period, samples were prepared for Western blotting (Section 2.10). Samples (20μg of protein) were run on a 12% gel and transferred for 30 min. PVDF membranes were incubated with an anti-Mcl-1, Bcl-2 or Bcl-X_L mAb overnight and enhanced with ECL. Actin is shown as a loading control. Results are representative of three independent experiments.

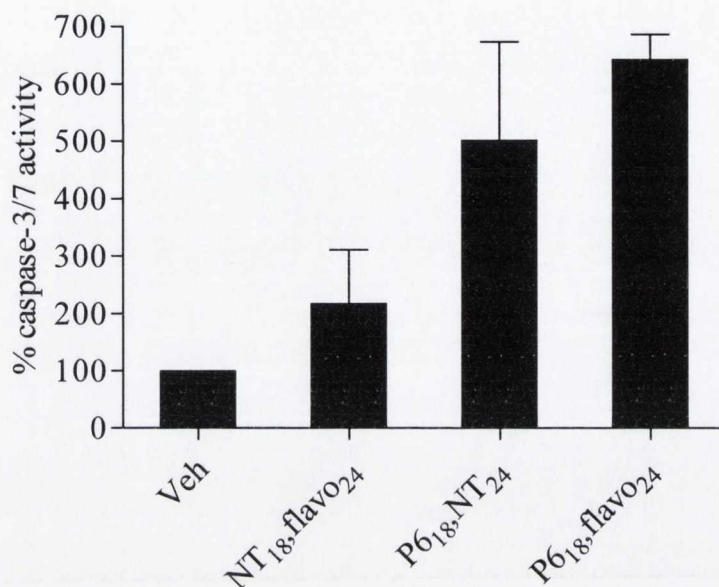


Fig. 4.14 The effect of combination treatment on caspase-3/7 activity.

K562 cells were seeded at a density of 2×10^5 cells/ml in 100 μ l medium in a 96-well plate and treated for 18 h with 5 μ M PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500 nM flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). After the 42 h incubation, 100 μ l of the Apo-ONE reagent was added to the cells. Plates were incubated at room temperature for 1 h in the dark on an orbital plate shaker. Plates were then read on a SpectraMax Gemini fluorescence plate reader at excitation and emission λ of 499 nm and 521 nm respectively. Values represent the mean $\pm$ S.E.M. of three independent experiments.

4.2.7 An accelerated exit from the G2/M transition with flavopiridol facilitates enhanced PBOX-induced apoptosis.

In order to determine the molecular basis of the enhancement of PBOX-induced apoptosis by flavopiridol, we characterised the cell cycle events associated with the combination treatment. Flow cytometry results showed PBOX-induced apoptosis to be preceded by a G2/M arrest in K562 cells. The number of cells in the G2/M phase began to increase after a 4 h treatment with 5 μ M PBOX-6 to $33.2 \pm 1.7\%$ reaching a peak after 18 h of $66.7 \pm 0.8\%$ before dropping back to similar levels observed in vehicle samples after 42 h of $19.4 \pm 2.5\%$ (Fig. 4.15A). The subsequent drop in the percentage of G2/M-arrested cells corresponded to an increase in the number of cells undergoing apoptosis, 14.6 ± 2.2 and $25.5 \pm 0.9\%$ after 26 and 42 h respectively (Fig. 4.15B).

When cells were treated with PBOX-6 for the longer period of 42 h (lack of G2/M arrest, Fig. 4.15A) the subsequent addition of flavopiridol for a further 24 h did not significantly enhance apoptosis. While some enhancement of apoptosis was still observed, the level of apoptosis was still lower than that of the combination treatment involving the shorter PBOX-treatment where cells had been arrested in the G2/M phase (Fig. 4.16).

Next, cells were treated with PBOX-6 for 18 h, the peak of G2/M arrest, and then subsequently resuspended in fresh medium with or without 500nM flavopiridol for up to 24 h and analysed for the number of G2/M associated cells. Even in the absence of flavopiridol, removal of PBOX-6 caused a reduction in the number of cells in the G2/M phase of the cell cycle suggesting a requirement for PBOX-6 in maintaining G2/M arrest. Treatment with flavopiridol enhanced the exit of cells from the G2/M phase of the cell cycle (Fig. 4.17A). Specifically, the number of G2/M-arrested cells reduced from 56.3 ± 3.8 to $35.5 \pm 4.2\%$ following just 2 h of treatment with flavopiridol when compared to cells incubated in drug-free medium for 2 h. This corresponded to a time-dependent increase in the number of cells undergoing apoptosis (Fig. 4.17B).

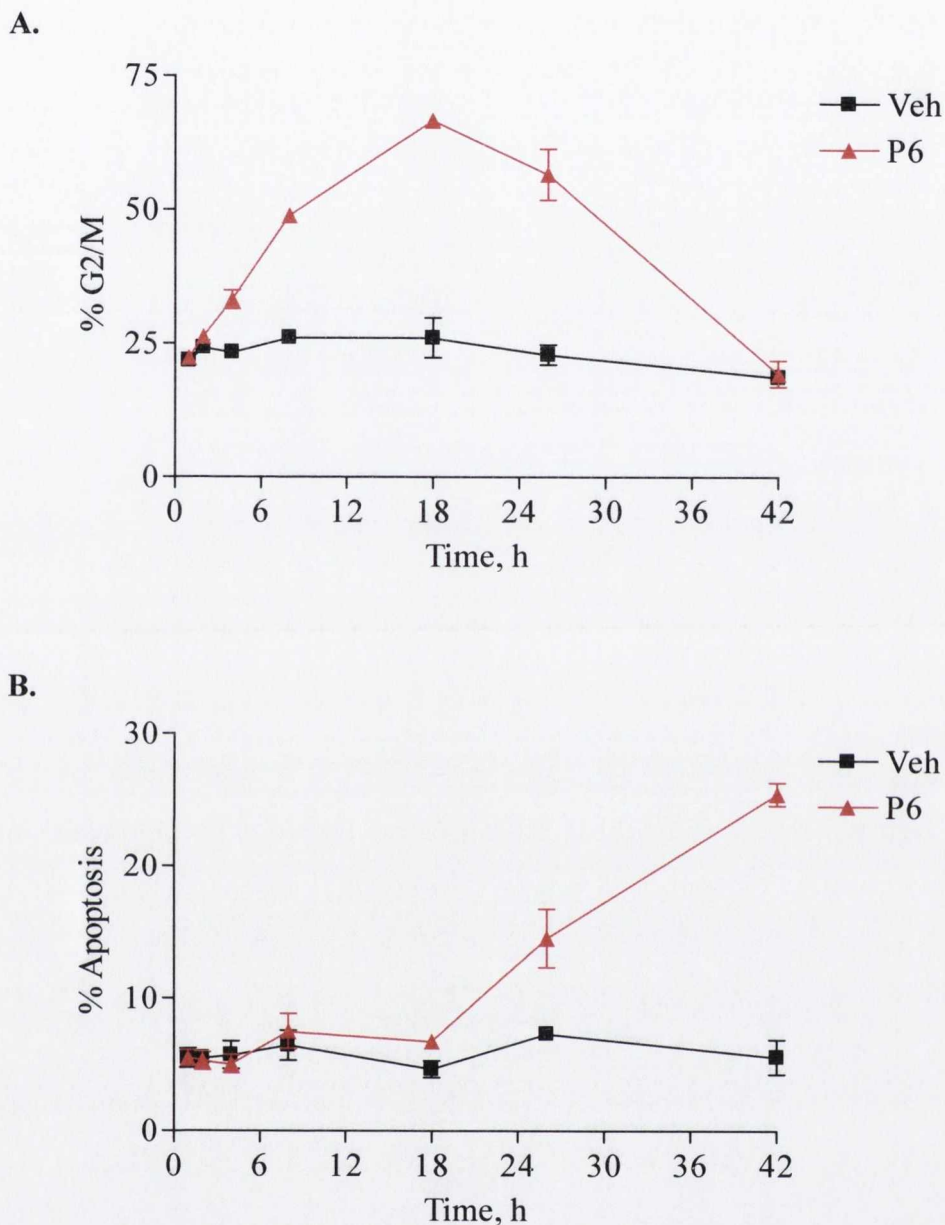


Fig. 4.15 PBOX-6-induced apoptosis in K562 CML cells is preceded by a G2/M arrest.

K562 cells were treated for up to 42 h with a vehicle (Veh) (0.1% (v/v) ethanol) or 5 μ M PBOX-6 (P6). After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry for **A.** the percentage of cells in the G2/M phase of the cell cycle and **B.** apoptosis as determined by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments.

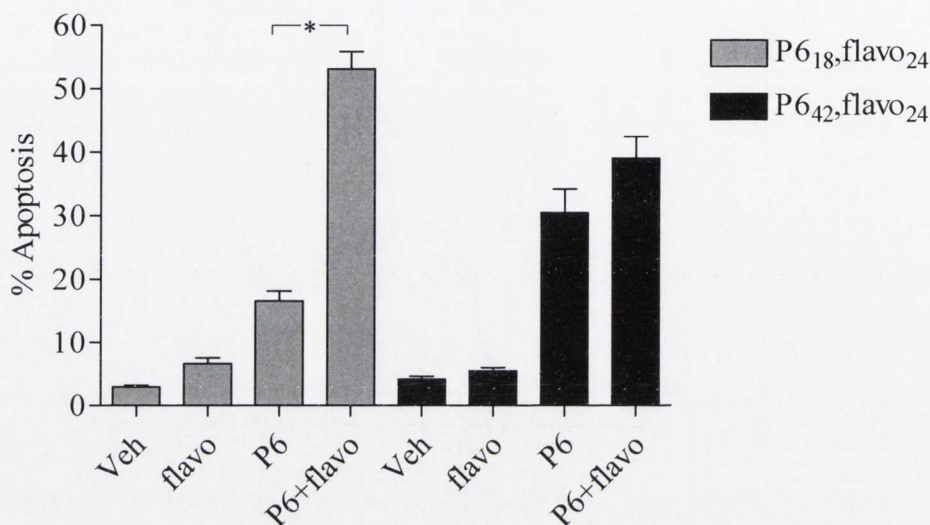


Fig. 4.16 G2/M-arrested cells show a significant increase in apoptosis following flavopiridol treatment compared to non-G2/M-arrested cells.

K562 cells were treated for up to 42 h with 5 μ M PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500nM flavopiridol (flavo) for a further 24 h or treated with the relevant controls. After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS, stained with propidium iodide and subsequently analysed by flow cytometry. The percentage of apoptosis was determined by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired t-test, *, $p < 0.05$.

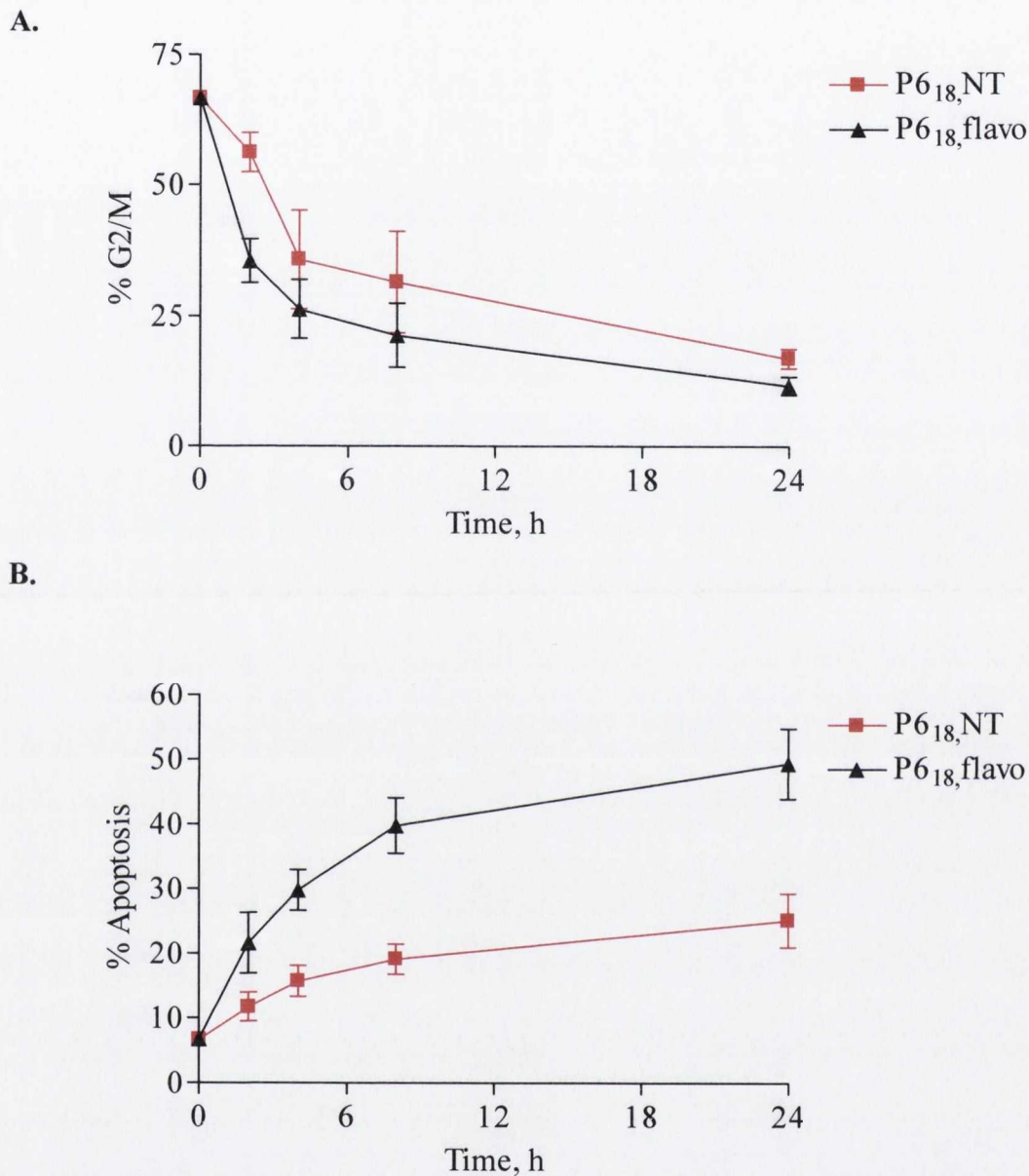


Fig. 4.17 Flavopiridol enhanced PBOX-6-induced apoptosis is associated with a time-dependent reduction in the number of cells in the G2/M phase.

K562 cells were treated for 18 h with 5 μ M PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500nM flavopiridol (flavo) for up to 24 h or treated with the relevant controls (NT). After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry for **A.** the percentage of cells in the G2/M phase of the cell cycle and **B.** apoptosis as determined by quantification of the pre-G1 peak. Values represent the mean $\pm$ S.E.M. of three independent experiments.

4.2.8 *The effect of PBOX-6 on the expression levels of cyclin B1, CDK1 and survivin.*

It is known that entrance to and exit from mitosis is regulated by CDK1/cyclin B1 activity. PBOX-6 has previously been shown to upregulate cyclin B1 levels in MCF-7 cells prior to the onset of a G2/M arrest¹⁹⁹. We sought to establish if PBOX-6 caused a similar effect in CML cells. K562 cells were subjected to SDS-PAGE and probed with an anti-cyclin B1 antibody (Section 2.10). Results showed cyclin B1 was upregulated following PBOX-6 treatment in K562 cells. This upregulation preceded G2/M arrest (Fig. 4.15) and was evident from 2 to 18 h. It may be suggested that cyclin B1-induced expression is at least partially responsible for G2/M arrest induced by PBOX-6. After 42 h PBOX-6 treatment the levels of cyclin B1 was substantially lower than that of the control sample (Fig. 4.18). This late downregulation of cyclin B1 correlated with loss from G2/M arrest and increased apoptosis by PBOX-6 at this time-point (Fig. 4.15).

We next examined the expression levels of CDK1. PBOX-6 had no effect on the expression levels of CDK1 until the late time-point of 42 h where CDK1 was seen to be downregulated. This may be a consequence of the increase in apoptosis observed at this time-point.

The IAP protein survivin is known to be upregulated in a large majority of cancers including CML. Activation occurs during mitosis via phosphorylation of Thr34 by the cell cycle complex CDK1/cyclin B1 and inhibition of this phosphorylation is associated with enhanced destruction of the protein. We hypothesised that PBOX-6, being an MTA, may have an effect on the expression levels of survivin. We therefore looked at the role of survivin in PBOX-6-induced apoptosis. Western blotting demonstrated that survivin was upregulated, with a maximum at 18 h, which correlated with the maximal G2/M arrest. At the later time-point of 42 h, full length survivin downregulation had occurred. Again this downregulation may be a consequence of the increase in apoptosis at this time-point (Fig. 4.18).

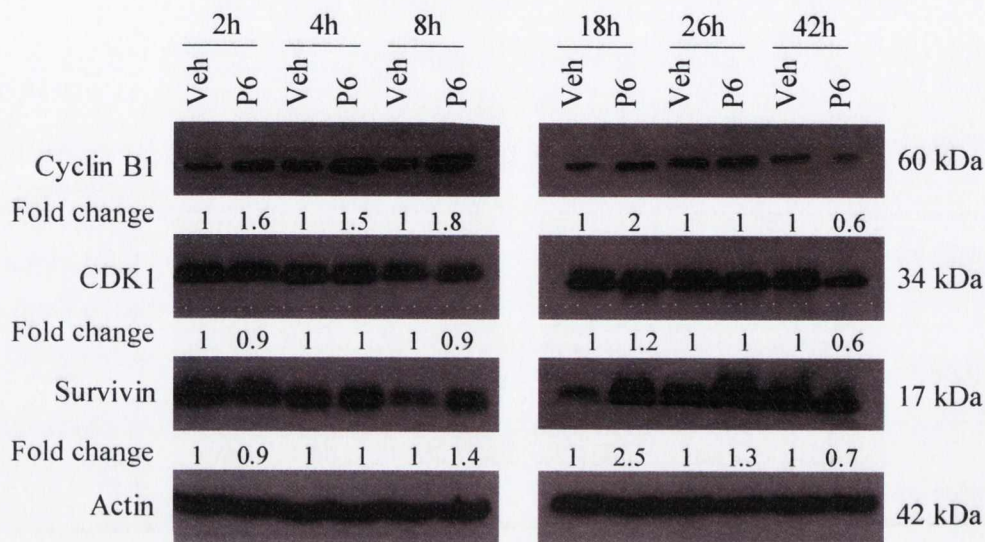


Fig. 4.18 The effect of PBOX-6 on the expression levels of cyclin B1, CDK1 and survivin.

K562 cells were treated for up to 42 h with a vehicle (Veh) (0.1% (v/v) ethanol) or 5 μ M PBOX-6 (P6). After the required incubation period, samples were prepared for Western blotting (Section 2.10). Samples (20 μ g of protein) were run on a 12% gel and transferred for 1 h. PVDF membranes were incubated with an anti-cyclin B1, CDK1 or survivin mAb overnight and enhanced with ECL. Actin is shown as a loading control. Band intensities were normalised by densitometry with respect to actin and expressed as fold change over vehicle control. Results are representative of three independent experiments.

4.2.9 PBOX-6/flavopiridol combination treatment is accompanied by an enhanced downregulation of cyclin B1 and survivin levels and decreased CDK1/cyclin B1 activity.

Combination studies in which cells were treated for 18 h with 5 μ M PBOX-6, the peak of G2/M arrest, and then subsequently resuspended in either PBOX-free medium or medium containing 500nM flavopiridol and incubated for 24 h showed an enhanced reduction in the expression levels of cyclin B1 compared to either compound alone (Fig. 4.19). A minimal reduction in the expression levels of CDK1 was also observed following PBOX-6/flavopiridol treatment as determined by Western blotting.

In the combination treatment survivin degradation was also enhanced in comparison with samples treated with PBOX-6 or flavopiridol alone (Fig. 4.19).

Samples were also immunoprecipitated with an anti-CDK1 mAb and subjected to a kinase activity assay using Histone H1 as a substrate (Section 2.10.9) to assess the activity levels of CDK1/cyclin B1. Results showed CDK1 activity had significantly decreased following the combination treatment (Fig. 4.20) when compared to samples treated with PBOX-6 alone (p value: 0.0198, *).

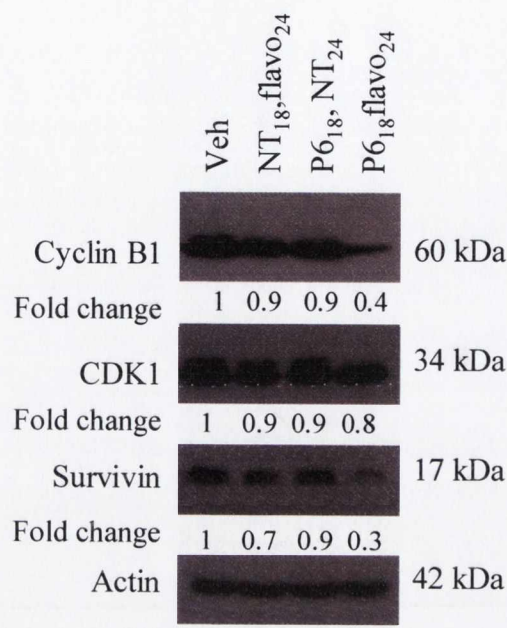


Fig. 4.19 PBOX-6/flavopiridol combination treatment is accompanied by an enhanced downregulation of cyclin B1 and survivin levels.

K562 cells were treated for 18 h with 5µM PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500nM flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). After the required incubation period, samples were prepared for Western blotting (Section 2.10). Samples (20µg of protein) were run on a 12% gel and transferred for 1 h. PVDF membranes were incubated with an anti-cyclin B1, CDK1 or survivin mAb overnight and enhanced with ECL. Actin is shown as a loading control. Band intensities were normalised by densitometry with respect to actin and expressed as fold change over vehicle control. Results are representative of three independent experiments.

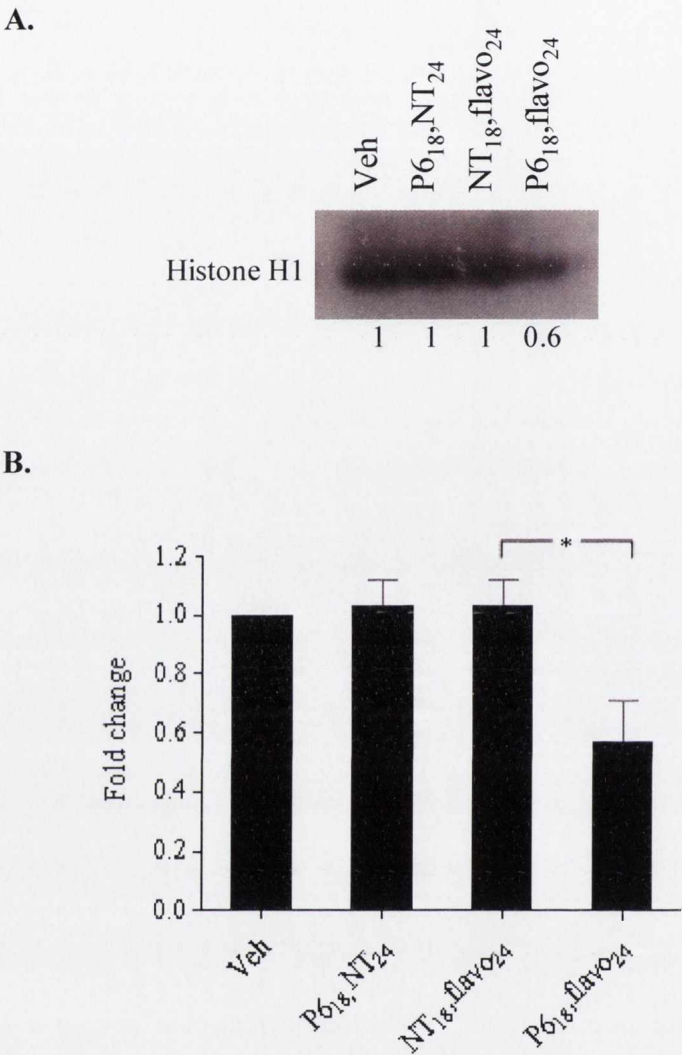


Fig. 4.20 PBOX-6/flavopiridol treatment reduces CDK1 kinase activity.

K562 cells were treated for 18 h with 5 μ M PBOX-6 (P6), centrifuged and resuspended in fresh medium containing 500nM flavopiridol (flavo) for a further 24 h or treated with the relevant controls (NT). After the required incubation period, samples were immunoprecipitated with an anti-CDK1 mAb and protein A beads and incubated with Histone H1 substrate and γ^{32} P-ATP (Section 2.10.9). Samples were then subjected to SDS-PAGE and detected by autoradiography. Band intensities are expressed as fold change over vehicle control. **A.** A representative autorad from three independent experiments. **B.** Bar-graph of autoradiography results.

4.2.10 *Inhibition of survivin by siRNA increases PBOX-6-induced apoptosis.*

To confirm the importance of survivin in PBOX-6-induced apoptosis, the survivin gene was knocked down for 24 h by transfection with siRNA sequences known to bind to and inhibit the targeted genes. Control cells were simultaneously transfected with a scrambled (sc) RNA sequence that does not bind to any known sequences, this was linked to the green fluorescent protein (GFP) gene to allow confirmation of efficient transfection rates with confocal microscopy after 24 h (Fig. 4.21A). Effective downregulation of the expression levels of the survivin protein was confirmed by Western blot analysis (Fig. 4.21B).

Following the knock-down of survivin levels, cells were sequentially treated with PBOX-6/flavopiridol. The number of apoptotic cells significantly increased from 25.0 ± 5.2 to $46.6 \pm 1.0\%$ (p value: 0.0380,*) in samples treated with $5\mu\text{M}$ PBOX-6. The number of apoptotic cells also increased from 51.7 ± 5.4 to $58.6 \pm 7.56\%$ in samples sequentially treated with PBOX-6 and flavopiridol however this difference was not significant (p value: 0.0602, ns). This further enhancement of apoptosis may be due to an even further reduction in survivin levels (Fig. 4.21C).

A proposed mechanism of action of flavopiridol enhanced PBOX-6-induced apoptosis is shown in Fig. 4.22. Artificial knock-down of survivin levels by siRNA evokes a similar enhancement of PBOX-6-induced apoptosis as flavopiridol treatment.

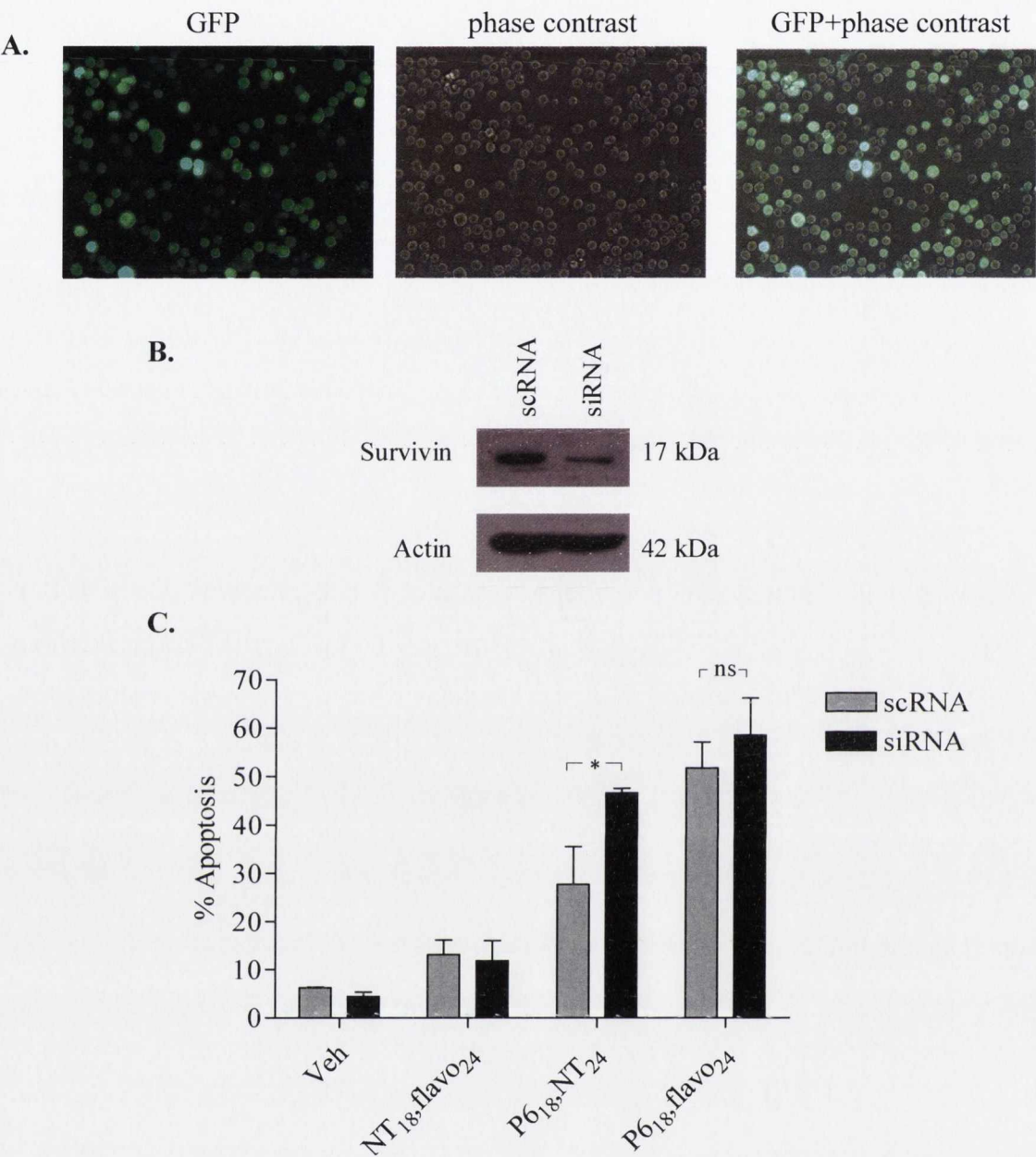


Fig. 4.21 A reduction in survivin levels by siRNA enhances PBOX-6-induced apoptosis.

K562 cells were transfected with siRNA targeting survivin or a control scRNA for 24 h. Following which samples were analysed for **A.** efficient transfection with GFP by confocal microscopy and **B.** efficient protein knock-down by Western blotting. **C.** Cells were subsequently treated for 18 h with 5µM PBOX-6 (P6) and a further 24 h with 500nM flavopiridol (flavo) or treated with the relevant controls (NT) and analysed by flow cytometry; apoptosis was determined by quantification of the pre-G1 peak. Values represent the mean ± S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired t-test, *, $p < 0.05$; ns, not significant.

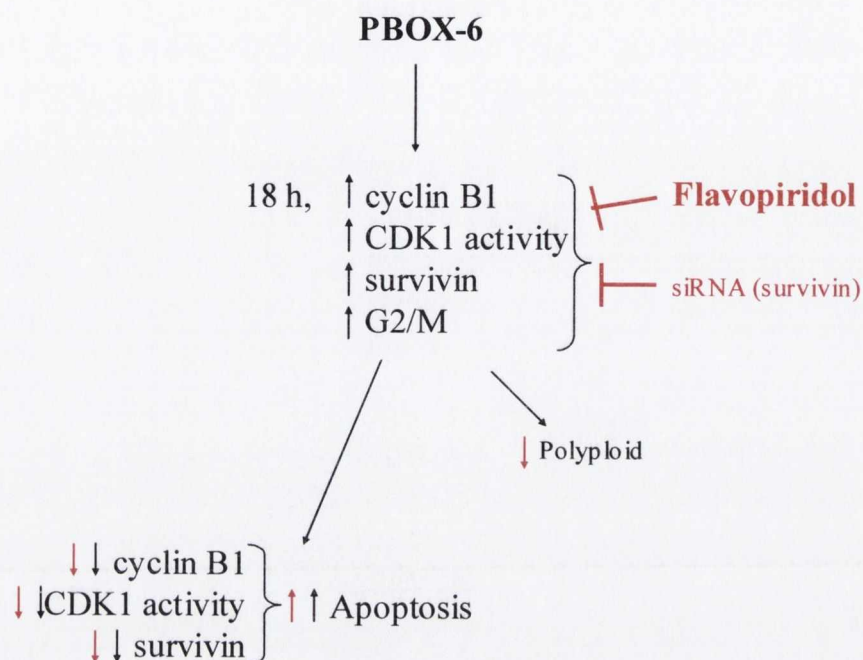


Fig. 4.22 Proposed mechanism of action of flavopiridol potentiation of PBOX-6-induced apoptosis.

PBOX-6 (black) administration causes a G2/M arrest and upregulation of the expression and activity levels of the CDK1/cyclin B1 complex and upregulation of survivin levels. This subsequently leads to the induction of apoptosis or a small amount of polyploidy. Sequential administration of flavopiridol (red) to PBOX-6 arrested cells accelerates the exit of cells from the G2/M arrest and the degradation of survivin leading to an enhanced level of apoptosis and reduced polyploidy. Inhibition of survivin by siRNA similarly enhances apoptosis.

4.3 Discussion

We have recently developed a series of novel compounds known as the PBOXs that induce apoptosis in numerous cancer cells including those derived from haematological malignancies such as CML and solid tumours such as breast carcinoma^{192; 231}. Furthermore we have demonstrated the apoptotic efficacy of the pro-apoptotic PBOX compounds in *ex vivo* CLL patient samples¹⁹¹ and significant anti-tumour activity *in vivo* in an aggressive murine model of mammary carcinoma²⁰¹, which further highlights their potential as anti-cancer agents. However, despite the potent activity displayed by the PBOXs against various cancer cell types a certain proportion of K562 CML cells remain resistant to PBOX-induced apoptosis. K562 cells are inherently resistant to a variety of chemotherapeutic agents due to the constitutive activation of Bcr-Abl. The possibility of enhancing the pro-apoptotic activity of the PBOXs by combination with other conventional chemotherapeutics has been shown. Our group has previously demonstrated that the Bcr-Abl kinase inhibitor STI571 enhances the apoptotic efficacy of PBOX-6 in human CML cells¹⁹⁰, whilst the previous chapter has also demonstrated PBOX-21 to similarly synergise with STI571. However, as a certain proportion of CML patients are now gaining resistance to STI571 it was decided to examine if alternative chemotherapeutics could similarly enhance the effects of the PBOXs.

Recent reports have demonstrated that the potent CDK inhibitor flavopiridol enhances MTA-induced tumour cell apoptosis *in vitro* and *in vivo*^{132; 131}. These reports prompted us to examine whether this CDK inhibitor could similarly enhance PBOX-induced apoptosis in CML cells. The aim of this chapter was therefore to evaluate the ability of flavopiridol to enhance the apoptotic efficacy of the pro-apoptotic PBOX compounds in CML cells and to uncover the molecular basis of this enhancement.

Apoptosis was synergistically enhanced in PBOX-treated CML cells following the sequential addition of flavopiridol. When flavopiridol was administered prior to or concomitantly with PBOX-6/-15, no enhancement of apoptosis was observed when compared to cells treated with either agent alone. Our results therefore suggest that the order in which PBOX and flavopiridol treatments take place in CML cells is critical for mediating a synergistic enhancement of apoptosis. This is in agreement with previously published papers showing flavopiridol treatment to be highly sequence-dependent particularly when combined with MTAs such as paclitaxel¹²⁵. In A549 lung carcinoma

cells, sequence-dependent synergy was also observed with cytarabine, topotecan, doxorubicin and etoposide when followed by flavopiridol¹²⁴.

The Bcl-2 family of proteins have long been identified as downstream executioners of mitochondrial-associated apoptosis¹⁴⁶ and have also previously been shown to be involved in PBOX-induced apoptotic pathways¹⁹⁷. In this report we show that sequential treatment of K562 cells with PBOX-6 and flavopiridol resulted in a marked reduction in the expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL and this may play a role in the potentiation of apoptosis. Konig *et al.*, (1997)¹⁰⁹ reported that flavopiridol induced growth inhibition and apoptosis in CLL cell lines with associated downregulation of the Bcl-2 protein suggesting this is an important way in which flavopiridol induces cytotoxicity. While Mcl-1 was downregulated following PBOX-6 treatment, no further downregulation was observed following combined treatment so it is unlikely to play a role in flavopiridol enhanced PBOX-6-induced apoptosis.

We have previously shown the involvement of caspases, key regulators of apoptotic pathways, is highly cell type specific in PBOX-induced apoptosis^{192; 196}, so it was important to establish their role in PBOX-6/flavopiridol-induced apoptosis. Activation of caspase-3/7 activity was detected in K562 cells in response to PBOX-6 and/or flavopiridol treatment. Caspase activity for the combination treatment also increased, although not significantly, when compared to PBOX-6 treatment, suggesting caspases may play a minor role in flavopiridol enhanced PBOX-6-induced apoptosis.

Previous research has shown that in response to MTAs, flavopiridol prevents endoreplication and development of polyploidy in cells with defective G1/S checkpoints²²⁶. A compromise in the G1 checkpoint results in the continuous activation of CDK2/cyclin E that leads to the endoreplication²²⁶. K562 cells are p53-deficient and therefore have a compromised spindle checkpoint. As we had previously shown that PBOX-6 increases the number of polyploid cells in CML cell lines¹⁸², it was of interest to see if flavopiridol administration could reduce this effect. Results showed that while the number of polyploid cells formed following PBOX-6/flavopiridol treatment decreased when compared to cells treated with PBOX-6 alone, this decrease was not significant and so a reduction in polyploid cells is unlikely to play a major role in the mechanism through which flavopiridol synergistically enhances PBOX-induced apoptosis.

Analysis of cell cycle data revealed that CML cells had undergone a G2/M arrest prior to the onset of apoptosis following treatment with PBOX-6, peaking at 18 h. The subsequent addition of flavopiridol to these PBOX-arrested samples resulted in fewer cells

in the G2/M phase when compared to cells left in drug-free medium correlating with a significant enhancement of apoptosis. Longer durations of PBOX-6 treatment (i.e. 42h) followed by flavopiridol did not result in a significant enhancement of apoptosis as the majority of cells treated for 42 h with PBOX-6 had progressed through this mitotic block. This observation confirms the hypothesis that flavopiridol synergises with compounds that arrest cells in the G2/M phase. This result is in agreement with a previous report demonstrating that flavopiridol facilitates exit from the mitotic block induced by paclitaxel in the human gastric cancer cell line, MKN-74¹²⁵. It also provides an explanation for the sequence dependency of the PBOX/flavopiridol combination treatment and for the ineffectiveness of the concurrent or reverse combination.

Progression through the G2/M phase is regulated by the complex of CDK1 and cyclin B1. It was therefore necessary to establish the effect PBOX-6/flavopiridol had on these cell cycle related proteins. Cyclin B1 was upregulated following a 2 h treatment with PBOX-6 alone which preceded the G2/M arrest, suggesting that cyclin B1-induced expression is at least partially responsible for the G2/M arrest elicited by PBOX-6 in K562 cells. This is in agreement with a previous report showing upregulation of cyclin B1 expression and a G2/M arrest in MCF-7 cells following treatment with PBOX-6¹⁹⁹. The PBOX-6/flavopiridol combination treatment was accompanied by an enhanced downregulation of cyclin B1 levels and decreased CDK1/cyclin B1 activity when compared to either drug alone. CDK1/cyclin B1 is responsible for regulating survivin, an IAP protein and mitotic regulator. Cells treated with PBOX-6 alone caused an increase in survivin expression which correlated with a PBOX-induced G2/M arrest. Consistent with this observation, it could be suggested that PBOX-induced microtubule destabilisation and mitotic arrest increased survivin expression which activated a cell survival pathway to counteract PBOX-6-induced apoptosis. The sequential addition of flavopiridol enhanced survivin degradation by inhibiting the CDK1/cyclin B1 complex thus enhancing the percentage of apoptotic cells. Interestingly, in K562 cells where survivin was knocked down using siRNA, the percentage of apoptosis observed was significantly enhanced after administration of PBOX-6 when compared to cells transfected with a scrambled scRNA. This is in agreement with other reports demonstrating an enhancement of apoptosis by chemotherapeutic agents in cells where survivin has been silenced²³². It is therefore likely that enhanced degradation of survivin through inhibition of the CDK1/cyclin B1 complex plays a key role in PBOX-6/flavopiridol-induced apoptosis. The accelerated inhibition of CDK1/cyclin B1 by flavopiridol prevents the phosphorylation and accumulation of

survivin, removing a viability checkpoint through which it enhances apoptosis. Transcriptional repressors of survivin such as YM155^{93; 94} and terameprocol⁹⁵ have recently displayed activity in clinical trials demonstrating the importance of targeting survivin in the fight against cancer.

The molecular basis of flavopiridol-mediated anti-cancer activity has not been fully elucidated to date. It may therefore also involve mechanisms unrelated to inhibition of CDK1 activity including broad suppression of gene transcription by interfering with CDK9/cyclin T function²³³. Furthermore, flavopiridol functions as a relatively broad inhibitor of various CDKs as well as non-cell cycle regulated kinases²³³, thus potentially producing a wide range of effects on cell cycle progression, gene transcription and on general cell signalling pathways. Despite these possible limitations, results presented herein demonstrate that one of the mechanisms by which flavopiridol enhances the pro-apoptotic effect of the PBOXs may involve loss of survivin expression. This model is consistent with other reports demonstrating the ability of flavopiridol to reduce the expression of other IAP family proteins²³⁴.

In conclusion, we have demonstrated the ability of flavopiridol to synergistically potentiate the apoptotic efficacy of the pro-apoptotic PBOX compounds PBOX-6 and PBOX-15 in CML cell lines. Potentiation of apoptosis was highly sequence dependent. Flavopiridol reduced the number of polyploid cells formed following PBOX-6-induced apoptosis and while this was not significant in our cells it may have contributed somewhat to the enhanced levels of apoptosis. Mechanistically, the enhanced downregulation of cyclin B1, decreased activity of CDK1/cyclin B1 activity and corresponding degradation of survivin were found to be of critical importance through which flavopiridol enhanced PBOX-induced apoptosis. The results obtained in this chapter represent the first example of successful combination of the PBOX compounds with a CDK inhibitor and highlight the potential of this novel combination as an effective strategy in treating CML.

Chapter 5.

The effect of the pro-apoptotic PBOX compounds in resistant mutant Bcr-Abl CML cell lines, in ex vivo patient samples and in an in vivo CML mouse model.

5.1 Introduction

The Ph chromosome is caused by the reciprocal translocation between two genes, Bcr and Abl, forming an aberrantly expressed oncogene known as Bcr-Abl⁶. Bcr-Abl is a constitutively active tyrosine kinase whose presence is responsible for the haematological stem cell disorder CML. CML cells, also referred to as blast cells are insensitive to apoptosis due to the continuous activation of signalling pathways downstream of Bcr-Abl. The majority of CML patients present with symptoms in the chronic phase of the disease which is easily managed with the novel tyrosine kinase inhibitor, STI571, which directly targets the oncogenic Bcr-Abl protein²⁶. However, some patients gain resistance to STI571 and CML can progress, usually unpredictably, into a much more aggressive accelerated phase where effective control of the disease is more difficult to achieve⁵. Patients finally enter into an acute blast crisis stage of CML which is fatal and largely resistant to STI571. Even in patients reaching a CCR, residual disease remains detectable by PCR techniques suggesting a continued threat of relapse¹⁶.

There are many different mechanisms through which patients become resistant to STI571 including an increase in the levels of Bcr-Abl mRNA expression and protein levels, amplification of the Bcr-Abl gene, an efflux of STI571 via MDR proteins such as P-gp³⁶ and activation of signalling pathways downstream of or independent to Bcr-Abl³⁷. Progression of the disease can also be associated with additional mutations in oncogenes and tumour suppressor genes such as p53, Ras and Rb. However, despite these numerous and diverse mechanisms of resistance the major factor influencing STI571 resistance is due to mutations at critical points in the Bcr-Abl gene. These Bcr-Abl mutations can occur in several functionally distinct regions within the kinase domain and confer various degrees of resistance to STI571. To date over 60 different amino acid substitutions have been found and many of these are mutated at more than one alternative residue. Mutations fall into two groups, those that alter amino acids that directly contact STI571 and those that prevent Bcr-Abl from achieving the inactive conformational state required for STI571 binding. Of the mutations that result from amino acid substitutions, the T315I and E255K mutations are the most prevalent⁴⁹. Crucially the T315I mutation is also associated with the highest degree of STI571-resistance, it prevents the formation of a critical hydrogen bond between STI571 and Bcr-Abl and changes the conformation of Bcr-Abl to prevent STI571-binding.

These mutations raise the possibility that additional drugs that target CML cells via alternative mechanisms to Bcr-Abl have the potential to be successful in treating STI571-resistant CML patients³⁵. As a result, a number of studies have been conducted with STI571 in combination with other anti-cancer therapeutics including farnesyltransferase inhibitors such as SCH66336^{57; 19; 13}, MAPK inhibitors PD184352, PD98059 and U0126^{60; 61} and histone deacetylase inhibitors MS-275 and SAHA amongst others^{64; 65; 66} with various degrees of success *in vitro* and *in vivo* animal models.

Another approach to overcome STI571-resistance has led to the development of second generation Bcr-Abl targeted therapies. Of these, dasatinib, an orally active, small molecule that works by inhibiting Bcr-Abl, the Src family, c-KIT, EPHA2 and PDGFR- β ⁷⁰ and nilotinib, an analogue of STI571, have recently been approved by the FDA for treatment of STI571-resistant CML patients⁷². Although third-line tyrosine kinase inhibitors with activity against T315I mutants are in clinical trials, it is likely that Bcr-Abl-independent resistance will arise in advanced CML, suggesting that agents that target CML cells via alternative mechanisms to Bcr-Abl have the potential to be successful in treating STI571-resistant CML patients⁴⁸.

Recently, some members of a novel set of compounds, the PBOXs, have been shown to induce apoptosis in a wide range of solid tumours and haematological malignancies^{192; 194}. Tubulin has been identified as a molecular target of these compounds which induce apoptosis in CML cells by bypassing Bcr-Abl¹⁹⁴. The PBOX compounds have also demonstrated efficacy in *ex vivo* CLL samples¹⁹¹ and an *in vivo* mouse mammary carcinoma model²⁰¹. Furthermore, we have also shown that PBOX-6 in combination with STI571 enhances apoptosis in STI571-resistant CML cell lines that overexpress the Bcr-Abl protein¹⁹⁰. It was therefore of interest to determine if the PBOX compounds could similarly induce/enhance apoptosis in CML cells that contain the highly resistant T315I mutation both *in vitro* and *in vivo*.

5.2 Results

5.2.1 Mutations in the *Bcr-Abl* gene confer various degrees of resistance to STI571 in transfected Baf/3 cell lines.

To confirm the status of the Bcr-Abl protein in Baf/3 cells, untreated samples were subjected to SDS-PAGE and Western blotting. Results confirmed the presence of the Bcr-Abl protein in transfected native and mutant cell lines and absence of Bcr-Abl in the parental, non-transfected Baf/3 cells (Fig. 5.1).

An alamar blue assay was utilised to show the various effects of STI571 on each cell line. As expected, STI571 displayed the highest level of toxicity against the native cell line followed by the H396P, E255K and finally the T315I cell lines.

Native cells were found to have an IC_{50} value of 658nM towards STI571. H396P cells were 2.9 times more resistant to STI571 than native cells while E255K cells were 11.1 times more resistant. Interestingly, the IC_{50} value for T315I cells could not be determined as these cells were found to be completely resistant to STI571 even at concentrations as high as 10 μ M (Fig. 5.2).

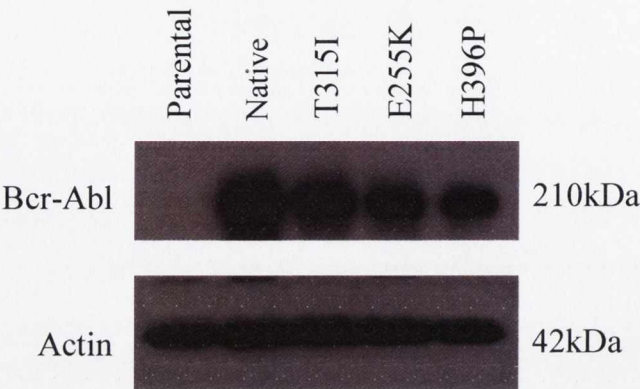
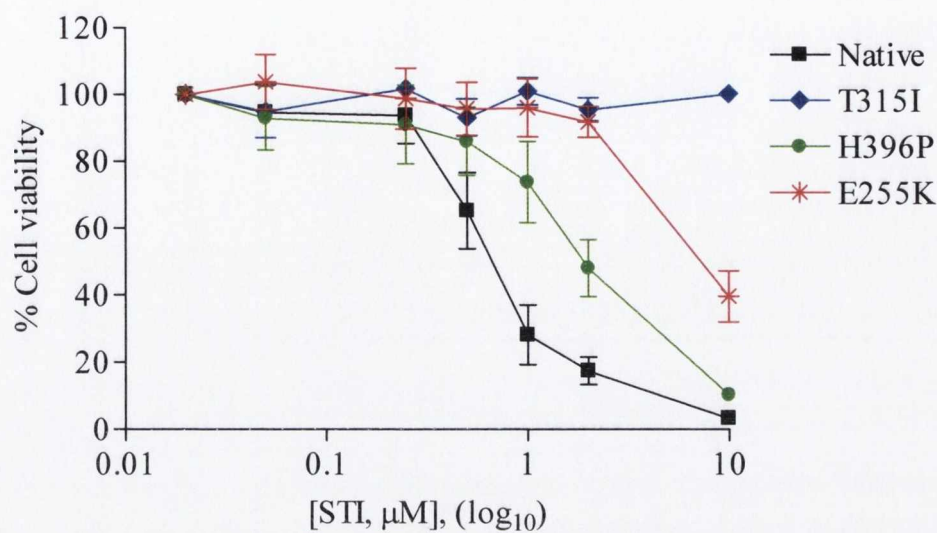


Fig. 5.1 Native and mutant Bcr-Abl Baf/3 cells contain the Bcr-Abl protein while parental Baf/3 cells do not.

Parental, native and mutant Bcr-Abl Baf/3 cells were seeded during the log phase of growth and incubated for 24 h. Samples were prepared for Western blotting (Section 2.10) and run on an 8% gel. Samples were transferred overnight to PVDF membranes and incubated for 1 h with an anti-Bcr-Abl mAb. Actin is shown as a loading control. Results are representative of three independent experiments.



	IC ₅₀ (STI, μM)	Fold difference
Native	0.658	1
T315I	N.D.	N.D.
H396P	1.9	2.9
E255K	7.28	11.1

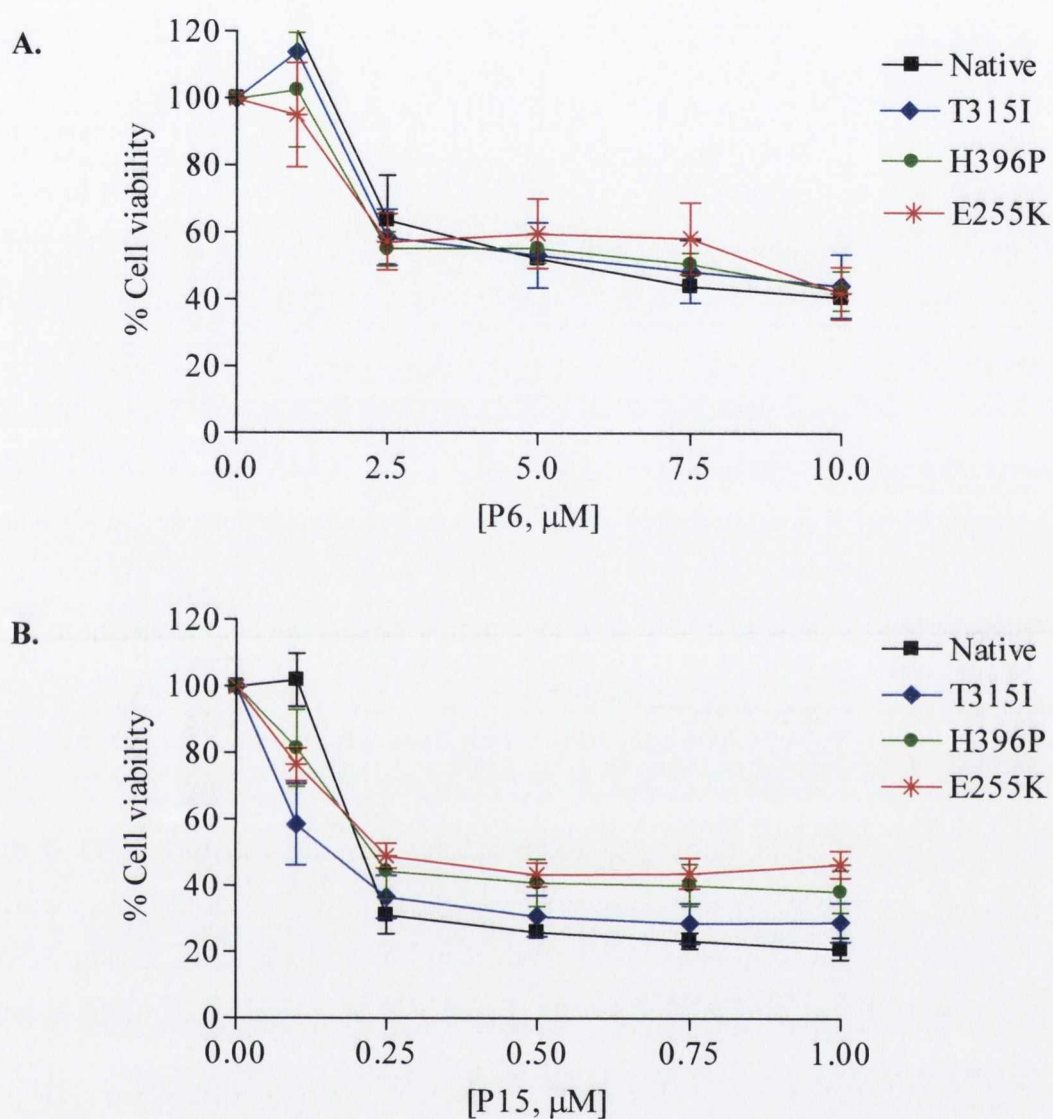
Fig. 5.2 Mutant Bcr-Abl Baf/3 cell lines display various degrees of resistance to STI571 when compared to the native cells.

Native and mutant Bcr-Abl Baf/3 cells were seeded during the log phase of growth and treated for 48 h with a vehicle (Veh) (0.1% (v/v) DMSO) or a range of STI571 (STI) concentrations (50nM to 10μM) in a 96-well plate. After the required incubation period, alamar blue dye (20μl) was added to each well and samples were incubated for 3 h. Cells were subsequently analysed for cell viability. Values represent the mean ± S.E.M. of three independent experiments performed in triplicate. N.D. - not determined.

5.2.2 *The PBOX compounds significantly reduce cell viability in STI571-resistant and native Bcr-Abl Baf/3 cell lines.*

The pro-apoptotic PBOX compounds were found to significantly reduce cell viability in all cell lines examined. This reduction in viability was equipotent across all four cell lines treated with PBOX-6. Viability was reduced to 40.7 ± 0.6 , 44.2 ± 9.4 , 42.8 ± 5.9 and $42.1 \pm 7.8\%$ in native, T315I, H396P and E255K cells treated with $10\mu\text{M}$ PBOX-6 (Fig. 5.3A).

PBOX-15 also significantly reduced cell viability in all the mutant Bcr-Abl Baf/3 cells. Specifically, PBOX-15 ($1\mu\text{M}$) reduced viability to 21.1 ± 3.4 , 29.0 ± 5.9 , 38.4 ± 6.4 and $46.8 \pm 4.1\%$ in native, T315I, H396P and E255K cells respectively (Fig. 5.3B).



5.3 PBOX-6 and PBOX-15 reduce cell viability in native and mutant Bcr-Abl Baf/3 cell lines.

Native and mutant Bcr-Abl Baf/3 cells were seeded during the log phase of growth and treated for 48 h with a vehicle (Veh) (1% (v/v) ethanol) or the indicated concentrations of PBOX-6/-15 (P6/P15) in a 96-well plate. After the required incubation period, alamar blue dye (20μl) was added to each well and samples were incubated for 3 h. Cells were subsequently analysed for cell viability. **A.** PBOX-6 and **B.** PBOX-15. Values represent the mean $\pm$ S.E.M. of three independent experiments performed in triplicate.

5.2.3 The PBOX compounds in combination with STI571 enhance apoptosis in native but not T315I-Bcr-Abl cells.

A recently published paper by our group demonstrated PBOX-6 to enhance the apoptotic efficacy of STI571 in K562 CML cells¹⁹⁰. It was therefore of interest to see if PBOX-6 and PBOX-15 could similarly enhance apoptosis in Bcr-Abl transfected Baf/3 cells. Results showed PBOX-6 and PBOX-15 in combination with STI571 did successfully enhance apoptosis in native cells. In more detail, 10 μ M PBOX-6 induced $8.4 \pm 3.7\%$ apoptosis in native cells, increasing to $27.1 \pm 1.5\%$ apoptosis when combined with 1 μ M STI571 following a 24 h treatment (p value: 0.0144,*) (Fig. 5.4A). After a 48 h PBOX-6/STI571 treatment, $43.2 \pm 5.5\%$ (p value: 0.0391,*) of cells underwent apoptosis when compared to PBOX-6 treatment alone, $23.4 \pm 8.8\%$ (Fig. 5.4B).

PBOX-15 (1 μ M) induced $13.8 \pm 2.9\%$ apoptosis in native cells, increasing to $26.0 \pm 2.1\%$ (p value: 0.0299,*) when combined with 1 μ M STI571 after 24 h (Fig. 5.4C) and from 38.7 ± 3.5 to $49.8 \pm 2.9\%$ apoptosis after a 48 h treatment (p value: 0.1586, ns) (Fig. 5.4D).

However, as the T315I Baf/3 cell line is intrinsically resistant to STI571 it was hypothesised that the PBOX compounds would not show an augmentation of apoptosis when combined with STI571 in these cells. Results confirmed this hypothesis (Fig. 5.5A-D). However, PBOX-6 and PBOX-15 did induce similar levels of apoptosis in these cells as was observed in native cells.

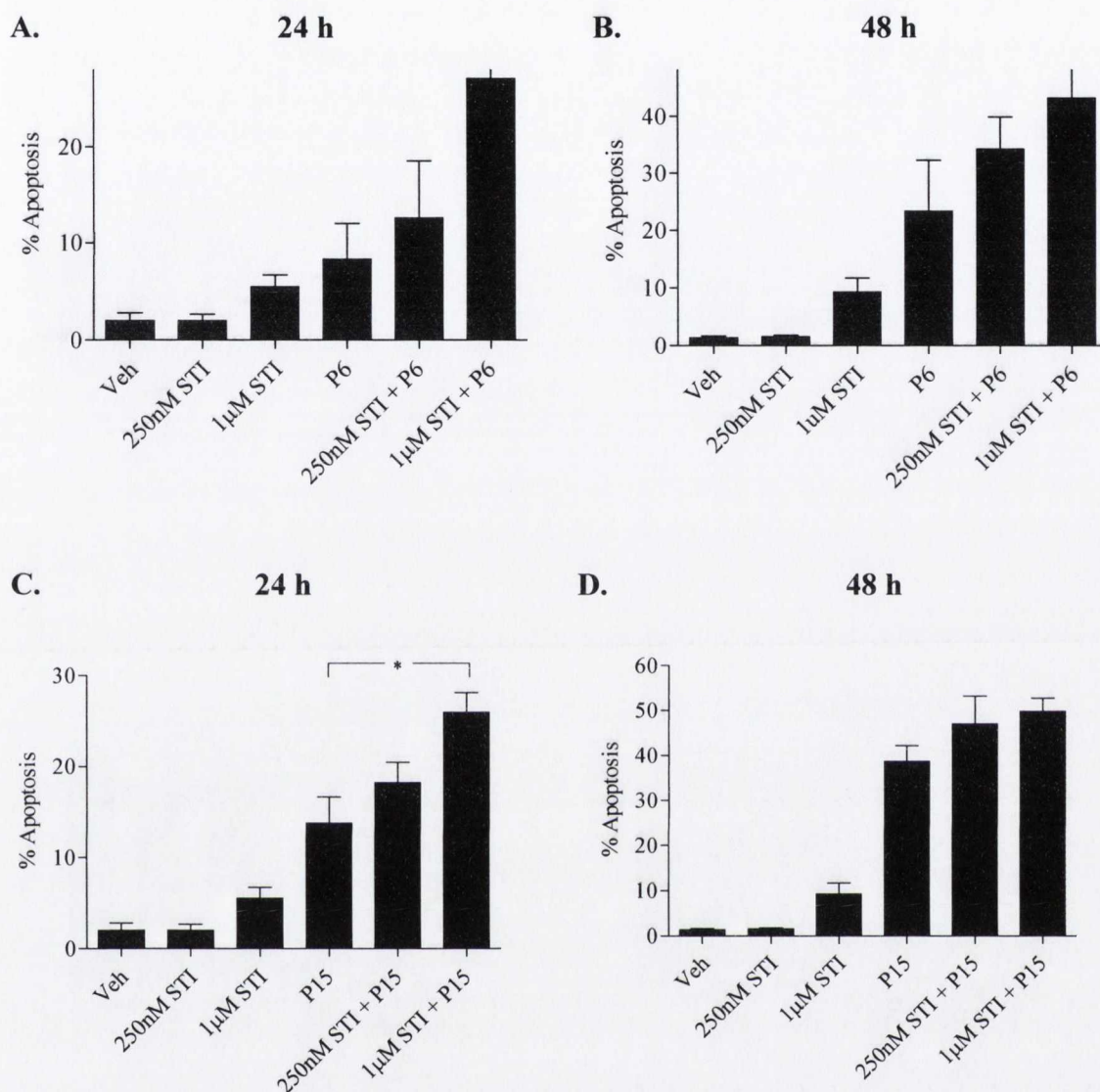


Fig. 5.4 PBOX compounds in combination with STI571 enhance apoptosis in native Bcr-Abl Baf/3 cells compared to each agent alone.

Native Bcr-Abl Baf/3 cells were seeded during the log phase of growth and treated for up to 48 h with a vehicle (Veh) (0.2% (v/v) ethanol or 0.01% (v/v) DMSO), PBOX-6/-15 (P6/P15), STI571 (STI) or a combination of both compounds. After the required incubation period, cells were fixed in 1ml ethanol and 100μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. **A., C.** 24 h and **B., D.** 48 h. Values represent the mean $\pm$ S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired t-test, *, $p < 0.05$.

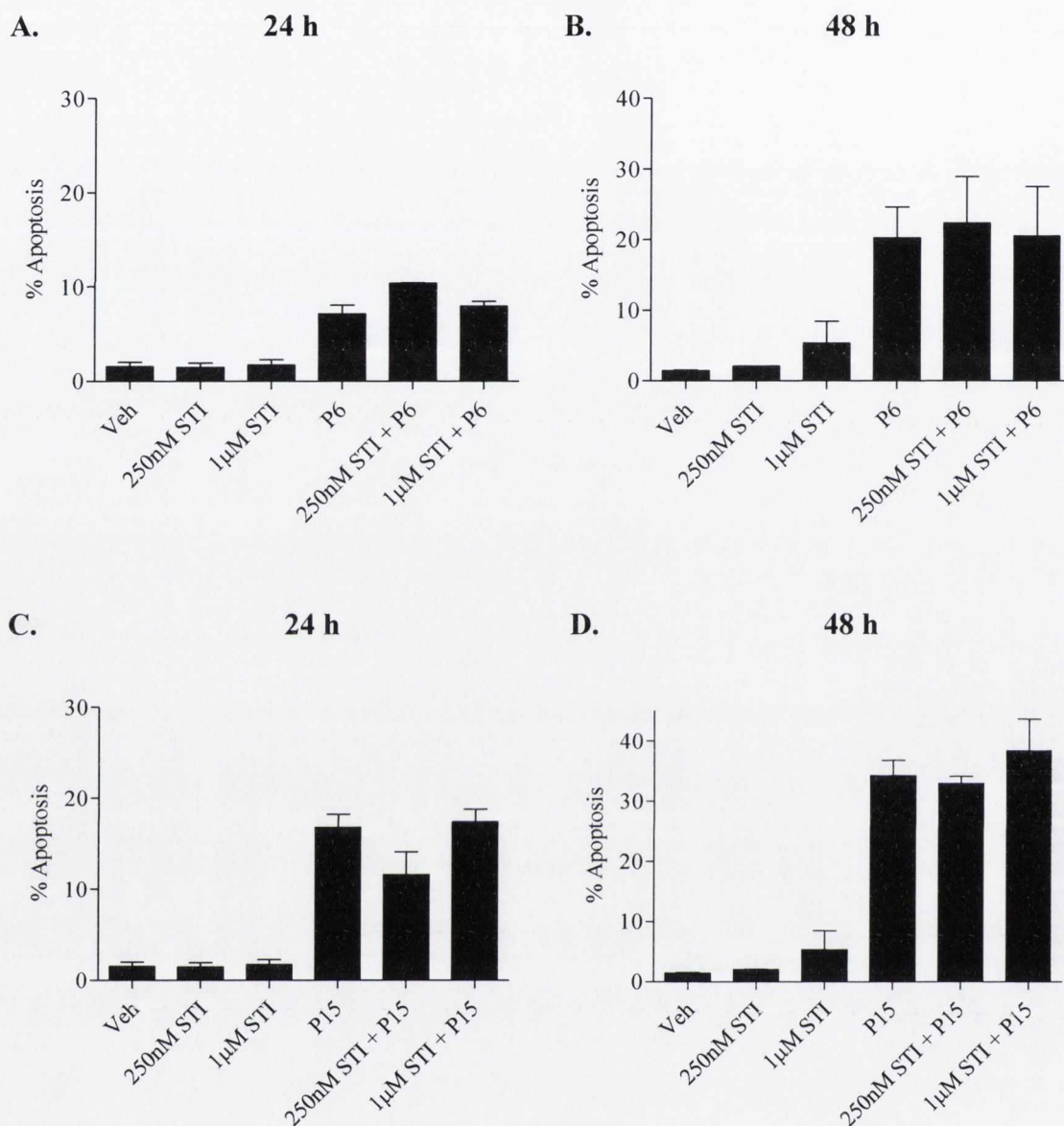


Fig. 5.5 PBOX compounds in combination with STI571 do not enhance apoptosis in T315I cells compared to each agent alone.

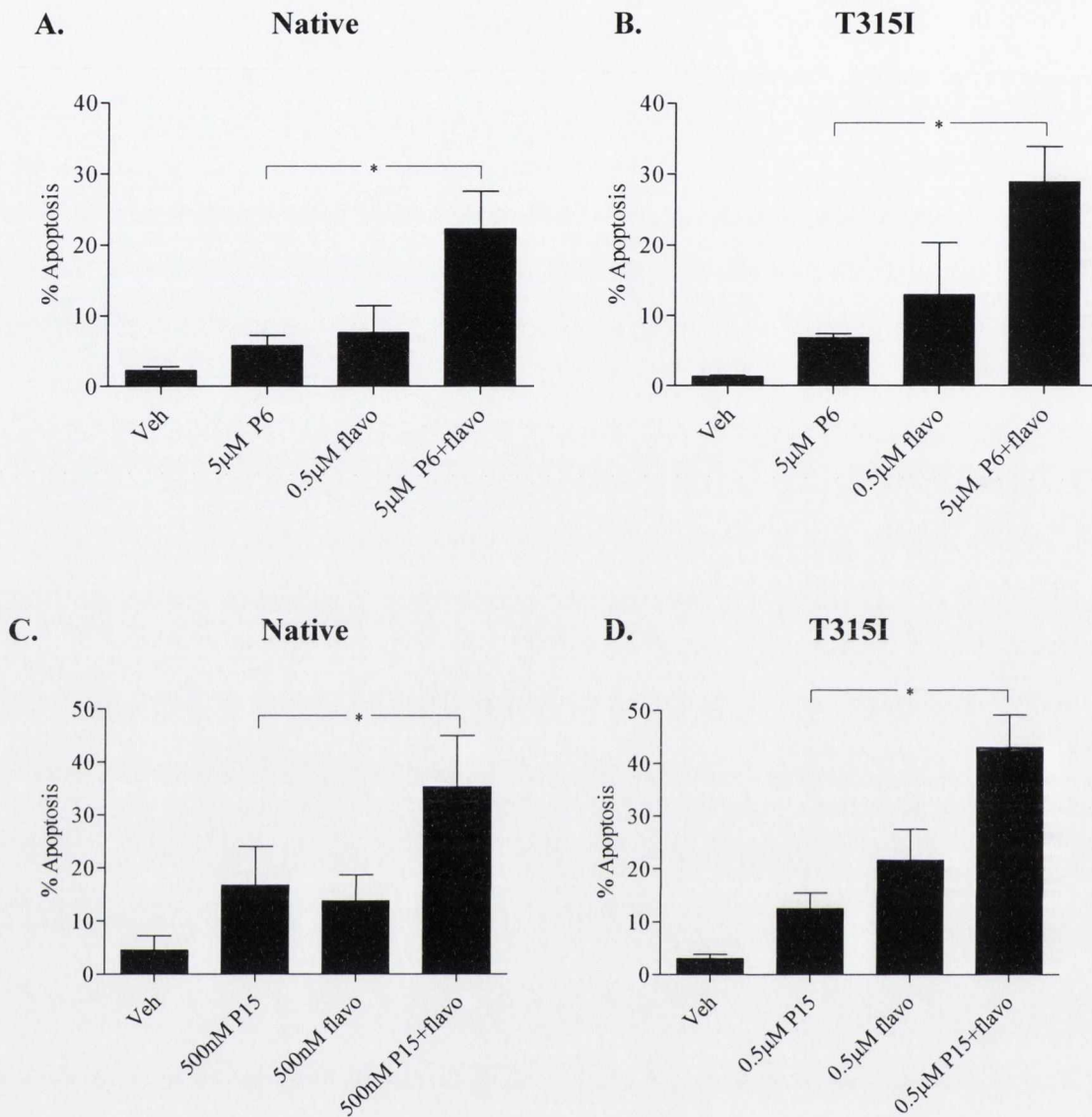
Mutant T315I-Baf/3 cells were seeded during the log phase of growth and treated for up to 48 h with a vehicle (Veh) (0.2% (v/v) ethanol or 0.01% (v/v) DMSO), PBOX-6/-15 (P6/P15), STI571 (STI) or a combination of both compounds. After the required incubation period, cells were fixed in 1ml ethanol and 100μl PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. **A., C.** 24 h and **B., D.** 48 h. Values represent the mean $\pm$ S.E.M. of three independent experiments.

5.2.4 Flavopiridol enhances the apoptotic effect of the PBOX compounds in native and T315I-Bcr-Abl cells.

The previous chapter of this report clearly demonstrates the synergistic effects of the pro-apoptotic PBOX compounds in combination with the CDK1 inhibitor flavopiridol. It was therefore desirable to determine if the novel PBOX/flavopiridol combination treatment may have potential in the treatment of STI571-resistant T315I cells.

Native cells treated for 18 h with PBOX-6 (5 μ M) followed by a further 24 h with drug free medium induced $5.8 \pm 1.5\%$ apoptosis, which rose to $22.3 \pm 5.4\%$ (p value: 0.0378,*) following the sequential additional of flavopiridol (500nM) (Fig. 5.6A). Similar, albeit slightly higher results were observed in T315I cells with apoptosis increasing from 6.8 ± 0.6 to $29.0 \pm 5.0\%$ for the same treatments (p value: 0.0468,*) (Fig. 5.6B).

Likewise, native and T315I cell lines treated with PBOX-15 (0.5 μ M) for 18 h followed by flavopiridol for 24 h resulted in an increase of apoptosis from 16.7 ± 7.3 to $35.3 \pm 9.7\%$ (p value: 0.0488,*) in native cells and from 12.5 ± 2.9 to $43.0 \pm 6.1\%$ in T315I cells (p value: 0.0109,*) (Fig 5.6C&D).



5.6 Flavopiridol enhances the apoptotic effect of PBOX-6 and PBOX-15 in native and T315I-Bcr-Abl Baf/3 cells.

Native and mutant T315I-Bcr-Abl Baf/3 cells were seeded during the log phase of growth and treated for 18 h with PBOX-6/-15 (P6/P15) followed by a 24 h treatment with flavopiridol or treated with the relevant controls. After the required incubation period, cells were fixed in 1ml ethanol and 100 μ l PBS and stained with propidium iodide. Cells were subsequently analysed by flow cytometry, the percentage of apoptosis was assessed by quantification of the pre-G1 peak. **A., C.** Native cells and **B., D.** T315I cells. Values represent the mean $\pm$ S.E.M. of three independent experiments. Statistical analysis was performed using the Student's paired t-test, *, $p < 0.05$.

5.2.5 The PBOX compounds induce apoptosis in *ex vivo* samples from newly diagnosed and STI571-resistant blast crisis phase CML patients.

The advent of cell lines has allowed us to gain insight into many molecular signalling pathways and they are a useful tool to help develop novel therapeutic anti-cancer agents. However as cell lines have been engineered to continuously and consistently divide they do not fully represent primary leukaemia cells. For this reason it is necessary to confirm the effects of potentially novel therapeutics found in cell lines on *ex vivo* patient samples. For this, informed consent was obtained from a cohort of newly diagnosed and treatment naïve ($n = 4$) CML patients along with those that had refractory disease ($n = 2$).

Patient samples #1 to #4 were newly diagnosed CML patients currently responding well to either STI571 or nilotinib, none have since progressed to later stages of CML. Patient samples #5 and #6 were obtained from STI571 and dasatinib insensitive patients in blast crisis (Table 5.1). Patient #5 tested 100% positive for the T315I mutation at time of death which occurred less than 1 mth after sample collection, while patient #6 who is currently on nilotinib treatment tested 100% positive for the F317L mutation at the time of sample collection and also had a minor T315I clone.

WBCs/PBMCs were isolated from freshly obtained blood samples, seeded and treated for 72 h with a PBOX compound (25 μ M PBOX-6 or 10 μ M PBOX-15) or 250nM STI571. Leukaemic cells were identified by their low to medium side scatter and low CD45 expression (Fig. 5.7). Annexin V staining was then used to identify apoptotic cells. Etoposide is shown as a positive control (Fig. 5.8). Specific results obtained were as follows:

Patient sample #1 was obtained from a newly diagnosed CML patient and so the cells were responsive to STI571 with the extent of apoptosis increasing from 20.0 ± 0.7 to $28.4 \pm 2.1\%$. Apoptosis also increased to 32.0 ± 7.9 and $38.0 \pm 0.6\%$ following treatment with PBOX-15 and -6 respectively (Fig. 5.9A).

For the second patient sample, the percentage of cells undergoing apoptosis rose from 1.4 ± 0.1 and $1.6 \pm 0.4\%$ in vehicle and STI571-treated cells to 17.4 ± 0.5 and $9.1 \pm 2.7\%$ following treatment with PBOX-15 and PBOX-6 respectively (Fig. 5.9B).

Background apoptotic cells accounted for $2.7 \pm 0.4\%$ of the total cell population in control samples from patient sample #3. This compared to $3.0 \pm 0.2\%$ of STI571-treated

samples. Treatment with PBOX-15 and PBOX-6 enhanced apoptosis to 44.5 ± 3.7 and $20.8 \pm 4.5\%$ (Fig. 5.9C).

PBOX-15 and PBOX-6 induced apoptosis of 43.5 ± 9.0 and $12.5 \pm 6.1\%$ in the final newly diagnosed patient sample. This compared to levels of 3.9 ± 0.9 and $3.3 \pm 0.2\%$ apoptosis for vehicle and STI571-stimulated cells (Fig. 5.9D).

Levels of apoptosis rose from $4.1 \pm 0.1\%$ in vehicle-treated cells from the fifth patient sample to 51.5 ± 10.2 and $23.1 \pm 5.2\%$ following treatment with PBOX-15 and -6 respectively (Fig. 5.10A). Administration of STI571 did not significantly enhance apoptosis ($4.9 \pm 0.2\%$) when compared to vehicle-treated cells.

The high level of background apoptosis observed with patient sample #6 illustrates the wide variation between clinical specimens. Results showed STI571 had a small effect on the number of cells undergoing apoptosis when compared to vehicle-treated samples, increasing from 40.2 ± 0.2 to $54.5 \pm 4.5\%$. Apoptotic levels increased to 61.6 ± 1.6 and $46.0 \pm 0.8\%$ following treatment with PBOX-15 and -6 respectively (Fig. 5.10B).

Collectively, when all samples were combined, PBOX-6 induced $24.7 \pm 5.0\%$ apoptosis (p value: 0.0050,**) while PBOX-15 induced $39.5 \pm 5.8\%$ apoptosis (p value: 0.0059,**) compared to $13.4 \pm 5.5\%$ in control samples. There was no significance difference between the control and the STI571-treated samples which underwent $18.2 \pm 7.7\%$ apoptosis (Fig. 5.11). This may have been due to the inclusion of the two STI571-resistant patient samples.

#	Sex	Age	Disease status	CML treatment	Current status	Received sample
1	M	46	Newly diagnosed	STI571	Currently on STI571, CCR within 1st year treatment	15 Jan 08
2	F	31	Newly diagnosed	STI571	Currently on STI571, CCR within 1st year treatment	15 Aug 08
3	M	26	Newly diagnosed	Nilotinib (As part of clinical trial)	Currently on nilotinib, MCR in 1st 3 mths treatment	9 Apr 09
4	F	42	Newly diagnosed	Nilotinib (As part of clinical trial)	Currently on nilotinib, MCR in 1st 3 mths treatment	26 May 09
5	F	73	Blast crisis	Failed STI571 + dasatinib treatment	Deceased 15 days later in 2007	18 Dec 07
6	F	62	Blast crisis	Failed STI571 + dasatinib treatment	Currently on nilotinib	25 Mar 08

Table 5.1. Characteristics of CML patients in *ex vivo* study.

Relevant medical and personal data was obtained from newly diagnosed and STI571-resistant CML patients enrolled in an *ex vivo* PBOX study carried out in the Haematology/Oncology Department, St. James’s Hospital, Dublin 8 in collaboration with Prof. Eibhlin Conneally. Information obtained included the sex of the patient, age at diagnosis and a short description of their medical and CML-specific treatment. M – Male; F – Female; CCR - Complete cytogenetic response means that no blood or bone marrow cells contained the Ph chromosome; MCR - Major (or partial) cytogenetic response with the presence of less than 35% Bcr-Abl-positive metaphase cells.

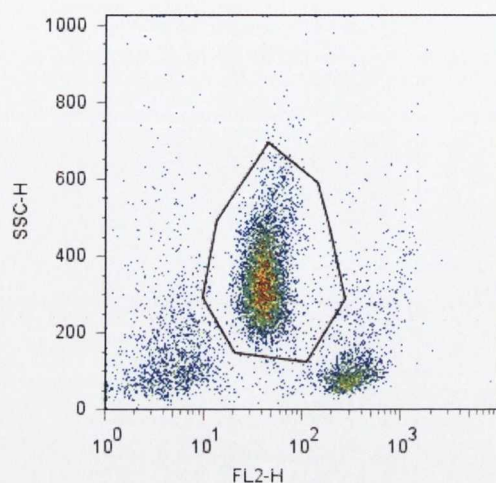


Fig. 5.7 Isolation of leukaemic cells from CML patient blood samples.

WBCs were isolated from the fresh peripheral blood of six CML patients by Ficoll gradient. Cells were seeded at a density of 1×10^6 cells/ml in 24-well plates and treated with a vehicle (0.5% (v/v) ethanol) for 72 h. The cells were then labeled with an anti-CD45 mAb and analysed by flow cytometry. Leukaemic cells were identified and gated based on their low to medium side scatter and low CD45 expression. A representative dot-plot is shown.

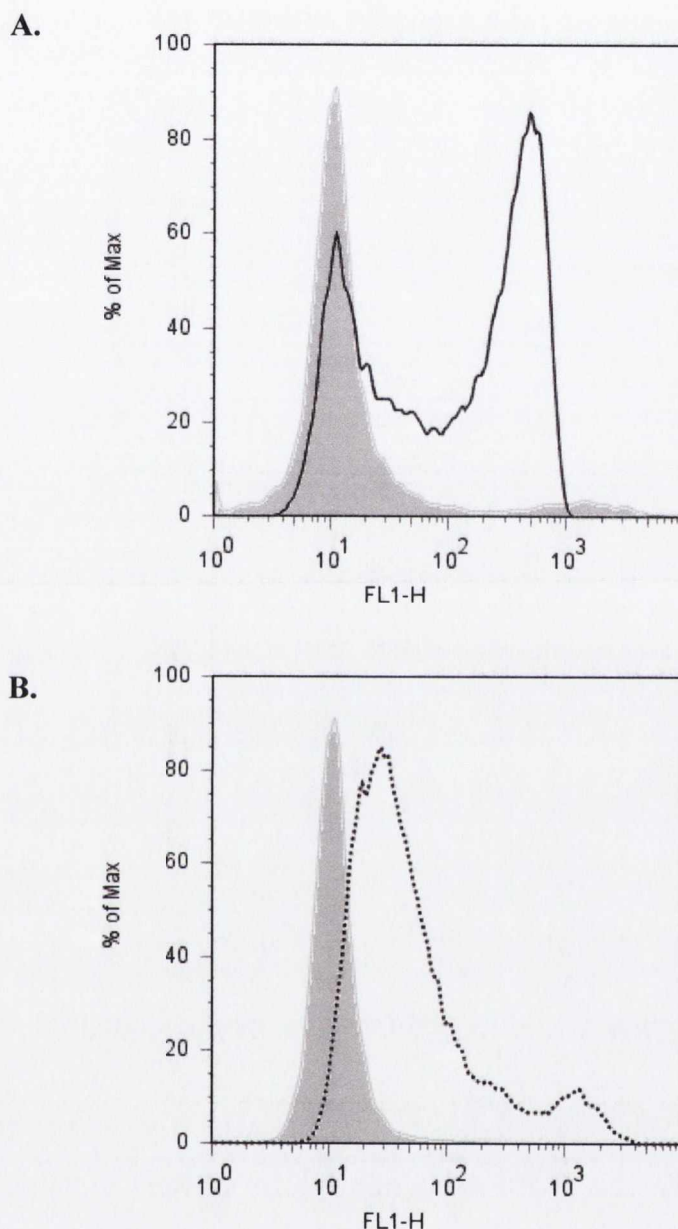


Fig. 5.8 Representative histogram plots of etoposide and PBOX-15-treated CML cells.

WBCs were isolated from the fresh peripheral blood of six CML patients by Ficoll gradient. Cells were seeded at a density of 1×10^6 cells/ml in 24-well plates and treated with the required vehicle/s or compound/s for 72 h. The cells were then labeled with an anti-CD45 mAb and FITC-annexin V and analysed by flow cytometry. Leukaemic cells were identified and gated based on their low to medium side scatter and low CD45 expression. Representative histogram plots of cells treated with **A.** etoposide (solid line) and **B.** PBOX-15 (dashed line) relative to vehicle-treated cells (shaded peak) are shown.

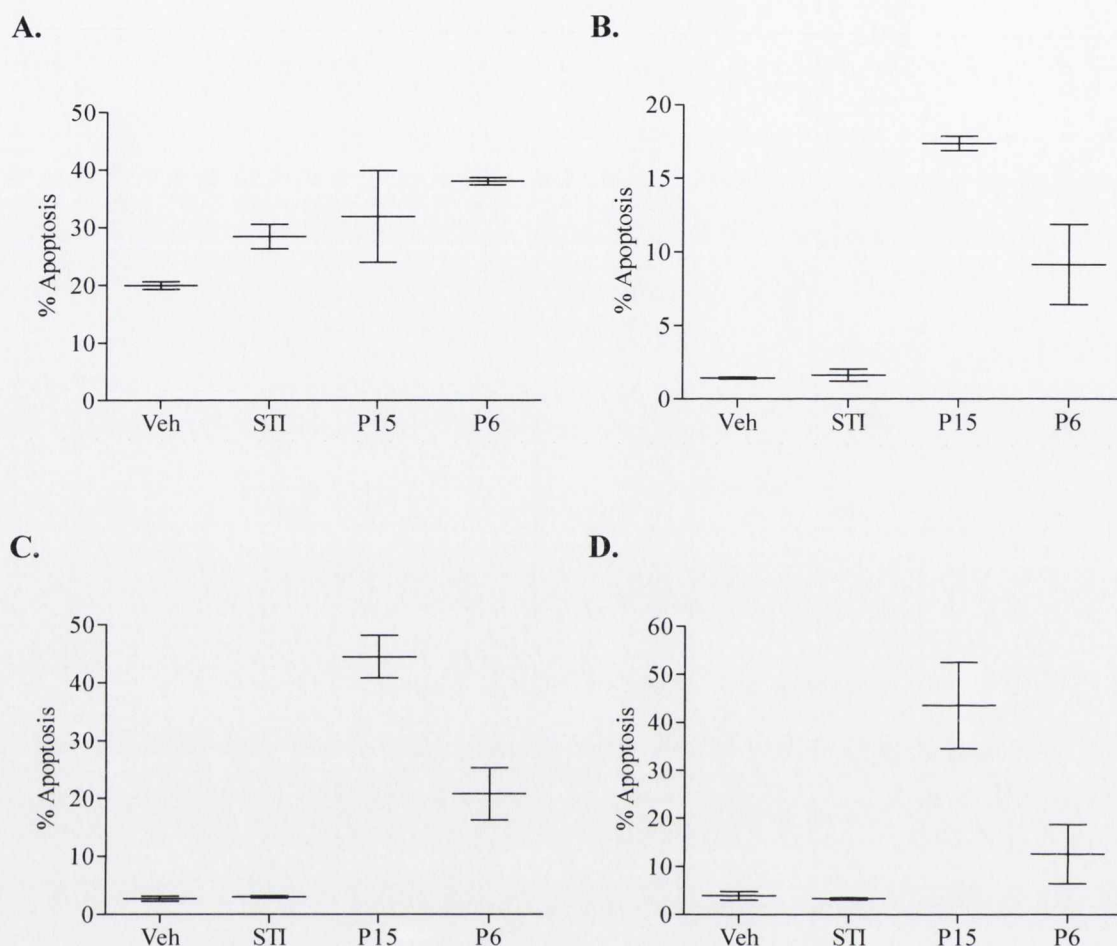


Fig. 5.9 The PBOX compounds induce apoptosis in newly diagnosed CML patient samples.

WBCs were isolated from the fresh peripheral blood of newly diagnosed CML patients by Ficoll gradient. Cells were seeded at a density of 1×10^6 cells/ml in 24-well plates and treated with a vehicle (veh) (0.5% (v/v) ethanol or 0.0025% (v/v) DMSO), 250nM STI571 (STI), 10μM PBOX-15 (P15) or 25μM PBOX-6 (P6) for 72 h. The cells were then labeled with an anti-CD45 mAb and FITC-annexin V and analysed by flow cytometry. Leukaemic cells were identified and gated based on their low to medium side scatter and low CD45 expression. **A.** Patient #1, **B.** patient #2, **C.** patient #3 and **D.** patient #4. Results represent the mean $\pm$ S.E.M. of experiments performed in duplicate.

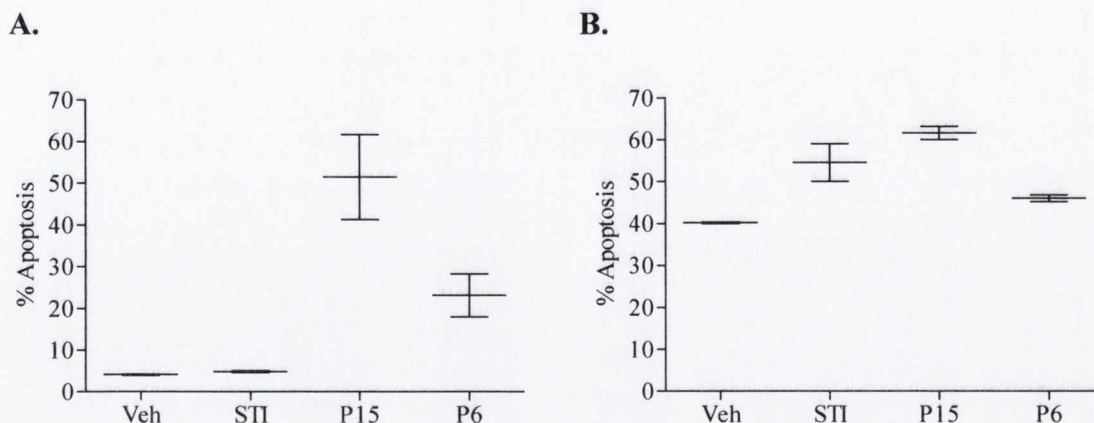


Fig. 5.10 The PBOX compounds successfully induce apoptosis in STI571-resistant CML patient samples.

WBCs were isolated from the fresh peripheral blood of STI571-resistant CML patients by Ficoll gradient. Cells were seeded at a density of 1×10^6 cells/ml in 24-well plates and treated with a vehicle (veh) (0.5% (v/v) ethanol or 0.0025% (v/v) DMSO), 250nM STI571 (STI), 10 μ M PBOX-15 (P15) or 25 μ M PBOX-6 (P6) for 72 h. The cells were then labeled with an anti-CD45 mAb and FITC-annexin V and analysed by flow cytometry. Leukaemic cells were identified and gated based on their low to medium side scatter and low CD45 expression. **A.** Patient #5 and **B.** patient #6. Results represent the mean $\pm$ S.E.M. of experiments performed in duplicate.

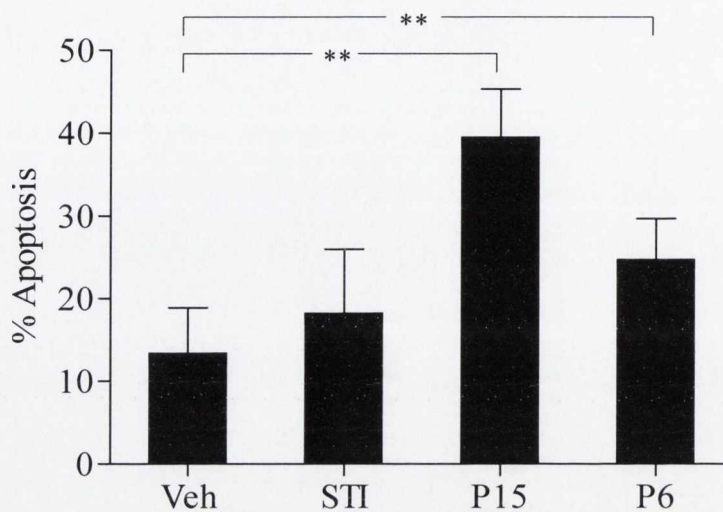


Fig. 5.11 PBOX-15 and PBOX-6 significantly induce apoptosis in all *ex vivo* CML patient samples collectively.

WBCs were isolated from the fresh peripheral blood of CML patients ($n = 6$) by Ficoll gradient. Cells were seeded at a density of 1×10^6 cells/ml in 24-well plates and treated with a vehicle (veh) (0.5% (v/v) ethanol or 0.0025% (v/v) DMSO), 10 μ M PBOX-15 (P15), 25 μ M PBOX-6 (P6) or 250nM STI571 (STI) for 72 h. Cells were then labeled with an anti-CD45 mAb and FITC-annexin V and analysed by flow cytometry. Leukaemic cells were identified and gated based on their low to medium side scatter and low CD45 expression. Experiments were performed in duplicate. Statistical analysis was performed using the Student's paired t-test, ** $p < 0.01$.

5.2.6 PBOX-6 administration significantly impedes tumour growth in a CML mouse model containing the STI-resistant T315I mutation.

Five to six week old female BALB/c-nude mice were inoculated s.c. into the right flank with 3×10^6 T315I-Baf/3 cells in the log phase of growth. Tumours started to appear by day 5/6 and were measureable by day 8. Mice were divided into groups with small, medium and larger sized tumours and then further randomly divided into two groups ($n = 6$) to ensure the average starting tumour size was equal for both groups. One group received daily i.t. injections of 7.5mg/kg PBOX-6 while the other group received control injections (10% (v/v) ethanol, 10% (v/v) cremophor EL in PBS).

Results showed that PBOX-6 did not adversely affect mouse weight. In fact, both groups put on a small amount of weight during the experiment increasing from 14.7 ± 0.7 and 14.8 ± 0.6 g to 16.5 ± 0.8 and 16.5 ± 0.3 g in control and PBOX-treated groups respectively. This would be expected with healthy mice of such an age (Fig. 5.12). Close inspection of the mice also ensured that no mouse suffered any form of distress such as failing to groom, hunching etc.

Tumour growth was measured every second day, the long (L) and short (S) axes were recorded and tumour volume (V) calculated using the following equation: $V = (S^2 \times L)/2^{209}$. Injections were initiated on day 8 when there was no significant difference in tumour volume between the control and the PBOX-treated groups, 44.8 ± 6.7 versus $43.9 \pm 9.25\text{mm}^3$ (p value: 0.9368,ns). By day 10, PBOX-6 had significantly reduced tumour volume from 69.6 ± 6.3 to $50.7 \pm 3.2\text{mm}^3$ (p value: 0.0251,*) when compared to vehicle-treated mice. By day 12, tumour growth had significantly reduced from 172.5 ± 39.0 to $81.1 \pm 9.3\text{mm}^3$ (p value: 0.0459,*) following PBOX-6 administration. Tumour burden was continuously and significantly inhibited from 232.2 ± 22.9 to $135.3 \pm 14.0\text{mm}^3$ (p value: 0.0047,**) on day 14, from 287 ± 23.4 to $194.1 \pm 29.4\text{mm}^3$ (p value: 0.0331,*) on day 16 and from 303.3 ± 20.6 to $227.5 \pm 35.2\text{mm}^3$ on day 18 (p value: 0.0418,*) for PBOX-6-treated mice compared to vehicle-treated mice (Fig. 5.13).

At the experimental end-point, day 18, mice were sacrificed by CO₂ asphyxiation and a splenectomy was performed. The vehicle-treated group showed gross enlargement of spleens or splenomegaly when compared to the PBOX-6-treated group, with a 3.7-fold increase in weight from 163.3 ± 39.51 to $608.7 \pm 105.3\text{mg}$ (p value: 0.0027,**) (Fig. 5.14). This is likely to be due to the reduced tumour burden of PBOX-treated animals. The average spleen weight in unchallenged Balb/c mice is approximately 96mg^{235} .

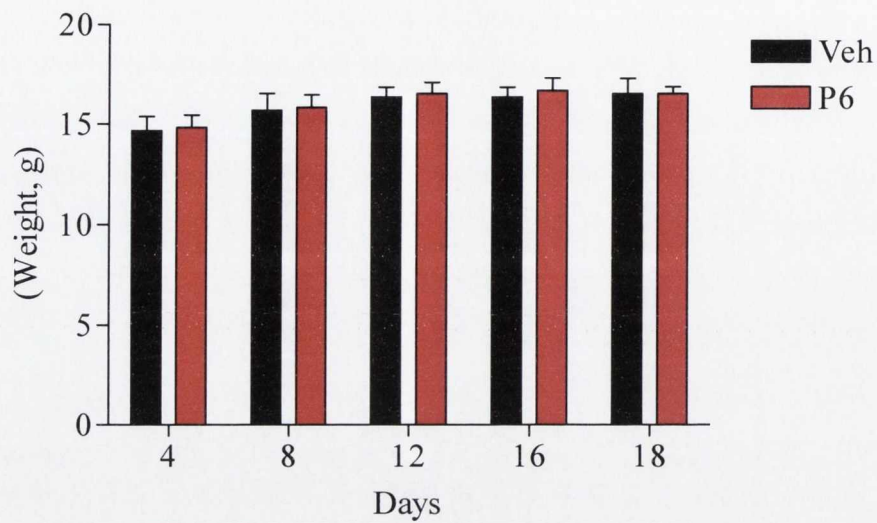


Fig. 5.12 PBOX-6 does not adversely affect weight gain *in vivo*.

Five to six week old female BALB/c-nude mice were inoculated s.c into the right flank with 3×10^6 T315I-Bcr-Abl Baf/3 cells in the log phase of growth. When tumours had reached a diameter of $\sim 4\text{mm}$ (day 8), mice were randomly divided into two treatment groups ($n = 6$) and received daily i.t. injections of PBOX-6 (P6) (7.5mg/kg) or the equivalent vehicle (Veh). Mice were weighed every four days and at the experimental end-point (day 18).

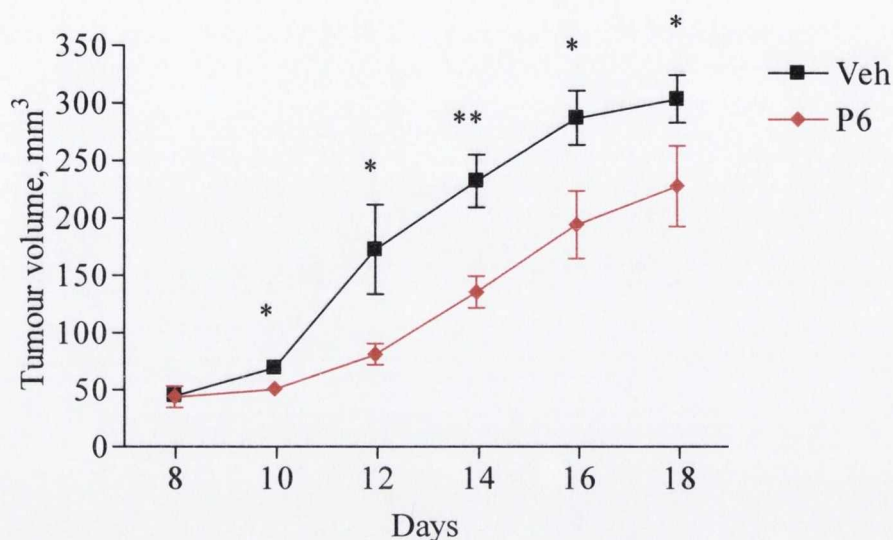


Fig. 5.13 Administration of PBOX-6 significantly inhibits tumour growth in an *in vivo* mouse model of CML containing the T315I mutation.

Five to six week old female BALB/c-nude mice were inoculated s.c into the right flank with 3×10^6 T315I-Bcr-Abl Baf/3 cells in the log phase of growth. When tumours had reached a diameter of ~4mm (day 8), mice were randomly divided into two treatment groups ($n = 6$) and received daily i.t. injections of PBOX-6 (P6) (7.5mg/kg) or the equivalent vehicle (Veh) (10% (v/v) cremophor EL, 10% (v/v) ethanol in PBS). Tumour growth was measured every second day, the long (L) and short (S) axes were recorded and tumour volume (V) calculated using the equation: $V = (S^2 \times L)/2^{209}$. Statistical analysis was performed using the Student's unpaired t-test, *, $p < 0.05$; ** $p < 0.01$.

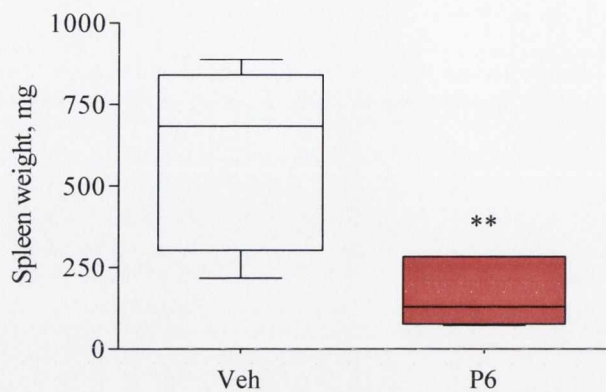


Fig. 5.14 Lack of spleen enlargement following PBOX-6 treatment in an *in vivo* mouse model of CML containing the T315I mutation.

Five to six week old female BALB/c-nude mice were inoculated s.c into the right flank with 3×10^6 T315I-Bcr-Abl Baf/3 cells in the log phase of growth. When tumours had reached a diameter of ~4mm (day 8), mice were randomly divided into two treatment groups (n = 6) and received daily i.t. injections of PBOX-6 (P6) (7.5mg/kg) or the equivalent vehicle (Veh) for 10 days. After which, mice were sacrificed, spleens were removed and weighed. Statistical analysis was performed using the Student's unpaired t-test, **, $p < 0.01$.

5.3 Discussion

Our group has recently developed a novel set of compounds known as the PBOXs which actively induce apoptosis in numerous haematological cancers and solid tumours^{192; 197; 199; 231}. Representative compounds have recently been shown to induce apoptosis in *ex vivo* CLL patient samples including samples which are comprised of cells with poor prognostic markers such as fludarabine-resistant cells with chromosomal deletions in 17p¹⁹¹. Furthermore, an *in vivo* study by our group has previously shown PBOX-6 to exhibit anti-tumour activity in a murine breast tumour model²⁰¹ highlighting their potential as novel anti-cancer agents. Recent data also suggests the PBOX compounds demonstrate activity in MDR resistant cell lines by bypassing P-gp¹⁹⁸.

The tyrosine kinase inhibitor STI571 has revolutionised CML treatment since its emergence on the clinical market in 2001⁵. In spite of this success, disease progression into a fatally acute leukaemia still occurs, albeit significantly delayed when compared to prior treatments such as hydroxyurea and IFN- γ . Disease progression is commonly associated with mutations within the Bcr-Abl gene that result in various degrees of resistance to STI571, these mutations have been identified as the major mechanism of acquired STI571-resistance. Therefore, the search for alternative drug treatments capable of overcoming such resistance is crucially important⁴⁷. For this reason it was necessary to analyse the effects of the PBOX compounds on CML cells harbouring such mutations.

The amino acid mutations chosen for this study represent several functionally distinct kinase domain regions, including the P-loop (E255K), the site of a hydrogen bond with STI571 (T315I) and the activation loop (H396P)³⁸. Transfected cells were firstly subjected to analysis by Western blot and confirmed the presence of the Bcr-Abl protein. A viability assay demonstrated that the mutant Bcr-Abl cells, displayed as expected, various degrees of resistance to STI571 of between 2.9- and 11.1- fold for H396P and E255K cells respectively when compared to native cells. T315I cells showed complete resistance to levels of STI571 as high as 10 μ M. IC₅₀ values and resistance fold difference could not therefore be determined for these cells. However a previous report suggests an IC₅₀ value of 18 μ M for T315I-Bcr-Abl cells treated with STI571 as determined by the MTT viability assay²³⁶.

In comparison to the wide range of effects of STI571 on the viability of Bcr-Abl mutants, PBOX-6 and PBOX-15 reduced cell viability to a similar extent in all four cell lines. As most of these mutations are found in advanced staged CML patients it was

originally assumed that the clones harbouring these mutations expand under the selective pressure of STI571 treatment. Intriguingly however, recent studies have demonstrated the existence of these mutations, particularly the prevalent mutations E255K and T315I, in some Bcr-Abl-positive ALL and CML patients before the onset of STI571 treatment^{41; 42; 43; 44; 45}. One recent study also showed a general hierarchy for proliferation rates amongst Bcr-Abl mutations with Y253F and E255K being the most aggressive, followed by T315I, H396P, native Bcr-Abl and M351T. Similar observations were made with transformation potency, Y253F > E255K > native Bcr-Abl > T315I > H396P > M351T, though results were dependent on cell type and growth conditions³⁸. The Bcr-Abl mutants also showed increased activities in *in vitro* kinase assays⁴⁶. The enhanced kinase activities and proliferation rates of the major STI571 resistance mutations E255K and T315I may confer leukaemia cells with a growth advantage, expanding in the absence of selective pressure from STI571 treatment and resulting in the clonal selection and multiplication of STI571-resistant CML cells and subsequent disease progression^{38; 46}. Mutations such as E255K are also associated with significantly shorter survival rates than other mutations, regardless of their sensitivity to STI571³⁸ resulting in a more aggressive phenotype. This highlights the importance of the PBOX compounds in overcoming these aggressive mutations.

One of the most common Bcr-Abl mutations found in CML patients is the T315I mutation. Currently, many of the STI571-resistant mutations are sensitive to the second generation inhibitors dasatinib and nilotinib however the T315I mutation remains insensitive to both drugs^{48; 47}. Thus it was decided to concentrate our efforts on the T315I mutation for further studies and analyse the effects of the PBOX compounds in combination with STI571 and flavopiridol, as discussed in chapters 3 and 4, on such a mutation. Results showed that PBOX-6/-15 in combination with STI571 increased the percentage of cells undergoing apoptosis in native Bcr-Abl cells but did not increase the levels of apoptosis in T315I cells when compared to treatment with the PBOX compounds alone. These results are not surprising and correlate with the high levels of STI571 resistance displayed by the mutant cells. It is therefore likely that any potential clinical benefit of PBOX compounds in combination with STI571 would be limited to chronic phase patients with no detectable point mutations or for patients whose resistance to STI571 is not linked to Bcr-Abl point mutations but instead related to overexpression and amplification of the Bcr-Abl protein or its downstream effects.

When PBOX-6 and PBOX-15-treated cells were subsequently treated with flavopiridol, the number of cells undergoing apoptosis increased in both native and T315I-

Bcr-Abl cell lines, suggesting the potential of this novel combination in the treatment of CML patients harbouring the T315I mutation.

While there are numerous reports of novel compounds effective at inducing apoptosis in T315I cells in the literature, many are at a pre-clinical stage such as ON012380²³⁷ a non-ATP-competitive inhibitor, SGX393¹⁶ a direct Bcr-Abl inhibitor and the histone deacetylase inhibitor LAQ824²³⁸. Compounds which have displayed activity against the T315I mutation in clinical trials include the aurora kinase inhibitor MK-0457²³⁹ and HHT²⁴⁰ which inhibits protein synthesis. Other compounds currently undergoing clinical assessment but whose anti-tumour activity in patients has yet to be fully determined include the histone deacetylase inhibitors SAHA and LBH589, the farnesyltransferase inhibitor BMS-214662 and the aurora kinase inhibitors PHA-739358 and AT-9283 amongst others^{237; 238}. However, to date no compound with activity against the T315I mutation has made it through trials and onto the clinical market.

While the effects of the PBOXs on Bcr-Abl mutations *in vitro* is clear, these results needed to be confirmed in *ex vivo* patient samples, critically important in predicting a clinical response to potential anti-cancer drugs. Of the newly diagnosed patients enlisted for PBOX evaluation, all *ex vivo* samples analysed (4/4) displayed a significant increase in apoptosis following PBOX-6 and PBOX-15 treatment when compared to STI571 and control treatments. These results suggest a potential role for the pro-apoptotic PBOX compounds in the treatment of newly diagnosed CML patients. Similar enhancements in apoptosis were also observed in two STI571-resistant *ex vivo* samples. One of these patients tested 100% positive for the F317L mutation suggesting the PBOX compounds also successfully induce apoptosis in cells harbouring this mutation. Although no firm conclusions can be drawn from the data of just two patients, these positive results do warrant a larger study assessing the effects of the PBOX compounds on further STI571-insensitive primary samples to be undertaken.

Notably, the PBOX compounds do not display cytotoxicity in blood samples obtained from healthy volunteers^{190; 197}. Furthermore, a low level of vasorelaxing activity and a lack of inhibition of nitrendipine binding, both measures of calcium antagonism suggest a general lack of cardiotoxicity of the PBOX compounds (unpublished results). A lack of *in vivo* toxicity was also observed in healthy Balb/c mice administered with PBOX-6 for 8 days as determined by analysis of the haematology parameters of platelets, WBCs and RBCs when compared to vehicle-treated mice (unpublished data).

Following the demonstration that the PBOX compounds induce apoptosis *in vitro* and in *ex vivo* patient samples we next sought to determine the effects of the PBOX compounds in an *in vivo* CML xenograft model. PBOX-6 was chosen as a representative PBOX compound for *in vivo* testing as it has previously been shown to restrict tumour growth in an *in vivo* mouse mammary carcinoma²⁰¹. Female Balb/c nude mice were injected s.c. with T315I-Baf/3 CML cells and randomly divided into two treatment groups. Results showed that PBOX-6 significantly impeded tumour growth whilst also preventing gross spleen enlargement when compared to mice receiving vehicle injections. Splenomegaly is usually associated with disease progression. Mice did not experience weight loss or exhibit any signs of distress during PBOX treatment suggesting PBOX-6 to be a viable anti-cancer agent *in vivo*. While tumour growth was significantly reduced, the tumour mass did still increase over the duration of the experiment, however the results from this and previous chapters suggest the true potential of the PBOX compounds *in vivo* could lie in their combination with other anti-cancer compounds, with the potential to reduce tumour burden even further¹⁸⁷.

The xenotransplant model has also been successfully used for the pre-clinical drug testing of SCH66336 and SGX393^{16; 19}, it is a relatively simple and straight forward model making it an excellent starting point for early drug discovery programmes like the PBOX compounds. However, the xenograft model is not without its limitations, tumour cell lines used in xenograft mouse studies might not be representative of the tumour in its native state due to being passaged for many generations. These cells may also lack the architectural and cellular complexity of real tumours. For these reasons, the PBOXs should also be tested on a retroviral transduction CML mouse model. This is considered the most accurate animal model of CML that most closely depicts the human form of the disease to date. This model is also the only way of testing the selectability and specificity of potential drugs on leukaemia stem cells^{21; 23}.

In conclusion, the PBOX compounds significantly reduced the cell viability of CML cells lines harbouring the E225K, H396P and T315I Bcr-Abl mutations. In addition, an enhancement of apoptosis was observed in native Baf/3 cells when the PBOXs were combined with the current CML treatment, STI571. The novel CDK inhibitor flavopiridol also significantly enhanced the apoptotic potential of the PBOXs in STI571-resistant T315I cells. Both PBOX-15 and PBOX-6 significantly increased the number of cells undergoing apoptosis in *ex vivo* patient samples while PBOX-6 also significantly reduced tumour burden in a mouse xenograft model. These results underline the potential of the PBOX

compounds in the clinical treatment of CML patients including those who develop the highly resistant T315I mutation.

Chapter 6.
General discussion

CML is a member of a group of myeloproliferative diseases and is currently one of the most extensively studied malignancies. Characterised by the genetic abnormality known as the Ph chromosome, the last decade has brought huge advances in survival rates and treatment options for CML patients mainly due to the discovery of the highly effective tyrosine kinase inhibitor STI571²⁶. However, as is inevitably the case, most patients will eventually become resistant to STI571 ultimately leading to a fatal disease progression. So at a time when cancer drug resistance is increasingly more prevalent a drive toward combination therapy is emerging. This prompted us to analyse the interaction between the PBOX compounds and other anti-cancer agents.

The PBOX compounds, originally synthesised as high-affinity ligands for the PBR receptor^{184; 189} can be subdivided into two groups with distinct mechanisms of action, G1-arresting agents and apoptosis-inducing compounds. Our results from Chapter 3 show that PBOX-21, a representative of the G1-arresting PBOX compounds, synergistically enhanced the apoptotic ability of STI571 through an enhanced downregulation or cleavage of Bcr-Abl and the anti-apoptotic proteins Bcl-X_L, Mcl-1 and Bcl-2 and upregulation of the pro-apoptotic Bcl-2 protein Bim. Both caspase-8 and chymotrypsin-like serine proteases were also found to be involved in PBOX-21/STI571-induced apoptosis suggesting a role for both caspase-dependent mechanisms, of which there is a great deal known^{140; 141}, and caspase-independent mechanisms, of which there is little known^{136; 167}. Reports demonstrating the involvement of a caspase-dependent and a separate caspase-independent pathway during apoptosis are limited^{221; 222} and this study will help to better our understanding of such complex signalling pathways.

As PBOX-21 does not induce apoptosis alone, this novel combination will likely only be effective against carcinomas in which STI571 has proven effective, however as STI571 has recently been approved for the treatment of a number of other malignancies²⁸ the co-administration of PBOX-21 and STI571 may also show enhanced activity in these diseases.

The second group of PBOX compounds are MTAs and actively induce apoptosis in a wide variety of malignancies. Publications demonstrating the potent CDK1 inhibitor flavopiridol, which is currently undergoing clinical evaluation, to enhance MTA-induced tumour cell apoptosis^{124; 125} prompted us to examine whether this CDK1 inhibitor could similarly enhance PBOX-induced apoptosis in CML cells. The next results chapter demonstrates that sequential treatment with the pro-apoptotic PBOX compounds and

flavopiridol synergistically enhanced apoptosis in CML cells compared to either agent alone. The addition of flavopiridol following PBOX-6 treatment resulted in an accelerated exit from the G2/M transition accompanied by an enhanced downregulation and deactivation of the CDK1/cyclin B1 complex and an enhanced degradation of survivin.

In cells where survivin was knocked down using siRNA, there was a significant enhancement of the levels of apoptosis observed after administration of PBOX-6 compared to cells transfected with a scrambled scRNA. This is the first account to date of the involvement of survivin in PBOX-induced apoptosis. Survivin, a key regulator of mitosis and programmed cell death, represents an ideal target for cancer therapies as it is uniquely overexpressed in cancer cells whilst only transiently expressed during mitosis in non-cancerous cells⁸⁴. Several therapeutic strategies targeting survivin have recently been described⁸⁴ including small chemical inhibitors such as YM155^{93; 94} and terameprocol⁹⁵ though it is likely that survivin inhibitors in combination with other anti-apoptotic agents, such as the PBOXs, may be a more attractive option with greater clinical potential. Studies such as the present ones which analyse part of the downstream signalling pathways induced by novel drug combinations should help to further clarify drug-drug interactions.

CML is the best example of an oncogene being solely responsible for the development of a cancer, other single mutations are rare in solid tumours. Exceptions are limited to NSCLC with mutations in the EGF receptor²⁴¹, exon 11 mutations in c-KIT-positive GIST tumours²⁴² and a hereditary form of papillary renal cell carcinoma in which von Hippel-Lindau mutations are responsible for the upregulation of angiogenic factors such as VEGF and PDGF²⁴³. Despite the presence of a single transforming oncogene, therapy with single agents directly targeting these mutations results in few complete remissions which are usually of short duration. While the majority of CML patients experience a long-lasting haematological and cytogenetic remission while receiving STI571 treatment, complete elimination of blast cells occurs in less than 20% of patients and there is a constant overall relapse rate of 1 to 2% per year due to residual disease. As a result, drug combinations have been employed widely in the treatment of such cancers²⁴⁴.

The fundamentals of combination therapies, as first described by Frei in the 1960s²⁴⁵ include using compounds with non-overlapping toxicities allowing each compound to be administered at near-maximal dose, combining agents that inhibit separate pathways known to be critical to the survival of the tumour (e.g. apoptosis and angiogenesis) and administering the full combination as early as possible in the disease²⁴⁵. However, recent studies such as the combination of PBOX and flavopiridol compounds

suggest that in some cases it may be of more benefit to administer the compounds sequentially. Ideally, the best chances of success will be when both agents in a combination inhibit multiple alternate forms of the target, eliminating resistance due to mutations in the target protein²⁴⁴.

Use of the maximum tolerated dose (MTD) of each compound ensures that maximal therapeutic activity will be achieved. While this may hold true for single agents, some agents are antagonistic when administered at certain doses in combination whilst being synergistic at other doses, so using combined drugs at MTD may not always be beneficial²⁴⁶. Maintaining drugs in the ratios required to produce synergistic effects requires strict control of the distribution, metabolism and excretion of compounds following administration *in vivo*. For example, using conventional aqueous formulations the majority of cisplatin is eliminated within 1 h of administration whilst the elimination half-life of paclitaxel is greater than 10 h²⁴⁶. The pharmacokinetics of both drugs ensures that co-treatment for NSCLC causes the ratio of the drugs to change dramatically. However new formulations and the advent of nanotechnology allows for greater control in this context and will be important to consider in the progression of the PBOX compounds through to more advanced clinical testing.

A typical treatment regime for CML patients entering into the accelerated phase of the disease will include increasing the dose of STI571, this will normally control the disease for around 12 mths before the number of blast cells begins to increase again²⁶. When this occurs and patients have entered into blast crisis, patients will again be offered a dose increase of STI571, however this is usually severely limited by dose-limiting side effects. Failing this, patients may be put on a high dose of either of the two second generation tyrosine kinase inhibitors recently approved for the treatment of STI571-resistant CML, dasatinib and nilotinib. However, how best should these inhibitors be administered, alone or in combination? It is yet to be determined whether either of the newer drugs have equal activity to STI571 in non-mutated Bcr-Abl cells as these drugs have not been approved for the treatment of the chronic phase of the disease. Administering STI571 until resistance develops and then switching to dasatinib or nilotinib in the blast crisis phase is not likely to be any more curative, as one would be facing a resistant disease whether with a single agent or multiple agents. However, potential overlapping toxicities of all three drugs together may represent a problem for their concurrent use. Thus, sequential use of two or three of these drugs against CML in a similar fashion to the PBOX and flavopiridol co-administration might therefore be a more

feasible strategy, either way more clinical studies would need to be investigated in order to determine the optimal treatment regime²⁴⁴.

The final results chapter focuses on the ability of the pro-apoptotic PBOX compounds to overcome STI571-resistance due to the presence of a number of Bcr-Abl mutations most notably the T315I mutation. The PBOX compounds were found to actively induce apoptosis in cells which contain the E225K and H396P mutations along with the highly resistant T315I mutation *in vitro* suggesting PBOX activity to be independent of the mutational status of Bcr-Abl. PBOX-6/15 in combination with STI571 did not enhance apoptotic levels any further in cell lines that contained the T315I mutation. However, the pro-apoptotic PBOX compounds in combination with flavopiridol did not display the same cross resistance to the T315I mutation and enhanced apoptosis when compared to either agent alone. While there are many compounds in clinical trials demonstrating activity against the T315I mutation, the current second generation tyrosine kinase inhibitors dasatinib and nilotinib do not, emphasising the importance of finding anti-cancer agents that do effectively inhibit these cells⁴⁷. The emergence of STI571 resistance highlights the possibility that any novel compound may potentially become the victim of intrinsic cellular resistance, including the PBOX compounds.

There are many different mechanisms through which cells can develop resistance to compounds including upregulation of the target protein, activation of signalling pathways downstream of the target protein or activation of altogether separate signalling pathways such as cell survival pathways. Cancer cells may also accumulate additional oncogenic mutations causing chromosomal instability. As the pro-apoptotic PBOXs function to depolymerise tubulin, point mutations within tubulin itself could potentially lead to PBOX-insensitivity. Acquired resistance to MTAs due to mutations in tubulin has previously been documented. Epothilone-resistant A549 and HeLa cell lines contain point mutations close to the M-loop, the nucleotide-binding site and the microtubule-associated protein binding sites of β -tubulin²⁴⁷ while paclitaxel-resistant chinese hamster ovary cells contain mutations in the H-loop. Interestingly, this study also found a tubulin mutation, L215, that enhanced the binding and sensitivity of paclitaxel²⁴⁸. However, we have previously shown, that the PBOXs bind to an as yet unidentified novel binding site on tubulin¹⁹⁹. Therefore it is unlikely that the tubulin mutations that confer resistance to some of the other MTAs would confer resistance to the PBOX compounds, however, we cannot eliminate the possibility that mutations may also occur in the PBOX binding site that may affect PBOX activity. Therefore it will be important to fully characterise this binding site.

Despite the success of MTAs, significant limitations with many compounds such as the taxanes exist, including acquired and intrinsic drug resistance, dose-limiting haematopoietic toxicity and cumulative neurotoxicity²⁴⁹. There is therefore a need to develop novel agents that target microtubules. Such novel agents include the epothilones such as patupilone and its derivatives, the novel synthetic compound indibulin (D-24851) and laulimalide which is derived from marine sponges. These agents are at varying stages of development^{181; 249}.

Whilst resistance to MTAs is mediated by multiple mechanisms including development of mutations within the MTA binding site of tubulin as outlined above, overexpression of P-gp, one of the main ABC transporters involved in the efflux of drugs is often the cause of acquired taxane resistance²⁵⁰. We have recently demonstrated that the PBOXs do not appear to be substrates for P-gp or another ABC transporter BCRP and actively induce apoptosis in MDR cell lines¹⁹⁸.

As we have demonstrated that the PBOXs do not appear to be substrates for drug efflux pumps, they may be able to bypass such pumps that are present in the choroid plexus to exclude potentially neurotoxic species from the CNS. Although we have yet to directly determine the neurotoxic effects of the PBOXs *in vivo* it is of interest to note that neither patupilone or indibulin, which are both currently undergoing clinical trials, appear to be susceptible to P-gp and similar drug efflux pumps and yet they exhibit minimal neurotoxicity^{249; 251}. Indibulin has been shown to discriminate between highly post-translationally modified tubulin present in mature neuronal microtubules and less-modified tubulin present in immature neuronal or non-neuronal microtubules²⁵². Indibulin appears to be specific for non-modified tubulin whereas vincristine acts on either tubulin equally well. It will be important in future studies to directly determine any neurotoxic effects of the PBOXs *in vivo*. However, what is promising is that in a previous study with a variety of MTAs such as vincristine, colchicine and indibulin, neurotoxicity was strongly correlated to loss of body weight in animal studies²⁵¹. We have shown that the PBOXs, at anti-tumoural efficacious doses *in vivo*, exert no effect on body weight suggesting that they may also exhibit limited neurotoxicity²⁰¹.

Chapter 5 also assessed the ability of the PBOXs to induce apoptosis in *ex vivo* patient samples and inhibit tumour growth in an *in vivo* CML mouse model. The pro-apoptotic PBOXs successfully induced apoptosis in both newly diagnosed and STI571-resistant CML patient samples again emphasising their potential for the treatment of STI571-resistant CML. While this study was restricted by the low number of patient

samples, these positive results warrant a further larger-scale study being undertaken. PBOX-6 also significantly reduced tumour burden in a xenograft mouse model harbouring the T315I mutation.

Xenograft mouse models use tumour cell lines that may not be representative of the tumour in its native state. These cells may lack the architectural and cellular complexity of real tumours, which incorporate inflammatory cells, vasculature and other stromal components. Additionally, the xenograft-host interactions under the skin in subcutaneous tumours may be different from those in the tissue of tumour origin²⁵³. Nonetheless, despite their limitations, xenograft models are an excellent starting point for early drug discovery programmes such as the PBOX compounds. Recent advances in xenograft models include the development of more advanced orthotopic models and imaging methods using fluorescent detection methods. However, animal models of cancer, when compared to animal models of other diseases such as cardiovascular diseases, tend to be poor predictors of the clinical outcome of novel compounds²⁵³. Many compounds that cause tumours to completely regress in cancer animal models fail at clinical trials highlighting the need for more accurate mouse models that more closely resemble the disease in humans such as the retroviral mediated mouse model²⁵³.

One suggested reason for the poor predictive success of some animal models is the presence of cancer stem cells. While the concept of leukaemia stem cells has been around for decades, it has only been in the last few years that experimental analysis of these cells has taken place. Recent studies have indicated that various forms of leukaemias arise from a small and biologically distinct subpopulation of tumour-initiating stem cells. While STI571 is successful at eradicating bulk disease, it has been suggested that relapses and residual disease may be due to the failure to eliminate leukaemia stem cells²³. As new methods emerge to analyse these cells it will be crucially important to ensure that novel compounds are selected to target these cells^{254, 255}. The effects of novel compounds on leukaemia stem cells are not currently being assessed in xenograft models. However, the development of a retroviral-mediated CML mouse model that originates from leukaemia stem cells provides the hope that more accurate mouse models for cancer may improve the clinical success of potential cancer therapies²³. It is yet to be seen whether the PBOX compounds will prove active in such a sophisticated model.

In conclusion, this study has revealed that the PBOX compounds exert activity against CML cells alone and in combination with STI571 and flavopiridol, two agents with different molecular targets and mechanisms of action. The PBOXs also display activity in

ex vivo CML patient samples and an *in vivo* mouse model of CML providing further insight into the use of these novel PBOXs as anti-cancer therapeutics for the treatment of CML, including drug refractory CML. This study should also help in the further understanding of the interaction between different compounds ultimately paving the way for potentially improved cancer therapies, prolonged remissions and enhanced quality of life for cancer patients.

Chapter 7.
Future work

The results of chapter 3 highlight part of mechanism through which PBOX-21 enhances the apoptotic effect of STI571. However further experiments to identify the molecular target of PBOX-21 and other G1-arresting compounds are warranted. We have previously shown that PBOX-21 causes a downregulation in cyclin D2 and D3 expression and a decrease in CDK2, 4 and 6 activity¹⁹³. It is possible that PBOX-21/STI571-enhanced apoptosis in CML cells is due to changes in cyclin D expression/activity which could be assessed through Western blotting. This information would allow us to further characterise the interaction between the PBOX-21 and STI571. Analysis of the signalling pathway upstream of caspase-8, for example by looking at the role of the death receptors in the PBOX-21/STI571-induced apoptotic pathway would also increase our knowledge of this novel combination. Expression levels of DR4 and DR5 could be examined by Western blotting. The use of neutralising antibodies to DR4/5 could also be used to determine their involvement in PBOX-21/STI571-induced apoptosis.

Similarly, further experiments looking at the PBOX-6/flavopiridol combination should be initiated. As flavopiridol is a general CDK inhibitor that can inhibit other CDKs, such as CDK9 which is involved in transcription²⁵⁶, it is also possible that there are other mechanisms through which flavopiridol can enhance PBOX-induced apoptosis apart from inhibiting the mitotic block that the PBOX compounds promote. Further experiments to rule in or out other pathways should therefore be undertaken using inhibitors of signalling pathways such as the PI3K inhibitors LY294002 and wortmannin and the MEK inhibitor U0126. It would also be of interest to determine if other CDK inhibitors such as olomucine, UCN-01 or xylocydine could also produce synergy when combined with the PBOXs. The involvement of the phosphorylated form of survivin could also be analysed using a nonphosphorylatable form of survivin (T34A) in comparison with a phosphomimetic version of survivin (T34E)²⁵⁷.

The link between depolymerising tubulin and the activation of apoptotic signalling pathways could also be analysed. The pro-apoptotic Bcl-2 protein Bim has previously been shown to be sequestered to microtubules under normal physiological conditions, however it has been reported that JNK activation can result in phosphorylation and release of Bim and translocation to the mitochondria²⁵⁸. As JNK activation is a crucial requirement for the induction of PBOX-6-induced apoptosis¹⁹⁵ this may explain in part the mechanism through which depolymerised tubulin activates apoptosis following PBOX treatment. Western blotting and subcellular fractionation experiments could be performed to determine

whether Bim becomes enriched in the mitochondrial fraction following PBOX treatment. It would also be of interest to knock-down Bim using siRNA to determine whether decreased Bim expression decreases the susceptibility of tumour cells to PBOX-mediated apoptosis.

The successful demonstration of the PBOX compounds working in synergy with both STI571 and flavopiridol as presented in this report warrants further analysis of PBOX compounds in combination with other chemotherapeutic agents. In particular, MTAs have also been shown to synergise with TNF-related apoptosis inducing ligand (TRAIL), a ligand of the TRAIL receptor in the extrinsic apoptosis pathway *in vitro* and *in vivo*. The TRAIL receptor is a member of the TNF- α superfamily of death receptors. The soluble form of the ligand is emerging as an attractive anti-cancer agent because of its cancer cell specificity and potent anti-tumour activity combined with its inactivity against normal healthy tissue²⁵⁹. Binding of TRAIL to the extracellular domain of agonistic receptors TRAIL-R1 or TRAIL-R2 culminates in apoptosis, other receptors TRAIL-R3, TRAIL-R4 and osteoprotegerin act as decoys or transduce anti-apoptotic signals²⁶⁰. *In vitro*, co-administration of TRAIL with DNA-damaging agents, irradiation, the proteasome inhibitor bortezomib, smac inhibitors, carboplatin, camptothecin, cisplatin, doxorubicin, 5-fluorouracil, the kinase inhibitor sorafenib or inhibitors of histone deacetylases significantly enhance apoptosis²⁵⁹. Arsenic trioxide, which employs a wide variety of mechanistic actions including targeting tubulin, has also been shown to enhance the effect of TRAIL²⁶¹. Anti-TRAIL-R1 (mapatumumab, HGS-ETR1) is currently in phase II clinical development as a single agent in patients with NSCLC and colorectal cancer²⁶² and also recently underwent clinical evaluation in combination with paclitaxel and carboplatin in a phase I trial against advanced solid tumours showing a preliminary clinical benefit²⁶³. Human antibodies to TRAIL-R2 (HGSETR2 and HGS-TR2J) have also entered the clinic and are currently in phase I clinical development²⁶². It is likely the PBOX compounds may also display similar effects when combined with reagents such as TRAIL and preliminary studies to determine if this is the case are currently ongoing.

As our results have shown that both pro-apoptotic and G1-arresting PBOX compounds synergise with STI571 in CML cells it is also possible that the PBOXs may similarly enhance the efficacy of STI571 in other cancers for which STI571 has proven effective, one such malignancy being GIST. GIST, are the most common mesenchymal neoplasm of the gastrointestinal tract and are highly resistant to conventional chemotherapies. GIST can arise anywhere along the gastrointestinal tract but are most commonly found in the stomach (50-65%), small bowel (20-30%), colon/rectum (5-10%)

and the oesophagus (5%) arising from interstitial cells of Cajal or their stem cell-like precursors. GIST occur with an incident rate of ~20 cases per million but varies depending on population. To date no risk or causative factors have been identified however 3/4 GIST are diagnosed in patients aged over 50²⁶⁴. Recent studies suggest that very small asymptomatic GIST are widespread in the population, the majority of which do not progress to major disease²⁶⁵. In 1998 the tyrosine kinase receptor c-KIT was identified as containing a gain-of-function mutation in more than 95% of GIST²⁶⁶. c-KIT encodes a transmembrane tyrosine kinase receptor which is activated by binding of SCF leading to receptor dimerisation and autophosphorylation. This causes activation of downstream intracellular signalling cascades involved in cell proliferation, adhesion and differentiation. Of the four different regions of c-KIT that have been found to be mutated in GIST, exon 11 is most frequently affected in up to 2/3 of patients. Other less commonly found mutations include PDGFR α and exon 9 of c-KIT²⁶⁷.

While surgery remains the frontline treatment for localised resectable GIST at least 50% of patients experience tumour recurrence. In 2002 STI571 was approved for their treatment of unresectable GIST prolonging median survival from 2 to 5 years. Despite the increase in survival duration, resistance to STI571 in GIST can occur²⁶⁴. Production of stable GIST lines, GIST882 and GIST48, derived from STI571-sensitive and STI571-resistant tumours have recently enabled testing of potential anti-cancer agents *in vitro*²⁶⁸. Our laboratory has recently acquired these cell lines and work is planned to analyse the effect of STI571 in combination with the PBOX compounds in these cells.

While two depolymerising MTA binding sites on tubulin have been identified, the binding sites of vinblastine and colchicine, previous research has shown the pro-apoptotic PBOX compounds bind to a novel binding site on tubulin. Other anti-tubulin agents such as estramustine²⁶⁹ and arsenic trioxide²⁷⁰ have also been shown to bind to as yet uncharacterised binding sites. X-ray crystallography using purified bovine brain tubulin will be used to characterise this binding site. Phosphoproteins of the stathmin family interact with tubulin and interfere with microtubule dynamics. Formation of a tubulin:stathmin complex stabilises tubulin in solution allowing for potential crystallisation. The x-ray structure of RB3, a neural protein containing a stathmin-like domain (SLD), in complex with tubulin has previously been reported²⁷¹. More recently, the structure of tubulin in complex with colchicine and RB3:SLD has been published providing insight into the mechanism of colchicines activity²⁷². Such an approach could be taken to produce tubulin-PBOX complex crystals for subsequent analysis using diffraction

techniques allowing for the identification and characterisation of the PBOX binding site on tubulin.

The PBOX compounds have also been shown to bind to the PBR receptor. Although the receptor is not implicated in their ability to induce apoptosis it does highlight the possibility that the PBOX compounds may target other molecules and thus display other mechanisms of action. Characterisation of the tubulin-PBOX binding motif would thus enable computer searches for similar binding motifs in other proteins allowing for the identification of further possible PBOX targets.

The rational design, synthesis and screening of second generation PBOX analogues by *in silico* virtual screening methods should be undertaken using both structure-based and ligand-based drug design. Knowledge of the characterised PBOX binding site on tubulin will help design compounds with improved potency and physiochemical properties. All molecular visualisation for hit finding and lead optimisation will be performed using MOE software. Informed by the binding and cellular potency data on the PBOX compound series to date, pharmacophore and QSAR models will be developed to describe their activity. In parallel with the ligand-based studies, protein binding features will be qualified and assessed for ligand accessibility. Protein-ligand contacts will be quantified and prioritised. Using both rigid and flexible docking, all known active and inactive ligands in the PBOX series will be computationally ‘docked’ in the tubulin binding site and their binding modes will be assessed through analysis of protein-ligand interactions. A model for ligand potency will be derived. Using classical medicinal approaches and *de novo* structure generation tools, libraries of modified potential PBOX compounds will be computationally enumerated and docked in the active site. These libraries will be pre-filtered using Daylight chemoinformatics software to optimise their pharmacokinetic and absorption, distribution, metabolism, elimination and toxicity (ADMET) properties. The overriding goal of this will be to “scaffold-hop” away from the PBOX molecular architecture and develop new chemistries with an equivalent biological effect^{273; 274}. Lead compounds will then be synthesised and biologically evaluated to determine their capacity to reduce cellular viability and induce apoptosis in a range of cancer cell lines including CML cells. This analysis should also help to determine any structure-activity relationships that may be present in the compounds.

Numerous novel PBOX analogues were produced by collaborating laboratories and analysed for apoptotic potential throughout the duration of this project including the short series of PBOX-STI571 hybrids created by Prof. Campiani’s laboratory developed using

the application of standard organic chemistry methodologies. Efforts to create further hybrids based on these preliminary results are continuing and while to date no compound has been identified as being more potent than either PBOX-6 or PBOX-15 it is hoped that more potent novel PBOX analogues can be identified and developed further.

While the present study highlighted the positive results of the pro-apoptotic PBOX compounds in *ex vivo* CML patient samples, the study was too small to make any definitive conclusions. A larger *ex vivo* study should be undertaken, similar to that of PBOX-15 in CLL samples where 55 patients were enrolled in the study¹⁹¹.

Further *in vivo* testing of PBOX compounds and optimisation of the CML retroviral stem cell mouse model will also be undertaken. A recent study has used this model for the characterisation of anti-tumour agents on CML stem cells. A plasmid containing the Bcr-Abl gene and additional mutations Nup98/HoxA9, thought to be associated with the blast crisis²², was used to transform an ecotropic packaging cell line, resulting in the formation of replication-deficient retroviruses containing the required genes. The viral supernatant was then added to freshly isolated haematopoietic stem cells from donor mice. Infected cells were then introduced i.v. into recipient mice inducing CML within 14 days²³. A similar experiment could be undertaken to analyse the effect of the PBOX compounds on a stem cell based CML mouse model with daily injections of PBOX-6 alone and in combination with STI571 or flavopiridol. For these combination studies, mice would be divided into groups treated with a vehicle only, a PBOX compound at three or four doses, STI571/flavopiridol at three or four doses and a combination of both compounds at several constant and non-constant combination ratios. Each group would contain six mice and the study would take 2 to 3 mths to complete.

As the PBOX compounds progress through to clinical testing it will be important to consider the properties of ADMET along with the formulation and solubility of the compounds *in vivo*. Many of these aspects of drug ADMET can be determined *in vitro* facilitating the selection of drug candidates with a high probability of clinical success²⁷⁵.

The easiest and preferred route for drug administration is oral administration, as a result high intestinal permeability is desired. The human colon carcinoma Caco-2 cell line represents the standard for drug permeability studies. This involves the use of semi-permeable membranes layered over chamber wells, permeability is then measured by quantifying of amount of the compound in the lower well^{276; 277}. MDCK kidney tumour cells stably transfected with P-gp are similarly used to evaluate the ability of a novel drug to penetrate through an intact cell layer²⁷⁸, while immobilised artificial membranes, known

as PAMPA are a less expensive, high throughput screening alternative for assessment of intestinal permeability²⁷⁹.

Once a drug enters the circulation system, it is likely to be subjected to hepatic metabolism and renal excretion. A short plasma half-life may require continuous i.v. infusion compared to, ideally, once or twice daily administration. Important drug metabolising cytochrome P450 (CYP) enzymes are CYP1A2, CYP2A6, CYP2B1, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. While they are mainly found in the liver, the intestine also contains a high concentration of CYP3A4. *In vitro* assays to assess metabolic stability involve human liver microsomes, subcellular membranes prepared from livers, designed to evaluate stability towards P450 oxidation²⁸⁰. They can also be used for drug-drug interaction studies. The compound to be evaluated is placed in a reaction mixture containing microsomes and the cofactors required for P450 oxidation. The disappearance of the compound is charted in relation to time. Human hepatocytes are similarly used representing a whole system that does not require exogenous cofactors, however difficulty in obtaining primary hepatocytes can be problematic^{280, 281}. It will be important to assess the permeability and metabolism of the PBOX compounds using assays such as those described.

Limitations associated with the use of MTAs such as the taxanes include dose-limiting hepatotoxicity and cumulative neurotoxicity. The possibility exists that the PBOX compounds may also cause such effects. It will therefore be important in future studies to directly determine any neurotoxic effects of the PBOXs *in vivo*, for example, by the use of a co-ordination (rota-rod) test or by measuring nerve conductance velocity following administration of the PBOXs. *In vivo* studies with the PBOX compounds did not result in loss of body weight suggesting no major systemic toxicities, however more specific testing on primary human hepatocytes can be used to identify hepatotoxicity, primary cardiomyocytes for cardiotoxicity and primary human kidney tubule cells for nephrotoxicity²⁸⁰.

Drug formulations and encapsulations offer the opportunity for direct access of a compound to the bloodstream as well as targeting to specific organ and tissue sites. The choice of drug delivery vehicle is critically important in maintaining drugs in their desired ratios and concentrations. Early drug delivery devices included mucosal inserts, sub-cutaneous or intra-muscular implants, ingestible capsules and topical skin patches²⁸². More commonly used vehicles today include liposomes and micelles while the recent advent of

nanotechnology has lead to the development of carriers such as nanoparticles and polymer-drug conjugates used for the delivery of anti-cancer drugs and other therapeutic agents²⁸³.

Liposomes, triglyceride emulsions and micelles are hydrophobic lipid formulations consisting of external phospholipids surrounding an internal aqueous core used for the delivery of water soluble agents. However limitations have included the small number of lipid delivery vehicles which are approved for clinical use, the increasing number of drugs that are not soluble or only partially soluble in lipid solvents and the requirement for more cancer targeted controlled drug releases²⁸³. Surface attachment of tumour directing peptides and antibodies to liposomes has shown positive results in overcoming the latter of these problems^{283; 284}. Nanotechnology is the latest era of controlled drug release. Nano-scale drug delivery vehicles such as nanoparticles, nanoemulsions, nanosuspensions and polymer-drug conjugates have a solid core composed of hydrophobic polymer and are used for the delivery of hydrophobic agents (e.g. paclitaxel). Advantages include delaying the excretion of a drug from the circulatory system, eliminating early-phase drug distribution, enhanced penetration of tumour tissues due to their leaky vasculature and poor lymphatic drainage^{285; 286} and allowing drug combinations to be delivered to the tumour at the desired synergistic ratio. These nano-scale delivery particles can also control the release rate of anti-cancer agents following injection thus maximising the therapeutic index²⁴⁶. *In vitro*, the PBOX compounds are soluble in ethanol, therefore it is more likely that would fall into the water soluble group requiring liposomal delivery²⁸³.

In summary, the current study has elucidated much of the molecular mechanisms underlying the PBOX compounds in combination with other anti-leukaemic agents STI571 and flavopiridol. Further *in vivo* characterisation of the compounds will be required as will elucidation of ADMET properties and formulation issues in order to translate the PBOX compounds from bench to clinic.

Chapter 8.

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Publications