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**STUDIES OF OVERCOMING ACQUIRED RESISTANCE:
MOLECULAR MECHANISMS AND DEVELOPMENT OF
NOVEL DRUGS**

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To my family

ABSTRACT

Chemotherapeutic agents have become widely applied for treatment of various types of malignancies. Drug resistance unfortunately remains as a major obstacle for the effectiveness of chemotherapy. Cancer drug resistance includes two broad categories: intrinsic and acquired. In this thesis I have examined the problem of acquired drug resistance and have aimed to develop novel approaches to overcome acquired resistance.

Clofarabine is a second-generation nucleoside analogue which has been employed primarily for the treatment of hematological malignancies. In paper I, we found that clofarabine inhibited [¹⁴C]-thymidine uptake, presumably by decreasing DNA synthesis. Clofarabine was also found to induce apoptosis of a solid tumor cell line, a finding which may open new fields of application of this drug.

In paper II, 5'-nucleotidases, a family of enzymes known to confer resistance to nucleoside analogues, were found to be expressed at various levels in samples from CLL patients. Moreover, degradation of fludarabine monophosphate was found to be associated with CN2 activity, and degradation of cladribine monophosphate was associated with CN1 activity. This result helps to explain why some leukemic patients may show resistance to different nucleoside analogues.

In paper III, we screened the RPMI 8226 myeloma cell line and its multidrug resistant subline 8226/Dox40 for the response to 3,000 chemically diverse compounds. We found one compound, designated VLX40, which showed strong cytotoxicity to the drug resistant cells. VLX40 was found to be cytotoxic to myeloid and lymphoid leukemia cells. Mechanistic studies showed that VLX40 is a novel microtubule inhibitor. The efficacy of the compound may potentially be improved leading to the development of novel tubulin active agents that are insensitive to common mechanisms of cancer drug resistance.

b-AP15 is a novel small molecule inhibitor of the ubiquitin-proteasome system. b-AP15 inhibits the USP14/UCHL5 deubiquitinases of the 19S proteasome and shows anti-tumor activity in a number of tumor models. In paper IV, we examined the anti-multiple myeloma activity of b-AP15. We found that b-AP15 has significant efficacy in multiple myeloma (MM) disease models, including cells resistant to the proteasome inhibitor bortezomib. The finding provides the framework for clinical evaluation of USP14/UCHL5 inhibitors to improve patient outcome in MM. In paper V, we show that despite the fact that b-AP15 is a reversible enzyme inhibitor, it induces rapid commitment to apoptosis/cell death. We show that the compound is rapidly taken up and enriched in cells, findings that explain the difference in potency of b-AP15 in biochemical and cellular assays.

Based on the effective anti-cancer activity of b-AP15, we performed a lead optimization procedure aimed to identify efficient b-AP15 analogues with improved solubility. In paper VI, we identified the analogue VLX1570 which has similar biochemical activity as b-AP15. VLX1570 has strong antineoplastic activity in multiple myeloma cells and is capable of overcoming bortezomib resistance. We conclude that VLX1570 is a promising candidate for the clinical management of multiple myeloma.

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- I. **Wang X**, Albertioni F.
Effect of clofarabine on apoptosis and DNA synthesis in human epithelial colon cancer cells.
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- II. Mirzaee S, **Wang X**, Bayat N, Fyrberg A, Lotfi K, Karlsson K, Juliusson G, Eriksson S and Albertioni F.
5'-Nucleotidase activities in blood cells from untreated patients with B-cell chronic lymphocytic leukemia: Correlation to the efficacy of nucleoside analogue therapy.
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- III. Fryknäs M*, Gullbo J*, **Wang X***, Rickardson L, Jarvius M, Wickström M, Hassan S, Andersson C, Gustafsson M, Westman G, Nygren P, Linder S, Larsson R.
Screening for phenotype selective activity in multidrug resistant cells identifies a novel tubulin active agent insensitive to common forms of cancer drug resistance.
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- IV. Tian Z, D'Arcy P, **Wang X**, Ray A, Tai YT, Hu Y, Carrasco RD, Richardson P, Linder S, Chauhan D, Anderson KC.
A novel small molecule inhibitor of deubiquitylating enzyme USP14 and UCHL5 induces apoptosis in multiple myeloma and overcomes bortezomib resistance.
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- V. **Wang X**, Stafford W, Mazurkiewicz M, Fryknäs M, Brjnic S, Zhang X, Gullbo J, Larsson R, Arnér E, D'Arcy P, and Linder S.
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- VI. **Wang X**, Mohanty C, Fryknäs M, D'Arcy P, Olofsson HM, Bossler F, Larsson R, Gullbo J, Linder S.
Development of the proteasome deubiquitinase inhibitor VLX1570 for treatment of multiple myeloma.
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LIST OF ABBREVIATIONS

5'-NT	5'-nucleotidases
AAA+ATPases	ATPases associated with diverse cellular activities
ABC	ATP-binding cassette
ADA	Adenosine deaminase
Admr1	Adrenomedullin Receptor
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
AK	Adenosine Kinase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
Apaf-1	Apoptotic protease activating factor 1
Ara-C	Cytosine arabinoside
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
AZT	azidothymidine
Bad	Bcl-2-associated death promoter
Bag-1	Bcl-2-associated athanogene
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
Bcl-xL	B-cell lymphoma extra large
BCRP	Breast cancer resistance protein
BH	Bcl-2 homology
Bid	BH3 interacting-domain death agonist
Bik	Bcl-2 interacting killer
Bim	B-cell lymphoma 2 interacting mediator of cell death
Bip	Binding immunoglobulin protein
BL	Binding loop
CAFdA	2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, clofarabine
caspase	CysteinyI aspartate proteinase
CdA	2-chloro-2'-deoxyadenosine, Cladribine
CDD	Cytidine deaminase
CDK	Cyclin dependent kinase

CDKI	Cyclin dependent kinase inhibitor
CHIP	Carboxyl terminus of Hsc70 Interacting protein
CHOP	C/EBP-homologous protein
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
CN	Cytosolic 5'-nucleotidase
CP	Core particle
C-terminal	Carboxy-terminal
CTP	Cytidine triphosphate
dCK	Deoxycytidine kinase
dCMP	Deoxycytidine monophosphate
dCTP	Deoxycytidine triphosphate
dFdC	2',2'-Difluorodeoxycytidine
dFdCTP	2',2'-Difluorodeoxycytidine triphosphate
dGK	Deoxyguanosine kinase
dGMP	Deoxyguanosine monophosphate
dIMP	Deoxyinosine monophosphate
DISC	Death-inducing signaling complex
DMB	Dynein motor binding
DNA	Deoxyribonucleic acid
dNKs	Deoxynucleoside kinases
dNT	Deoxynucleotidase
dNTP	Deoxynucleoside triphosphate
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
DUBs	Deubiquitinating enzymes
dUMP	Deoxyuridine monophosphate
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
eIF2 α	Eukaryotic initiation factor 2 α
FADD	Fas-associating death domain
Fara-A	9- β -D-arabinofuranosyl-2-fluoroadenine, Fludarabine
Fara-AMP	Fara-A monophosphate
Fara-ATP	Fara-A triphosphate
FDA	Food and drug administration
GFP	Green fluorescent protein

GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
hCNT	Human concentrative nucleoside transporter
hENT	Human equilibrative nucleoside transporter
HCL	Hairy cell leukemia
HPLC	High performance liquid chromatography
Hsp	Heat shock protein
IAPs	Inhibits the inhibitor of apoptosis proteins
I κ B	Inhibitor of kappa-light-chain-enhancer of activated B cells
IU1	USP14 inhibitor 1
JAMM	JAB1/MPN/Mov34 metalloenzyme
JNK	C-Jun N-terminal kinase
Lys	Lysine
IMP	Inosine monophosphate
M	Mitosis
MAPs	Microtubule associated proteins
MCPIP	Monocyte chemotactic protein-induced protein
Mdm2	Mouse double minute 2 homolog
MDR	Multi-drug resistance
MHC	Major histocompatibility complex
MMR	Mismatch repair
MP	monophosphate
MRP1	MDR-associate protein1
MTOCs	Microtubule organizing centers
MTs	Microtubules
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NAs	Nucleoside analogues
NAD	Nicotinamide adenine dinucleotide
NDPK	Nucleoside diphosphate kinase
NF- κ B	Nuclear Factor kappa B
NHL	non-Hodgkin's lymphoma
NMPK	Nucleoside monophosphate kinase
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
NSCLC	Non-small cell lung cancer
NT	Nucleoside transporter
ODC	ornithine decarboxylase

OTU	Ovarian tumor protease
PARP	Poly (ADP-ribose) polymerase
PERK	PKR-like ER kinase
P-gp	P-glycoprotein
PHDs	Prolyl hydroxylase domain proteins
POH1	Pad one homolog-1
Pru	Pleckstrin-like receptor for the ubiquitin
PSMB5	Proteasome subunit β 5
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Regulatory particle triple-A protein
Rpn	Regulatory particle non-ATPase subunit
Rpt	Regulatory particle triple-A protein
RR	Ribonucleotide reductase
SAH	S-adenosylhomocysteine
Smac	Second mitochondria derived activator of caspase
STAT3	Signal transducer and activator of transcription 3
TGF- β	Transforming growth factor- β
TK	Thymidine kinase
TP	Triphosphate
Ub	Ubiquitin
UBA	Ubiquitin-associated
Ubal	Ubiquitin aldehyde
UBL	Ubiquitin-like
Ubp	Ubiquitin specific protease
UbVS	Ubiquitin vinyl sulfone
Uch	Ubiquitin carboxy-terminal hydrolase
UCHL5	Ubiquitin carboxy-terminal hydrolase L5
UIM	Ubiquitin interacting motif
UPP	Ubiquitin proteasome pathway
UPR	Ubiquitin proteasome response
UPS	Ubiquitin proteasome system
USP	Ubiquitin specific protease
UTP	Uridine triphosphate
Xbp1	X-box-binding protein-1

1. BACKGROUND

1.1 What is cancer?

Cancer is not a single disease, but a term used to describe hundreds of diseases [1]. It is characterized by either uncontrolled cell growth and/or prolonged survival of cells. Cancer may originate from almost any type of tissue and cancer cells may spread to distant tissues [2]. It is mainly induced by interaction of genetic susceptibility and environmental factors [3, 4]. In 2012, the estimates of the World Health Organization (WHO) for global cancer incidence and associated mortality were 14.1 million and 8.2 million, respectively. In Sweden, 57,726 individuals were diagnosed with cancer in 2011 [5]. Furthermore, it is estimated that > 1,665,000 new cancer cases and > 585,000 cancer deaths will occur in the United States during 2014 [6]. In the United Kingdom, one person in three will develop a malignancy by the time they reach the age of 70 [7]. Although cancer can affect all ages, there is a steep increase in incidence with age.

The hallmarks of cancer include eight biological capabilities acquired during the multistep development of human tumors: sustaining proliferative signalling, evading growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, activation of the propensity to invade and metastasize, reprogramming of energy metabolism and evasion of immune destruction [8]. A major problem in the clinical management of cancer is that malignant cells are not confined to their tissue of origin, but can spread to other parts of body via the lymphatic system and bloodstream, creating secondary deposits known as ‘metastases’ [9-11].

Loss of cell cycle regulation and loss of regulation of cell death induction are central features in the etiology of the cancer and will be briefly reviewed below.

Cell cycle

Non-dividing cells are in G_0 phase. Cells reproduce by a series of well orchestrated events where cellular macromolecular contents are duplicated, followed by division into two daughter cells. Dividing cells pass through four distinct phases during the cell cycle (Figure 1): (1) the G_1 -phase where cells increase in size and prepare to copy their DNA; (2) the S-phase during which DNA is replicated (S for DNA synthesis); (3) the G_2 -phase during which cells further grow; (4) M-phase when chromosomes separate and cells divide (M for mitosis). After one cycle, the daughter cells can continue to enter a new cycle, enter G_0 phase or become terminally differentiated [12, 13].

Cell death

According to morphological appearance of cells, cell death is mainly classified apoptosis and necrosis [14].

Apoptosis

Apoptosis, programmed cell death, is a fundamental process that occurs during development and is required for maintenance of tissue homeostasis. Deregulation of apoptosis may lead to diseases such as neurodegenerative disorders, diabetes, or cancer [15]. Apoptosis is an active, energy requiring process involving a characteristic series of morphological changes that accompany the degradation of the cell. Early features of apoptosis include chromatin condensation, DNA fragmentation and cell shrinkage.

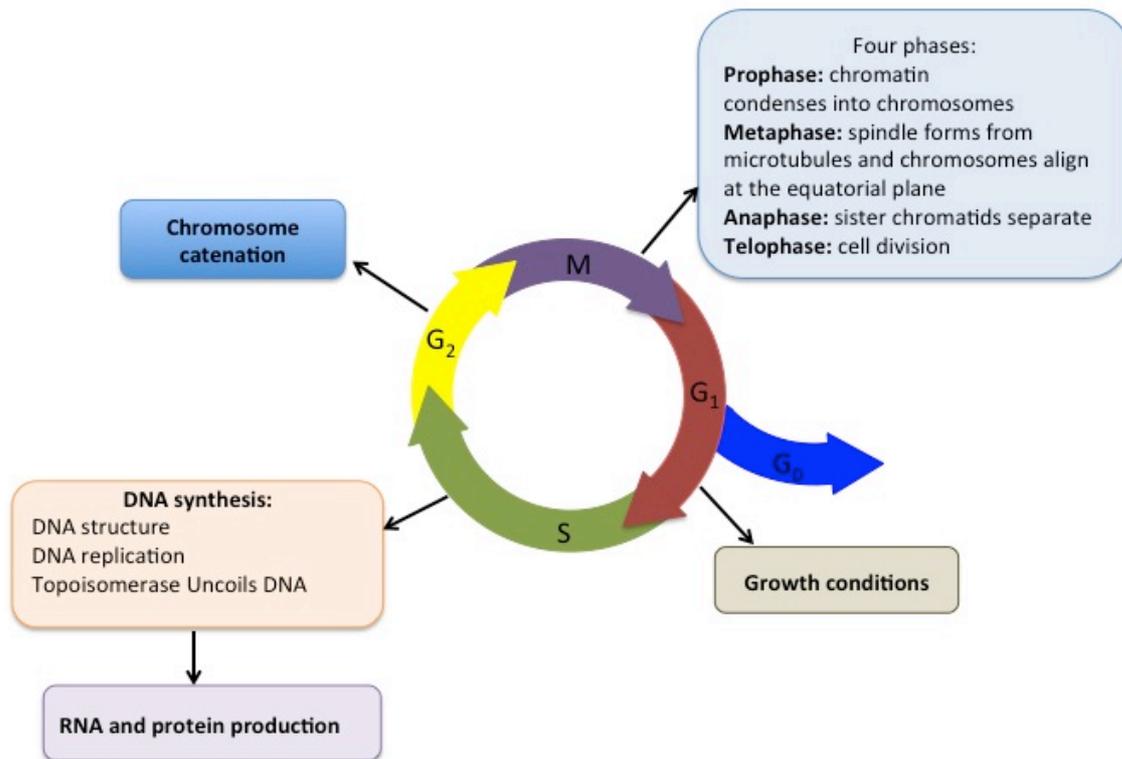


Figure 1. Cell cycle: potential targets for chemotherapeutic drugs.

During the late stages of apoptosis, cellular components are packed into apoptotic bodies which are phagocytised by macrophages and neighbouring cells, thus avoiding an inflammatory response [16]. Cytotoxic anticancer agents and radiotherapy induce mutations in cancer cells that may not be directly lethal, but the damage induces signaling events which trigger apoptosis.

There are two main pathways which trigger apoptosis: death receptor (extrinsic) and mitochondrial (intrinsic) pathways. The death receptor pathway is triggered when an extracellular death ligand (e.g. FasL) binds to a death receptor (e.g. Fas) on the cell surface, which in turn recruits the adaptor protein FADD (FAS-associated death domain) to form a complex called the death-inducing signalling complex (DISC). DISC then recruits and activates the initiator procaspase-8. Activated procaspase-8 subsequently triggers a caspase cascade where downstream executioner caspases such as caspase-3 and caspase-7 are activated, ultimately leading to DNA fragmentation and apoptotic cell death [17]. The mitochondrial pathway can be triggered by a variety of cellular stresses (e.g. DNA damage, hypoxia, depletion of survival factors and ER stress), which can lead to release of molecules such as cytochrome c, apoptosis inducing factor (AIF) and Smac/DIABLO from mitochondria. When cytochrome c is released into the cytosol, it associates with Apaf-1 to form the apoptosome, a complex that activates procaspase-9. In the presence of cytochrome c and the nucleotide dATP/ATP, procaspase-9 is auto-catalytically activated and then induced downstream executioner caspases, such as caspase-3 and caspase-7 [18]. Smac/DIABLO (and probably Htra2/Omi) binds to/inhibits the inhibitors of apoptosis proteins (IAPs), a different class of caspase inhibitors (Figure 2) [19].

The Bcl-2 family of proteins plays an important role in the apoptosis. The Bcl-2

family comprises both proapoptotic and antiapoptotic members, the balance of which determines apoptosis. These proteins are regulating cytochrome c release from mitochondria. Upregulation of antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xL and/or loss of function of proapoptotic proteins such as BAX and BAK is common in many human tumors. Dysregulation of the Bcl-2 family of proteins can prevent cell death in response to a variety of apoptotic stimuli. In mammals, the Bcl-2 family consists of at least 20 members, all of which share at least one conserved Bcl-2 homology (BH) domain [20]. Proapoptotic proteins, such as BAX and BAK, act mainly at the mitochondrion and are believed to induce the permeabilization of the outer mitochondrial membrane allowing efflux of cytochrome c [21]. Antiapoptotic members block apoptosis by guarding mitochondrial integrity and thus prevent the release of cytochrome c, as well as other mitochondrial molecules (Smac/DIABLO and Omi/Htra2) [22-24].

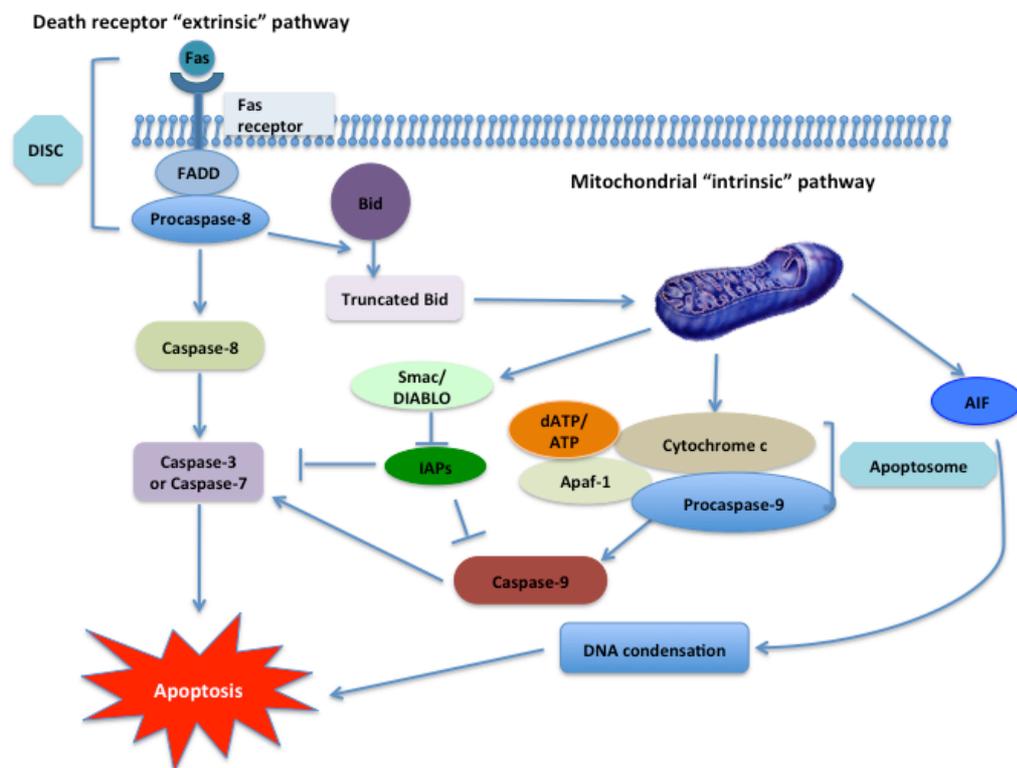


Figure 2. A schematic illustration of the death receptor and mitochondrial pathways for apoptosis. The detailed description refers to the main text.

Necrosis

The morphological features of necrosis are swollen organelles, plasma membrane rupture and loss of intracellular materials. Necrosis is usually caused by gross cell injury and severe cellular ATP depletion. It is thought as an accidental form of cell death in response to supra-physiological conditions. However, some studies have suggested that necrosis may also be tightly controlled and initiates either inflammatory or reparative responses in the host [25, 26].

1.2 Cancer treatment

Current cancer therapies include four major types: surgery, radiotherapy, chemotherapy and immunotherapy. Surgery has a highest chance to achieve a complete cure. But surgical removal of the primary tumor is not always successful, due to irregular shape of some tumors, the inaccessibility of some tumors (e.g. brain tumors) or poor health and old age of the patients [27-29]. Furthermore, many tumors have metastasized at the time of diagnosis, leading to widespread disease which is no longer treatable by surgery. If surgery is not a good choice, other therapies must be considered. *Radiotherapy* has been used to the treatment of cancer for a hundred years. Ionizing radiation deposits energy that injures or destroys cells in the target tissue by damaging their genetic material, making it impossible for these cells to continue to grow [30-33]. Although radiation damages both cancer cells and normal cells, the latter are able to repair themselves and function properly. Radiotherapy may be used to treat localized solid tumors and some hematologic malignancies. *Chemotherapy* uses drugs to inhibit the proliferation of cancer cells or, preferably, to destroy cancer cells. Drugs are distributed via the vascular system and will be able to target both the primary tumor as well as any cancer cells that have spread to distant tissues [34]. Chemotherapy can be given orally, subcutaneously, intravenously, directly into a body cavity or topically applied to the skin. *Immunotherapy* uses the immune system to fight disease. This can be done by stimulating the patients' own immune system or by administering immune system components. Immunotherapy is also a systemic therapy [35]. These different types of therapies are often combined during clinical management of cancer. Reducing cancer morbidity and mortality still requires prevention and earlier detection.

1.3 Principles of cancer chemotherapy

Chemotherapy can be used alone, but is usually used in combined therapy modalities, along with surgery and/or radiotherapy, to achieve and maintain remission. Both single agent and combination chemotherapy are given at intervals in pulsed doses or in cycles. The patients are monitored during the process in order to evaluate response to therapy, and treatment is adjusted accordingly. The complexity of the disease leads to considerable heterogeneity between patients with regard to response, leading to the necessity to individualize therapy according to tumor and patient characteristics. Large effort are being made in the fields of pathology, oncology and pharmacology to individualize therapy [34].

The aim of chemotherapy is to completely eradicate disease. Tumors nevertheless commonly relapse, and such relapses may occur locally or at a distance (metastasis) from the primary tumor site. Chemotherapy is usually classified as follows:

- (1) *Induction chemotherapy*: initial therapy administered with the aim of achieving significant reduction and ideally complete remission of the disease. The outcome may be a complete or partial response;
- (2) *Consolidation/intensification chemotherapy*: administered after remission with the aim to prolong disease-free survival. Consolidation chemotherapy uses the same drugs for induction of remission;
- (3) *Adjuvant chemotherapy*: administered in combination with localized treatment such as surgery or radiotherapy;

- (4) *Neoadjuvant chemotherapy*: given prior to local therapy with the aim to reduce tumor burden prior to surgery;
- (5) *Maintenance chemotherapy*: continuous, low-dose chemotherapy is used to extend the duration of remission and with curative intent;
- (6) *Salvage chemotherapy*: may be given after failure of other treatments to control the disease and/or to provide palliation;
- (7) *Combination chemotherapy*: although single agent chemotherapy may be used in some situations, combination chemotherapy achieves more profound effects on tumors [36].

Most chemotherapeutic drugs target the process of cell proliferation and division to explore since cancer cells divide at higher frequency than normal cells. [12, 37].

1.4 Classification of chemotherapeutic agents

Since the discovery of the toxic action of nitrogen mustards on blood cells [38], many chemotherapeutic agents have been discovered by screening the cytotoxic potency of a large number compounds *in vitro* or *in vivo* models. They are generally classified according to their mechanism of action as described below.

1.4.1 Alkylating agents

This class of agents include nitrogen mustards, alkyl alkane sulphonates, nitrosureas, tetrazines, and platinum agents. They cause cell death by cross-linking DNA strands, which may result in inhibition of DNA, RNA and (ultimately) protein synthesis. This type of drugs are cell cycle-nonspecific agents [39, 40].

1.4.2 Antimetabolites

Antimetabolites have structural similarities to natural existing substances, such as vitamins, nucleosides or amino acids. They mainly comprise folic acid antagonists, pyrimidine analogues and purine analogues. They usually act by competing with natural substrates for the active site on key enzymes or receptors. Some antimetabolites are incorporated directly into DNA or RNA, resulting in inhibition of DNA, RNA and protein synthesis. Most of them are cell cycle-specific agents [41-43].

1.4.3 Anti-microtubule agents

Vinca alkaloids and taxoids are the two main groups of anti-microtubule agents. Although both types of agents cause microtubule dysfunction, resulting in blocking of cell division, they have opposite mechanisms of action. The vinca alkaloids prevent the formation of the microtubules, whereas taxoids prevent microtubule disassembly [44, 45].

1.4.4 Topoisomerase inhibitors

There are two broad classes in topoisomerase inhibitors: topoisomerase I inhibitors and topoisomerase II inhibitors. Topoisomerases are involved in all DNA-dependent events, such as DNA replication, transcription, recombination, repair and nucleosome remodelling, chromosome condensation and segregation [46]. Inhibition of topoisomerase activity results in induction of DNA strand breaks, resulting in inhibition of cell proliferation and/or apoptosis. This class of drugs are phase-specific and prevent cells from entering mitosis [47].

1.4.5 Cytotoxic antibiotics

Most cytotoxic antibiotics are derived from bacteria and fungi. They have various mechanisms of action. This group of drugs include anthracyclines, actinomycin, bleomycin and mitomycin c. They affect the function and synthesis of nucleic acids in various ways [12, 42].

In addition to the above classes of chemotherapeutic agents, there are a number of miscellaneous agents that target different points of cell growth pathways, and that may induce cell death.

In this thesis, I have studied a few different chemotherapeutic agents: nucleoside analogues, anti-microtubule agents and proteasome inhibitors.

1.5 Resistance to chemotherapeutic agents

Drug resistance remains as a major obstacle for the effectiveness of chemotherapy. Cancer drug resistance includes two broad categories: intrinsic and acquired. *Intrinsic resistance* is a term used to describe the fact that tumor cells may show natural resistance to certain therapies due to expression of factors that interfere with therapy efficacy. *Acquired drug resistance* means that tumor cells that are initially sensitive to drugs become resistant due to mutations and various adaptive responses during the process of treatment [48, 49].

Tumor cells are constantly selected for survival and proliferation [50]. In general, cancer cells develop drug resistance due to the mechanisms outlined below (Figure 3):

1.5.1 Drug influx and drug efflux

Both decreased drug uptake and increased drug efflux can induce drug resistance. Drug efflux is mediated by the ATP-binding cassette (ABC) transporter family. Three members of this family are associated with multidrug resistance (MDR): ABCB (also known as ABCB1/MDR1/P-glycoprotein), the MDR-associated protein 1 (MRP1; also known as ABCC1), and breast cancer resistance protein (BCRP; also known as ABCG2/MXR). All three proteins have broad and overlapping substrate specificity and promote the elimination of various hydrophobic compounds [48]. MDR1 is a membrane-bound glycoprotein that is expressed in most tissues. MDR1 is also overexpressed in many tumors and can also be induced by chemotherapy. Many studies have shown that overexpression of MDR1 is associated with chemotherapy failure in various types of cancer [51-53]. MRP1 overexpression is also associated with drug resistance in lung, breast and prostate cancer [54-56] and BCRP is identified to correlate with chemo-resistance in breast cancer and leukaemia [57, 58].

1.5.2 Drug activation and inactivation

Resistance mechanisms related with drug metabolism are usually specific for each class of drugs. Nucleoside analogues (NAs) can be inactivated by deoxycytidine kinase (dCK) which catalyse the rate-limiting step of conversion of most NAs to their corresponding monophosphates. Decreased or absent dCK activity will therefore confer resistance to NAs [59-63]. Platinum drugs and alkylating agents can be inactivated by glutathione [64], and the conversion of antimetabolites such as 5-fluorouracil (5-FU) and methotrexate to their active forms does not occur when the relevant cellular enzyme activities are absent [65, 66].

1.5.3 Alterations in drug targets

Mutation or alteration of the expression of a drug target can reduce the effectiveness of inhibitors of the target, leading to resistance. For example, mutation and/or overexpression of the gene encoding proteasome subunit $\beta 5$ (PSMB5) lead to resistance to the proteasome inhibitor bortezomib [67]. Similarly, alterations in MAPK and mutation of MEK1 or/and MEK2 have been shown in patients with acquired resistance to RAF or MEK inhibitor monotherapy [68].

1.5.4 Adaptive response

After active drug has accumulated and inhibited its target(s), the effectiveness of treatment depends on how the cancer cell responds. Many anticancer drugs act via inducing DNA damage, directly or indirectly. The cell may respond to DNA damage in two ways: by repair or cell death. The response of cancer cells to DNA damage is a major factor determining the effectiveness of DNA-damaging drugs. Examples are deficiencies or mutations in mismatch repair (MMR) genes or/and p53, which may confer resistance to DNA damaging drugs [48, 50].

1.5.5 Deregulation of apoptosis

Mutations, amplifications, chromosomal transactions and overexpression of anti-apoptotic proteins, such as anti-apoptotic BCL-2 family members, inhibitor of apoptosis proteins (IAPs) and the caspase 8 inhibitor FLIP, are also associated with resistance to chemotherapy [48-50]. Moreover, these genes may also be transcriptional targets for prosurvival transcription factors, for example nuclear factor- κ B (NF- κ B) and signal transducer and activator of transcription 3 (STAT3), the expressions of which are frequently activated during tumorigenesis [48].

1.5.6 Autophagy

Autophagy is a lysosomal degradation pathway that degrades damaged organelles and proteins in order to maintain cellular biosynthesis and viability under conditions of metabolic stress [14, 69]. Although autophagy has been shown to play a clear role in tumor suppression, its role during cancer treatment is still controversial. The ability of autophagy to promote cell survival during nutrient deprivation and other types of stress suggests that it may promote resistance to cytotoxic therapy. Many studies have indeed shown that tumor cell autophagy induced by anticancer treatment inhibits tumor cell killing [53, 70, 71].

In this thesis, we identified a novel tubulin active agent insensitive to common multidrug resistant models. Multiple drug resistance (MDR) describes a phenomenon whereby resistance to one drug is accompanied by resistance to several drugs completely differing in chemical structure and/or mechanism of action. It is a major factor in the failure of many forms of chemotherapy. In the clinic, the problem of MDR can include two main types: one acquired during treatment and the other pre-existing at the time of diagnosis. The identification of mechanisms of MDR is important for development of therapeutic agents to overcome the MDR. In addition to the mechanisms discussed above, the concept that the proliferation of human tumor may be driven by a minor population of self-renewing tumor cells, often termed tumor stem cells, may be of large importance for MDR. Tumor stem cells with MDR properties may repopulate tumors between cycles of cancer therapy, representing a major problem in clinical oncology [72, 73].

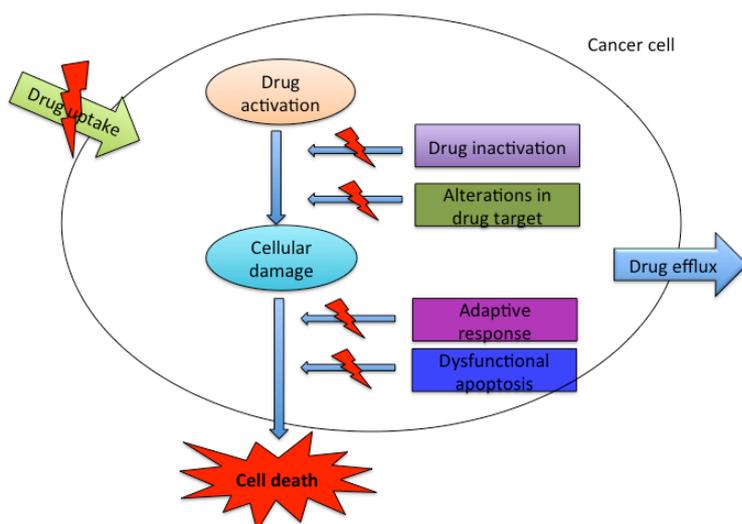


Figure 3. Cellular mechanisms of drug resistance.

The anticancer activity of a drug can be affected by poor drug uptake or/and excessive drug efflux, drug inactivation or limited activation, mutations or alterations of expression of drug targets, activation of adaptive pro-survival responses and dysfunctional apoptosis that block cell death [48].

1.6 Nucleoside analogues

The effectiveness of nucleoside analogues (NAs) as antitumor drugs was established in the 1960's with the nucleobase antimetabolites 5-fluorouracil [74] and the thiopurines [70]. These agents mimic physiological nucleosides in terms of uptake and metabolism and are incorporated into newly synthesized DNA, resulting in synthesis inhibition and chain termination. Some NAs can also inhibit key enzymes involved in the generation of purine and pyrimidine nucleotides and RNA synthesis, and some can directly activate the caspase cascade. In addition, these compounds may potentiate their action by indirectly inhibiting ribonucleotide reductase (RR), thus lowering the availability of endogenous nucleosides [75, 76]. All of these effects may lead to cell death [75].

Nucleoside analogues are transported into the cell either by active transport via specific transporters, including nucleoside transporters such as hCNT and hENT, or by passive diffusion. Once they have entered into cells, NAs are phosphorylated to mono-, di- and triphosphate forms by the enzymes dNK, NMPK and NDPK, respectively (Figure 4). The action of these enzymes is counteracted by cytoplasmic 5'-nucleotidase activity which can dephosphorylate monophosphate derivatives. NAs can also be rapidly deaminated by cytidine deaminase to non-toxic metabolites [75]. Most nucleoside analogues exert their cytotoxic effects by interfering with DNA replication with their active form. Although NAs share common general characteristics, each drug also has specific properties in terms of drug-target interactions, which helps to explain the variation in their efficacies toward different diseases.

The NA family includes both pyrimidine and purine analogues. During the past decades, new NA agents have been introduced into clinical use. Furthermore, the use of NAs has expanded into the field of solid tumors.

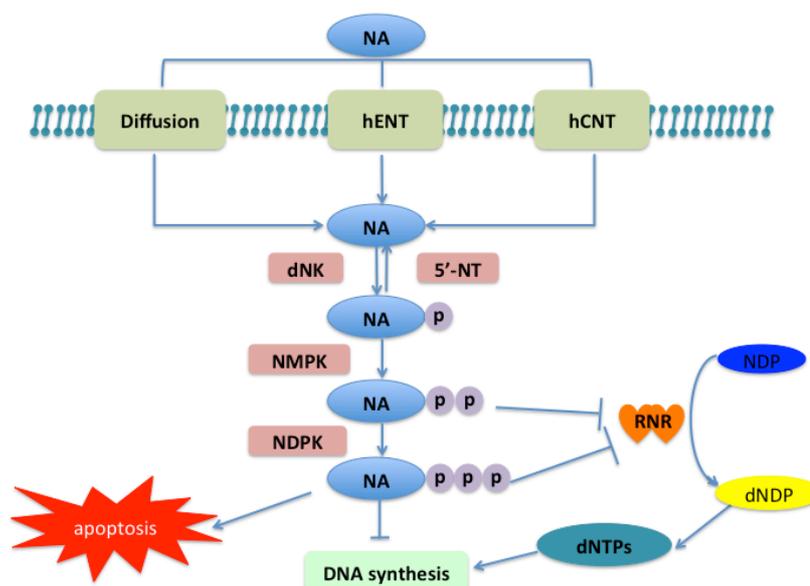


Figure 4. Schematic presentation of metabolism and mechanisms of action for NAs. NAs enter cells via specific nucleoside transporters. Once inside the cells, NAs are phosphorylated by dNK, NMPK and NDPK to the active triphosphates. 5'-NTs can dephosphorylate 5-monophosphate derivatives, preventing the production of the active forms. NAs exert their action by inhibition of DNA synthesis resulting in chain termination and cell death. Also, some NAs indirectly block DNA replication by inhibiting the RR enzyme, leading to reduced production of deoxyribonucleotide diphosphates (dNDPs). The decrease of deoxyribonucleotides triphosphate (dNTP) pools favors incorporation of NA active 5-triphosphate derivatives into DNA to block DNA synthesis. Furthermore, NA-triphosphates can directly activate the caspase cascade by stimulating apoptosome formation.

1.6.1 Purine nucleoside analogues

Cladribine (2-chloro-2'-deoxyadenosine, CdA)

Cladribine is a deoxyadenosine analogue [77]. Cladribine received FDA approval in the 1980s for treatment of hairy cell leukaemia [78]. CdA has activity in a variety of hematologic malignancies, autoimmune conditions and multiple sclerosis [79-86]. It was recently shown that in addition to its purine nucleoside analogue activity, cladribine possesses epigenetic properties, by inhibiting S-adenosylhomocysteine hydrolase (SAH) and DNA methylation [87-92].

CdA is phosphorylated to CdATP by dCK, AMP kinase, and nucleoside diphosphate kinase. In mitochondria, it is phosphorylated by dGK [93]. CdAMP can be dephosphorylated by 5'-NTs. The accumulation of CdATP has been shown to depend on the relative concentrations of dCK and 5'-NTs [94]. CdA is cytotoxic both to replicating and resting cells [95, 96]. In replicating cells, it inhibits DNA synthesis by incorporation of its triphosphate form into DNA [97, 98], resulting in chain termination and S-phase-specific apoptosis [99]. CdA also inhibits DNA replication indirectly through inhibition of ribonucleotide reductase [100]. In addition, CdA can disrupt the integrity of mitochondria [101], leading to the release of the pro-apoptotic mitochondrial protein, thereby initiating the caspase cascade. Moreover, CdATP can cooperate with cytochrome c and Apaf-1 to activate caspase-3 and trigger the caspase pathway [102, 103]. In resting cells, CdATP is incorporated into DNA by the repair machinery and terminates the nucleotide excision repair process leading to the

progressive accumulation of DNA single-strand breaks which eventually initiate apoptosis by p53-dependent or p53-independent pathways [104, 105]. Incorporation of CdA into DNA may also alter gene transcription with a consequent depletion of proteins required for cell survival [106].

Fludarabine (9- β -D-arabinosyl-2-fluoroadenine, Fara-A)

FaraA is a purine (adenosine) nucleoside [107]. Fludarabine has been extensively used in lymph proliferative malignancies, including various types of non-Hodgkin's lymphoma [108].

Similar to other NAs, it requires phosphorylation to Fara-ATP for interruption of DNA and RNA synthesis and induction of apoptosis [109, 110]. Similar to CdA, Fara-A is cytotoxic to both dividing and resting cells. In dividing cells, it inhibits DNA and RNA synthesis and RR. DNA synthesis inhibition is mediated by competition of the triphosphate metabolite with dATP for incorporation into DNA [111]. RR inhibition causes a reduction of dNTPs pools. The mechanism of action of Fara-A in replicating cells is mainly cell cycle-specific, and incorporation of Fara-ATP into DNA during S phase is required for the induction of apoptosis [112]. In resting cells, FaraA can block cellular DNA repair. In addition, Fara-AMP and Fara-ATP can be incorporate into RNA, resulting in premature termination of the RNA transcript, impairing its function as a template for protein synthesis [113]. Fara-ATP also inhibits RNA synthesis by suppressing the activity of RNA polymerase II [114]. Fara-ATP is also a nucleotide activator of Apaf-1, with the consequent activation of the caspase-9 and caspase-3 pathways. Moreover, it was reported that Fara-A downregulate Bcl-2 mRNA expression, thereby favouring apoptosis [115].

Clofarabine (2-chloro-2'-fluoro-deoxy-9- β -D-arabinofuranosyl adenine, CAFdA)

Clofarabine, a second-generation purine nucleoside analogue, was designed to overcome the limitations of fludarabine and cladribine [116]. Similar to other nucleoside analogues, clofarabine enters cells by nucleoside transporters. Once clofarabine is transported into the cell, it is phosphorylated to its monophosphate derivatives by deoxycytidine kinase (dCK) or deoxyguanosine (dGK), a process that is counteracted by dephosphorylation by 5'-nucleotidases. CAFdA-monophosphate is further phosphorylated to its active form, clofarabine triphosphate [117]. CAFdA-triphosphate is effective against proliferating, quiescent and resting cells and is a potent inhibitor of DNA polymerase- α and - ϵ [118]. In addition, the action of CAFdA-triphosphates may involve RNA-dependent mechanisms [119-121]. CAFdA-triphosphate also strongly inhibits RR activity. Furthermore, CAFdA-triphosphate can replace dATP and directly affects cytosolic apoptotic protease-activating factor 1 (Apaf-1), then leading to caspase activation [102]. CAFdA-triphosphate also has direct actions on mitochondria leading to the release of proapoptotic factors, such as cytochrome c and apoptosis-inducing factor, to activate pro-apoptotic pathways [122].

Clofarabine was approved by the United States Food and Drug Administration for treatment of pediatric patients with relapsed or refractory acute lymphoblastic leukemia (ALL) in 2004, and by the European Commission in 2006 [123]. With the rapid development of clofarabine in hematologic malignancies, the anti-cancer activity of clofarabine toward other types of tumors has attracted interest.

1.6.2 Pyrimidine nucleoside analogues

Cytarabine (1- β -D-arabinofuranosylecytosine, cytosine arabinoside, cytarabine, araC)

Cytarabine is a pyrimidine nucleoside-based anticancer drug widely used for the treatment of leukemia [124]. It may be used alone or in combination with other anticancer agents, such as daunorubicin, doxorubicin, thioguanine, or vincristine [124].

In the cell, ara-C is phosphorylated by dCK and pyrimidine kinases to the active 5-triphosphate derivative ara-CTP [125, 126] to inhibit DNA polymerase by competing with deoxycytidine triphosphate and thus reduce the cell replication [125]. Cytarabine acts on rapidly dividing cells and inhibits DNA synthesis at the S-phase of the cell cycle and also hinders progression of cells from the G1-phase to the S-phase.

Gemcitabine (2', 2' -difluoro 2' deoxycytidine, dFdC)

Gemcitabine is a pyrimidine analogue [127]. Gemcitabine is effective in inhibiting the growth of human neoplasms and is used for the treatment of a broad range of solid and hematological cancers.

Gemcitabine first requires cellular uptake by nucleoside transporters and is then phosphorylated to dFdCMP by dCK. The monophosphate form is subsequently converted dFdCDP and dFdCTP by pyrimidine kinases. The cytotoxic activity of gemcitabine is thought to related to inhibition of DNA polymerase and the incorporation of the drug into DNA [128], leading to the termination of chain elongation [129]. The dFdCDP metabolite also inhibits RR, resulting in a decrease in competing deoxyribonucleotide pools necessary for DNA synthesis. Additionally, dFdCTP incorporates into RNA, although the consequences of this incorporation has not been extensively studies [127].

1.6.3 Metabolism of nucleoside analogues

Anabolic enzymes

This family of enzymes is involved in two pathways: the *de novo* pathway and the salvage pathway.

Deoxycytidine kinase (dCK)

dCK is expressed throughout the cell cycle. The activity of dCK can be increased by exposure to deoxyribonucleoside, NAs, genotoxic agents and UV/ γ -irradiation [130-134]. dCK is highly expressed in quiescent cells where it phosphorylates nucleosides necessary for DNA repair and the expression may, depending on the cell type, increase several-fold when the cells enter S-phase [135-138]. dCK is believed to be the most important enzyme for activation of NAs due to its high expression in hematopoietic cells [139]. Low enzyme activity can lead to resistance to NAs [140-143].

Deoxyguanosine kinase (dGK)

dGK shows sequence homology with dCK [144]. dGK is mainly located in mitochondria, but can be translocated into the cytosol during apoptosis. Mitochondrial dGK is responsible for phosphorylation of purine deoxyribonucleosides and their analogues in the mitochondrial matrix, providing the dNTPs necessary for mitochondrial DNA synthesis. Cytoplasmic dGK may also contribute to the supply of purine deoxynucleotides for nuclear DNA replication and repair. Nucleoside analogues phosphorylated by dGK may therefore exert their cytotoxic effects by interference with

both mitochondrial and nuclear DNA synthesis [75].

Thymidine kinase-1 (TK1)

TK1 plays an important role in the pyrimidine salvage pathway. The expression of TK1 is regulated following the cell cycle, high-level expression during S-phase. TK1 is expressed in leukemia cell lines and numerous malignant tumors [145]. Some important nucleoside analogues, such as zidovudine (AZT), stavudin and floxuridine, can be phosphorylated by TK1 [146, 147].

Thymidine kinase-2 (TK-2)

TK-2 is a mitochondrial enzyme which is expressed in all tissues and throughout the cell cycle, being the only pyrimidine deoxyribonucleoside kinase expressed in non-proliferating cells [148]. TK-2 has a broad substrate specificity, phosphorylating dCyt, thymidine and dUrd It also phosphorylates anti-cancer and anti-viral NAs such as AZT, Ara-C and dFdC [149]. In humans, deletion of the TK-2 gene leads to a myopathic form of mitochondrial DNA depletion syndrome [150].

Catabolic enzymes

5'-Nucleotidase (5'-NT)

This family of enzymes dephosphorylates nucleoside monophosphates and cytotoxic mononucleotides. In vitro, increased 5'-NT activity has been consistently associated with nucleoside drug resistance [62, 96, 151].

Cytosolic 5'-nucleotidase-1 (CN1)

CN1-A is located in the cytosol and expressed at a high level in brain, skeletal and heart muscle [152]. This enzyme has a broad range of substrates, AMP and pyrimidine deoxyribonucleotides, and also for monophosphates of adenosine, cytidine and guanosine [153]. When CN1-A over-expressed in the cells, the cells become less sensitive to several NAs. CN1-B shows the highest expression in testis and lowest expression in skeletal muscle and the brain. CN1-A and CN1-B show sequence homology and have similar substrate specificities [154].

Cytosolic 5'-nucleotidase-2 (CN2)

CN-2 is ubiquitously expressed in human tissues. CN-2 has been reported to have high activity in lymphoblastoid cells [155]. Downregulation of CN-2 using siRNA results in significantly decreased sensitivity of patient cells to NA treatment [156] and a high CN-2/dCK ratio is associated with NA resistance both in the leukemic patients [157] and in cell lines [158, 159].

Cytosolic 5' (3')-deoxyribonucleotidase-1 (dNT1)

dNT1 is ubiquitously expressed in human tissues [160]. The enzyme uses all deoxyribonucleotide monophosphates except deoxythymine monophosphate as substrates [161]. Low levels of dNT1 may increase cellular concentrations of dCTP to compete with the triphosphates of active cytidine analogues resulting in decreased cytotoxic effects [162].

Cytosolic 5' (3')-deoxyribonucleotidase-2 (dNT2)

dNT2 has a similar structure as dNT1. The mRNA level of dNT2 is high in brain, heart and skeletal muscles and low in kidney and pancreas. dNT2 shows specificity for dUMP and dTMP. In non-cycling cells with minimal *de novo* synthesis, the dNT2/TK2 substrate cycle is important for regulation of dTTP pools [163].

Ecto-5'-nucleotidase

Ecto-5'-NT gene is expressed in many tissues and is frequently expressed in acute leukemia [164, 165]. The enzyme dephosphorylates purine mononucleotides to nucleosides, commonly AMP to adenosine.

Cytidine deaminase (CDD)

Cytidine deaminase catalyses the inactivation of cytidine and dCyd to uridine and deoxyuridine, respectively. CDD also deaminates some NAs, i.e. ara-C and gemcitabine [166]. Several studies have indicated that increase levels of CDD is associated with the development of resistance to ara-C [167-169].

1.6.4 Ribonucleotide reductase

Human ribonucleotide reductase (RR) plays a vital role in the synthesis of the four deoxyribonucleosides required for DNA synthesis and repair by catalyzing ribonucleoside diphosphates to deoxyribonucleoside diphosphates [170]. This rate-limiting step in synthesis of DNA precursors is regulated at several different levels, including allosteric control of activity and specificity of RR by nucleoside triphosphates [171]. RR is a tetramer composed of two non-identical homodimers, designated R1 and R2. Transcription of the R2 gene occurs exclusively during the S-phase [172], whereas R1 is virtually constant throughout the cell cycle. When R2 is not available, R1 will associate with p53R2 to form active RR that supplies quiescent cells with deoxyribonucleotides for repair of damaged DNA [173, 174].

1.7 Tubulin inhibitors

Tubulin inhibitors are an important class of anticancer agents. Since the first known compound binding tubulin, colchicine, was identified, a large number of tubulin-binding agents have been isolated or synthesized and used for cancer treatment.

1.7.1 Tubulin and the microtubule system

Microtubulin structure and function

The structural backbones of microtubules are tubulin dimers and microtubule associated proteins (MAPs). There are 5 forms of tubulin: α -, β -, γ -, δ - and epsilon-tubulin [175, 176]. α - and β -tubulin are the main subunits of microtubules. Both α - and β -tubulin contain binding sites, the N- and E-sites, for the nucleotide guanosine triphosphate (GTP). One GTP molecule is tightly bound to the α -tubulin and cannot be exchanged, while another GTP molecule is bound to β -tubulin and can be free to exchange with guanosine diphosphate (GDP) [45, 177]. Microtubules grow from discrete assembly sites in the cells called microtubule organizing centers (MTOCs), which are a network of MAPs. MAP is a complex family of proteins, including MAP2, MAP4, Mip-90, tau and STOP, most of them are related with regulation of tubulin

polymerization and function [45, 177].

The process of microtubule formation includes three phases: nucleation, elongation and steady state. Nucleation is the slow formation of a small microtubule nucleus from soluble tubulin. Following formation of this nucleus, microtubules rapidly elongate. Elongation involves the rapid addition of heterodimers to the plus end of the microtubule. After elongation, the microtubule enters a steady state. Lengthening and shortening of the microtubule are balanced in steady state [178].

Microtubules are essential components of the cytoskeleton of eukaryotic cells and play important roles in key cellular functions such as cell signaling, proliferation, cell shape and structure maintenance, formation of mitotic spindles, intracellular migration and transport [177]. Furthermore, microtubules are associated with the permeability transition pore that is involved in cytochrome c release during apoptosis [45].

1.7.2 Mechanism of action of microtubulin inhibitors

Because the microtubule system is important for cell division, development of anticancer agents that target microtubule dynamic has become an attractive strategy. According to the mode of action and binding site, tubulin inhibitors can be classified three groups: taxane-like microtubule stabilizing compounds, vinca alkaloid site binding agents and colchicine site binding agents [45].

Tubulin inhibitors act via interfering with the microtubule polymerization or depolymerization. Depolymerization inhibitors suppress microtubule dynamics by induction of polymerized tubulin, enhancement of microtubule assembly and increasing the microtubule polymer mass at high concentration in the cells. They act as microtubule-stabilizing agents, and are referred to as "depolymerization inhibitors". An example is paclitaxel (Taxol[®]). MT polymerization inhibitors include colchicine binding site inhibitors and vinca alkaloids binding site inhibitors. Both of them bind to β -tubulin. This class of inhibitors suppresses microtubule dynamics by causing microtubule depolymerization. They act as microtubule-destabilizing agents, and are referred to as "polymerization inhibitors". Examples are colchine analogues and vinca alkaloids [179-182].

Paclitaxel

Paclitaxel was initially isolated from the bark of the yew tree *Taxus brevifolia* [180]. In 1979, Schiff and Horwitz found that paclitaxel causes microtubule stabilization and increased polymer mass [183]. Paclitaxel has been found to be of clinical benefit for the treatment of breast, ovarian, skin, head and neck and non-small cell lung carcinomas [184]. Paclitaxel can directly bind to β -tubulin of MTs, which result in the stabilization of the lateral interactions between heterodimers. This leads to inhibition of mitosis, chromosome segregation, and cell division [182, 185].

Vincristine

Vincristine was first isolated from the leaves of the periwinkle plant *Catharanthus roseus*. It was approved by the US FDA in 1963 as Oncovin. As a tubulin inhibitor, vincristine is mainly used in children leukemia, adult hematological malignancies and neuroblastoma [186]. It is also occasionally used as an immunosuppressant. Vincristine directly binds to the β -subunit of the tubulin dimer near the exchangeable nucleotide. Vincristine blocks microtubule assembly and induces microtubule depolymerization, prevents GTP hydrolysis and decreases polymer [179]. These mechanisms induce the arrest of mitosis in the metaphase [177, 182].

Based on the properties of many of the currently used tubulin inhibitors, development of new antimicrotubule agents is an active field of research.

1.8 Proteasome inhibition

Intracellular protein turnover is essential for the regulation of cellular functions and maintenance of cellular homeostasis. Two major protein degradation systems are used by cells: the lysosomal system and the ubiquitin-proteasome system (UPS) [187]. More than 80% of the degradation of cellular proteins was estimated to be preformed by the UPS [188]. Tumor cell growth and survival are dependent on the ubiquitin proteasome pathway (UPP) and the development of compounds that target the UPP is attracting increasing interest from cancer drug pharmacologists.

1.8.1 Ubiquitin-proteasome pathway (UPP)

The critical role of the UPP in biology was recognized by Avram Hershko, Aaron Ciechanover and Irwin Rose, who were awarded with the Nobel Prize for Chemistry in 2004. The UPP is the major system for protein degradation in the mammalian cytosol and nucleus. It is involved in a broad variety of cellular processes, including apoptosis, cell cycle regulation, DNA transcription and repair, antigen presentation, cell differentiation and protein quality control [189-191]. Defects in the UPP can result in the pathogenesis of several important human diseases, such as neurodegenerative disorders, viral diseases and cancer [192].

UPP includes two discrete and successive processes: (i) tagging of the substrate protein by the covalent attachment of a polyubiquitin chain (conjugation); (ii) the subsequent degradation of the tagged protein by the 26S proteasome (degradation) (Figure 5).

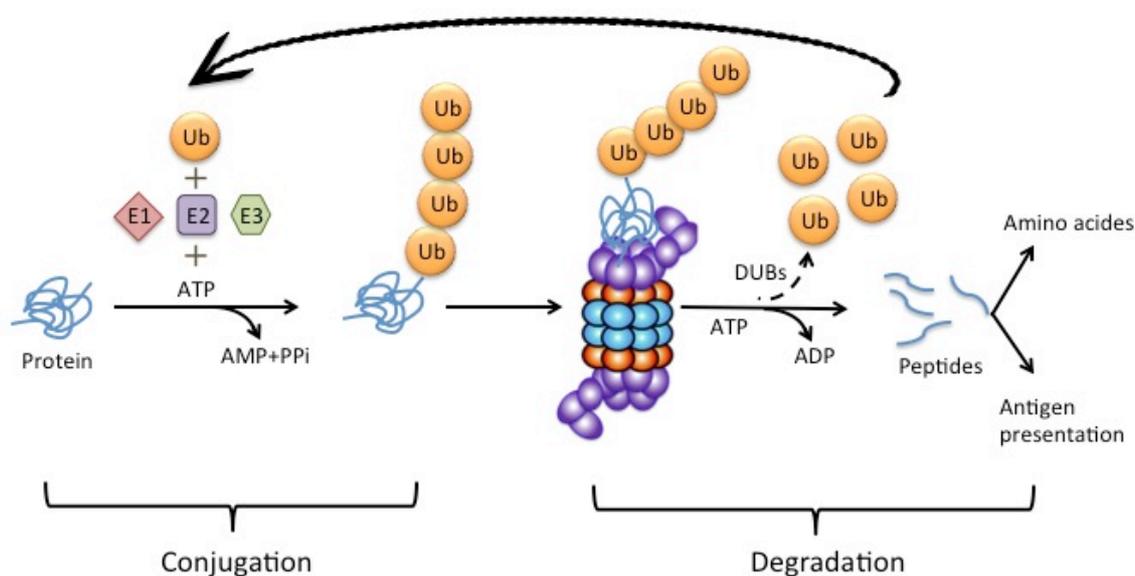


Figure 5. Ubiquitin proteasome pathway.

1.8.1.1 Ubiquitination

Ubiquitin (Ub) is a tightly packed globular protein consisting of 76-amino acids which is strongly expressed in cells [193]. Ubiquitination is a posttranslational modification that depends on the sequential action of three enzymes: ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The human genome encodes two isoforms of E1, dozens of E2 enzymes and more than

1000 types of E3 ligases. The different types of E3 ubiquitin ligases confer specific substrates to the UPP [194].

Ubiquitination of target protein is performed in three steps. First, ubiquitin is activated by the formation of a thiol ester bond between its C-terminal glycine residue and an active site cysteine residue in the E1 enzyme. Secondly, the activated ubiquitin molecule is transferred to an active cysteine residue of an E2 enzyme to generate a new thiol ester intermediate. Finally, E3 ligases catalyze the transfer of ubiquitin from the E2 to a lysine residue of a target protein. Following the addition of the first ubiquitin to the substrate, the process is repeated by attachment of additional ubiquitin monomers, resulting in the formation of a polyubiquitin chain (Figure 6) [195, 196]. Most substrates require polyubiquitination to be delivered to the proteasome. Ubiquitin chains can be formed on one or more of the seven lysine residues of ubiquitin at position 6, 11, 27, 29, 33, 48 and 63, resulting in ubiquitin chains of various lengths, types and functions. Lys48-linked chains represent the most abundant ubiquitin chain type in human cells (~ 28%) [197]. They are required for protein degradation by the proteasome. Recent studies show that Lys6, Lys11, Lys27, Lys29, and Lys33 linked chains are also involved in proteasomal degradation of proteins [198]. Lys63-linked chains are associated mainly with non-proteolytic processes such as transcription, intracellular protein trafficking, autophagy, DNA-damage response and cell signalling. [199-202]. Recently, Lys63-linked chains were also shown to be targets for proteasome degradation [203-205]. Ubiquitination can be reversed by specific deubiquitinating enzymes (DUBs). Deubiquitination can occur at any time after addition of ubiquitin to a target substrate, regulating protein stability [206].

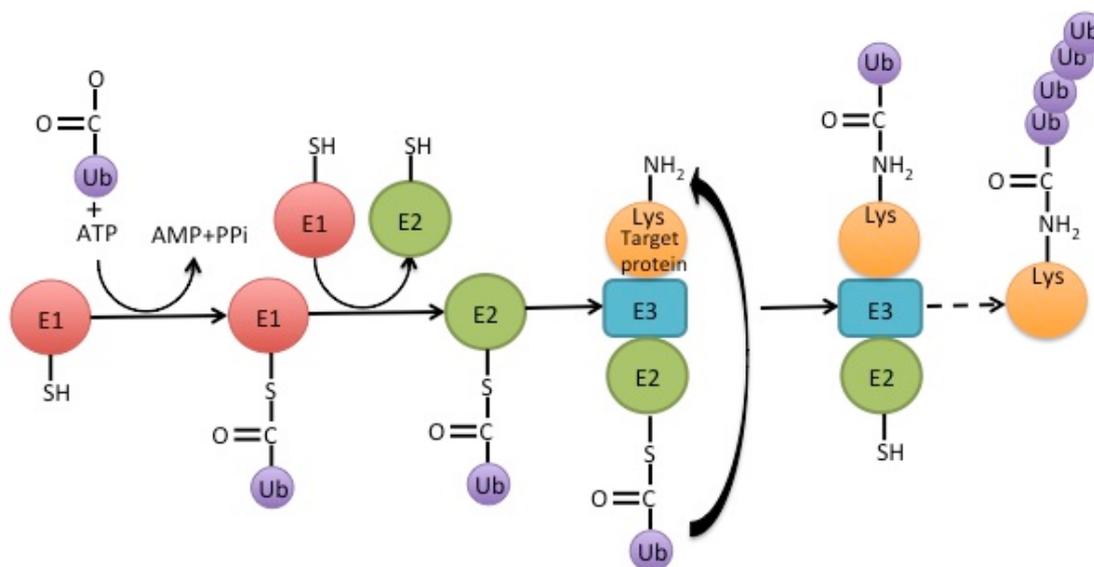


Figure 6. The process of ubiquitination.

Most proteasomal substrates bind to ubiquitin receptors on the proteasome. The enzyme ornithine decarboxylase (ODC), a key role in the polyamine biosynthesis, is an exception to this rule. Instead of binding poly-ubiquitin chains, ODC is degraded by the proteasome via binding of polyamine-induced protein (antizyme) [207, 208].

1.8.1.2 26S proteasome

The 26S proteasome is a large multicatalytic enzyme complex and found in nucleus and cytosol of all eukaryotic cells. It consists of a central 20S core particle (CP) capped with one or two 19S regulatory particles (RP) [209, 210].

The *19S regulatory particle* plays multiple roles in regulating the activity of the proteasome including recognition of ubiquitinated substrate and deubiquitination, substrate unfolding and translocation into the 20S catalytic chamber [211]. The 19S RP is composed of at least 19 subunits with molecular masses ranging from 10 to 110 kDa and can be divided into two sub-complexes: the base and the lid [212].

The base includes six AAA+ ATPases, Rpt1/S7, Rpt2/S4, Rpt3/S6, Rpt4/S10b, Rpt5/S6 and Rpt6/S8, which form a ring structure [213]. The base also includes four non-ATPase subunits: Rpn1/S2, Rpn2/S1, Rpn10/S5a and Rpn13/hRpn13 [213-216]. The ATPases provide the energy required for substrate unfolding, necessary for enter of substrates into the 20S CP catalytic chamber. Rpt2/S4 is involved in the process of opening of the pore into the 20S CP and Rpt5/S6 plays a role in recognizing poly-ubiquitin chains [217-219]. In the base, Rpn1/S2 and Rpn2/S1 are the largest subunits of the proteasome [220, 221]. Most substrates dock at the proteasome by binding specific ubiquitin receptors. Rpn1/S2 binds ubiquitin receptor-like Rad23, Dsk2 and Ddil, members of the UBL-UBA family. These proteins contain an ubiquitin-like (UBL) domain that can bind to Rpn1, Rpn10 and Rpn13 in the proteasome and one or more ubiquitin associated (UBA) domains which bind to poly-ubiquitinated chains [222]. UBL-UBA proteins have been proposed to function as shuttling factors that selectively deliver polyubiquitinated substrates to the proteasome [223]. Rpn10/S5a and Rpn13/hRpn13 are two ubiquitin receptors located in the base of the 19S RP. They bind ubiquitin chains. Rpn10/S5a contains two C-terminal ubiquitin-interacting motifs (UIM), which cooperate in binding multiple ubiquitins in chains [220, 224]. Rpn13 has high affinity to bind Lys48-linked di-ubiquitin and serves as a receptor for the Uch37/UCHL5 deubiquitinating enzyme, linking chain recognition and disassembly [225].

The lid complex includes 9 non-ATPase subunits, Rpn3, Rpn5–Rpn9, Rpn11, Rpn12, and Sem1 [212]. The connection of the lid complex and the base complex is via interactions between Rpn12 and Rpn2, and between Rpn11 and Rpn1 [226].

A major task of the lid and the base is deubiquitination. Rpn11/S13 (also referred to as POH1) is the essential DUB for this function and other two additional DUBs also participate the deubiquitination process, UCH37/UCHL5 and Ubp6/USP14. While Rpn11/S13 promotes substrate degradation, UCH37/UCHL5 and Ubp6/USP14 are believed to antagonize substrate degradation. These latter DUBs trim the polyubiquitin chain from the distal end to regulate the time of substrate-proteasome interaction and then the rate of degradation [227-229].

The *20S core particle* is a degradation unit which is made up of four stacked rings, forming a central cavity. Seven different α subunits form the two outer rings, the main structure, and cap the two inner rings which contain seven different β subunits [230]. The catalytic activities of the proteasome are divided into three major classes: the caspase-like, trypsin-like, and chymotrypsin-like activities. These activities are associated with the β 1, β 2, and β 5 subunits, respectively, and confer the ability to cleave peptide bonds at the C-terminal side of acidic, basic, and hydrophobic amino-acid residues [191]. The three peptidase activities can be probed using specific fluorogenic peptides. Proteasome inhibitors have been developed that target these activities [231]. The channel leading into the catalytic chamber is narrow (~ 13 Å in diameter) and is mainly composed of α 2, α 3 and α 4 subunits. In order to be degraded into oligopeptides, substrate proteins pass this narrow channel to access the active sites of catalytic enzymes at the center of the α -ring [232-234].

1.8.1.3 Deubiquitinating enzymes of the 26S proteasome

Like many other protein modifications, ubiquitylation is a reversible process. The conjugated polyubiquitin chains are removed and cleaved by deubiquitinases (DUBs)

to facilitate substrate translocation and degradation. The human genome encodes at least 98 DUBs that are divided into six classes: the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Machado-Joseph disease protein domain proteases, JAMM/MPN domain-associated metallopeptidases (JAMMs) and monocyte chemotactic protein-induced protein (MCPIP) families. All of these enzymes are cysteine proteases, with the exception of the JAMM/MPN+ DUBs which are zinc metalloproteases [235]. Deubiquitination is carried out by the hydrolysis of the isopeptide bond in ubiquitin–protein conjugates. Three DUBs are associated with the proteasome: Rpn11/POH1, Ubp6/USP14 and Uch37/UCHL5 (yeast/human nomenclature).

Rpn11/POH1

Rpn11/POH1 is a Zn^{2+} -dependent DUB that belongs to the JAMM domain family and is a part of the lid of the 19S RP [236]. Rpn11 is essential for substrate degradation during the process of deubiquitination. Rpn11/POH1 is localized above the entrance to the ATPase ring. This location is beneficial for the subsequent removal of ubiquitin from substrates prior to translocation of the substrate into the CP. Rpn11 cleaves the ubiquitin chain in an ATP-dependent manner. To avoid disassembly from the receptor during the cleavage process, an ubiquitin chain must consist of at least four ubiquitin units to span the distance between the receptors and the Rpn11 [227, 237, 238]. Defects of Rpn11/POH1 block cell growth, cellular protein degradation and the assembly of the 26S proteasome, resulting in the inhibition of the activity of the proteasome [239, 240]. Rpn11/POH1 is essential for viability of cancer cells, but detailed mechanistic studies in the proteasome context are lacking due to difficulties in purification, which has prevented the development of potent inhibitors for Rpn11 [241]. Lys63-linked polyubiquitin chains are the preferable substrates for Rpn11/POH1 [242].

Rpn11/POH1 is an obvious target for development of anticancer agents [243]. Knockdown of Rpn11/POH1 has same effect as proteasome inhibition, leading to the accumulation of polyubiquitinated substrate proteins [240, 243]. Overexpression of Rpn11/POH1 confers cellular resistance to vinblastine, doxorubicin and paclitaxel, possibly via modulation of AP-1-mediated stress pathways [244]. Rpn11/POH1 can also increase the expression of ErbB2, a receptor tyrosine kinase which is associated with poor prognosis in breast cancer [245].

Ubp6/USP14

Ubp6/USP14, a cysteine protease, is a member of the ubiquitin specific proteases (USP) family. Lys-48-linked poly-ubiquitin chains are the preferable substrates for Ubp6/USP14 and are cleaved from their distal end or within the chain [240]. Unlike Rpn11/POH1, Ubp6/USP14 is not an integral subunit of the proteasome. The full-length human Ubp6/USP14 contains 494 amino acids, with a 9-kDa UBL domain at its N-terminus followed by a 45-kDa catalytic domain, which resembles an extended right hand comprised of three domains: fingers, palm, and thumb. These three domains form a binding surface for ubiquitin. Two surface loops, blocking loop 1 and 2 (BL1 and BL2), partially fill the predicted binding pocket for the C-terminus of ubiquitin. These loops are believed to block access of the C-terminus of ubiquitin to the active site of USP14 and the BL2 and BL1 loops must be removed for enzymatic activity [246, 247]. Ubp6/USP14 catalytic activity is increased several hundred-fold when it is associated with the proteasome and it can be dissociated from the proteasome under high-salt conditions [228, 240].

Ubp6/USP14 is believed to be involved ubiquitin recycling. Deficiency of ubiquitin pools impairs the function of the UPP, resulting in protein accumulation [248-250].

Ubp6/USP14 has also been shown to inhibit proteasome activity in a manner

independent of its DUB activity by delaying the breakdown of proteins by the proteasome. Proteins are docked at the proteasome for a longer time, which leads to more extensive trimming of ubiquitin chains, thus reduces the affinity of protein to the proteasome [248, 251]. The interaction of Ubp6/USP14 and ubiquitin conjugates also can stimulate gate opening of the 20S core particle, enabling the substrate to be degraded [252]. The majority of the cellular content of Ubp6/USP14 is not associated with the proteasome, indicating that the enzyme may be involved in other cellular processes [252].

High expression of USP14 has been found in several hematopoietic and solid tumor cell lines, for example leukemic, colon cancer and multiple myeloma cells [253-255]. The overexpression of Ubp6/USP14 was reported to be beneficial for cell proliferation via induction of β -catenin and was found to be correlated with overall survival rate of lung adenocarcinoma and colorectal cancer patients [256].

Uch37/UCHL5

Uch37/UCHL5 is a cysteine protease and a member of ubiquitin C-terminal hydrolases (UCH) family. Uch37/UCHL5 can cleave both Lys48- and Lys63-linked poly-ubiquitin chains [257]. Similar to the Ubp6/USP14 enzyme, it is associated with the base complex of the 19S RP and reversibly associates with the proteasome. The estimated molecular mass of UCHL5 is 37.6 kDa [258]. It includes two functional domains, a catalytic domain (UCH-domain) and a C-terminal domain. In distinction to Rpn11/POH1, Uch37/UCHL5 is not essential for the activity or the structure of the 26S proteasome. It can bind to 26S proteasome via the Rpn13/Admr1 receptor located in the 19S RP base complex. Uch37/UCHL5 suppresses proteasome degradation through disassembly of distal polyubiquitin chains and releases mono-ubiquitin from chains [238, 259, 260]. A recent study has shown that Uch37/UCHL5 promotes the degradation of specific proteasome substrates, nitric oxide synthase and I κ B- α [261]. Above indicates that Uch37/UCHL5 suppresses or promotes proteasome degradation may differ between substrates. UCHL5 has also been shown to associate with the Ino80 chromatin-remodeling complex in the cell nucleus, which is related with nucleosome position during transcription or DNA repair [262]. Both knockdown of USP14 and UCHL37 can block cell growth, decrease protein degradation and accumulate polyubiquitinated proteins [240].

The activity of UCHL5 was also found to be increased in several carcinomas, including cervical carcinoma, hepatocellular carcinoma and multiple myeloma [255, 263-265]. UCHL5 was reported to affect cell survival via its interaction with Smad7, a transcription factor involved in transforming growth factor- β (TGF- β) signaling [266, 267].

1.8.2 Cellular responses to proteasome inhibition

As the proteasome plays essential role in the cell function, the proteasome inhibitors as therapeutic agents attracted more and more attention. Proteasome inhibitors exert broad effect on cancer cells by various mechanisms to induce cell death in many types of tumors.

NF- κ B

NF- κ B is a transcription factor induced by various signals, such as pathogens, growth factors and cytokines [268]. NF- κ B regulates various immune and inflammatory responses, cell survival and proliferation. NF κ B is constitutively active in a large proportion of advanced cancers to promote tumor cell growth, and NF κ B overexpression has been linked to resistance anticancer drugs [269]. NF κ B is activated via proteasomal degradation of its inhibitor I κ B. Proteasome inhibitors inhibit NF- κ B

activity by preventing degradation of I κ B, resulting in tumor cell apoptosis [270, 271]. However, the role of NF κ B in mediating the effects of proteasome inhibition is controversial [191]. One study showed that proteasome inhibitors actually activate NF κ B in endometrial carcinoma cells [272]. Another report showed that the proteasome inhibitor bortezomib can activate two upstream NF κ B activating kinases (RIP2 and IKK β) in multiple myeloma cells, promoting non-proteasomal degradation of I κ B and increases NF κ B DNA binding [273]. In addition, some reports also suggested that NF- κ B activity enhances tumor cell sensitivity to apoptosis. NF- κ B transcriptionally up-regulated pro-apoptotic target genes, for example p53 and Bax, leading to induce cell death [274-276]. However, one study showed that NF- κ B is not involved in the response of HCT-116 cells to bortezomib [277].

Cell cycle

Progression through the cell cycle is tightly controlled by regulatory proteins which are degraded in UPP. Three key classes of regulatory molecules are included in the process of the cell cycle: cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs) [278]. Proteasome inhibitors induce cell cycle arrest by blocking the degradation of cyclins (D, E, A, B) and cell cycle regulatory proteins and accumulation of CKIs (p21 and p27) in malignant cells [279-281].

Regulation of apoptosis

Inhibition of proteasome activity leads an upregulation of proapoptotic factors such as p53, pro-apoptotic members of the Bcl-2 family (Bax, Bak, Bad, Bim, Bik, and Bid) and NOXA, and to downregulation of anti-apoptotic proteins such as Bcl-2 and IAP (inhibitor of apoptosis) proteins [282].

The tumor suppressor p53 is an important regulator of apoptosis induced by DNA damage and transforming oncogenes. p53 is regulated by interaction with its negative regulator Mdm-2, which is an E3 ubiquitin ligase. In cancer cells, Mdm-2 is often over activated, leading to proteasomal degradation of p53 [283-285]. Proteasome inhibition can upregulate p53 and activate p53 downstream target genes such as p21, Fas ligand, PUMA and Bax to block cell growth and induce apoptosis [286].

Endoplasmic reticulum stress (ER stress)

Inhibition of proteasome can induce accumulation of unfolded or misfolded proteins in ER lumen leads to induction of ER stress [287]. In order to cope with the stress, cells elicit a signal network termed unfolded protein response (UPR). The goal of the UPR is to restore ER homeostasis and promote cell survival. However, if proteasome inhibitors maintain accumulation of misfolded proteins, signalling can switch from pro-survival to pro-apoptotic [288]. Tumor cells usually have higher protein synthesis rates than their normal counterparts, leading them more prone to accumulation and aggregation of protein and more sensitive to proteasome inhibitor-induced apoptosis. Induction of pro-apoptotic ER stress by proteasome inhibitors has been found in different cancer cell types including, multiple myeloma, head and neck cancer and non-small cell lung carcinoma [289, 290].

Oxidative stress

Oxidative stress reflects an imbalance between the production of reactive oxygen species (ROS) and antioxidant defences [291]. Superoxide anions, hydrogen peroxide, and hydroxyl radicals all have higher reactivity than molecular and can severely damage DNA, protein, and lipids [292]. ROS is mainly produced by the electron transport chain in the mitochondria [293]. Antioxidant defences include enzymatic antioxidant defenses (superoxide dismutase, catalase, glutathione peroxidase,

thioredoxin reductase) and non-enzymatic antioxidants (Vitamin C, Vitamin E and others) [294, 295].

Proteins oxidation may lead to misfolding and aggregate formation [296]. The UPP can remove oxidized proteins [297]. Moreover, oxidized proteins can be degraded by the 20S CP in an ATP-independent manner without involvement of ubiquitin [298]. Elevated level of ROS can increase p53 expression and in induction of the JNK pathway, resulting in induction of apoptosis [299]. Usually tumor cells have higher levels of ROS than normal cells in order to promote cell survival and tumor progression [189]. Proteasome inhibitors have been shown to induce ROS in various cancer cells [300-303].

DNA repair

Proteasome inhibitors may affect DNA repair by the depletion of available nuclear ubiquitin [304]. Inhibition of proteasome activity leads to accumulation of non-degraded polyubiquitinated proteins and a reduction in the amount of free ubiquitin. A decreased ubiquitin pool will result in a decrease in histone ubiquitination, and impaired DNA-damage responses. Proteasome inhibitors have been proved to sensitize tumor cells to various anticancer therapies such as radiation, camptothecin and topoisomerase inhibitors, all of which induce DNA damage [191].

1.8.3 Inhibitors of the ubiquitin proteasome pathway

Bortezomib

Bortezomib (PS-341, Velcade ®; Millennium Pharmaceuticals) was chosen from a series of boronic acid analogues screened against the National Cancer Institute's (NCI's) panel of 60 cancer cell lines, according to its potency of cytotoxicity [305]. Bortezomib was extensively studied in vitro and in vivo in several tumor types, including non-small cell lung cancer, prostate cancer, multiple myeloma and mantle cell and follicular non-Hodgkin's lymphoma. Bortezomib was found to show particularly strong cytotoxic activity against multiple myeloma. It was approved for third-line treatment of multiple myeloma by the FDA in 2003 [306], and was later approved for first-line treatment and for treatment of mantle cell lymphoma [307]. Bortezomib is a reversible inhibitor of the chymotryptic-like and caspase-like activities of the 20S CP [308]. Many researches have been shown that bortezomib can induce growth arrest and apoptosis in a wide range of tumor cell lines, animal models or/and patients of hematologic and solid tumor malignancies by the different mechanism of proteasome mentioned above. Such as, the inhibition of the NF- κ B activation [309], induction of p53 [310], and the CKIs p27 and p21 [311], activates the stress kinase JNK, and induces pro-apoptotic proteins [312, 313]. However, as I mentioned above, there are conflicting reports concerning the role of NF- κ B activation [314]. Bortezomib not only shows considerable activity as a single agent, but shows additive/synergistic activity in combination with other chemotherapeutic agents [296, 315]. The activity of bortezomib might not entirely be due to proteasome inhibition. Thus, when proteasome subunit expression was knocked-down using siRNA, bortezomib still induced an increase in the number of apoptotic cells without a further reduction in proteasome activity [279]. But similar to other chemotherapeutic agents, acquired drug resistance and serious side effects limit the efficacy of bortezomib in clinical use. The toxic side effects of bortezomib include peripheral neuropathy, low platelet and erythrocyte counts and joint pain [266]. Large efforts are devoted to the development of new proteasome inhibitors, with the goal to overcome bortezomib resistance, reduce toxicity, enhance anti-tumor activity and improve effectiveness of proteasome inhibition.

A new generation of proteasome inhibitors

Carfilzomib, a second generation of proteasome inhibitor, is an irreversible inhibitor of the chymotrypsin-like activity of the proteasome [316]. In clinic, marizomib has been shown the activity on bortezomib-refractory multiple myeloma patients and is well tolerated with no peripheral neuropathy side effects [317]. In addition, new generation of proteasome inhibitors such as NPI-0052, MLN9708, CEP-18770 and ONX0912 have been shown to show activity on bortezomib-resistant cells, extending the range of application and enhancing clinical outcomes [191].

Targeting deubiquitinating enzymes

The importance of DUBs for proteasomal degradation have fueled interest in these enzymes as targets for anticancer agents [318]. A number of pan-DUB inhibitors targeting both proteasomal and non-proteasomal DUBs have been described. WP1130 (degrasyn), a small-molecule DUB inhibitor, can inhibit USP9x, USP5, USP14 and UCHL5. By inhibiting these DUBs, WP1130 treatment leads to accumulation of poly-ubiquitinated proteins, down-regulation of anti-apoptotic mediators and up-regulation of pro-apoptotic proteins in cells [319]. WP1130 has shown antitumor activity in lymphoma animal models [320]. IU1 is a reversible small molecule inhibitor of USP14. At high concentrations, IU1 increases the degradation of proteasome substrates. Since IU1 increases proteasome activity, this compound is not a candidate for anticancer drug development. However, it has been suggested as a treatment option for neurodegenerative diseases that are associated with the accumulation of misfolded and aggregated proteins [251]. AC17 is a curcumin analogue which has been shown to irreversibly inhibit the deubiquitinase activity of the 19S RP. The anticancer activity of AC17 was investigated both *in vitro* and *in vivo* and is associated with the inhibition of NF- κ B and activation of p53. However, the specificity of AC17 with regard to DUBs inhibition is unclear [321]. b-AP15 is a novel small molecular reversible inhibitor of the proteasomal DUBs USP14 and UCHL5. It was found to have antineoplastic activity in a number of tumor models representing both hematological and solid tumor [322]. b-AP15 was demonstrated to be cytotoxic to bortezomib resistant cells [255].

2. AIMS

The overall purpose of this thesis was to examine the phenomenon of anticancer drug resistance and to identify and characterize drugs that are effective on tumor cells that have acquired resistance to conventional cancer therapy.

Specific aims:

- ❖ To investigate the effect of clofarabine on apoptosis and DNA synthesis in human epithelial colon cancer cells.
- ❖ To measure 5'-NT activities and CdAMP and Fara-AMP degradation in samples from patients with chronic lymphocytic leukemia and to investigate the relationship between the levels of dCK mRNA and the cytotoxicity of five nucleoside analogues (dFdC, CdA, FaraA, Ara-C and CAFdA).
- ❖ To identify novel active agents insensitive to multidrug resistance by drug screening.
- ❖ To investigate the action of the deubiquitinase inhibitor b-AP15 in multiple myeloma and the ability of this drug to overcoming bortezomib resistance.
- ❖ To investigate the cellular uptake and metabolism of b-AP15 and to further characterize its mechanism of action.
- ❖ To perform lead optimization of b-AP15 and to evaluate the activity of the optimized lead VLX1570 in multiple myeloma.

3. RESULTS AND DISCUSSION

3.1 Paper I: Effect of Clofarabine on Apoptosis and DNA Synthesis in Human Epithelial Colon Cancer Cells

Clofarabine, a new-generation purine nucleoside analogue, has shown significant efficacy in pediatric relapsed/refractory acute lymphoblastic leukemia (ALL) and hematologic malignancies in adults. Based on its broad activity, the effect of clofarabine on the DNA synthesis of human colon carcinoma cells (HCT116) was investigated in this study.

We used a LigandTracer White instrument to investigate the uptake and retention of clofarabine in cells. At 100 nM, the uptake is detectable and at 10 μ M a strong signal is achieved and approaches equilibrium after 1~2 hours. Thymidine incorporation into cells (and presumably DNA) was rapidly stopped by incubation with 10 μ M clofarabine and a 3-fold increase in apoptosis induction in HCT116 cells by clofarabine was detected.

The results show that clofarabine is capable of inducing apoptosis of solid tumor cells, presumably due to interference with DNA synthesis. The experimental approach to drug uptake in real-time was found to be quite useful and was also used in paper V.

3.2 Paper II: 5'-Nucleotidase activities in blood cells from untreated patients with B-cell chronic lymphocytic leukemia: Correlation to the efficacy of nucleoside analogue therapy

The primary aim of this study was to uncover the significance of 5'-NT activities for the sensitivity of chronic lymphocytic leukemia (CLL) cells to nucleotide analogues. We measured the activities of 5'-NTs in peripheral blood cells from 59 CLL patients by using HPLC-based procedures. Considerable inter-individual variations in the activity of different 5'-NTs were observed. The median value of CN1 was 132 pmol/mg protein/min (95% CI=119-157), the median value of CN2 activity was 478 pmol/mg protein/min (95% CI=427-616) and median dNT activity was 3265 pmol/mg protein/min (95% CI 2690-3573). A clear relation between the degradation of fludarabine monophosphate (Fara-AMP) and cytosolic CN2 activity was observed, and a relation between the degradation of cladribine monophosphate and CN1 activity. Although Fara-AMP and CdA have similar chemical structures and mechanisms of action, there are also some important differences, especially with respect to the enzymes involved in their metabolism [323, 324]. These differences may explain why certain CLL patients who experienced relapse with Fara-AMP treatment may still respond to CdA [325-327] as previously described [328, 329]. The results suggest that treatment with different NAs can be individualized.

Although the role of 5'-nucleotidases in resistance to NAs has not yet been extensively investigated, observations using cell lines as well as clinical studies have indicated that these enzymes are important in decreasing the clinical efficacy of NAs [330, 331]. For instance, an Ara-C-resistant cell line with a high level of CN2 and reduced level of dCK was found to be less sensitive to FaraA compared to another cell line, which only had a low level of dCK [332]; a series of K562 cell lines resistant to dFdC, CdA, Ara-C and Fara-A exhibited elevated CN2 and low dCK activities [333]; elevated levels of CN2 mRNA were found to be correlated with poor prognosis for patients with acute myeloid leukemia (AML) [334, 335]; high levels of CN2 mRNA and low levels of CN3 mRNA were found to be correlated with poor clinical outcome for AML patients treated with Ara-C [336]; dCK and CN2 mRNA expression in leukaemic blasts at diagnosis is correlated with clinical outcome and may play a functional role in the resistance to ara-C in patients with AML[337]; low levels of dCK, dNT1 and ecto-NT mRNA and over-expression of hCNT3 mRNA have been found to be correlated with more rapid disease progression in patients with CLL receiving Fara-AMP [338] and among patients with CLL or hairy cell leukemia, non-responders exhibited higher CN2 and /or lower dCK activity [339].

By phosphorylating nucleoside analogues, dNKs oppose the action of 5'-NTs and are therefore likely to play an important role in determining dNTP pool sizes. We found that the sensitivity of leukemic MOLT4 cells to purine analogues was attenuated by down-regulation of dCK using siRNA (Figure 7, 8). Thus, the efficacy of cladribine and fludarabine treatment in CLL may be dependent on the level of cellular dCK, CN1 and CN2 activities. In conclusion, the expression of anabolic and catabolic enzymes may be of important to consider in predicting the response to CLL treatment.

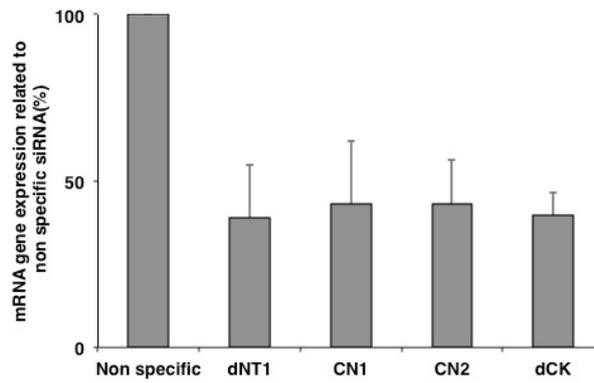


Figure 7. The effects of siRNA transfection on levels of dCK and 5'-NT mRNA in MOLT4 cells. Cells were transfected with siRNA designed to silence dCK or one of various 5'-NT genes, and mRNA levels subsequently measured. The levels obtained were normalized to the levels of housekeeping GAPDH mRNA and are expressed as percent down-regulation in comparison to control cells transfected with non-specific siRNA.

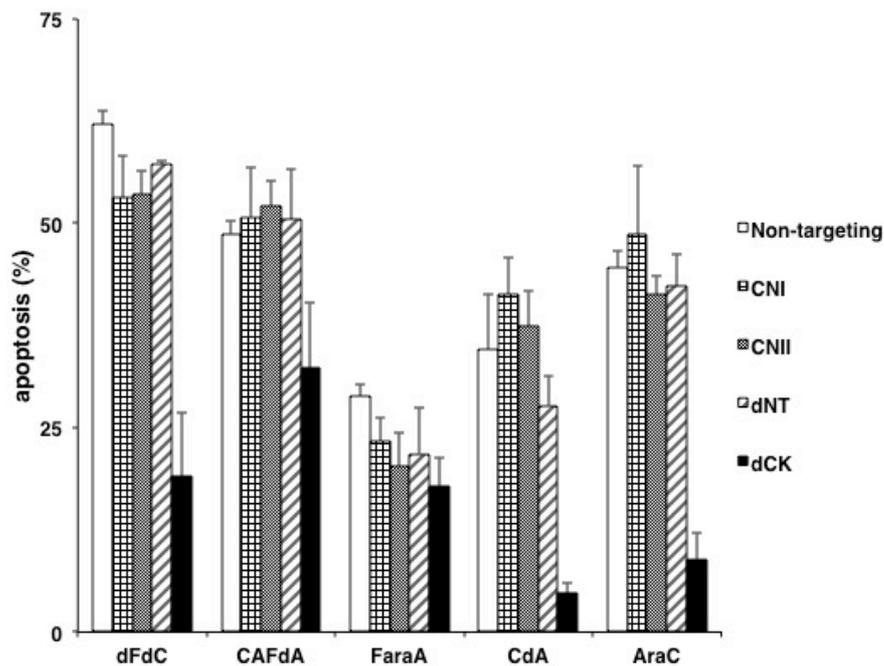


Figure 8. The sensitivities of siRNA-transfected cells to nucleoside analogues. Cell transfected with siRNA designed to silence the expression of 5'-NTs (dNT1, CN1 and CN2) or dCK were incubated for 24 hours with one of five purine analogues, dFdC, CAFdA, Fara-A, CdA or AraC. The results are expressed as percent apoptotic cells in comparison to the control cells transfected with non-specific siRNA (NS).

3.3 Paper III: Screening for phenotype selective activity in multidrug resistant cells identifies a novel tubulin active agent insensitive to common forms of cancer drug resistance

Drug resistance is a major obstacle to successful treatment of many cancers. This study was designed to identify drugs effective on multidrug resistant cells.

A well characterized MDR model was used, the multidrug resistant myeloma cell line RPMI 8226/Dox40. This cell line was originally selected for resistance to doxorubicin and show cross-resistance to mitoxantrone, acronycine, etoposide, and vincristine. The resistant subline strongly overexpresses the MDR1 gene product P-gp170. Screening a library of 3,000 chemically diverse compounds resulted in the identification of a compound, designated VLX40, which demonstrated slightly higher activity against 8226/Dox40 cells compared to its parental counterpart. We found that VLX40 inhibited proliferation of the breast cancer cell line MCF7 and induced apoptosis in myeloma and myeloid leukemia cell lines. In order to understand the molecular mechanisms of action, we used a bioinformatic approach based on drug-specific gene expression signatures [340]. The results suggested that VLX40 was a tubulin-active agent; in vitro assays subsequently confirmed that VLX40 inhibits the polymerization of tubulin monomers. VLX40 was also found to induce G2/M cell cycle arrest.

Several antitubulin drugs are in clinical use, such as paclitaxel and vincristine. We found that VLX40 showed a favorable pharmacological profile compared to vincristine being active against a multidrug resistant myeloma cell line with little sensitivity to other common forms of vinca alkaloid resistance. In addition, VLX40 showed a relatively narrow spectrum of activity in PCPTCs of various tumor types, particularly in leukemias and lymphomas. In contrast, vincristine is often insensitive to myeloid blast cells. Compared to its high activity in leukemias and lymphomas, VLX40 showed very limited activity on ex vivo solid tumor cells from breast, ovary, lung, colon and renal cancer patients. The reason for the low activity observed in the PCPTC solid tumor models may be due to a poor drug penetration in the latter model system, consisting of multicellular clusters. This hypothesis was supported by the modest antitumor activity obtained in the 3-D spheroid model cell line. In addition to poor penetration, low rates of proliferation of PCPTCs could contribute to the low solid tumor activity observed.

We also investigated the effect of VLX40 and six standard cytotoxic drugs on solid and hematological tumor samples, expressed as the solid/hematological (S/H) ratio. VLX40 had a ratio of 0.28 indicating a modest activity against solid tumors compared to cisplatin (S/H ratio 1.2). Other drugs showed S/H ratios < 0.5. The results for the standard drugs are consistent with their main clinical use. In addition, for evaluation of tumor cell specificity, drug effects were compared in cells from CLL and normal PBMCs. VLX40 expressed a significantly higher activity against malignant phenotypes with a PBMC/CLL median IC₅₀ ratio of 12.2. Among the tested standard cytotoxic drugs only vincristine was more active in CLL than in PBMC. The high PBMC/CLL IC₅₀ ratio indicates a potentially high therapeutic index. It should be considered that both the PBMC/CLL ratio and S/H ratios are in vitro indicators for therapeutic index and clinical activity spectra and should be evaluated in relative rather than absolute terms. A ratio of 1 indicates equal sensitivity for PBMC vs. CLL and solid vs hematological activity, respectively.

In vivo activity of VLX40 was investigated in hollow fiber cultures of myeloid U-937 cells subcutaneously implanted in mice.

VLX40, a 2-phenyl-4-hydroxyquinoline, is a flavone-like element that has previously been used in medicinal chemistry, for example to design inhibitors of bacterial cell membrane pumps [341] or to inhibit cyclo-oxygenases [342]. However, several studies have reported that 2-phenyl-4-quinolones, the isomers of 2-phenyl-4-hydroxy-quinolines, have cytotoxic activities on human cancer cells [343-347]. The 2-phenyl-4-hydroxy-quinolines are structurally unrelated with other tubulin inhibitors, and may have different characteristics of importance for cytotoxic activity and resistance. Vincristine is a substrate for both MRP and P-gp170, but VLX40 appears unaffected by both mechanisms. AML cells are relatively insensitive to vincristine, but are sensitive to VLX40. VLX40 may therefore be useful for development of novel tubulin active agents that are insensitive to common mechanisms of cancer drug resistance and an agent for AML.

3.4 Paper IV: A novel small molecule inhibitor of the deubiquitylating enzymes USP14 and UCHL5 induces apoptosis of multiple myeloma cells and overcomes bortezomib resistance

Proteasome inhibitors have shown antiproliferative activity in multiple myeloma by targeting protein degradation. In this study we found that b-AP15, a novel small molecular inhibitor of the USP14/UCHL5 DUBs of the 19S RP, decreases viability in MM cell lines and patient MM cells, inhibits proliferation of MM cells even in the presence of bone marrow stroma cells, and overcomes bortezomib resistance.

By western blotting and immunohistochemistry analysis, we found that USP14 and UCHL5 are highly expressed in MM cells compared to normal plasma cells and PBMCs. We also found that the knockdown of USP14 and UCHL5 can decrease MM cell viability. These findings suggested that USP14 and UCHL5 are required for viability of MM cells and that these enzymes are valid anticancer targets.

b-AP15 induced significant accumulation of polyubiquitin conjugated proteins in MM cells. b-AP15 did not inhibit the proteolytic activities of the proteasome and did not inhibit total DUB activity. We observed inhibition of USP14 and UCHL5 activity in treated cells using the active site probe ubiquitin vinyl sulphone. The results suggest that b-AP15 targets the deubiquitylating function of USP14 and UCHL5 in the cellular environment. It should be noted, however, that the efficacy of inhibition of particularly UCHL5 varied between experiments. We do not quite understand the reason for this variability, but believe it to be due to b-AP15 being a reversible inhibitor. During preparation of cell extracts for labeling experiments, the compound may detach from the target enzymes, perhaps by a temperature-dependent manner.

Peripheral neuropathy associated with bortezomib therapy is believed to partly result from blockade of the neuronal cell survival protease HtrA2/Omi. In this study, we found no significant inhibition of HtrA2/Omi in response to b-AP15 treatment, whereas bortezomib inhibited HtrA2/Omi activity. These data further suggested the selectivity of b-AP15.

We also found b-AP15 not only decreased viability in MM cell lines and primary MM cells from patients, but also had antitumor activity in distinct human MM xenograft models. At the IC₅₀ for MM cells, b-AP15 did not significantly affect the viability of normal PBMCs. In addition, we found that anti-MM activity of b-AP15 is associated with growth arrest via downregulation of CDC25C, CDC2, and cyclin B1 as well as induction of caspase-dependent apoptosis, and activation of UPR signaling pathway. Importantly, ANBL-6 and its bortezomib-resistant subline ANBL-6.BR showed similar sensitivities to b-AP15. We also found similar responses to b-AP15 in tumor cells from patients in whom MM was resistant to therapies such as bortezomib, lenalidomide, and dexamethasone. These data suggest that b-AP15 (or related compounds) can be used for treatment of patients with bortezomib resistant MM.

We also examined anti-MM activity of b-AP15 *in vivo*. A significant inhibition of tumor growth was observed in two distinct human MM xenograft mouse models treated with b-AP15. In addition, b-AP15 treatment was not associated with any detectable toxicity as evidenced by no significant effects on body weight and/or alteration in blood chemistry. By IHC analysis of caspase 3 cleavage, Ki67, VEGF,

VEGFR2, LYVE1 and CD31 in the tumor sections from tumors harvested from control and b-AP15-treated mice, we further confirm the anti-MM activity of b-AP15 in vivo at aspect of apoptosis, proliferation and angiogenesis.

Finally, we also examined whether b-AP15 can be combined with other anti-MM agents. Our results show synergistic anti-MM activity of b-AP15 with HDAC inhibitor SAHA, dexamethasone and lenalidomide.

In summary, this study showed the efficacy of b-AP15 in MM disease models by targeting DUBs in the ubiquitin proteasomal cascade and had the ability to overcome proteasome inhibitor resistance, which provides the framework for clinical evaluation of USP14/UCHL5 inhibitors to improve patient outcome in MM.

3.5 Paper V: The 19S deubiquitinase inhibitor b-AP15 is enriched in cells and elicits rapid commitment to cell death

In this study we characterized several questions related to the molecular pharmacology of the b-AP15 compound.

b-AP15 contains an α , β -unsaturated carbonyl unit that is likely to serve as a Michael acceptor to interact with the thiol group of cysteines in deubiquitinating enzymes, leading to inhibition of deubiquitinase activity. b-AP15 contains an additional Michael acceptor (an acrylamide residue). We here tried to understand the relative roles of these Michael acceptors plays a role for the biological activity of b-AP15. The acrylamide was not required for induction of cell death. In contrast, decreasing the reactivity of the unsaturated carbonyls lead to reduced cytotoxic effects. The result suggested that the biological activity of b-AP15 is governed primarily by the reactivity of α , β -unsaturated carbonyl pharmacophore.

The reversibility of b-AP15 was investigated in this study. We used the suicide probe Ub-VS to determine the activity of USP14 and UCHL5 and confirmed that both proteasomal DUBs are inhibited by b-AP15. Dilution of b-AP15 in cell extracts lead to recovery of the active USP14, indicating b-AP15 to be a reversible enzyme inhibitor. We were interested in determining whether inhibition of proteasome function and induction of apoptosis by b-AP15 would also be reversible. To address this question, we used a reporter cell line expressing a proteasome targeted substrate, ubiquitin fused to yellow fluorescent protein (Ub^{G76V}-YFP). Treatment of cells with a low concentration of b-AP15 for one hour resulted in the induction of polyubiquitin conjugates, which started to decrease after 4 hours from drug removal. A similar pattern was observed for the reporter protein Ub^{G76V}-YFP and the proteasome substrate p21^{Cip1}, indicating that b-AP15 was a reversible inhibitor of proteasome function. Interestingly, cleavage of caspase-3 and PARP was observed 24 hour after drug removal, indicating that despite reversible inhibition of the proteasome function cells became committed to apoptosis after only one hour of b-AP15 exposure.

Using the LigandTracer[®] White technique (used in Paper I), we observed that b-AP15 was rapidly uptaken by cells during ~30 min. After washing out of the drug, we found a ~40% decrease of the content of radiolabeled b-AP15 over two hours and a ~50% decrease after ten hours. These findings suggested that drug is available for proteasome inhibition even washout after a short treatment with a high drug concentration. Consistent with this, treatment of reporter Ub^{G76V}-YFP expressing cells with a higher b-AP15 concentration resulted in the accumulation of polyubiquitin conjugates that were still abundant after 8 hours from drug removal. In addition, we found low concentrations of free intracellular b-AP15, suggesting that majority of the drug molecules may bind to cellular macromolecules. As mentioned above, Michael acceptors react with thiol groups in cysteines of proteins, suggesting that b-AP15 was binding to intracellular thiols. We pretreated cells with N-ethylmaleimide, a covalent inhibitor of DUB activity, before treatment of cells with radiolabeled b-AP15. Interestingly, the cellular uptake of b-AP15 was inhibited by pre-treatment 10 μ M NEM. This result showed that uptake into cells was thiol-dependent. Whether inhibition is due to inactivation of a thiol-dependent transport system, or due to NEM blocking a subset of hyper-reactive cysteines which also bind b-AP15, is not known at present. It appears likely that, similar to b-AP15, NEM is enriched intracellularly and may block a large fraction of reactive cysteines when used at 10 μ M.

Higher concentrations of b-AP15 were required to inhibit deubiquitinase activity in biochemical assays than in assays of proteasomal function on cells [322]. The reason for this difference was further investigated in this study. When Ub-AMC was used as a DUB substrate, an $IC_{50} \sim 17 \mu\text{M}$ of b-AP15 was observed. The use of other substrates and assays resulted in somewhat lower IC_{50} values ($\sim 5 \mu\text{M}$). These values are higher than the b-AP15 concentrations of $< 1 \mu\text{M}$ that were sufficient to inhibit proteasome function in colon carcinoma cells. The reason for this apparent discrepancy appears to be the effective uptake of b-AP15 from the medium and enrichment of this compound in cells.

b-AP15 is expected not to only bind to thiols present in the proteasomal deubiquitinases, but also to other enzymes and proteins. We here found that b-AP15 inhibits thioredoxin reductase. The thioredoxin enzymatic system is important for redox regulation of cellular function. The inhibition of thioredoxin reductase may help to explain the strong induction of oxidative stress observed by b-AP15 [348]. Our results show, however, that thioredoxin reductase inhibition is of limited importance for the cytotoxicity of b-AP15. Another important enzymatic system in regulating cellular redox-homeostasis is the glutathione system. We observed that glutathione reductase was not inhibited by b-AP15, suggesting selective binding of b-AP15 to cellular cysteines (i.e. to thioredoxin reductase and proteasomal DUBs, but not to glutathione reductase). Somewhat surprisingly, b-AP15 was found to irreversibly inhibit thioredoxin reductase, in distinction to the reversible inhibition of USP14. We do not understand the underlying difference with regard to inhibition, but believe it to be related to strong binding to the selenocysteine of thioredoxin reductase.

Almost one hundred cysteine DUBs have been identified in the human genome. Surprisingly, we have only been able to demonstrate inhibition of the activities of proteasomal DUB by b-AP15 [255, 322]. The Michael acceptors of the compound are expected to preferentially react with "hyper-reactive cysteines", demonstrated to encompass a minor fraction of all cellular cysteine [349, 350]. It is possible that USP14 and UCHL5 share some features which render them preferred targets for b-AP15. Both USP14 and UCHL5 are activated by mechanism involving displacement of loops to expose the active site cleft to ubiquitin [247]. Alternatively, and perhaps more likely, also other DUBs are inhibited by b-AP15, but these interactions do not generate the same degree of cytotoxicity compared to the delirious consequences of inhibiting proteasomal DUBs.

In conclusion, even b-AP15 is a reversible inhibitor of deubiquitinating activity and proteasome function, it can be enriched in cells and rapidly committed to cell death within one hour of drug treatment. Importantly, cell death is intimately associated with proteasomal blocking as evidenced by induction of cell death only in cells which accumulate a proteasomal reporter.

3.6 Paper VI: Development of the proteasome deubiquitinase inhibitor VLX1570 for treatment of multiple myeloma.

In this study, we describe and characterize the compound VLX1570, an optimized lead of the 19S deubiquitinase inhibitor b-AP15.

Similar to b-AP15, VLX1570 inhibits the enzymatic activities of both USP14 and UCHL5, leading to accumulation of high molecular weight polyubiquitinated proteins. VLX1570 was slightly more cytotoxic than b-AP15 on colon cancer cells and myeloma cells. Using the substrate Ub-AMC to determine 19RP DUB activity, VLX1570 showed a lower IC₅₀ compared to b-AP15 (13.0 + 2.7 μ M compared to 16.8 + 2.8 μ M). VLX1570 induces a similar gene expression profile as b-AP15 in MCF-7 cells. This response is characterized by induction of chaperones, induction of ER stress and oxidative stress. Furthermore, we compared the cytotoxicity of VLX1570 to that of b-AP15 in 14 human tumor cell lines. A correlation coefficient of 0.93 was observed between the recorded IC₅₀ values, indicating that VLX1570 induces a similar cellular response as b-AP15.

b-AP15/VLX1570 induce gene expression profiles which are very similar to those induced by 20S proteasome inhibitors, and cell death is closely linked to proteasome inhibition in cells (Paper V). We were nevertheless concerned that the reactivity of b-AP15/VLX1570 will lead to a wide range of off-target activities. To begin to address this issue we examined the ability of VLX1570 to inhibit the activity of cellular kinases. We did not, however, find significant inhibition (Cdk4 being the only kinase significantly inhibited at 10 μ M). This result implies that off-target activities may not be as extensive as may have been envisioned.

In paper IV, we found that b-AP15 has activity in multiple myeloma in vitro and in vivo. We here investigated the cytotoxicity of VLX1570 on three multiple myeloma cell lines, RPMI8226, KMS-11 and OPM2. We found that VLX1570, similar to b-AP15, was effective in inducing apoptosis/cell death of myeloma cells. Apoptosis was confirmed by determining cleavage of caspase-3 and PARP in these cell lines. Similar to b-AP15 and the 20S proteasome inhibitor bortezomib, VLX1570 induced p21^{Cip1}, the chaperone Hsp70B' and the oxidative stress marker Hmox-1. VLX1570 also activated the JNK pathway in the multiple myeloma cell lines.

In paper IV, we also reported that b-AP15 can overcome resistance developed to the proteasome inhibitor bortezomib [255], providing a basis for future clinical use. In this study, we found that a multiple myeloma cell line (OPM2) selected for resistance to bortezomib, OPM2/BZ cells, showed a similar sensitivity to VLX1570 as its parental derivative. Interestingly, OPM2/BZ cells were partially resistant to VLX1570-induced apoptosis. These findings are consistent with our previous notion that b-AP15 is able to overcome apoptosis resistance [322, 351]. The mechanism(s) underlying the strong induction of cell death by b-AP15 and VLX1570 are unclear at present, but may be due to induction of organelle damage by accumulated misfolded proteins.

We also studied the anti-multiple myeloma activity of VLX1570 in vivo. Growth inhibitory effects by VLX1570 were observed in two different human multiple myeloma xenograft-mouse models. These findings are consistent with our previous results showing anti-tumor activity of b-AP15 in multiple myeloma and solid tumor

xenograft models [322, 352]. Immunohistochemical analysis showed decreased ERK phosphorylation in tumors treated with VLX1570. In paper IV we were able to show increased levels of K48-linked polyubiquitin in tumors from b-AP15-treated animals. This result has been difficult to reproduce, probably due to different properties of antibodies. Further studies are required to determine which biomarker will be optimal in future pharmacodynamic studies.

We conclude that VLX1570 is a candidate agent for treatment of multiple myeloma. VLX1570 shows improved solubility compared to the hit molecule b-AP15. The compound is currently undergoing toxicological studies in preparation for future clinical trials.

4. CONCLUSIONS

Paper I

- ❖ Clofarabine may inhibit DNA synthesis and can induce apoptosis in HCT116 colon cancer cells.
- ❖ Real-time analysis of drug uptake is useful for characterization of drug action *in vitro*.

Paper II

- ❖ The activities of different 5'-NTs are different in blood samples from patients.
- ❖ The degradation of fludarabine monophosphate was associated with CN2 activity, and the degradation of cladribine monophosphate was associated with CN1 activity.
- ❖ The sensitivity of leukemic Molt4 cells to the purine analogues was attenuated by down-regulation of dCK.
- ❖ The expression of anabolic and catabolic enzymes in tumor cells should be considered during treatment of CLL with nucleotide analogues.

Paper III

- ❖ VLX40 has identified as showing antiproliferative activity in a multidrug resistant model of myeloid leukemia.
- ❖ VLX40 is a novel tubulin active agent that may be further developed for treatment of malignancies that are resistant to common chemotherapeutic agents.

Paper IV

- ❖ The proteasome DUB inhibitor b-AP15 shows anti-MM activity *in vitro* and *in vivo*.
- ❖ This study provides proof of concept for evaluation of proteasome DUB inhibitors, alone and in combination, as potential therapy to improve patient outcome in MM.

Paper V

- ❖ The ability of b-AP15 to inhibit proteasome function and DUB activity is reversible.
- ❖ b-AP15 is rapidly taken up and enriched in cells and elicits rapid commitment to apoptosis/cell death.
- ❖ b-AP15 inhibits thioredoxin reductase. This activity is likely to contribute to oxidative stress but does not appear important for cell death.

Paper VI

- ❖ VLX1570 shows a similar cytotoxic profile and similar biochemical activity as b-AP15.
- ❖ VLX1570 shows significant anti-MM activity *in vitro* and *in vivo*.

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