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Targeting the Colchicine Binding Site on Tubulin to Overcome Multidrug Resistance and Anticancer Efficacy of Selective Survivin Inhibitors

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Abstract

Tubulin inhibitors are widely used as chemotherapeutic agents, and their success is attributed to their ability to target microtubule dynamics and disrupt critical cellular functions including cell signaling, motility, intracellular trafficking, and mitosis. Interference with microtubule dynamics consequently disrupts mitotic progression and ultimately leads to apoptosis, validating microtubule dynamics as an excellent target for anticancer agents. While this class of drug has proven to be effective against many cancer types, the clinical efficacy of current tubulin inhibitors is often limited by the development of multidrug resistance. The most common form of resistance to these agents arises from the overexpression of drug efflux transporters. Extensive research efforts have attempted to develop colchicine binding site inhibitors, which are reported to be significantly less susceptible to multidrug resistance and have therapeutic advantages over agents that target the taxane and vinca alkaloid site. Herein, we evaluated the anticancer activity of novel small-molecules that target the colchicine binding site, focusing on the most promising compounds from several structural scaffolds including indolyl-imidazopyridines (DJ95 and DJ101), VERU-111 analogs with a modified indole moiety (10ab and 10bb) or 3,4,5-trimethoxyphenyl moiety (13f), and heterocyclic pyrimidines (4a, 6a, 5a, and 5b). We demonstrated the cytotoxic potency of these compounds against a variety of cancer cell lines, including malignant melanomas, taxane-resistant prostate cancer cells, and drug efflux pump-overexpressing cell lines. Their mechanism of action was revealed through tubulin polymerization inhibition, disruption of microtubule networks and mitotic spindle formation, and confirmed through X-ray crystallography, which detailed their specific molecular interactions with tubulin in the colchicine binding pocket. Furthermore, these compounds exhibited hallmark characteristics of colchicine binding site agents, such as arresting cells in the G2/M phase of the cell cycle, inducing apoptosis in a concentration-dependent manner, and impeding cancer cell proliferation and migration. Finally, the compounds were efficacious *in vivo* against melanoma and taxane-resistant prostate cancer xenograft tumors. Several agents were evaluated for ability to prevent melanoma metastases to the lungs in experimental mouse models, and they potently inhibited the development of metastatic foci. Safety assessment by pharmacological profiling demonstrated minimal interactions to physiologically important targets and pathophysiological analysis of major organs from the *in vivo* treatment groups did not expose apparent drug-related injury. Several of the investigated compounds also demonstrated vascular disrupting properties by targeting tumor vasculature and inhibiting capillary-like network formation of endothelial cells. Ultimately, these compounds exhibit strong anticancer efficacy, specifically target the colchicine binding site, and have great potential as cancer therapeutics, particularly for multidrug resistance phenotypes.

Another target we explored for anticancer intervention was survivin. Survivin is the smallest member of the inhibitor of apoptosis protein family and its overexpression in tumor cells is been positively correlated with the development of multidrug resistance and radiation resistance. Because it is differentially expressed in healthy tissues and tumors, it is an attractive therapeutic target. Using the scaffold of UC-112, which was previously identified through virtual screening, we evaluated a series of analogs designed to optimize potency and improve selectivity to survivin over other inhibitor of apoptosis proteins. We identified compound 10f, which was highly cytotoxic to melanoma and Pglycoprotein overexpressing cell lines, induced apoptotic cascades in a concentration-dependent manner, specifically downregulated survivin protein levels, and significantly inhibited tumor growth *in vivo*. Ultimately, these results validated our in-depth biological investigation of novel scaffolds of survivin inhibitors and verified the anticancer efficacy of 10f.

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Resistance and Anticancer Efficacy of Selective Survivin Inhibitors**

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Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Kinsie E. Arnst
December 2018

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DEDICATION

To my family and friends who have unconditionally supported my endeavors.

And to my mom, whose strength reinvigorated my passion for science.

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ABSTRACT

Tubulin inhibitors are widely used as chemotherapeutic agents, and their success is attributed to their ability to target microtubule dynamics and disrupt critical cellular functions including cell signaling, motility, intracellular trafficking, and mitosis. Interference with microtubule dynamics consequently disrupts mitotic progression and ultimately leads to apoptosis, validating microtubule dynamics as an excellent target for anticancer agents. While this class of drug has proven to be effective against many cancer types, the clinical efficacy of current tubulin inhibitors is often limited by the development of multidrug resistance. The most common form of resistance to these agents arises from the overexpression of drug efflux transporters. Extensive research efforts have attempted to develop colchicine binding site inhibitors, which are reported to be significantly less susceptible to multidrug resistance and have therapeutic advantages over agents that target the taxane and vinca alkaloid site. Herein, we evaluated the anticancer activity of novel small-molecules that target the colchicine binding site, focusing on the most promising compounds from several structural scaffolds including indolyl-imidazopyridines (DJ95 and DJ101), VERU-111 analogs with a modified indole moiety (**10ab** and **10bb**) or 3,4,5-trimethoxyphenyl moiety (**13f**), and heterocyclic pyrimidines (**4a**, **6a**, **5a**, and **5b**). We demonstrated the cytotoxic potency of these compounds against a variety of cancer cell lines, including malignant melanomas, taxane-resistant prostate cancer cells, and drug efflux pump-overexpressing cell lines. Their mechanism of action was revealed through tubulin polymerization inhibition, disruption of microtubule networks and mitotic spindle formation, and confirmed through X-ray crystallography, which detailed their specific molecular interactions with tubulin in the colchicine binding pocket. Furthermore, these compounds exhibited hallmark characteristics of colchicine binding site agents, such as arresting cells in the G₂/M phase of the cell cycle, inducing apoptosis in a concentration-dependent manner, and impeding cancer cell proliferation and migration. Finally, the compounds were efficacious *in vivo* against melanoma and taxane-resistant prostate cancer xenograft tumors. Several agents were evaluated for ability to prevent melanoma metastases to the lungs in experimental mouse models, and they potently inhibited the development metastatic foci. Safety assessment by pharmacological profiling demonstrated minimal interactions to physiologically important targets and pathophysiological analysis of major organs from the *in vivo* treatment groups did not expose apparent drug-related injury. Several of the investigated compounds also demonstrated vascular disrupting properties by targeting tumor vasculature and inhibiting capillary-like network formation of endothelial cells. Ultimately, these compounds exhibit strong anticancer efficacy, specifically target the colchicine binding site, and have great potential as cancer therapeutics, particularly for multidrug resistance phenotypes.

Another target we explored for anticancer intervention was survivin. Survivin is the smallest member the inhibitor of apoptosis protein family and its overexpression in tumor cells is been positively correlated with the development of multidrug resistance and radiation resistance. Because it is differentially expressed in healthy tissues and tumors, it is an attractive therapeutic target. Using the scaffold of UC-112, which was

previously identified through virtual screening, we evaluated a series of analogs designed to optimize potency and improve selectivity to survivin over other inhibitor of apoptosis proteins. We identified compound **10f**, which was highly cytotoxic to melanoma and P-glycoprotein overexpressing cell lines, induced apoptotic cascades in a concentration-dependent manner, specifically downregulated survivin protein levels, and significantly inhibited tumor growth *in vivo*. Ultimately, these results validated our in-depth biological investigation of novel scaffolds of survivin inhibitors and verified the anticancer efficacy of **10f**.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCB1	Multidrug Resistance Protein 1/P-glycoprotein
ABCC1	Multidrug Resistance-Associated Protein 1
ABCG2	Breast Cancer Resistant Protein
ABI	2-aryl-4-benzoyl-imidazole
AIRCAR	5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside
Akt	Protein Kinase B
ANOVA	Analysis of variance
AVPI	Ala-Val-Pro-Ile tetrapeptide
BCRP	Breast Cancer Resistant Protein
BIR	Baculoviral IAP repeat
BIRC	Baculoviral IAP repeat-containing
CA-4	Combretastatins A-4
CARD	Caspase Activation Recruitment Domain
c-GAS	cyclic GMP–AMP synthase
cIAP	cellular IAP1
CIK	Cytokine-induced killer cells
DC	Dendritic cells
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signaling complex
DU-145/TxR	Docetaxel-resistant DU-145
FADD	Fas-associated death domain
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
H&E	Hematoxylin and Eosin
HUVEC	Human umbilical vein endothelial cell
IAP	Inhibitor of apoptosis protein
IHC	Immunohistochemistry
IKK	Inhibitor of κ B kinase
ILP	IAP-like protein 2
I κ B	Inhibitor of NF- κ B
KB-C2	Colchicine resistant/ABCB1-overexpressing KB
KB-CV60	Vincristine resistant/ABCC1-overexpressing
LUBAC	Linear ubiquitin chain assembly complex
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MDR1	Multidrug resistance protein 1
ML-IAP	Melanoma IAP
MRP1	Multidrug Resistance-Associated Protein 1
MTD	Maximum tolerable dose
NCI	National Cancer Institute
NCI-H460/MX20	Mitoxantrone-resistant/ABCG2-overexpressing

NIAP	Neuronal IAP
NIK	NF- κ B inducing kinase
NLRP3	NOD-like receptor pyrin domain containing 3
PARP	Poly(ADP-ribose) polymerase
PC-3/TxR	Paclitaxel-resistant PC-3
P-gp	P-glycoprotein
RI	Resistance index
RING	Really Interesting New Gene
RIP1	Receptor-interacting protein 1
SAR	Structure-activity relationship
SW620/Ad300	P-gp overexpressing SW620
T2R-TTL	Tubulin-RB3-SLD-TTL
TAK1	TGF- β activated kinase 1
TGI	Total growth inhibition, Tumor growth inhibition
TMP	3,4,5-trimethoxyphenyl
TNF α	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TTL	Tubulin tyrosine ligase
UAB	Ubiquitin-associated
UBC	Ubiquitin-conjugating
VDA	Vascular disrupting agent
XAF1	XIAP-associated factor 1
XIAP	X chromosome-linked IAP
2-Me	2-Methoxyestradiol

CHAPTER 1. INTRODUCTION TO TUBULIN INHIBITORS

Microtubule Dynamics as an Anticancer Drug Target

Microtubules are key components of the cellular cytoskeleton and have essential roles in proliferation, intracellular trafficking, migration, and mitosis. Microtubules are composed of α and β tubulin protein heterodimers that bind in a head-to-tail fashion and form cylindrical polymeric tubes of 13 protofilaments that measure approximately 25 nm in diameter (1, 2). Microtubules undergo stochastic phases of growth and shrinkage in a phenomenon termed “dynamic instability.” Dynamic instability includes the rates of polymerization and depolymerization and the frequencies of the transition between polymerization to depolymerization (“catastrophe”), and vice versa (“rescue”) (1). Another characteristic of microtubules involves “treadmilling,” or the net change in growth on one end and shrinkage on the opposite end of the protofilament. Each α and β tubulin subunit can accommodate one molecule of GTP; the α bound-GTP is not hydrolyzed or exchanged, but the GTP bound to the β tubulin can be hydrolyzed after being merged into the polymeric tubulin (3). Microtubules cycle through stages of adding GTP-tubulin dimers to their growing end, hydrolyzing GTP to GDP in the microtubule protofilament, and dissociating the GDP dimer from the microtubule, though the conformations affiliated with each of these mechanical states are complex. Free GTP-tubulin dimers naturally have a 12 degree kink at the intradimer surface but convert to a straight, expanded conformation when they bind to the microtubule end, introducing strain energy into the microtubule lattice (4, 5). Once the GTP is hydrolyzed to GDP, there is a conformational change and an accompanying relaxation in strain energy (6). **Figure 1-1** shows the cycling between microtubule polymerization and depolymerization states. In the polymerization state, microtubules adopt a straight structure, stabilized through lateral interactions, but hydrolyzed GDP-tubulin in the depolymerization state bow outward from the protofilament (7). It is generally accepted that a GTP rich “cap” on the end of the microtubule will allow it to stabilize and grow, however, when the cap is lost through hydrolysis to GDP, the core of the unstable protofilament rapidly shortens as the GDP tubulin subunits are released from the microtubule ends (1, 8, 9).

Both polymerizing and depolymerizing microtubules are observed within a cell population, supporting that dynamics are an intrinsic property of the polymer, and while the exact biomechanics of the phenomenon are not completely understood, dynamic instability is linked to the hydrolysis of GTP (2, 3, 10). Microtubule dynamics are also governed by a variety of regulator proteins and mechanisms, and they function both spatially and temporally (11). Furthermore, they are essential for successful mitotic events governed by mitotic spindles and mitotic organizing centers. Mitotic spindle microtubules are significantly more dynamic than the interphase cytoskeleton microtubule networks. The mitotic spindles radiate outwards from centrioles and attach to the centromeres of sister chromatids in dividing cells (12). This is followed by a simultaneous addition of tubulin at the kinetochore and is accompanied by a comparable rate of tubulin loss at the opposite poles (13, 14). If a chromosome is unable to achieve

Figure 1-1. Microtubule structure and dynamics.

A) Conformational changes of microtubules during catastrophe and rescue phases. During polymerization, GTP-tubulin is added to the growing end of the microtubule. During depolymerization, GTP is hydrolyzed to GDP and the GDP-protofilaments peel away from the microtubule and are released. A GTP rich cap stabilizes the microtubule and acts as a primer for tubulin polymerization. B) Binding sites on the tubulin dimer. The α and β tubulin dimer each contain a site that can accommodate a GTP molecule. The α tubulin has a non-exchangeable N site and the β tubulin has an exchangeable E site. The vinca alkaloids bind near the E site on the β tubulin dimer. Taxanes act at a site on the lumen of GDP- bound β tubulin and promote the stable, straight conformation. The colchicine site is at the interface of the α and β tubulin dimer and ligand binding causes interdimer bending that favors destabilization.

bipolar attachment to the spindle, the cell is unable to continue through the cell cycle and is blocked in the metaphase, eventually to succumb to apoptosis (15).

Interfering with microtubule dynamics is an attractive anticancer strategy, and many drugs employing this tactic are therapeutically effective in a wide range of malignancies. Mitotic arrest in the G₂/M phase, a hallmark of microtubule targeting agents, is thought to occur through the perturbation of mitotic spindle machinery and failure to pass mitotic checkpoints. There are several sites on the tubulin heterodimer to which microtubule targeting agents can bind, the most common being the vinca alkaloid, taxane, and colchicine binding sites (**Figure 1-1B**). These drugs are generally divided into one of two classes; stabilizing agents, which enhance polymerization, or destabilizing agents, which inhibit tubulin polymerization.

Stabilizing Agents

Stabilizing agents are able to promote polymerization of tubulin even when the unfavorable GDP molecule is bound and are more effective than endogenous microtubule-associated proteins at stabilizing the microtubule (16, 17). There are several drug categories within this class, including taxanes, epothilones, and laulimalide binding site agents (18). Representative drugs in this class can be found in **Figure 1-2**.

Taxanes. The taxane binding site is located at the lumen of the β tubulin subunit in the intact heterodimer. Taxanes use the H6/H7 loop on β tubulin as a hinge to translocate from an intermediate position on the outside of the microtubule to the final binding site on the lumen of the microtubule (19). This motion is supported by a hydrogen bond in Ser277 in the M loop present in most tubulin isoforms. However, in the class β III isoform, this Ser277 is replaced by an arginine residue, and overexpression of the β III tubulin isoform is associated with resistance to taxanes, which will be further explained in greater detail (19, 20). The first stabilizing agent identified, paclitaxel (Taxol), was approved by the FDA in 1992 and has been available clinically since 1996 (17). Docetaxel (Taxotere), a semi-synthetic analog of paclitaxel, is another early generation taxane that was FDA approved for breast cancer in 1996, non-small-cell lung cancer in 1999, metastatic hormone-refractory prostate cancer in 2004, and head and neck cancer in 2006 (21). While these drugs achieved much success in treating various cancers, the development of drug resistance often limits their efficacy. Another issue hindering the clinical efficacy of taxanes is their limited solubility. Formulations such as Cremaphor EL and polysorbate are vehicles that allow for the solubilization of hydrophobic drugs but can induce a range of negative biological side-effects in patients (22). Development of Abraxane, also known as paclitaxel protein-bound, is a delivery method that has mitigated this problem to some degree. High toxicity, immunosuppression, and peripheral neuropathy that accompany long-term use of taxanes prove that there is still a great need to develop agents with higher specificity, improved efficacy, and minimal off-target toxicities for this class of drugs (23).

Figure 1-2. Examples of different classes of microtubule stabilizing and destabilizing agents.

Pothilones. Epothilones make up another class of tubulin targeting agents that are distinct from taxanes, though they share a common binding site. While epothilones compete with paclitaxel for β tubulin binding, their common pharmacophore differs in that each class exploits the binding pocket in an independent manner and involves distinct amino acids (24, 25). Epothilones, originally isolated from the myxobacterium *Sorangium cellulosum*, are more amenable to synthetic modifications than taxanes, more potent in taxane-resistant and sensitive cell lines, and are poor substrates for P-glycoprotein (P-gp) drug efflux pumps (26). Epothilones contain a macrolide ring with a methylthiazole side chain, and naturally occurring epothilones are characterized as either epoxides (epothilone A, B, E, F) or olefins (epothilone C, D) (27). Semi-synthetic compounds have been developed to improve their pharmacologic properties and biological activities. For example, Ixabepilone, a second-generation derivative of Epothilone B, is less susceptible to degradation by carboxylesterase and has increased water solubility. It was the first epothilone derivative to be FDA approved for cancer treatment (25). Sagopilone is the first fully synthetic analog of Epothilone B and has reached phase II clinical trials (28, 29).

Laulimalide Binding Site Agents. Another binding site on tubulin is the laulimalide binding site. This site was discovered when it was observed that laulimalide possessed a similar mode of action as taxanes but was completely unable to inhibit the binding of [3H] paclitaxel or Flutax-2, paclitaxel derivative to tubulin, confirming that it did not compete with the taxane binding site (30, 31). Peloruside A also directly binds to tubulin without competing with paclitaxel but can be displaced by laulimalide, supporting that Peloruside A shares a binding site with laulimalide (32, 33). Both laulimalide and Peloruside A synergistically enhanced microtubule assembly induced by stabilizing agents, including discodermolide, dictyostatin, paclitaxel, Epothilones A and B, eleutherobin, and cyclostreptin (32). Furthermore, laulimalide and Peloruside A stabilize the M loop at the taxoid binding site, which contributes to crosstalk and explains the observed synergism between these agents and taxane site ligands (34).

Destabilizing Agents

The other class of microtubule targeting agents are the destabilizing agents and include drugs that bind to the vinca alkaloid binding site and colchicine binding site. Their major mechanism of action is to interfere with tubulin dynamics, as opposed to just reducing overall polymerization. Representative drugs in this class can also be found in **Figure 1-2**.

Vinca Alkaloids. Vinca alkaloids are a natural product or semi-synthetic extraction derivative from the periwinkle plant, *Catharanthus roseus* (35). The major vincas in clinical use are vinblastine, vincristine and semisynthetic derivatives vinorelbine, vindesine, and vinflunine. The last two are only approved for use in Europe, while the rest are FDA approved in the US. In addition to their microtubule depolymerizing effects, vincas have also been reported to increase oxidized glutathione, alter lipid metabolism and membrane lipid content, elevate cAMP, and inhibit cAMP

phosphodiesterase (36). Vincas bind near the exchangeable GTP site and inhibit GTP to GDP hydrolysis and GDP exchange, causing a change in the tubulin dimer from the straight conformation (which is favored for polymerization) to the curved confirmation (23). Maytansinoids and halichondrins also induce depolymerization of microtubules at the vinca site, while dolastatins, spongistatins, and cryptophycins bind to the “peptide site” of the vinca binding domain (23, 37). Though vincas are amongst the most commonly used microtubule targeting agents, they also are susceptible to development of drug resistant mechanisms and toxic side effects.

Colchicine Binding Site Agents. The final major category of tubulin inhibitors are colchicine binding site agents. Colchicine, from the meadow saffron *Colchicum autumnale* L., was the first agent discovered in this group as a tubulin depolymerizing agent. The colchicine binding domain is located at the interface between the α and β tubulin dimer. When colchicine binds in the binding domain, it displaces the M loop and then the bound colchicine-tubulin complex becomes incorporated into microtubule and interferes with the formation of lateral contacts at the end of protofilaments. This causes steric clashes and the tubulin can no longer maintain a straight conformation (38). As the concentration of colchicine increases, more lateral contracts are broken, ultimately leading to destabilization of microtubules. Colchicine and colchicine binding site inhibitors are therefore able to suppress tubulin dynamics at lower concentrations and induce destabilization of microtubules are higher concentrations. Colchicine itself is an approved drug for the treatment of gout and has implications in other inflammatory conditions such as familial Mediterranean fever pericarditis and Behçet's disease. However, there are currently no colchicine binding site agents that have been approved for cancer therapy due to limitations such as poor drug solubility, narrow therapeutic window, and harsh side effects including neurotoxicity. Despite these shortcomings, colchicine binding site agents have several advantages over other tubulin agents targeting different binding sites. First, drugs binding to the colchicine binding pocket may overcome mechanisms of drug resistance, which will be discussed herein. Also, colchicine binding site inhibitors can target tumor vasculature and prevent formation of new blood vessels or disrupt existing microvessels (39, 40). For these reasons, the colchicine binding site is an attractive target for the development of chemotherapeutic drugs and numerous scaffolds are being investigated.

Combretastatins are naturally occurring stilbenoid phenols isolated from the bark of *Combretum caffrum* (41). Combretastatins A-4 (CA-4) demonstrates cell-cycle dependent arrest, potent antiangiogenic activity, and cytotoxicity by apoptosis rather than necrosis (42, 43). The water soluble phosphate prodrug, CA-4P, phenostatin, and many other CA-4 derivatives have been developed to optimize its potency, vascular disrupting potential, drug-like properties, and anticancer efficacy (44). Based on the knowledge gained from combretastatin and phenostatin, 1,1-diarylethene isomers of combretastatin A were developed called isocombretastatin A (45). CC-5079, an isocombretastatin A analog, was determined to be an inhibitor of tubulin polymerization and TNF α . It showed cell cycle arrest in G₂/M phase, increased phosphorylation of G₂/M checkpoint proteins, and stimulated apoptosis. Additionally, its effect on TNF α production also diminished phosphodiesterase type 4 enzymatic activity (46). Alkenyldiarylmethanes are a class of

non-nucleoside reverse transcriptase inhibitors that are structurally related to CC-5079, and several compounds in this class also demonstrate potent tubulin destabilization (47).

2-Methoxyestradiol (2-ME), a metabolite of estradiol-17 β , suppresses microtubule dynamics at the colchicine binding site, induces G₂/M phase arrest, inhibits blood vessel formation at several stages in the angiogenic process, and has potent anticancer activity (48, 49). It inhibits tubulin assembly in a concentration-dependent manner, and higher concentrations are needed to depolymerize microtubules. In addition to showing efficacy in a variety of cancer types (breast, ovarian, lung, prostate, and colorectal carcinomas), 2-ME is active against estrogen-dependent and -independent cancerous cells. In addition to disrupting microtubule dynamics, 2-ME also acts through triggering the apoptotic cascade via reactive oxygen species, superoxide dismutase, and nitric oxide synthase (50).

Nocodazole is another natural product that inhibits tubulin polymerization by binding to the colchicine binding site as a reversible inhibitor (51). It also binds with varying affinities to the different tubulin isoforms, with the weakest affinity for β III tubulin (52). Nocodazole did not advance through clinical trials due to toxic side effects, however, it is frequently used in cell culture to block mitosis and as a reference compound for colchicine binding site agents (16, 53). Several colchicine binding site agents have made it to clinical trials including ABT-751, BNC105 and verubulin (36, 54). One of the hallmarks of colchicine binding site agents is that they accommodate a wide range of structurally diverse scaffolds, and there is still much room for improvement to limit toxicities, improve solubility, and increase efficacies. Future directions for colchicine binding site agents aim to optimize structural based design, utilize targeting strategies, and increase specificity.

Mechanisms of Multidrug Resistance

One of the major hurdles that continues to limit current anticancer agents is the development of drug resistance. Multidrug resistance (MDR) is the simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic drugs and is a substantial obstacle impeding the success of anticancer agents (55). Many cancers initially respond well to chemotherapy early on during treatment but subsequently develop acquired resistance, and more than 90% of patients with metastatic cancer fail to respond to or relapse from chemotherapeutics (56). For these reasons, rigorous efforts have been devoted to elucidating both inherent and acquired MDR mechanisms over the past few decades. Although a complete understanding of MDR is still far from being uncovered, two major mechanisms have been proposed: non-cellular and cellular mechanisms.

Non-cellular drug resistance is the inherent capacity of tumor cells to survive chemotherapy. It is always mediated by the tumor microenvironment and is typically associated with the unique properties possessed by solid tumors, such as heterogeneous tumor vasculature, high interstitial fluid pressure, acidic microenvironment, and increased

presence of non-cycling tumors caused by insufficient supply of nutrients and oxygen (57-61). Cellular MDR mechanisms arise inside solid tumors, and compared to non-cellular MDR mechanisms, cellular MDR mechanisms are more important for novel drug development. Some examples in which cellular mechanisms are modified to escape drug action include elevated DNA repair, increased drug metabolism, altered apoptotic pathways to bypass drug targets, loss or change of drug target proteins, and increased efflux of anticancer drugs (e.g. altered activities of membrane transporters such as ATP-binding cassette transporters)(62, 63) (**Figure 1-3**). Metabolic changes of drugs that promote drug degradation are also critical to MDR. For instance, overexpression of glutathione, an important metabolic antioxidant, promotes MDR by converting drugs to drug-conjugates or by generating inactive metabolites that are expelled as waste (64). Mutation of key apoptotic regulators is another primary reason for cellular MDR. Tumor suppressor protein p53 is crucial to many physiological processes, especially in regulating apoptosis. Several studies have shown that mutation of p53 and overexpression of BAX, an anti-apoptotic protein in the Bcl-2 family, are involved in MDR (62, 65).

Among all the MDR mechanisms, the increase of drug efflux mediated by ATP-binding cassette (ABC) transporters is the most significant factor leading to MDR. Many studies demonstrated that the ABC transporters comprise seven distinct classes of structurally related membrane proteins including ABCB, ABCC and ABCG. Overexpression of ABC transporters in tumor cells promotes MDR by reducing sufficient accumulation of anticancer drugs through increasing drug efflux. P-gp, encoded by the ABCB1/MDR1 gene, is the best characterized transporter protein mediating drug resistance and many drugs (e.g. docetaxel, paclitaxel, doxorubicin, etoposide, vinblastine, vincristine, and teniposide) are susceptible to resistance incurred from elevated P-gp (65-67). P-gp overexpression was reported to lead to failure of chemotherapy in various cancers, such as ovarian, lung, breast and acute myeloid leukemia cancer (68). ABCC1/Multidrug Resistance-Associated Protein 1 (MRP1) is responsible for transporting organic anions and phase II metabolic products and causes resistance to anticancer agents. It was first shown to be overexpressed in a doxorubicin-resistant cell line, but also accommodates a wide range of substrates in addition to doxorubicin, including anthracyclines, epipodophyllotoxins, vinca alkaloids, and camptothecins (69). Finally, ABCG2/Breast Cancer Resistant Protein (BCRP) is a well-defined efflux transporter first identified in a breast cancer cell line selected for resistance to mitoxantrone. Similar to the other MDR transporters, BCRP serves as an efflux pump for many diverse scaffolds of xenobiotics, including 7-ethyl-10-hydroxycamptothecin (SN-38)(70), tyrosine kinase inhibitors (71), methotrexate (65, 72), and is involved in drug resistance in colon, breast cancer and gastric carcinomas (73, 74).

Strategies to Overcome Multidrug Resistance

Strategies to overcome inherent or acquired drug resistance are essential to the treatment of cancers. Using inhibitors of ABC transporters is one of the common approaches to overcome P-gp-mediated MDR, rendering tumor cells more sensitive to

Figure 1-3. Drug-resistance mechanisms in tumor cells.

1) Loss or change of surface receptor. 2) Mutations in drug targets. 3) Enzymatic deactivation. 4) Altered drug metabolism. 5) Change in apoptotic pathways. 6) Increased drug efflux. 7) Decreased drug influx (solute carriers). 8) Increased DNA repair.

chemotherapeutics. Some strategies for overcoming MDR include developing novel anticancer drugs that are not the substrates of P-gp, suppressing the MDR-related genes through destruction of mRNAs by using microRNA and RNA interference (75, 76), decreasing intracellular GSH levels via hammerhead ribozymes against glutamate cysteine ligase or L-buthionine-(S,R)-sulfoxime to repress glutathione synthesis (77, 78), targeting P-gp to reverse MDR with anti-P-gp monoclonal antibodies (e.g. MRK16 and MRK17), and using nanotechnology to deliver anticancer drugs to specific targets efficiently (79). Development of novel drugs that overcome MDR continues to be a major area of research in drug discovery and development. Herein, we briefly summarize some strategies in developing tubulin inhibitors that overcome MDR.

Taxane Binding Site. Paclitaxel and docetaxel, members of taxane family, are chemotherapeutics used to treat patients with various solid tumors, including ovarian, breast, head and neck, lung, and prostate cancers. However, severe adverse effects and development of MDR have largely compromised the use of paclitaxel and docetaxel. In addition to transporter-mediated resistance, overexpression of the class III β -tubulin isoform also limits efficacy of many taxanes. Differences between β I-tubulin, the most commonly represented and constitutively expressed isotype, and β III-tubulin are limited to substitution within only a 13 amino acid region, where β III has an Arg277 instead of the Ser277 present in β I-tubulin (80). This substitution prevents stable paclitaxel binding and creates more dynamic microtubules, minimizing the polymerization-inducing activity of taxanes and increasing resistance to stabilization (81). Creation of newer generations of taxanes that are poor substrates of P-gp such as cabazitaxel, drug combination therapy, and drug conjugates for tumor-targeted delivery are all strategies that attempt to address the issue of MDR and develop more effective treatments (21).

Ixabepilone (BMS-247550), an epothilone derivative, was first marketed under the trade name Ixempra for the treatment of drug-resistant metastatic breast cancer. The efficacy of ixabepilone in resistant tumors may result from its low susceptibility to alteration in tubulin isotypes, tubulin mutations, and overexpression of P-gp/MDR1 drug. In a phase II clinical trial of metastatic breast cancer patients treated with 40 mg/m² ixabepilone as a 3-hour infusion every 3 weeks, ixabepilone demonstrated promising antitumor activity and had an acceptable safety profile (82, 83). Taccalonolides are taxol-like microtubule stabilizing agents isolated from plants of the genus *Tacca* that increase the density of cellular microtubules, block mitotic progression, and induce apoptosis. Similar to ixabepilone, taccalonolides can circumvent MDR mechanisms due to their low susceptibility to P-gp overexpression and tubulin alteration-mediated resistance (84, 85). Recently, new taccalonolides AF and AJ were obtained and demonstrated potent IC₅₀ values of 23 nmol/L and 4 nmol/L in HeLa cell lines. Taccalonolides AF and AJ were generated from their parent taccalonolides A and B, by epoxidizing the C22–C23 double bond. This simple epoxidation dramatically increased the potency over 200- and 700-fold, respectively (86). Due to the unique structural characteristics and mechanism of taccalonolides, Susan I. Mooberry's group modified the precursor structure and reported on a semi-synthetic derivative, taccalonolide AI, which showed an IC₅₀ below 1 nM (87, 88). These studies are particularly informative for the synthesis of taccalonolide derivatives. Due to their strong cytotoxicity and unique

mechanisms of covalent bonding, taccalonolide semi-synthetic derivatives are a very promising generation of irreversible tubulin inhibitor for resolving the drug resistance dilemma.

Vinca Alkaloid Binding Site. Hemiasterlin, a natural product, is a member of tripeptides derived from marine sponges. Hemiasterlin and its analog HTI-286 bind to vinca-peptide site in tubulin, disrupt normal microtubule dynamics, and trigger the cell apoptotic process (89). Total synthesis of hemiasterlin and its analogs has been accomplished and the SAR studies have been explored. HTI-286 showed potent activity with an IC_{50} of about 2 to 5 nM against a panel of tumor cell lines and was less sensitive to the P-gp drug transporter pump than current anti-microtubule agents including paclitaxel, docetaxel, vinorelbine, and vinblastine (90). Additionally, resistance to HTI-286 was not detected in cells overexpressing the multi xenobiotic resistance drug pump. However, side effects of HTI-286 included neutropenia, hair loss, and pain, which ultimately terminated phase II trials. Further optimization is needed to address the current limitations of anticancer therapies and side effects.

Tubulysins, originally isolated from myxobacterial cultures, are tetrapeptides composed of D-methylpipercolate, L-isoleucine, L-tubuvaline, and L-tubuphenylalanine residues (91). Tubulysin D demonstrated the most potent activity, exceeding other marketed chemotherapeutics such as Ixabepilone, vinblastine, and paclitaxel, by 20- to 1000-fold. The mechanisms of action of tubulysins resembled dolastatin-10 and hemiasterlin, which bind to the vinca domain site, arrest cancer cells in the G_2/M phase, and trigger subsequent cell apoptosis (92, 93). Tubulysins are highly active in MDR cell lines that either overexpress P-gp pumps or have tubulin mutations, providing an alternative solution to treating MDR cancers.

Colchicine Binding Site. While microtubule targeting agents often face shortcomings due to the development of resistance, colchicine binding site agents have several advantages over other classes of tubulin inhibitors. First, many drugs in this class are less susceptible to MDR mechanisms that limit the efficacy of so many other tubulin inhibitors (54, 94). For example, one of the main mechanisms of drug resistance is due to the overexpression of the class III β tubulin that alters the conformation of the taxane binding site but does not confer resistance to colchicine binding site agents (95). Humans have at least 7 expressed β tubulin isoforms, and alterations in their expression are linked to acquired drug resistance (95). Furthermore, paclitaxel-resistant mutants often show elevated sensitivity to drugs that bind to alternative tubulin sites, such as colchicine site agents (95). There is also evidence that the efficacy of the vinca drug, vinorelbine, is reduced when βIII tubulin is overexpressed, whereas colchicine binding site agents were unaffected, suggesting that the resistance from βIII tubulin isoforms are binding site specific (81, 96). This offers an advantage for chemotherapeutic drugs targeting the colchicine binding site.

To address conventional multidrug resistance to many microtubule-inhibiting agents, there have been numerous efforts to develop colchicine binding site inhibitors because of their therapeutic advantages over taxanes and vinca alkaloids.

Recently, 2-aryl-4-benzoyl-imidazoles ABI-274 and ABI-231 were discovered by Miller and Li's group and they specifically target the colchicine binding site (97, 98). The compounds exhibited similar potency to MDR cells compared to corresponding parent cells. (99). They also demonstrated synergistic action through the combination of ABI-274 and vemurafenib to overcome vemurafenib-acquired resistance in BRAF(V600E) melanoma. The combination treatment effectively decreased the levels of phosphorylated and total AKT, activated the apoptosis cascade, and reduced cleaved caspase-3 and cleaved PARP (100). These contributions offer new strategies to alleviate the problem of drug-resistance and they provide practical implications for further investigation of colchicine binding site agents. Bai et al. reported a similar colchicine binding site inhibitor, BZML, that showed potent cytotoxic activity against both A549 and A549/Taxol-resistant cells (99). Mechanistic studies showed that BZML decreased P-gp expression at the protein and mRNA levels. Cell morphology changes and the expression of apoptosis-related proteins denoted that BZML induced mitotic catastrophe to A549/Taxol-resistant cells in a p53-independent, apoptotic-like pathway, whereas BZML caused apoptosis to A549 cells (99). Verubulin (MPC-6827) is a quinazoline derivative that showed potent antineoplastic activities against diverse tumors and promoted apoptosis in both sensitive and MDR cancer cell phenotypes (101). Other verubulin analogs have been developed in attempt to maintain potency and avoid resistance, and these compounds have reported vascular disrupting activities and low IC₅₀ values against various cancer cells (102). Several other colchicine binding site inhibitors investigated include indanocene, millepachine, SSE15206, IMB5046, D4-9-31, and their respective synthetic derivatives. Indanocene is a derivative of indanone and a selective inducer of apoptosis to stationary multidrug-resistant cancer cells. One approach to eradicate tumor cells involved combination therapy with indanocene, which was selectively cytotoxic to non-cycling, multidrug-resistant cells and other chemotherapeutic drugs, which targeted abnormalities of cell cycle tumors (103). Yang et al. reported the ability of millepachine to evade multidrug resistance in an A2780CP (P-gp overexpressing) model and demonstrated that it irreversibly interacted with the colchicine site of β -tubulin and retained full activity toward MDR cells (104). SSE15206 also circumvented drug resistance in KB-V1 and A2780-Pac-Res cell lines that overexpressed P-gp (105). IMB5046 was not a P-gp substrate and displayed potent cytotoxicity against a panel of tumor cell lines and multidrug-resistant cell lines that were resistant to colchicine, vincristine and paclitaxel treatment (106). D4-9-31, a pyridine-pyrimidine amide, showed strong efficacy in isogenic paclitaxel-resistant breast cancer cells and also evaded P-gp-mediated drug efflux (107). Another major mechanism of drug resistance to tubulin inhibitors that is a critical clinical concern is due to the overexpression of certain β -tubulin isoforms. Humans have at least 7 expressed β -tubulin isoforms, and alterations in their expression is linked to acquired drug resistance (95). Studies have shown that overexpression of class III β -tubulin reduces the efficacy of taxanes and vinca alkaloids, but drugs that target the colchicine binding site may circumvent resistance (81).

Further efforts evaluating novel tubulin inhibitors will help elucidate the drug resistance mechanisms and thus guide drug design that can overcome inherent or acquired drug resistance. In addition, discovery of tubulin inhibitors with different inhibition mechanisms (such as targeting two pathways with one compound) will open

alternative avenues to bypass the problem of drug resistance. Another strategy involves utilizing antibody-drug conjugates of tubulin inhibitors for targeted drug delivery to circumvent MDR, improve the therapeutic window, and limit toxicities (108). While significant strides have been made to address MDR, resistance is often multifactorial and heterogeneous. Therefore, efforts to gain insight into tumor dependence and the relationship of the individual drug resistance effectors may assist in combating the complex phenomenon of MDR (109).

Microtubule Targeting Agents' Potentiation of Immune Response and Implications in Cancer

Dendritic Cells: Induction of Maturation and T Cell Priming

In addition to their antimitotic effects, microtubule targeting agents can also affect many non-mitotic mechanisms, including eliciting an immune response following the disruption of intact microtubules. The perturbation of microtubules from anti-tubulin agents can then induce anti-inflammatory responses and leukocyte action (110). For example, dendritic cells (DC) are antigen-presenting cells and are important regulators in the initiation of innate and adaptive immunity. While they have long been heralded for their defensive action to foreign pathogens, they are more recently being appreciated for their role in regulation of antitumor response. However, tumor-altered DC differentiation can interfere with their ability to potentiate an immune response (111). This can lead to DCs and their precursors accumulating in the tumor microenvironment and exacerbate the immunosuppressive action of the tumor. Therefore, intervening and converting the immature DCs to mature antigen presenting cells is an important target for immune-therapy based cancer treatment (112). Microtubule destabilizing agents including ansamitocin P3, dolastatin, and antibody-drug conjugates containing dolastatin 10 analogs have been shown to induce functional DC maturation and activation, and also increase the capacity of priming antigen-specific CD4⁺ and CD8⁺ T cells (113, 114). It has also been reported that other classes of microtubule-destabilizing agents can activate DCs, unlike microtubule stabilizing agents which only invoked minimal activation in murine cells and caused no observed effect in DCs (112). Another group also investigated the effects of microtubule destabilizing agents on DC maturation and T-cell activation in relation to immunogenic tumor cell death. They reported that dendritic cell vaccines pulsed with colchicine- or 2-phenyl-4-quinolone analog- treated cell lysates mitigated tumor growth, revealed cytotoxic T-lymphocyte activity in tumors, and extended survival of treated mice inflicted with tumor xenografts (115).

Inflammation

Inflammation can play a role in malignant transformation, cancer progression and pathogenesis in numerous tissues. For example, inflammatory bowel diseases such as ulcerative colitis and Crohn's disease contribute to the development of colon cancer,

because chronic inflammation can endorse the production of tumors. It has been reported that the use of nonsteroidal anti-inflammatory drugs lowers mortality caused by sporadic colon cancers, representing a link between cancer and inflammation (116-118). The interaction of tumor cells with a variety of aspects within the tumor microenvironment, including macrophages, B and T cells, mast cells, fibroblasts, myofibroblasts and extracellular matrix components, has been shown to propagate tumor progression. Furthermore, tumors are capable of secreting cytokines, chemokines, growth factors, and proteases to remodel the stroma microenvironment (119). Inflammation is driven by these soluble factors from the tumor cells or from cells recruited to the tumor microenvironment such as macrophages and mast cells, which can then drive tumor cell growth, interfere with differentiation, and increase cancer cell survival. Elevated influx of angiogenic cytokines from the surrounding immune cells to the tumor microenvironment can also contribute to tumor metastasis (120). Since levels of cytokines such as Tumor necrosis factor α (TNF α), IL-8, IL-6 and vascular endothelial growth factor are often elevated in the serum of colorectal carcinoma patients, they represent potential anticancer therapeutic targets (121, 122). Oncogenic signaling pathways are also activated by cytokines that induce NF- κ B signaling and turn on oncogenic mediators that promote inflammation (119). While aberrantly expressed inflammation is linked to cancer promotion and disease progression, targeting pro-apoptotic signals from an inflammatory immune response to induce apoptosis of tumor cells could utilize these targets in anticancer treatment. Cell death imposed by an immune response can be from cell killing by cytotoxic T cells and natural killer cells, and can also be stimulated by apoptotic ligands, such as TNF α . There have been reports on microtubule targeting agents sensitizing cells to TNF α and other inflammatory apoptotic ligands (123, 124). AK301, a colchicine binding site tubulin inhibitor, was found to cause treated cells to express elevated levels of TNF receptor 1 (TNFR1) on the cell surface and increase caspases-8, -9, and -3 activation in the presence of TNF α . It also induced Fas- and Tumor necrosis factor ligand-dependent apoptosis (123). Interestingly, it caused greater TNF α sensitization than other tubulin binding agents including colchicine, nocodazole, and vincristine, though it achieved a lower degree of tubulin polymerization inhibition from tubulin binding assays. Nonetheless, these studies explore the relationship between microtubules and apoptotic ligand-sensitizing agents and how these agents can exploit immune response elements for cancer therapy. This may be particularly useful for colon or other cancers that have high levels of inflammatory cell infiltrate or following treatment with an immune stimulant or vaccines.

Colchicine, which is historically used in the treatment of gout, has been shown to induce an anti-inflammatory response through destabilization of microtubules, which subsequently interferes with the assembly of the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome (125). Activation of the NLRP3 inflammasome within macrophages is commonly implicated in innate immune inflammatory response (125). NLRP3 uses a mitochondrially-bound adaptor protein (apoptosis-associated speck-like protein) containing a caspase recruitment domain to recruit caspase-1 to the complex, followed by autocatalytic processing and activation, ultimately facilitating the cleavage of pro-inflammatory cytokines IL-1 β and IL-18 to their activated forms (126). The generation of optimal sites for activation of the NLRP3 inflammasome, correct

localization, and its direct activation is mediated by microtubules, and microtubule disrupting agents block inflammasome activation (127).

Polyploidy and Micronucleation

Another way that microtubule targeting agents may provoke an immune response is related to their ability to cause polyploidization (128-130). This can happen through a process called mitotic slippage, where cells arrested in the M phase can enter interphase without undergoing proper chromosome segregation and cytokinesis, resulting in tetraploid multinucleated cells (131). An increase in the number of chromosome sets (>4) has been shown to elevate endoplasmic reticulum stress and cause translocation of calreticulin to the cell surface. This exposure promotes phagocytosis of stressed cells by macrophages and dendritic cells of the immune system and stimulates immunosurveillance (130).

Microtubule targeting agents induce mitotic arrest via disruption of the mitotic spindle, and interestingly, they have achieved greater success than mitosis-specific inhibitors. This may be in part attributed to their multifaceted roles in other interphase functions including intracellular trafficking, migration, angiogenesis, and cell signaling, suggesting that their anticancer efficacy is due to their roles in both mitotic and non-mitotic events (132, 133). A relatively new theory as to why tubulin targeting agents, particularly those in the taxane class, have achieved greater clinical success than mitosis-specific inhibitors is their capacity to promote multiple micronuclei (134). These form from chromosome segregation errors during mitosis and recruit a nuclear envelope during telophase which does not incorporate into the main nucleus. This phenomenon happens when cells are treated with subtoxic drug concentrations that fail to induce complete mitotic arrest. These micronuclei formed from double stranded DNA breaks can initiate the activation of cyclic GMP–AMP synthase (cGAS) inflammatory signaling (135). While this may only occur in a small population of dividing cells, it can have substantial implications on whole tumor regression through amplified inflammatory activation. Micronucleated cells can accumulate within the tumor and are not cleared by phagocytosis in the same manner as apoptotic cells. Fragmented DNA is exported through nuclear pores into the cytoplasm and binds to the cytosolic DNA sensor cGAS. cGAS consequently turns on the endoplasmic reticulum membrane protein STING through the synthesis of 2'3'-cGAMP (136). This activation triggers the transcription factors NF- κ B and IRF3 through the kinases IKK and TBK1, eventually leading to the activation of inflammation. The cGAS-STING pathway activation has been shown to restrain oncogenes and increase pro-inflammatory genes in cancer cells (137). Ultimately, cGAS–STING-dependent inflammatory signaling can play a role in antitumor drug response.

Summary

Because of their major role in mitosis and other critical cellular processes, targeting microtubules is an excellent strategy for developing anticancer agents. While some microtubule targeting agents that exploit the taxane and vinca alkaloid binding sites have been FDA approved for cancer treatment, there are still many hurdles to overcome to find a more satisfactory treatment. Targeting the colchicine binding site on tubulin may circumvent many MDR mechanisms affiliated with taxanes and vinca alkaloids, such as overexpression of drug efflux pumps and alterations in β tubulin isoforms. Although there are currently no marketed anticancer agents that target the colchicine binding site, immense efforts have been made to advance small molecules in this class. Additionally, drugs targeting this site often are effective as vascular disrupting agents and can accommodate a large range of structurally diverse scaffolds. While these drugs show promise for the future of anticancer therapeutics, poor solubility, metabolic stability, and dose limiting neural and cardiotoxicities continue to be major issues limiting their clinical potential. While the search for acceptable compounds targeting microtubules continues, newer generations of tubulin inhibitors will need to optimize efficacy, capitalize on combination therapies, and effectively escape the shortcomings of current anticancer drugs.

Hypotheses for Novel Tubulin Inhibitors

In the following Chapters 2-4, we disclose the properties and anticancer activities of newly synthesized tubulin inhibitors with the following hypothesis in mind: Tubulin inhibitors that target the colchicine binding site will be able to potently inhibit tubulin polymerization and disrupt microtubule dynamics, interfere with cellular processes such as migration and proliferation, evade multidrug resistance, and show strong efficacy in animal models harboring cancer xenografts and taxane-resistant tumors. This is the overall goal for each of the different series of compounds evaluated, but we specifically tested different scaffolds in each of the chapters.

In Chapter 2, we investigated two novel indolyl-imidazopyridines. These new analogs were designed from diaryl-ketone chemotypes that were shown to possess tubulin polymerization inhibition properties (138-142). To optimize this scaffold, the ketone was cyclized to a pyridine ring and further introduced an imidazole, leading to a series of series of indolyl-imidazopyridines. We previously reported that the lead compounds in this series caused substantial cytotoxicity against several cell lines and enhanced the metabolic stability in liver microsomes (143). Therefore, based on those initial findings, we were determined to expand our biological assessment and explore the drugability of the two most promising compounds, DJ95 and DJ101. We hypothesized that these compounds would show broad anticancer action in expanded cell lines and disrupt microtubule networks and mitotic spindle formation. We also hypothesized that they would optimally target the colchicine binding site, maintain a low resistance index

against ABC transporter overexpressing cell lines, and show significant efficacy against taxane-resistant tumors in animal models.

In Chapter 3, we explored a different scaffold in a similar manner. Previously, we reported the discovery of the 2-aryl-4-benzoyl-imidazole (ABI) scaffold (140). Subsequent structural optimization of the benzoyl moiety, the aryl moiety, and the imidazole fragment in this scaffold resulted in analogs with improved antiproliferative activities and tubulin inhibiting properties (98, 141, 143, 144). ABI-231 (VERU-111) was determined to be the most potent from those modifications, and it is currently under clinical development by VERU Healthcare. We hypothesized that we could improve on VERU-111 by focused structural-based optimization of the indole moiety and the 3,4,5-trimethoxyphenyl moiety. In doing so, we hypothesized we could obtain analogs with lower IC_{50} s, enhanced tubulin depolymerization capabilities, and strong efficacy against melanoma and taxane-resistant prostate cancer xenograft tumors in mice without causing toxicity or eliciting off-target adverse reactions.

In Chapter 4, we unveil the final anti-tubulin series we evaluated, which was based off the Azixa (Verubulin/MPC-6827) scaffold (101). We chose this scaffold because of Azixa's potent tubulin depolymerization properties and its vascular disrupting attributes (39, 40, 101, 102). Other reported indole derivatives of Azixa demonstrated significant disturbance of tumor vasculature and induced necrosis (102). We selected our starting point from an Azixa derivative with reduced conformational flexibility compared with other Azixa analogs (145). Because it showed strong potency and tubulin depolymerization action but poor metabolic stability and high toxicity, it provided us with an opportunity for significant improvement. Therefore, we hypothesized that we could optimize this scaffold and identify novel compounds with greater metabolic stabilities and reduced toxicities. We also surmised that these compounds would demonstrate significant efficacy against clinically relevant MDR xenograft models and display drastic morphological changes to tumor vasculature, similar to other vascular disrupting agents.

CHAPTER 2. INDOLYL-IMIDAZOPYRIDINES DJ95 AND DJ101 ARE TUBULIN INHIBITORS THAT OVERCOME MULTIDRUG RESISTANCE¹

Introduction to DJ95 and DJ101

Microtubules are cytoskeletal structures that are essential for a multitude of cellular events including movement, intracellular transport, cell signaling, and mitosis (146-149). Microtubules are composed of α - and β -tubulin proteins, which readily undergo polymerization and depolymerization in a phenomenon known as dynamic instability (150). They also form the highly dynamic mitotic spindles that are responsible for the alignment and segregation of chromosomes in the cell during mitosis (151). Interference with microtubule dynamics consequently disrupts mitotic progression and ultimately leads to apoptosis and cell death (152). Therefore, interfering with microtubule dynamics by targeting tubulin is a validated anticancer strategy, and many agents are already used clinically or are undergoing development (148, 153-155).

Tubulin inhibitors can be broadly classified as stabilizing or destabilizing agents. Currently, all approved tubulin inhibitors for cancer therapy target the taxane or vinca alkaloid binding site. However, many tubulin inhibitors, including paclitaxel (Taxol®), are associated with MDR mechanisms such as overexpression of drug efflux pumps or the β III tubulin isoform (54, 156-158). Drug efflux mediated by ABC transporters is the most commonly observed mechanism responsible for inhibiting the intracellular accumulation of therapeutic agents in resistant cell lines (72). Of the known human ABC transporters, resistant melanoma cells have been shown to overexpress ABCB1 (MDR1, P-gp), ABCC1 (MRP1), ABCC2 (MRP2), and ABCB5 amongst others (159-162). ABCG2 (BCRP) is also well-characterized and its overexpression causes resistance to a variety of anticancer drugs including paclitaxel and docetaxel (163, 164). Extensive research efforts have addressed the issue of MDR, and numerous studies have demonstrated that colchicine binding agents can overcome ABCB1 overexpression and β III tubulin-mediated drug resistance (81, 94, 97, 165-169). While colchicine is not employed as an anticancer agent due to its toxic side effects, other colchicine binding inhibitors have demonstrated promising potential and some are currently being investigated as anticancer candidates (23, 54).

More recently, there has been increasing interest in the vascular-disrupting capabilities possessed by some microtubule binding agents (40, 170, 171). It is well-known that tumor progression is dependent on blood vessels to supply oxygen, essential nutrients, and growth factors. Vascular disrupting agents act on the tumor endothelium and induce destructive changes that decrease blood flow, promote vascular collapse,

¹ Modified with permission from American Association for Cancer Research. Kinsie E. Arnst, Yuxi Wang, Dong-Jin Hwang, Yi Xue, Terry Costello, David Hamilton, Qiang Chen, Jinliang Yang, Frank Park, James T Dalton, Duane D. Miller and Wei Li. A Potent, Metabolically Stable Tubulin Inhibitor Targets the Colchicine Binding Site and Overcomes Taxane Resistance. *Cancer research*. 2018; 78(1): 265-277.

initiate hypoxia within the tumor, and cause necrosis (171). There is accumulating evidence that microtubule binding agents can act selectively on tumor endothelial cells, and this supports the pursuit of targeting tumor vasculature (170).

We previously reported the discovery of diaryl-ketone chemotypes, including a phenyl ring as linker (I-387) (138), 4-substituted methoxybenzoyl aryl thiazoles (SMART)(142), phenylaminothiazoles (PAT) (139), arylbenzoylimidazoles (ABI) (98), and reverse arylbenzoylimidazoles (RABI)(141), that interfere with tubulin polymerization by binding to the colchicine domain. While these compounds displayed potent anticancer activity in human melanoma and prostate cancer cell lines, the ketone moiety in their structure presented a metabolically labile site (172). Further structural optimization to incorporate this metabolic soft spot into a stable ring produced a novel class of indolyl-imidazopyridines, with DJ95 and DJ101 representing the two most promising drug candidates in this series (structures shown in **Figure 2-1**) (143, 173). DJ95 and DJ101 not only possessed excellent metabolic stability as designed, they also showed improved anticancer potency in initial cytotoxicity testing and strong polymerization effects in a cell-free tubulin assay(143, 174, 175).

To further preclinically evaluate the DJ compounds, we expanded our investigation to additional malignant melanoma cell lines, the NCI-60 cell line panel, taxane-resistant cell lines, and ABC-transporter overexpressing cell lines. We also evaluated their effect on cancer cell migration, clonogenic potential, and endothelial cell tube formation *in vitro*. Their depolymerization effects were demonstrated through visualization of the microtubule networks as well as mitotic spindles formation. The binding of both DJ95 and DJ101 to the colchicine site was confidently confirmed through X-ray crystallographic analyses. Prior to *in vivo* studies, compounds were screened by separate pharmacological profiling services to identify potential off-target interactions to physiologically important targets. Antitumor efficacy was demonstrated for both compounds in a melanoma xenograft model, and DJ101 was also evaluated in a metastatic melanoma model and taxane-resistant prostate cancer xenograft model. To determine if the compounds possess vascular disrupting properties, immunohistochemistry (IHC) analysis of the tumor sections was utilized to determine alterations to the tumor endothelium.

Materials and Methods

Cell Culture and Reagents

Human melanoma cell lines, A375, RPMI-7951, WM-164, WM115 and SK-MEL-1, and adult primary dermal fibroblasts cells (American Type Culture Collection or ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic/antimycotic mixture

DJ95

DJ101

Figure 2-1. Structures of compounds DJ95 and DJ101

(Sigma-Aldrich, St. Louis MO). Murine melanoma B16F10 cells (ATCC, Manassas, VA, USA) were cultured in Minimum essential medium (Invitrogen, Carlsbad, CA), supplemented with 5% heat-inactivated Hyclone FBS (Thermo Scientific, Rockford, IL), 1% antibiotic-antimycotic mixture (Sigma-Aldrich, St. Louis MO), 1% Mem-sodium pyruvate (Invitrogen, Carlsbad, CA), 1% Mem-vitamin (Invitrogen, Carlsbad, CA), L-Glutamine (2 mM final concentration) (Invitrogen, Carlsbad, CA), and 1% Mem NEAA (Invitrogen, Carlsbad, CA). Parental prostate cancer PC-3, its paclitaxel-resistant daughter line PC-3/TxR, parental prostate cancer DU-145, and its docetaxel-resistant daughter line DU-145/TxR were gifts from Dr. Evan Keller at the University of Michigan Medical School. PC-3 and DU-145 cell lines were cultured in RPMI 1640 media (Gibco® by Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis MO). Taxane-resistant PC-3/TxR and DU-145/TxR cell lines were cultured in the same media and additionally supplemented with 10 nM paclitaxel or docetaxel, respectively. Paclitaxel or docetaxel was not included in the media for PC-3/TxR or DU-145/TxR for at least one week prior to *in vitro* and *in vivo* testing. HUVECs were cultured in Endothelial Cell Growth Medium containing growth supplement (Promocell, Heidelberg, Germany) and 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis MO). The human epidermoid carcinoma cell line KB-3-1, its drug-selected ABCB1-overexpressing KB-C2 cell line (maintained in medium with 2 µg/mL colchicine) and ABCC1-overexpressing KB-CV60 cell line (maintained in medium with 1 mg/mL of cepharanthine and 60 ng/mL of vincristine) (176) were kindly provided by Dr. Shinichi Akiyama at Kagoshima University, Japan. The human non-small cell lung cancer cell line NCI-H460 and its mitoxantrone-selected ABCG2-overexpressing NCI-H460/MX20 cells (maintained in medium with 20 nM of mitoxantrone) (177), and the transfected cell lines HEK293/pcDNA 3.1, HEK293/ABCB1, HEK293/ABCC1, and HEK293/ABCG2-R482 were kindly provided by Drs. Susan E. Bates at Columbia University, NY, and Robert W. Robey (NIH, Bethesda, MD). These cell lines were established by transfecting HEK293 cells with either the empty pcDNA3.1 vector or the vector containing full length ABCB1, ABCC1, and wild type ABCG2, respectively (178). These cell lines were cultured in DMEM, penicillin/streptomycin (Corning, Manassas, VA) and FBS or Hyclone bovine calf serum (GE Healthcare Life Science, Pittsburgh, PA). All cell lines were authenticated by ATCC by short tandem repeat profiling. Cultures were maintained to 80-90% confluency at 37 °C in a humidified atmosphere containing 5% CO₂. Compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to make a stock solution of 20 mM. Compound solutions were freshly prepared by diluting stocks with cell culture medium before use.

Cytotoxicity Assays

The cytotoxic effect against melanoma and fibroblast cell lines was previously described (179). Briefly, cells were seeded in 96-well plates at a density of 1,000–3,500 cells per well, depending on the growth rate of the cell line. After overnight incubation, test compounds were added to the wells at 10 concentrations ranging from 0.03 nM to 1

μ M plus a media-only control for 72 hrs in four replicates. Following treatment, the MTS reagent (Promega, Madison, WI) was added to the cells and incubated in dark at 37 °C for at least 1 hour. Absorbance at 490 nm was measured using a plate reader (BioTek Instruments Inc., Winooski, VT).

IC₅₀ values were calculated by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA). In addition, DJ101 and DJ95 were evaluated in one dose and five dose assays against the NCI-60 cell line panel by the National Cancer Institute Developmental Therapeutics Program (NCI/DTP).

The cytotoxic effects of DJ95 to KB-3-1, KB-C2, KB-CV60, NCI-H460 and NCIH460/MX20 cell lines, and the transfected cell lines HEK293/pcDNA3.1, HEK293/ABCB1, HEK293/ABCC1, and HEK293/ABCG2-R482, was obtained by Zi-Ning Lei (St. John's University). The IC₅₀ was determined using the MTT reagent (Thermo Fisher Scientific Inc., Haverhill, MA) as previously described (180). Known tubulin inhibitors with different mechanisms of actions, including paclitaxel (microtubule-stabilizing agent, targeting the taxane-binding site in tubulin), colchicine (microtubule-destabilizing agent, targeting the colchicine-binding site in tubulin), and vincristine (microtubule-destabilizing agent, targeting the vinca alkaloid-binding site in tubulin) (148, 166), were selected as positive controls for comparison with DJ59. Paclitaxel, colchicine and vincristine, which are existing substrates of ABCB1 and ABCC1(181-183), also served as positive substrate controls for the experiments involving ABCB1- or ABCC1-overexpressing cell lines in this study. Mitoxantrone, a known substrate of ABCG2(184), was used as positive substrate control in ABCG2-overexpressing cells. Cisplatin was used as negative control since it is not a substrate of ABCB1, ABCC1 or ABCG2 (180).

Colony Forming Assay

Two plating methods were used. To test the effects of DJ95, A375 cells were seeded in 6 well plates at a density of 500 cells/well (n=4). To test the effects of DJ101, A375 and RPMI-7951 cells were seeded in 24 well plates at a density of 200,000 cells/well (n=4). After overnight incubation in at 37 °C cells were treated with the compound or media only control and incubated for 10 days. Cells were then fixed with methanol and stained with 0.5% crystal violet. Images were taken, and colony area was quantified with ImageJ software (NIH, Bethesda, MD).

Scratch Migration Assay

A375 and RPMI-7951 cells were seeded in 24 well plates (200,000 cells/well) in replicates of four and incubated overnight. A 200 μ L pipette tip was used to scratch a straight line through the cell monolayer to remove an area of cells, then washed several times to remove any debris and uprooted cells. Media was replaced containing equivalent vehicle (DMSO) control or DJ compounds at 25 nM concentrations. Images were

obtained at the start of the experiment and after 24 hrs with Evos Fl Imaging System (LifeTechnologies, Carlsbad, CA). The analysis was performed with ImageJ software (NIH, Bethesda, MD).

Endothelial Cell Tube Formation Assay

Matrigel (Corning, Manassas, VA) was thawed on ice overnight then diluted with serum-free media for a final concentration of 10 mg/mL. Matrigel was plated in 48 well plates and incubated at 37 °C for 1 hour. Low passage number HUVECs (<5) in logarithmic growth phase were trypsinized and suspended in endothelial cell growth media. Cells (7×10^4) were plated on the Matrigel plates in quadruplicate containing the desired drug concentrations. Images were captured after 6 hr incubation with Evos Fl Imaging System (LifeTechnologies, Carlsbad, CA). Analysis was performed with angiogenesis tool plug-in with ImageJ software (NIH, Bethesda, MD).

Immunofluorescent Staining

A375, RPMI-7951, or WM-164 cells were seeded 2.5×10^5 - 5×10^5 on glass coverslips in 6 well plates and incubated overnight. Media was changed, and cells were treated with DJ95, DJ101, paclitaxel, docetaxel or media only control for 18 hrs. Cells were then fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X (Sigma-Aldrich, St. Louis MO) in Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific Inc., Haverhill, MA) and blocked with 3% Bovine serum albumin (Cell Signaling Technology, Danvers, MA) prior to staining. Microtubules and mitotic spindles were visualized after incubating with anti- α -tubulin antibody (Thermo Scientific, Rockford, IL) and Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes, Eugene, OR). The coverslips were mounted with Prolong Diamond Antifade mounting media containing DAPI (Invitrogen, Eugene, OR) and images acquired with a Keyence BZ-X700 fluorescence microscope and BZ-X analyzer software (Keyence, Osaka, Japan).

X-Ray Crystallography

Protein Expression and Purification. X-Ray crystallography was performed by Dr. Yuxi Wang (Sichuan University, in China). The clones of the stathmin-like domain of RB3 and TTL were generous gifts provided by Dr. Benoît Gigant (CNRS, France) and Dr. Michel O. Steinmetz (PSI, Switzerland). The stathmin-like domain of RB3 (RB3-SLD) from rat was transformed into and overexpressed in *E. coli*. The protein was purified by anion-exchange chromatography and gel filtration chromatography. The peak fractions from the gel filtration column were concentrated to 10 mg/mL and stored at -80 °C (51, 154, 185). The TTL protein from chicken was expressed and purified from an *E. coli* expression system as described previously (186). Briefly, the protein was expressed in *E. coli* using LB, purified through Ni-NTA affinity chromatography and gel filtration chromatography (buffer: Bis-Tris Propane pH 6.5, 200 mM NaCl, 2.5 mM MgCl₂, 5 mM

β Me, 1% glycerol). The peak fractions were concentrated to 20 mg/mL and saved at -80 °C. The SDS-PAGE was performed to check the purity of RB3 and TTL. Porcine brain tubulin (Catalog # T-238P) (Cytoskeleton Inc., Denver, CO) was supplied at 10 mg/mL in G-PEM (General tubulin buffer: 80 mM PIPES pH 6.9, 2 mM MgCl₂, 0.5 mM EGTA and 1 mM GTP) as a frozen liquid and saved at -80 °C until use.

Crystallization and Crystal Soaking. The previously published process of obtaining crystals of the tubulin-RB3_SLD-TTL (T2R-TTL) complex was applied (186, 187). In brief, tubulin (10 mg/mL), TTL (20 mg/mL) and RB3 (10 mg/mL) were mixed at the molar ratio of 2:1.3:1.2 (Tubulin: RB3_SLD: TTL) and incubated on ice with 1 mM AMPPCP, 5 mM tyrosinol and 10 mM DTT, and the mixture was concentrated to 20 mg/mL at 4 °C. The crystallization of the T2R-TTL complex was carried out at 20 °C using the sitting-drop vapor diffusion method by mixing an equal volume of protein complex and crystallization buffer containing 6% PEG 4000, 5% glycerol, 0.1 M MES, 30 mM CaCl₂, 30 mM MgCl₂, pH 6.7. Seeding was used to obtain the well diffracting crystals. Initial crystals were observed after two days of incubation and reached a final length of 200-300 μ m within 3-5 days. Morphologically superior crystals were selected, cryoprotected with crystallization buffer containing 20% glycerol, and flash frozen in liquid nitrogen.

X-Ray Data Collection and Structure Determination. Diffraction data were collected at 100K on the beamlines BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF) in Shanghai, China. The native dataset was collected at a wavelength of 0.97853 Å using MX225 CCD detector. Data were indexed, integrated and scaled using HKL2000 (188). The initial phase was determined by molecular replacement using the apo structure T2R-TTL (PDB code: 4I55) as the searching model. The structure of T2R-TTL-DJ95 was determined by molecular replacement using the previously published T2R-TTL structure (PDB ID: 4I55) as a search model. The rotation and translation function searches were performed by the program PHASER (189). The model was further built with Coot (190) and refined using the phenix.refine module Phenix (191). The model quality was checked with PROCHECK and shows a good stereochemistry according to the Ramachandran plot. PyMol was used to generate the figures. Analysis was determined in coordination with Dr. Stephen White's group (St. Jude Children' Research Hospital).

Pharmacological Profile Screening

Screening of potentially significant off-target effects to DJ95 binding and enzyme targets was performed via SafetyScreen44 offered by Eurofins Cerep-Panlabs. DJ95 was tested at 1.0E-07 M. Compound binding was calculated as a % inhibition of the binding of a radioactively labeled ligand specific for each target. Compound enzyme inhibition effect was calculated as a % inhibition of control enzyme activity. Results showing an inhibition (or stimulation for assays run in basal conditions) higher than 50% are considered to represent significant effects of the test compounds. Results showing an inhibition (or stimulation) between 25% and 50% are indicative of weak effects. Results

showing an inhibition (or stimulation) lower than 25% are not considered significant and mostly attributable to variability of the signal around the control level. In each experiment, the respective reference compound was tested concurrently with DJ95, and the data were compared with historical values determined at Eurofins. The experiment was accepted in accordance with Eurofins validation Standard Operating Procedure. Assessment of potentially significant off-target effects of DJ101 to 47 major physiologically important targets was performed by DiscoverX (DiscoverX Corporation, Fremont, CA) in 78 assays using its Safety47™ Panel and standard protocols. DJ101 was assayed at 1 μ M concentrations in two replicates. Hit responses < 70% are considered negligible.

LC-MS/MS Plasma Concentration Analysis

LCMS/MS Parameters. The LC-MS/MS data was obtained by Dr. Dejian Ma (UTHSC). The LC-MS/MS system comprised a Sciex (Framingham, MA) 5500 triple quadrupole mass spectrometer, equipped with a Turboionspray™ ionization interface and Analyst software version 1.6.3. Chromatographic separation was carried out using a ZORBAX SB-C18 column of 150 x 4.6 mm i.d., and 3.5 μ m particle size (Agilent Technologies, Santa Clara, CA) maintained at 35°C using Shimadzu (Columbia, MD) Nexera XR HPLC system and SIL-20ACXR autosampler. The mobile phase (A: Milli-Q water, B: methanol) was eluted at a flow rate of 0.5 mL/min. The gradient started at 50% of mobile phase B and maintained for 0.5 min, then linearly rose to 100 % B over 1 min. Subsequently, the eluent composition was maintained at 100% B from 1.5 to 6 min before it was decreased to initial condition 50 % mobile phase B for re-equilibration in 0.1 min. The total run time was 6.5 min plus a pre-equilibrate of 0.5 min. A switching valve directed the mobile phase to the MS system between 4.5 and 6.1 min. The electrospray ion source was operated in a positive ionization mode for all the experiments. The typical parameters were: capillary 5.5 kV; entrance potential (EP) 10 V; channel electron multiplier (CEM) 1800 V; source temperature 600°C.

LC/MS Sample Preparation. Protein precipitation was used to extract DJ95 from plasma. Plasma (50 μ L) was added in 150 μ L precipitation solution, methanol including 26.5 nM ABI-231 as IS, vortexed for 15 seconds, and then centrifuged for 15 minutes at 4°C (circa 12,000 rpm). Supernatant (120 μ L) was then transferred to a 96-well plate and 1 μ L sample was injected into the LC-MS/MS system. Calibration standards were prepared by mixing DJ95 stock solution in pooled human plasma, resulting in matrix concentrations of 1, 10, 100, 200, 1000, 2000, 10,000 nM. Blank samples were prepared using blank plasma. All samples were stored at -20°C prior to use. The lowest standard of 1 nM was not detected. The LLOQ was determined to be 10 nM.

***In Vivo* Mouse Models and Treatments**

All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National

Institutes of Health and were approved by the Institution's Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center (UTHSC, Memphis, TN). All animals were maintained in a room with a 12 h light/dark cycle and provided food and water ad libitum.

Plasma Concentration and Pharmacokinetic Analysis. Thirty CD-1 ICR mice from Charles River Laboratories (Wilmington, MA) of approximately 6 weeks of age were used for the study. Both males and females were used, and at least one animal of each sex was collected at each time point. Animals (n=3 per time point) were dosed with 15 mg/kg DJ95 via intraperitoneal injection and drug vehicle was PEG300 (Sigma-Aldrich, St. Louis MO) and PBS at a 1:1 ratio.

Blood was collected at the following time points: 0, 15, 30, 60, 90, 180, 360, 480, 720 and 1440 min. A blood volume of 0.6 mL was collected from each mouse at respective time point via cardiac venipuncture using heparinized syringes and collected into lithium heparinized tubes. Samples were centrifuged at 3,000 rpm for 10 min. Plasma was collected into 1.5 mL centrifuge tubes and frozen at -80C until analysis by LC/MS. Pharmacokinetic parameters area under the curve (AUC_{inf}), half-life (t_{1/2}), clearance, volume of distribution and maximum concentration (C_{max}) were measured.

MTD and Xenograft Studies. We first performed an acute maximum tolerable dose (MTD) study for DJ101 formulated in the vehicle (equal parts PEG300: PBS) by progressively increasing daily injection doses via i.p. route to ICR mice (n=2) (Evigo Corporation, Denver, CO), and estimated the MTD to be at least 65 mg/kg. To ensure a safety margin during the repeated treatment, the dose was scaled down to 30 mg/kg in the animal experiments. We also performed a longer MTD study by subjecting nude mice (Evigo Corporation, Denver, CO) to 30 mg/kg doses of DJ95 or DJ101 formulated in the vehicle by i.p. injection for 5 consecutive days. Because mice receiving DJ95 treatment began to show signs of toxicity beyond this time point, we scaled the dose back farther to 15mg/kg for the xenograft study.

Nude mice, age 6–8 weeks old, were purchased from Evigo Corporation (Denver, CO) for the xenograft studies. Logarithmic growth phase A375, PC-3, or PC-3/TxR cells were prepared in phenyl red-free, FBS-free media and mixed with thawed matrigel prior to injecting into mice. Tumors were established by injecting 100 μ L of matrigel/cell suspension containing 2.5×10^6 cells subcutaneously in the hind flank of each mouse. After tumors were established, mice were ranked on tumor size and randomized into control or treatment groups, with each group receiving half males and half females. 100 μ L of the drug treatment or vehicle control solution was administered via i.p. injection 5x a week for the duration of the studies.

Tumor volume was measured three times a week with a caliper and calculated by using the formula $a \times b^2 \times 0.5$, where a and b represent the larger and smaller diameters, respectively. Tumor growth inhibition (TGI) at the conclusion of the experiments was calculated as $100 - 100 \times ((T - T_0)/(C - C_0))$, where T, T₀, C and C₀ are the mean tumor volume for the specific group on the last day of treatment, mean tumor volume of

the same group on the first day of treatment, mean tumor volume for the vehicle control group on the last day of treatment and mean tumor volume for the vehicle control group on the first day of treatment, respectively (100). Animal activity was monitored, and body weights were recorded throughout the study to assess potential acute toxicity. At the end of the experiment, mice were sacrificed, and the tumors were dissected out, weighed, and fixed in 10% neutral buffered formalin solution prior to pathology staining analysis.

B16F10 Melanoma Lung Metastasis Model. C57BL/6 mice from Charles River Laboratories International, Inc., age 7–8 weeks old, were used to study the inhibition effect of DJ101 on lung metastasis of melanoma cells. Murine B16F10 melanoma cells growing in a logarithmic growth phase were suspended in the conditioned media at a density of 1×10^6 per mL. Each mouse was inoculated with the tumor cells (100 μ L containing 1×10^5 cells) via the lateral tail vein. The treatment started on the third day after the inoculation to ensure the initiation of metastasis before beginning treatment. DJ101 (30 mg/kg) and vehicle were formulated as described above. Doses (100 μ L) of DJ101 or vehicle solution were administered via i.p. injection every other day for 2 weeks. Animal activity and body weights were monitored during the entire experiment period to assess acute toxicity. Mice were sacrificed 15 days after the initiation of the experiment, and the lungs were separated, expanded and preserved in 10% neutral buffered formalin. The number of lung metastasis nodules was recorded. Major organs were also preserved in the same manner for subsequent toxicological examination.

Histology and Immunohistochemistry

The fixed tumor xenograft tissues were embedded in paraffin. Serial sections were obtained for Hematoxylin and Eosin (H&E) and immunohistochemistry (IHC) analysis. Staining was performed with rabbit anti-cleaved caspase 3 antibody (Cell Signaling Technology Inc., Danvers, MA) and rabbit anti-CD31 (Cell Signaling Technology Inc., Danvers, MA) following ABC-DAB methods. Antigen retrieval was performed with H-3300 antigen unmasking solution (Vector Laboratories, Burlingame, CA). Images were captured with a Keyence BZ-X fluorescent microscope (Keyence Corporation, Itasca, IL) at 10x and 20x magnification. Five representative fields of view from three tumors per group at 20x magnification were analyzed in ImageJ to calculate positive stained area. Pathological tissue sections from major organs (heart, liver, kidney, lung and spleen) were examined similarly to identify any potential drug-related effects by Dr. Frank Park (UTHSC). Images were obtained with EVOS XL Core microscope (Life Technologies, Carlsbad, CA).

Statistical Analysis

Data were analyzed using Prism Software 5.0 (GraphPad Software, Inc., San Diego, CA). IC₅₀ values were calculated by nonlinear regression. The statistical significance ($P < 0.05$) was calculated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, comparing each treated group to the

corresponding control group for studies comparing 3 or more groups. An unpaired Student's t-test was used to calculate significance for studies comparing two groups.

Results

Potency Against Malignant Melanomas and the NCI-60 Cell Panel

Our preliminary studies showed that DJ101 and DJ95 have potent cytotoxic activity against a variety of melanoma and prostate cancer cell lines (143). To expand upon this observation and determine their *in vitro* efficacy against additional metastatic melanoma cell lines that harbor clinically relevant mutations, we first performed cytotoxicity assays for the DJ compounds against a panel of human metastatic melanoma cell lines including A375, RPMI-7951, WM-164, WM115, and SK-MEL-1 (**Table 2-1**). These cell lines possess diverse genetic complexity and contain genomic mutations in one or more of the following genes: BRAF, CTNNB1, CDKN2A, PTEN, and TP53. DJ101 demonstrated comparable potency against these lines to colchicine, with IC₅₀ values ranging from 7-12 nM in each of the tested cell lines. DJ95 was the most potent against A375 cells, though less potent than DJ101 or colchicine.

DJ95 and DJ101 were also tested in the NCI-60 cell panel, assaying a diverse selection of cancer types, in a single-dose and 5-concentration assay to see if they were effective against other kinds of cancer (**Appendix Figures A-1 and A-2**). DJ compounds were highly active and demonstrated low GI₅₀ values for the majority of melanoma cell lines, and were especially potent against leukemia, colon, CNS, and prostate cancers. The average total growth inhibition (TGI) and LC₅₀ was greatest for colon cancer and melanoma. The dose-response curves of each individual melanoma cell line tested revealed that the cell lines most sensitive to DJ95 treatment were LOX IMVI and SK-MEL-5 and to DJ101 were MDA-MB-435 and also SK-MEL-5.

DJ Compounds Overcome Taxane Resistance and ABC Transporter-Mediated Resistance

In addition to melanoma cell lines, we also tested DJ compounds against prostate cancer cell lines, including parental PC-3 and DU-145, paclitaxel-resistant PC-3 (PC-3/TxR) and docetaxel-resistant DU-145 (DU-145/TxR) cell line pairs to understand if DJ95 can overcome mechanisms associated with taxane resistance (**Table 2-2**). In the PC-3 cell line, DJ95 and DJ101 were mildly less toxic (8.5 ± 0.7 and 17.0 ± 2.6 nM) than the colchicine-treated cells (5.31 ± 1.86 nM). Paclitaxel and docetaxel treatment demonstrated greater cytotoxicity, with IC₅₀s of 3.7 ± 0.3 and 0.5 ± 0.1 nM, respectively. However, in the paclitaxel-resistant PC-3/TxR cell line, DJ101 and DJ95 were much more potent than in the parental cell line (8.5 ± 0.7 and 4.8 ± 1.7 nM) and even outperformed colchicine (28.1 ± 1.2 nM). There was a drastic reduction in efficacy for

Table 2-1. Cytotoxic effects of DJ compounds against malignant melanoma cancer cell lines.

Treatment	IC ₅₀ ± SEM (nM)				
	A375	RPMI-7951	WM-164	WM115	SK-MEL-1
DJ95	19.2 ± 2.3	46.0 ± 6.5	92.2 ± 12.7	92.2 ± 12.7	62.5 ± 9.9
DJ101	7.6 ± 0.5	10.1 ± 0.9	12.1 ± 2.4	10.3 ± 1.8	9.6 ± 0.4
Colchicine	11.7 ± 0.4	17.8 ± 1.9	7.8 ± 0.9	7.8 ± 0.9	8.3 ± 1.9

Table 2-2. Cytotoxic effects of DJ95 and DJ101 against parental and taxane-resistant prostate cancer cell lines.

Treatment	IC ₅₀ ± SEM (nM)		^a RI	IC ₅₀ ± SEM (nM)		RI
	PC-3	PC-3/TxR		DU-145	DU-145/TxR	
DJ95	17.0 ± 2.6	4.8 ± 1.7	0.3	32.4 ± 9.7	222.9 ± 28.0	6.9
DJ101	8.5 ± 0.7	6.4 ± 0.3	0.75	18.2 ± 0.9	63.3 ± 7.2	3.5
Colchicine	8.0 ± 0.8	28.1 ± 1.2	3.5	25.7 ± 1.4	>1,000	>40
Paclitaxel	3.7 ± 0.3	110.7 ± 3.7	30	1.1 ± 0.1	>1,000	>1,000
Docetaxel	0.5 ± 0.1	27.9 ± 1.1	55.8	0.10 ± 0.01	352.3 ± 118.2	>3,523

^a RI: resistance index is calculated by dividing the IC₅₀ value of compound in resistant cell lines by the IC₅₀ in the corresponding parental cell lines.

the taxanes, showing 30-fold increase in resistance to paclitaxel and a 55.8-fold increase for docetaxel. The DJ compounds demonstrated substantially better resistance indexes (RI) of 0.3 for DJ95 and 0.8 for DJ101. Similar results were observed in the DU-145 and DU-145/TxR cell pair except that the differences were more profound: while paclitaxel and docetaxel had IC_{50} s of 1 nM or less, and the DJ compounds were slightly less potent than in the PC-3 cell lines, the taxanes showed greater than 1000 fold decrease in efficacy to the DU-145/TxR cells while DJ101 had only a slight increase in the resistance index to 3.5. DJ95 showed some resistance, but much less than colchicine, which showed more than a 40-fold increase in resistance DU-145/TxR compared to the parental cells. These results demonstrate that DJ compounds, particularly DJ101, can overcome some mechanisms of taxane resistance that developed by cancer cells.

Because we were particularly interested in the ability of DJ95 to overcome transporter-mediated drug resistance, we tested DJ95 against genetically engineered HEK293 cells lines, that stably transfected full length ABCB1, ABCC1, wild type ABCG2, or the pcDNA3.1 blank vector. Cisplatin, which is not a substrate for any of these ABC transporters, was used as negative control. Greater than 100-fold resistance was observed for the ABCB1 overexpressing HEK293 cells against paclitaxel, colchicine, and vincristine (**Table 2-3**). On the other hand, HEK293/ABCB1 resistant cells showed virtually no resistance to DJ95. Slight resistance (RI= 3.7) was revealed for the ABCC1 overexpressing HEK293 transfected cells against DJ95 compared to the vector-only cells, but all other microtubule targeting drugs that were tested (paclitaxel, colchicine, vincristine) demonstrated significantly increased resistance, with IC_{50} s greater than 10 μ M against HEK293/ABCC1. Similarly, against the HEK293/ABCG2-R482 cells, resistance was evident for all tubulin inhibitors, though less resistance was developed against the DJ95 compound (about 8.7-fold). This was still lower than mitoxantrone, a known substrate of ABCG2, showing about 11.2-fold decrease in potency in the ABCG2 overexpressing cell line.

We also wanted to assess the effects of DJ95 against drug-selected resistant cell lines. From the parental epidermoid carcinoma cell line KB-3-1, the colchicine resistant/ABCB1-overexpressing (KB-C2) cell line and vincristine resistant/ABCC1-overexpressing (KB-CV60) cell line were developed. The mitoxantrone-selected ABCG2-overexpressing cell line NCI-H460/MX20 was generated from the parental non-small cell lung cancer cell line NCI-H460 and utilized to further evaluate the effects against ABCG2 upregulation. Compared to the existing tubulin inhibitors paclitaxel, colchicine and vincristine, DJ95 exhibited greater cytotoxicity against drug-selected ABCB1-overexpressing KB-C2 cells and ABCC1-overexpressing KB-CV60 cell lines, with IC_{50} values of 719.54 ± 181.48 nM and 35.90 ± 4.50 nM, respectively (**Table 2-4**). Although there was still some increase in resistance against the KB-C2 (colchicine selected/ABCB1 overexpressing) cells compared to the parental KB-3-1 cells, both KB-C2 and KB-CV60 were much less resistant to DJ95 than they were against the three other tubulin inhibitors tested. Since no resistance to DJ95 was revealed from ABCB1 transfected cell line, the increase in resistance observed for DJ95 against the colchicine-selected KB-C2 may be related to mechanisms affiliated with the colchicine binding site and not solely ABCB1 overexpression.

Table 2-3. Cytotoxic effects of DJ95 against gene transfected ABCB1-, ABCC1- or ABCG2-overexpressing cancer cell lines.

Treatment	IC ₅₀ ± SD (nM)			
	HEK293/ pcDNA3.1	HEK293/ ABCB1	HEK293/ ABCC1	HEK293/ABCG2 -R482
DJ95	330.0 ± 44.1	314.7 ± 73.1	1213.9 ± 241.6	2869.2 ± 123.3
Paclitaxel	34.6 ± 6.3	3496.0 ± 657.8	>10000	>10000
Colchicine	112.1 ± 41.5	>10000	>10000	>10000
Vincristine	99.4 ± 23.1	>10000	>10000	>10000
Mitoxantrone	108.0 ± 27.5	—	—	1209.7 ± 111.4
Cisplatin	2510.1 ± 362.6	2583.1 ± 474.5	2584.1 ± 151.8	2475.3 ± 127.4

Data obtained by Zi-Ning Lei (St. John's University).

Table 2-4. Cytotoxic effects of DJ95 against parental and drug-selected ABCB1-, ABCC1- or ABCG2-overexpressing cancer cell lines.

Treatment	IC ₅₀ ± SD (nM)				
	KB-3-1	KB-C2 (ABCB1)	KB-CV60 (ABCC1)	NCI-H460	NCI- H460/MX20 (ABCG2)
DJ95	23.5 ± 2.2	719.5 ± 181.5	35.9 ± 4.5	673.0 ± 111.7	5361.5 ± 645.5
Paclitaxel	9.2 ± 2.1	1918.5 ± 106.3	120.0 ± 14.6	>10000	>10000
Colchicine	23.9 ± 4.9	7445.3 ± 446.3	91.7 ± 7.7	>10000	>10000
Vincristine	1.3 ± 0.2	748.8 ± 99.8	218.6 ± 1.9	>10000	>10000
Mitoxantrone	—	—	—	118.7 ± 40.2	2456.5 ± 420.4
Cisplatin	2024.5 ± 463.7	2871.2 ± 115.3	1796.5 ± 198.8	2226.6 ± 179.5	2870.6 ± 281.7

Data obtained by Zi-Ning Lei (St. John's University).

In both NCI-H460 and its mitoxantrone-selected ABCG2 overexpressing cell line, DJ95 demonstrated better anticancer activities than paclitaxel, colchicine and vincristine, with IC₅₀ values of 673.04 ± 111.71 nM and 5361.50 ± 645.45 nM, respectively, whereas IC₅₀ values of the three other tubulin inhibitors were higher than 10,000 nM in both cell lines. However, low resistance against DJ95 was observed in NCI-H460/MX20 cell line compared to its parental cell line (about 8-fold resistance), though the resistance index was not as high as that of the substrate control mitoxantrone (about 20-fold resistance). The results from the drug-selected resistant cells are consistent with those obtained from transfected ABCG2-overexpressing cell line HEK293/ABCG2-R482 and its vector control, which indicated that DJ95 might be a weak substrate of ABCG2. Further study is needed to verify if ABCG2 would be a factor mediating resistance to DJ95.

Interaction at the Colchicine Binding Site on Tubulin

We previously reported the design and synthesis of DJ95 and DJ101(143) and a number of potent tubulin inhibitors related to this scaffold (98, 141, 142). While mechanistic studies suggested that these agents produce their antitumor activities by interacting with the colchicine binding site on tubulin, the detailed molecular interactions had not previously been fully elucidated. Recently, high-resolution (<3.0 Å) crystal structures of tubulin in complex with several known colchicine site inhibitors were reported (51, 192). Using these newly established procedures, we obtained the high-resolution X-ray crystal structure of DJ101 in complex with αβ-tubulin (deposited to the Protein Databank with PDB code: 5H7O, resolution 2.8 Å). This high-resolution crystal structure confirms the direct binding of DJ101 in the colchicine binding site (**Figure 2-2A**). DJ101 forms three hydrogen bonds with the αβ-tubulin dimer: one hydrogen bond from T179 in loop five in the α-monomer to the imidazole nitrogen, one hydrogen bond from N349 in loop nine in the β-monomer to the indole nitrogen, and one hydrogen bond between the oxygen of the 4-methoxyl moiety and the thiol moiety in C241 in helix seven of the β-monomer (**Figure 2-2B**). A tight hydrophobic “sandwich” formed by sidechains from C241, L255, L248, and N258 wraps the trimethoxyphenyl and the imidazopyridine moieties firmly in the colchicine binding domain. Additionally, the sidechain M259 in helix eight of the β-monomer serves as a “wedge” between sheet 9 and DJ101 to lock its curved conformation. It is also evident that DJ101 overlaps with colchicine at its binding site (**Figure 2-2C**). These results provide the first direct evidence of DJ101’s direct interaction with the colchicine binding site in tubulin to inhibit tubulin polymerization.

We also determined the crystal structure of the T2R-TTL complex bound with compound DJ95 at 2.5 Å resolution, and the coordinates and structure factors have been deposited in the Protein Data Bank with the PDB ID: 6C77. In the crystal structure, the compound is bound only at the interface of the first α- and β-tubulin heterodimer, unlike colchicine where the interfaces of both α- and β-tubulin heterodimers were occupied (**Figure 2-2D**). In the case of the unliganded T2R-TTL complex reported earlier, the β-T7 loop of β-tubulin at the interface of the α- and β-tubulin heterodimer is in a closed conformation (PDB ID: 4I55). The DJ95 binding pocket is created by the outward

Figure 2-2. X-ray crystal structures of DJ101 and DJ95 in complex with tubulin protein.

A) Surface representation of the overall structure of α -tubulin (black) and β -tubulin (grey). DJ101 is shown in sphere representation (cyan). B) Detailed molecular interactions between the DJ101 molecule (cyan) and the tubulin dimer. Hydrogen bonds are indicated with black dash lines. C) Superimposition of the binding sites for DJ101 (cyan)-tubulin complex (5H7O) with colchicine (yellow)-tubulin complex (4O2B). D) Cartoon showing the organization of α -tubulin (green), β -tubulin (salmon), RB3-SLD (cyan), TTL (grey) proteins, GTP, GDP, and DJ95. E) 2Fo-Fc electron density map for DJ95 contoured at 1 sigma shown as blue mesh, and hydrogen bonds are shown as red dashed lines. F) Superimposed tubulin-DJ95 (green sphere) complex and tubulins as found in straight protofilaments (PDB ID: 1JFF; cyan) showing that binding is not compatible with the straight conformation. Conformational changes of the secondary elements are labeled. Crystallographic data obtained by Yuxi Wang (Sichuan University, China) and interpreted by Dr. Stephen White and Dr. Gyanendra Kumar (St. Jude Children's Research Hospital).

movement or opening of this loop and the same motion has previously been observed when colchicine binds (PDB ID: 4O2B). Looking closely at the bound DJ95, we find that the 3-methoxyphenyl group of DJ95 occupies a deep pocket in the β -tubulin subunit that is lined with residues Val236, Cys239 and Leu253 (**Figure 2-2E**). The side chain of Leu253 makes a pi-H interaction with the phenyl ring of 3-methoxyphenyl group. The imidazopyridine ring forms two hydrogen bond interactions; a nitrogen atom with the main chain NH of β -tubulin Asp249 and an NH with the main chain carbonyl oxygen of Thr179 from α -tubulin across the interface. Finally, the indole ring of DJ95 sits in a pocket lined by Asn347, Lys350, Asn256 and Met257 of β -tubulin and Val181 from α -tubulin. The electron density is consistent with this ring having two conformations rotated by $\sim 180^\circ$ – one where the nitrogen faces the Met257 from β -tubulin, and the other where the nitrogen faces the Val181 from α -tubulin. The refinement suggests that the latter conformation is preferred 2:1 over the former. Comparison with the unliganded T2R-TTL complex revealed that a significant conformational change not only occurs in loop β -T7 to accommodate DJ95 but also in loop α T5 (**Figure 2-2F**). These two loop conformations are incompatible with the ‘straight’ structure of the tubulin filament and are therefore consistent with DJ95 acting as a destabilizer of filament formation.

Disruption of Microtubule Networks and Spindle Formation

To characterize their effects on microtubule networks and cell morphology, we used confocal microscopy to visualize several melanoma cell lines treated for 18 hours with either DJ compounds or a microtubule stabilizing agent such as paclitaxel or docetaxel, which promote polymerization. The alteration in microtubule rearrangement in the treated cells can clearly be observed (**Figure 2-3A**). Control cells appeared to exhibit well-organized microtubule networks extending throughout the cell to the cell periphery. Treatment with DJ95 or DJ101 led to fragmented microtubule networks and an increase in soluble, cytoplasmic tubulin. At higher concentrations, there appeared to be very little polymeric tubulin framework left intact. These observations are consistent with a mechanism of action of tubulin depolymerization. In contrast, taxane-treated cells demonstrated highly condensed, polymeric tubulin. With increasing concentrations, the aggregation of the filaments was more pronounced, consistent with increased stabilization of microtubules.

Microtubules also make up the mitotic spindle and utilize the dynamic properties for chromosome segregation during mitosis (151). Therefore, tubulin targeting agents also interfere with the formation and organization of mitotic spindles (193-196). We investigated the effects of DJ95 in a concentration-dependent manner compared to untreated control cells (**Figure 2-3B**). While control cells exhibited normal, bilateral spindle formation extruding from centrosomes, DJ95 treatment caused aberrant spindle development as evidenced by multi-polar and disorganized spindle morphology. These abnormalities were exacerbated with increasing drug concentrations. This is further proof that DJ95 inhibits the progression of mitosis by interfering with microtubule dynamics.

Figure 2-3. DJ compounds interfere with microtubule networks and mitotic spindle organization.

A) Microtubule networks of melanoma cells (either A375, RPMI-7951, or WM-164) after treatment with DJ101, DJ95, docetaxel or paclitaxel at 40x magnification. The concentration of the drug treatment is indicated in the bottom of each image. The top row of images are the untreated control cells for comparison. B) The effect on mitotic spindle organization of cells undergoing mitosis obtained at 63x magnification. All confocal images of all cells were obtained after 18 hr drug treatment. Tubulin (red) is visualized by α -tubulin primary antibody and Alexa Fluor 647 secondary antibody and DNA (blue) is stained with DAPI.

Inhibition of Colony Growth and Cell Migration

DJ95 and DJ101 were tested separately in colony-forming assays to evaluate the long-term inhibitory growth effects on different melanoma cell lines. We first tested the ability of DJ95 to inhibit colony formation in a concentration-dependent manner compared to untreated A375 control cells. (**Figure 2-4A**). A significant decrease was observed for all treated cells, and concentrations as low as 10 nM caused a $26.78 \pm 1.9\%$ inhibition of colony area compared to control ($P < 0.001$) and higher concentrations of 25 nM and 50 nM inhibited colony formation by $44.73 \pm 1.9\%$ and $65.01 \pm 3.08\%$, respectively ($P < 0.0001$). At 100 nM, DJ95 almost completely eliminated colony formation, and only $1.25 \pm 0.07\%$ of the area compared to the control remained.

We also tested DJ101 and colchicine in both A375 and RPMI-7951 cells lines using lower concentrations (**Figure 2-4B and 2-4C**). Treatment with 4 nM of DJ101 resulted in colonies covering only $30.6\% \pm 0.8\%$ of the total surface area, representing a $59.5\% \pm 2.0\%$ inhibition compared to the untreated control cells, which inhabited $75.6\% \pm 3.7\%$ of the total area. Colchicine was used as a reference control because its ability to potently and persistently inhibit colony formation is well-documented (197, 198). The effect of DJ101 on A375 cell colony formation was similar to colchicine at the same concentration, which had colonies covering a total area of $34.7\% \pm 1.5\%$ ($54.1\% \pm 3.5\%$ inhibition). Control RPMI-7951 cells formed colonies occupying a surface area of $83.2\% \pm 2.1\%$ and comparable reductions in colony formation were measured for treated cells, where DJ101-treated cells occupied $47.1\% \pm 5.7\%$ ($43.5\% \pm 11.8\%$ inhibition) and colchicine-treated cells covered $50.9\% \pm 3.4\%$ ($38.8\% \pm 7.0\%$ inhibition). Higher concentrations (20 nM) of DJ101 or colchicine resulted in complete colony obliteration. Dunnett's multiple comparison tests after one-way ANOVA analysis gave an overall P value of less than 0.0001 for each treated group compared to the control, indicating that they are statistically different.

To demonstrate that the DJ compounds interfere with cell migration through microtubule destabilization, we performed scratch migration assays against A375 and RPMI-7951 cell lines. Cells were treated with 25 nM of the drug and images were taken at 0, 12 and 24 hours (**Figure 2-4D and 2-4E**). After 24 hours, untreated A375 cells migrated into $83.8\% \pm 6.1\%$ of the wound channel, nearly closing the gap. DJ101 and DJ95 treated cells migrated into only $40.8\% \pm 6.0\%$ and $35.6\% \pm 1.4\%$, respectively. A greater reduction in cell migration was observed in the RPMI-7951 cell line, where control cells reclaimed $86.4\% \pm 10.5\%$ of the wound channel and DJ compound treated cells occupied less than 25% of gap area. Similar but less inhibition of the scratch area was observed colchicine-treated cells, achieving $52.0\% \pm 2.7\%$ closure for the A375 cell line and $52.3\% \pm 3.4\%$ for the RPMI-7951 cell line. One-way ANOVA analysis of both cell lines gave P values in both cell lines were less than 0.0001. There was no significant difference in the scratch channel area for each group at the beginning of the experiment (time 0), and the % closed was based on this average area. Taken together, these *in vitro* studies demonstrate that the DJ compounds strongly reduce aberrant cancer cell proliferation at low concentrations and hinder cell migration at least as efficiently as colchicine in these melanoma cell lines.

Figure 2-4. Inhibition of cell proliferation and migration.

A) Colony formation assay of A375 cells treated with DJ95 or colchicine (n=3). Colony area was quantified by VisionWorks software and represented by total area (in pixels) $\pm$ SD. B) Colony assay formation of A375 and C) RPMI-7951 cells treated with DJ101 or colchicine (n=4). Colony area is represented as percent of the total surface area $\pm$ SD as determined by ImageJ software. Colony assays were treated for 10 days in 6-well plates. D) Scratch migration assay of A375 and E) RPMI-7951 cell lines (n=4). Cell migration is presented as mean percent of the area closed $\pm$ SD after 24 hrs compared to the scratch area at time 0. ImageJ software was used to calculate the total scratch area at each of the time points. Scale bar = 1000 μ m. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test, comparing each treatment group to the control group for the above experiments. (** p < 0.01, *** p < 0.001, **** p < 0.0001).

Anti-Angiogenic Activity on HUVEC Tube Cell Formation

In recent years, there has been extensive research on the vascular disrupting properties of tubulin targeting agents and their ability to selectively target tumor vasculature (39, 40, 97, 199-201). To see whether DJ95 would exhibit these characteristics, we tested the *in vitro* effect on the formation of capillary-like networks of human umbilical vein endothelial cells (HUVECs) plated on Matrigel. The Matrigel basement membrane allows endothelial cells to form tubules with tight cell-to-cell and cell-to-membrane contacts (202). After 6 hrs of incubation in the presence of the test compounds, it was clearly observed that both DJ95 and colchicine disrupted the tube cell formation in a concentration-dependent manner (**Figure 2-5**). At concentrations of 100 nM and 200 nM, DJ95 disrupted networks by $35.7\% \pm 5.6\%$ and $51.8\% \pm 3.3\%$ based on total calculated network length ($P < 0.01$ and 0.0001 , respectively). This was similar to the effect observed from colchicine treatment at the same concentrations, inhibiting HUVEC tube cell formation by $60.4\% \pm 9.0\%$ and $81.6\% \pm 8.0\%$, respectively ($P < 0.0001$). Since this result was observed in a short timeframe, we expect that the drug action on tube cell formation was not a result of antiproliferative activity but rather from anti-angiogenic action.

Pharmacological Profiling

In vitro pharmacological profiling is increasingly being used to identify undesirable off-target activity profiles early in the drug discovery process. It involves screening the compound of interest against a wide range of targets such as receptors, ion channels, transporters and enzymes other than the intended therapeutic target in order to identify specific interactions that may elicit adverse drug-related side effects (203). About 3/4ths of all adverse drug reactions are dose-dependent, and pharmacological profiling of the drug of interest can predict the risk and help reduce drug attrition rate. To further test for potentially significant off-target effects and determine the drug safety profile, DJ101 was evaluated at 1 μM in the Safety47™ Panel using functional assays for human targets (**Figure 2-6A**). This *in vitro* pharmacological profiling service includes the assessment of the functional response of 47 major physiologically important targets across 78 assays to the drug of interest, normalized to respective controls. Only a value higher than |70%| response is considered significant. DJ101 only showed hit responses in 2 of the 78 assays, namely norepinephrine transporter (NET) blocking and glucocorticoid receptor (GR) antagonism. Since the concentration tested was about 100 times greater than the average IC_{50} value for DJ101, which is expected to be well above its practical physiological concentration, results from this Safety47™ Panel screening strongly suggest that DJ101 has minimal potential off-target effects.

DJ95 also underwent pharmacological profiling for off-target assessment but was screened through a different safety panel. DJ95 was evaluated in binding and enzyme uptake assays performed by Eurofins Cerep Panlabs. DJ95 demonstrated nominal specific binding inhibition or stimulation against 37 radioactive labeled ligand targets (**Figure**

Figure 2-5. *In vitro* inhibition of angiogenesis.

Representative images of HUVEC capillary-like formation on Matrigel taken after 6 hr incubation with indicated concentrations of DJ95 or colchicine. Quantification of total tube length was performed with ImageJ analysis and expressed as mean $\pm$ SD (n=4). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. (** $p < 0.01$, **** $p < 0.0001$).

Figure 2-6. Pharmacological profiles for DJ95 and DJ101.

A) Assessment of DJ101 in the Safety47™ panel. Graph represents mean percent response $\pm$ SD. Values in between -70% and +70% (indicated with dashed lines) are considered insignificant. B) Assessment of DJ95 in the SafetyScreen 44 panel. DJ95 binding was calculated as a % inhibition of the binding of a radioactively labeled ligand specific for each target. Compound enzyme inhibition effect was calculated as a % inhibition of control enzyme activity. Results showing an inhibition or stimulation less than 50% are considered insignificant. Both screens were performed in duplicate at 1 μ M concentrations.

2-6B). Additionally, DJ95 did not induce significant alterations in enzyme function for COX1, COX2, PDE3A, PDE4D2, Lck kinase or ACHE. Results showing an inhibition or stimulation higher than 50% are considered significant in this panel, none of which were observed at any of the targets studied in this screening. These results from 2 separate off-targeting screening services suggest that the DJ compounds minimally interact with some of the key off-target liabilities responsible for cessation of clinical drug development.

Pharmacokinetics and Plasma Concentration

Based on the preliminary *in vitro* data suggesting that DJ compounds are potentially active against melanoma cell lines and do not significantly interfere with off-targets, we investigated their *in vivo* anticancer activity. We determined that concentrations up to 30 mg/kg of DJ95 or DJ101 administered by i.p. injection daily was well-tolerated for at least 5 days in nude mice, but additional treatments with DJ95 caused a decrease in mouse weight and decline in behavioral activity. Therefore, we scaled back the dose to 15 mg/kg of DJ95 for the pharmacokinetic and xenograft study. To determine if DJ95 could reach therapeutically relevant biological concentrations at doses of 15 mg/kg, we collected blood samples from 15 minutes to 24 hrs following i.p. injection of the drug and analyzed the plasma concentrations by LC-MS methods. The C_{max} for DJ95 was 13.65 μ M and the detected concentrations stayed above 13 μ M for at least 1.5 hrs in mouse plasma (**Figure 2-7A**). While the concentration of DJ95 gradually decline over the course of the 24 hrs during when samples were collected, there was still an average of 126.5 nM at 12 hrs and 8.1 nM at 24 hrs. This data, along with the AUC (50500 hr*nM) suggests acceptable exposure for DJ95 over the course of a day. We also determined additional pharmacokinetic parameters including half-life (3.28 hrs), volume of distribution to (3.51 L/kg), and clearance (0.744 L/hr/kg). We reasoned that these results supported a dosing regimen of 5 treatments/week, allowing for 2 recovery days to avoid accumulating toxicities.

Tumor Growth Inhibition and Reduction of Metastasis in Melanoma Models in Mice

The antitumor efficacy of the DJ compounds was determined in an A375 xenograft model in nude mice. Tumors were established by subcutaneous inoculation of A375 cells, and treatment began after viable tumors developed. Groups were dosed by i.p. injection with either 15 mg/kg treatments of DJ95, 15 or 30 mg/kg of DJ101, or vehicle solution only. After 2 weeks and a total of 10 treatments, the group of mice receiving 15 mg/kg doses of DJ95 or DJ101 had an average tumor growth inhibition (TGI) of 61.4% and 66.4%, respectively (**Figure 2-7B**). At the higher doses 30 mg/kg of DJ101, the treatment group averaged 92.8% inhibition compared to vehicle control. Dunnett's multiple comparison test indicated that treatments with 15 mg/kg of DJ95 ($P < 0.01$), 15mg/kg of DJ101 ($P < 0.001$), and 30 mg/kg doses of DJ101 ($P < 0.0001$), were

Figure 2-7. Inhibition of tumor growth and metastasis in melanoma mouse models.

A) Plasma concentrations at each timepoint following i.p. injection 15 mg/kg concentration of DJ95 shown as individual replicates connected by mean (n=3 per time point). B) A375 melanoma xenograft model in nude mice. Mice were dosed by i.p. injection 5 times a week for 2 weeks with 15 mg/kg DJ95, 15 or 30 mg/kg of DJ101, or vehicle solution only. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test for the final tumor volumes compared to the control. C) Mean mouse weight change $\pm$ SD in the xenograft model. D) B16F10 melanoma lung metastasis model in C57BL/6 mice. Mice were dosed by i.p. injection 5 times a week for 2 weeks with 30 mg/kg of DJ101 or vehicle solution only. Graph represents mean number of nodules with individual number for each mouse plotted $\pm$ SD (n=11). Representative photos of lungs with melanoma nodules (black dots) are shown below. Statistical significance ($P=0.0001$) was determined with an unpaired student's t-test. E) Mean mouse weight change $\pm$ SD in the metastasis model.

significantly better than the vehicle based on the percent increase in final tumor volume. Animal behavior and mouse body weights were measured and recorded throughout the course of the experiment to assess for acute toxicities, and major deviations were not observed (**Figure 2-7C**). This *in vivo* study demonstrates the antitumor efficacy of DJ compounds in a melanoma xenograft model, and DJ101 for further *in vivo* experiments because of its enhanced antitumor activity without dose-limiting toxicities.

Since a major obstacle in treating melanoma is tumor metastasis, we next assessed the efficacy of DJ101 in suppressing melanoma metastasis using a B16F10 experimental lung metastasis model in mice. After two weeks of treatment with 30 mg/kg doses of DJ101, the average number of tumor nodules that developed on the lungs was 1.7 ± 0.3 , whereas the vehicle-treated group averaged 10.5 ± 2.0 nodules, demonstrating a 6.2-fold decrease in lung metastasis for those receiving DJ101 treatment (**Figure 2-7D**). An unpaired Student's t-test gave a *P* value of < 0.001 , representing a significant decrease in lung metastasis for the treatment group. Body weights and physical activities of mice were normal in both groups (**Figure 2-7E**). These results demonstrate that DJ101 is well-tolerated for doses up to 30 mg/kg in mice and can efficiently reduce the potential for lung metastasis of murine melanoma.

Overcoming Paclitaxel Resistance *In Vivo*

We next compared the efficacy of DJ101 and paclitaxel *in vivo* using both PC-3 and PC-3/TxR xenograft models. Both paclitaxel (15 mg/kg) and DJ101 (30 mg/kg) inhibited tumor growth in the parental PC-3 xenograft model as determined by average tumor volume (**Figure 2-8A, left panel**). The TGI for the group receiving paclitaxel treatment was 101.1% and 78.8% for the DJ101 treated group in PC-3 xenografts (*P* values of < 0.0001). Their efficacy against tumors was also evident based on final tumor weight (**Figure 2-8B**) and representative tumor images (**Figure 2-8C**), where the DJ101- and paclitaxel-treated groups showed a 47.1% ($P < 0.001$) and 79.3% ($P < 0.0001$) reduction in tumor weight compared to the control group, respectively. No acute toxicities were observed based on physical activity and body weights (**Figure 2-8D**). In contrast, using the same dosing regimen and frequency as we did in the parental PC-3 model, DJ101 caused a TGI of 104.0%, remarkably demonstrating an overall reduction in tumor volume in the PC-3/TxR xenograft model, whereas paclitaxel only modestly inhibited tumor growth by 37.8% (**Figure 2-8A, right panel**). These results were corroborated by final tumor weights, where DJ101 caused a 77.3% reduction ($P < 0.0001$) and paclitaxel showed only a 35.6% reduction ($P < 0.05$) compared to the control group (**Figure 2-8B**). No acute toxicities were observed in this model based on body weight (**Figure 2-8D**).

Elevated Apoptosis and Vascular Disruption in Xenograft Tumors

Hematoxylin and eosin (H&E) stains of representative tumor sections in the vehicle control group showed aggressive growth with numerous mitotic cells in different

Figure 2-8. Inhibition of taxane-resistant prostate cancer tumors in mouse models.

A) Tumor volumes for PC-3 (left panel) and PC-3/TxR (right panel) xenograft model in nude mice. Graphs represent mean tumor volume percent increase $\pm$ SEM (n=7). B) Final tumor weights $\pm$ SEM for PC-3 and PC-3/TxR mice. C) Representative images for PC-3 and PC-3/TxR tumors. D) Mouse weight change $\pm$ SEM for PC-3 and PC-3/TxR mice. One-way ANOVA analysis was performed for final tumor volumes and weight followed by Dunnett's multiple comparison test of each treated group with the corresponded results of vehicle group. (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$)

stages (**Figure 2-9A**). The increase of metaphase cells and decrease of anaphase cells in the DJ101 treatment groups indicate mitotic blocking in the G₂/M phase, consistent with our previous cell cycle analysis (143). Tumor sections from both low and high dose treatment groups showed significantly less proliferation, and there were abundant apoptotic cells showing dense nuclear pyknosis and cytoplasmic karyorrhexis. There was also extensive central tumor necrosis in the treatment groups. Additionally, CD31 labeled endothelial cells in the vehicle control tumor sections exhibited well-developed networks of capillaries or small blood vessels around tumors, while the DJ101 treatment groups displayed severely distorted blood vessels or absence of CD31 positive stains, suggesting potential vascular disrupting properties of DJ101. Finally, cleaved caspase-3 IHC staining of tumor sections in the control group showed very few positive regions, but tumor sections from the DJ101 treatment groups showed a profound increase in positive labeled cells, confirming enhanced apoptosis due to DJ101 treatment.

Separate and more extensive analysis of tumor vasculature was performed on the DJ95 treated xenografts. CD31 staining of tumor sections revealed the change in microvessels of the DJ95 treated group compared to the control group (**Figure 2-9B**). The control group displayed more abundant and in-tact microvessels throughout the tumors, whereas DJ95 treatment tumors showed vessel fragmentation and decreased overall density and occupied area. Quantification of the positive CD31-stained area revealed that there was a 50.5% decrease in total microvessel area, representing a significant difference ($P= 0.0038$) (**Figure 2-9C**). These findings validate the *in vitro* results and suggest that the anti-vascular capacities portrayed by DJ95 and DJ101 may contribute to their anticancer efficacy.

Toxicology Analysis of Major Organs

To assess whether DJ101 treatment produces potential organ toxicities, pathological analysis was performed on major organs collected from both melanoma *in vivo* studies including the heart, kidney, liver, lung and spleen. H&E stained sections revealed no apparent drug-related injury or pathological changes in the cellular structure of the various tissues in the melanoma xenograft mouse models (**Figure 2-9D**) and the experimental lung metastasis mouse model (**Figure 2-9E**), providing further support of an overall acceptable safety profile.

Discussion

Importance of Overcoming Drug Resistance

Melanoma is the most aggressive form of skin cancer and is one of the most rapidly increasing cancers worldwide (204). Malignant melanoma is characterized by resistance to chemotherapy and is incurable in most affected patients (205, 206). Despite

Figure 2-9. Immunohistochemistry analysis of tumors and pathological assessment of major organs.

A) IHC of DJ101 treated A375 tumors. H&E insert (2 fold) shows white arrows indicating metaphase and black arrows indicating anaphase. CD31 expression shows blood vessel disruption and expression of cleaved caspase-3 is indicative of apoptosis. B) Representative IHC images of DJ95 treated tumors stained with CD31 (10x magnification). C) CD31 area represented as mean pixels $\pm$ SD. Statistical significance was determined by student's t test (20x magnification, 5 images per tumor, 3 separate tumors per group) (** $p < 0.01$). D) Representative images of H&E stained sections of major tissues (heart, kidney, liver, lung, and spleen) from the A375 xenograft and E) B16F10 lung metastasis study.

recent advances in treating melanoma with targeted therapies and immunotherapies, significant obstacles still exist for finding satisfactory treatments. Intrinsic and acquired resistance are the major causes of treatment failure, and it is crucially important to discover and develop agents that can overcome drug resistance, improve response rates, and extend survival for melanoma patients.

Interfering with tubulin dynamics is a validated approach for anticancer treatment and many antimetabolic microtubule stabilizers and destabilizers are widely used clinically or are undergoing clinical development (207). However, many of these agents cause neurotoxicity, exhibit chemical instability, or have elevated metabolic clearance (172). Additionally, the clinical efficacy of many FDA approved tubulin inhibitors is often limited by drug efflux pumps or overexpression of certain tubulin subtypes, most notably the β III tubulin isotype (54). Design and development of new tubulin inhibitors targeting the colchicine binding site represent an attractive approach for improving and advancing tubulin inhibitors.

We previously identified DJ95 and DJ101, small-molecule tubulin destabilizing agents, synthesized from a series of indolyl-imidazopyridines, that demonstrated high potency and improved metabolic stability *in vitro* (143). Because genetic heterogeneity among cancer cell lines of the same cancer type has emphasized the necessity to study multiple cell lines in a panel, we expanded our investigation of the compounds in a more extensive array of malignant melanomas with varying degrees of genetic complexity based on genomic mutations (BRAF, CTNNB1, CDKN2A, PTEN, or TP53)(208). The DJ compounds were also screened in the NCI-60 panel, which includes numerous extensively characterized cell lines from nine different tumor types, providing additional evidence to support their broad-spectrum anticancer efficacy.

While taxanes are some of the most clinically effective chemotherapy drugs available, their clinical success is often limited by the emergence of intrinsic and acquired drug resistance (209). One of the major culprits responsible for contributing to MDR is the overexpression of ABC transporters such as ABCB1 (MDR1), ABCC1 (MRP1), and ABCG2 (BCRP) (210). Agents that target the taxane and vinca binding sites of tubulin are particularly susceptible to resistance from the overexpression ABCB1 and are effectively extruded from the cell (209, 211-213). These agents are also substrates to MRP and BCRP proteins that also decrease their intracellular concentrations (214). We discovered that DJ95 had a lower resistance index than the other tested tubulin inhibitors in both the drug-selected and gene-transfected cell lines overexpressing ABCC1. While it did not show resistance to the transfected ABCB1 overexpressing cells, it did display some resistance to KB-C2 drug-selected cell line overexpressing ABCB1. This might be due to the more complex MDR mechanisms in drug-selected MDR cells in comparison with gene-transfected cells. As KB-C2 cells were established by continuous selection with colchicine (2 μ g/mL), they may have incurred additional drug-resistance mechanisms besides ABCB1 upregulation. For example, mutations to the colchicine binding domain could account for the cross-resistance to DJ95 or other agents targeting this site.

We also investigated the effect of DJ compounds against drug-selected taxane resistant cell lines. In the paclitaxel-resistant PC-3/TxR prostate cancer cell line, more than 200 genes are upregulated, in addition to P-gp overexpression, which representing numerous paclitaxel resistance mechanisms (174, 175). Both DJ compounds maintained potency in the resistant PC-3/TxR cell line and had significantly lower resistance indexes to both PC-3/TxR and DU-145/TxR cells than did colchicine and paclitaxel. We also demonstrated the excellent potency of DJ101 against PC-3/TxR xenograft tumors in mice. Other groups have reported elevated sensitivity of paclitaxel resistant cancer cells to colchicine binding site drugs. One such study investigated a variety of microtubule targeting agents that bind to the taxane site, vinca alkaloid binding site, and colchicine binding site for docetaxel resistant MCF-7 breast cancer cells. They reported that while MCF-7_{TXT} cells demonstrated cross-resistance to vinca alkaloids, they were more sensitive to colchicine binding site agents, including 2MeOE2, ABT-751, CA-4P, and colchicine, than the non-resistant counterpart MCF-7 cells (215). This also supports the notion that colchicine binding agents such as the DJ compounds, particularly DJ101, may be an alternative treatment when tumors acquire resistance to treatment by taxanes.

Optimizing Scaffolds Through X-Ray Crystallography

Tubulin inhibitors interacting with the colchicine binding site have widely diverse structures. Thus, it has been very challenging to reliably decipher the molecular interactions between an inhibitor and tubulin using molecular modeling with crystal structures containing a different class of tubulin inhibitors. Furthermore, available X-ray crystal structures often have low resolution which further impedes reliable molecular modeling studies.

We obtained the high-resolution X-ray crystal structures of DJ101 and DJ95, showing their interaction with colchicine binding pocket at the interface of α - and β -tubulin heterodimer. These X-ray crystallography results not only provide the structural basis for the anticancer mechanism of action of the DJ compounds, they underline the benefit of utilizing high-resolution crystal structures over molecular modeling in guiding structure optimizations for tubulin inhibitors interacting at this site. For example, three hydrogen bonds formed between DJ101 and the tubulin dimer and additional strong hydrophobic interactions from surrounding residues firmly anchored DJ101 in this colchicine binding site. Interestingly, our previous molecular modeling studies using the crystal structure of 1SA0 showed a different binding pose for DJ101, in which the top portion of DJ101 was rotated (143). Examination of the crystal structures of 1SA0 and the current DJ101 complex clearly revealed significant conformational changes for the T4 loop in the tubulin α -subunit and T7 loop in the tubulin β -subunit. It is apparent that the “closing up” of the T4 loop to the colchicine binding pocket in the 1SA0 structure prevented the top moiety of DJ101 in adopting its true binding pose, and thus forced its top moiety to rotate 180 degrees.

It is well known that while the colchicine site can accommodate a wide variety of structurally distinct molecules, and seemingly insignificant changes to many of these

compounds can result in a total loss of activity (54, 94). These results highlight critical contributions to the optimal bindings of different scaffolds at the colchicine site in tubulin. The detailed crystallographic analysis presents opportunities for designing better analogs of these compounds that may show improved potency.

Targeting Microtubule Dynamics

The main function of microtubule targeting agents is to inhibit mitosis through disruption of microtubule dynamics, leading to mitotic arrest and eventual apoptosis. Paclitaxel and docetaxel are microtubule stabilizing agents that target the taxane site within the lumen of polymerized microtubules and alter their conformation to the more stable GTP-bound β -tubulin structure, thereby locking them in the polymerized state (23). The DJ compounds are destabilizing agents that bind to the colchicine site, which causes interdimer bending and promotes destabilization (23). To further confirm the mechanism of action of the DJ compounds and evaluate the physical change in microtubule networks, we performed immunofluorescent imaging studies of melanoma cells that had been treated with the DJ compounds or taxanes. In the untreated cells, the microtubule networks displayed an arrangement of organized, fibrous microtubules extending throughout the elongated cells. Treatment with the DJ compounds led to fragmented microtubules and a dramatic decrease of visible tubulin filaments. On the contrary, paclitaxel treated cells demonstrated rigid and condensed polymeric microtubules. The immunofluorescent images clearly differentiated the stabilizing and destabilizing effects on microtubule structure and cellular framework by these two opposing classes of compounds.

Microtubules constitute the mitotic spindle in cells undergoing mitosis. Disruption of the mitotic spindle through suppression of treadmilling and dynamic instability are the primary means by which microtubule inhibitors thwart cellular functions and induce apoptosis (216). Typical features of mitotic spindle suppression and aberrant formation such as multiple asters in mitotic cells were evident in the DJ95 treated cells, whereas the control cells demonstrated polar spindle formation and centrally aligned chromosomes.

In addition to their well-defined roles in supporting cellular structure and their involvement in mitosis, microtubules are also implicated in cell migration and motility. It has been suggested that the dynamic instability imposed on the microtubules by suppressing and interfering with tubulin behavior causes cell immobility, as the cell is less able to remodel and respond to change the cell shape demanded for cell migration (198). The antimitotic and anti-migratory effects of the DJ compounds were demonstrated by their ability to inhibit anchorage-dependent colony propagation and suppress cell motility in a wound healing assay. The cessation of colony formation and cell movement was attributed to disruption of microtubule dynamics, though additional studies are needed to further elucidate the mechanism of microtubule binding drugs on different facets of cellular function.

Minimizing Failure in Drug Development

Off-target adverse drug reactions often contribute to the high attrition rate in the drug discovery and development process. Reducing the failure rate of potential pharmaceutical drug candidates is an important step in drug discovery and development and will help to mitigate the risk of expensive failure in late stages. Identification of off-target, adverse drug reactions for drug candidates to important pharmacological targets such as GPCRs, drug transporters, ion channels, nuclear receptors, kinases and non-kinase enzymes through the use of predictive safety panels is supported by major pharmaceutical companies such as AstraZeneca, GlaxoSmithKline, Novartis and Pfizer (203). DJ101 and DJ95 underwent this pharmacological profiling in two separate panels (DiscoverX Safety47™ Panel and Eurofins SafetyScreen44™ Panel). DJ95 did not show significant inhibition or activation to of the receptors or enzymes tested in the screening, DJ101 only elicited a functional response to NET and GR antagonism when tested at concentrations significantly higher than its IC₅₀. This suggests that the DJ compounds minimally interact with or activate some of the key targets that potentially cause adverse drug reactions, suggesting a good safety profile for this scaffold.

Anticancer Efficacy

We proceeded with *in vivo* studies and found that the DJ compounds were able to hinder melanoma tumor growth at doses of 15 mg/kg, and at higher concentrations of 30 mg/kg, DJ101 dramatically inhibited tumor growth and reduced metastasis without causing signs of toxicity.

DJ101 was able to induce tumor regression in a taxane-resistant prostate cancer xenograft models in mice, whereas paclitaxel had almost no effect. Other groups have also reported elevated sensitivity of resistant cancer cells to colchicine binding site drugs. One such study investigated a variety of microtubule targeting agents that bind to the taxane site, vinca alkaloid binding site, and colchicine binding site for docetaxel resistant MCF-7 breast cancer cells. They reported that while MCF-7_{TXT} cells demonstrated cross-resistance to vinca alkaloids, they were more sensitive to colchicine binding site agents including 2MeOE2, ABT-751, CA-4P, and colchicine than the non-resistant counterpart MCF-7 cells (215). This also supports the notion that colchicine binding agents such as DJ101 may be an alternative treatment when tumors acquire resistance to treatment by taxanes.

Because antimitotic drugs exploit different sites on tubulin and microtubules, synergistic combinations could be investigated to optimize their clinical usefulness. This assertion is supported by several reported studies that combined paclitaxel with other tubulin targeting drugs (217-220). It will also be important to combine novel tubulin inhibitors such as the DJ compounds with agents exploiting different cancer targets. Towards this end, we previously demonstrated that strong synergy can be achieved by combining ABI-274 (a less metabolically stable analog of DJ101) and vemurafenib in a BRAF inhibitor resistant-melanoma xenograft model (100). Continued exploration of

combination therapies is warranted to maximize the clinical potential of the DJ compounds.

Dual Inhibitors of Tubulin and Tumor Vasculature

It is well-appreciated that tumor growth and metastasis require a reliable system of blood vessels to support the tumor microenvironment, and interfering with this process is an attractive strategy for inhibiting tumor growth (39, 221). This has prompted research in the development of anti-angiogenic and vascular disrupting agents, and many tubulin inhibitors that target the colchicine binding site demonstrate this capability (39, 40, 97, 222). Microtubule stabilizing agents such as paclitaxel, Peloruside A, and laulimalide also have reported vascular disrupting properties, but they have other shortcomings, such as the development of resistance and dose-limiting toxicities (223-226). We demonstrated that DJ95 treatment suppressed capillary-like networks *in vitro* in HUVECs in a concentration-dependent manner. We also discovered vascular disrupting action evident in mouse xenografts after treatment with the DJ compounds, where there was significant disturbance of endothelial vasculature and an overall decrease in microvessel area for these treatment groups compared to control tumors. These *in vitro* and *in vivo* data support the dual role of the DJ compounds as tubulin inhibitors and vascular disrupting agents, and they may exert their anticancer effects through multiple mechanisms.

Conclusions

In our examination of DJ101 and DJ95, we found that they were potent against a variety of melanoma cell lines and other cancer types and were able to overcome ABC-transporter mediated drug resistance and acquired taxane resistance. We obtained the high-resolution crystallography of both compounds in complex with tubulin, confirming their direct interaction with the colchicine binding domain and demonstrated their depolymerizing effect microtubule morphology, which was distinct from stabilizing agents. Both compounds show antitumor efficacy in melanoma xenograft models without causing apparent toxicity to major organs and display good pharmacological safety profiles. DJ101 treatment also reduced lung metastasis nodules in a metastatic model and caused tumor reduction in the paclitaxel-resistant xenograft model. The DJ compounds also disrupted endothelial cells and tumor vasculature, indicating that they may play a role as vascular disrupting agents in addition to tubulin inhibitors. There are currently no colchicine binding site inhibitors approved for chemotherapy, and intervention with the DJ compounds could prove to be an effective alternative treatment when other tubulin inhibitors fail to show efficacy due to MDR.

CHAPTER 3. OPTIMIZING VERU-111 ANALOGS FOR IMPROVED ANTICANCER EFFICACY²

Introduction to VERU-111 Analogs

The microtubule targeting agents have achieved great success in cancer treatment in the last decade. However, acquired drug-resistance is often developed over the course of treatment and becomes a tremendous obstacle. It is estimated that 22 million new cancer cases will be diagnosed worldwide in the coming two decades, and therefore an urgent need exists to develop new anticancer agents to treat resistant phenotypes (157, 227). Mechanisms that mediate drug-resistance against microtubule targeting agents include, but are not limited to, the overexpression of membrane-bound drug efflux proteins, such as P-glycoprotein; the overexpression of β -tubulin isotypes; and microtubule mutations (146, 228).

Colchicine binding site inhibitors target tubulin at the interface between α and β -tubulin heterodimers and mechanistically decrease cellular motility, alter cell morphology, impair protein assembly, and arrest mitosis (229, 230). Colchicine has wide-scope mechanisms of action, but unfortunately, it induces systemic toxicities and multiorgan dysfunction that have prevented its clinical use for cancer treatment (229). Other colchicine binding site inhibitors have also been disclosed in the last decade. Encouragingly, extensive preclinical studies have proven that drugs targeting the colchicine binding site may suppress overexpression of tubulin isotypes and surmount drug-resistance mediated by P-gp, MRP1, and MRP2 (81, 94, 231).

The 3,4,5-trimethoxyphenyl (TMP) is a common moiety shared by many drugs targeting the colchicine site and is crucial for maintaining suitable molecular conformations that are needed for optimal interactions with tubulin and for producing the maximum antiproliferative activities (232). Attempts to modify this TMP moiety usually lead to significantly reduced anticancer potency (94, 97, 165, 233). For example, substituting the methoxy with a bulky group or demethylating the methoxy on the TMP moiety of colchicine were reported to reduce the potency (234, 235). In addition, removing or adding methoxy to TMP significantly impaired the antimitotic activity (>10-fold) (236). However, some isosteric modifications of the TMP in colchicine or CA-4 have been shown to maintain the potency to that of parent natural products. For instance, Semenov et al. have demonstrated that isosteric replacement of the TMP of CA-4 with [1,3]dioxole or [1,4]dioxane did not significantly reduced antimitotic activity (236).

²Modified with permission from American Chemical Society. Qinghui Wang*, Kinsie E. Arnst*, Yuxi Wang*, Gyanendra Kumar, Dejian Ma, Hao Chen, Zhongzhi Wu, Jinliang Yang, Stephen W. White, Duane D. Miller, and Wei Li. Structural Modification of the 3,4,5-Trimethoxyphenyl Moiety in the Tubulin Inhibitor VERU-111 Leads to Improved Antiproliferative Activities. *Journal of Medicinal Chemistry*. 2018; 61 (17), 7877-7891.

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Cornigerine, a [1,3]dioxole analog of colchicine, displayed equipotent antimitotic activity to that of the parent compound (237).

In our prior reports, we described the discovery of several scaffolds of potent tubulin inhibitors, including the discovery of a 2-aryl-4-benzoyl-imidazole (ABI) scaffold (140). Subsequent structural optimization of the benzoyl moiety, the aryl moiety, and the imidazole fragment in this scaffold resulted in analogs with improved potency (98, 141, 143, 144). VERU-111 is the best analog from that series of structural optimizations. VERU-111 is currently under clinical development as 3rd line hormonal therapy by VERU Healthcare. Interestingly, it also exhibits potent antiproliferative activities against melanoma cell lines and acts as a colchicine binding site tubulin inhibitor. Due to an unproductive chemical synthesis, its in-depth medicinal programmatic investigation was nevertheless hampered. The TMP moiety in VERU-111, like many other colchicine binding site inhibitors with this shared moiety, is critical for its high binding affinities to tubulin.

In this chapter, we report the extensive structure-activity relationship (SAR) study of VERU-111 analogs by focusing modifications to the indole moiety and TMP moiety, leading to the identification of the 3 most potent compounds, **10ab**, **10bb**, and **13f**. Mechanism studies confirmed that the new VERU-111 analogs inhibit tubulin polymerization and promote microtubule fragmentation and disassembly. Direct binding to the colchicine binding domain on tubulin is revealed through X-ray crystallography, and specific molecular interactions and overlay with colchicine are detailed. The compounds demonstrated good *in vitro* metabolic stability, and **10bb** and **13f** showed significant anticancer efficacy in melanoma mouse models without demonstrating toxicity to animals or significantly inducing off-target interactions based on pharmacological safety screening. Finally, **10bb** was highly effective in a taxane-resistant prostate cancer xenograft model, suggesting its ability to overcome resistant mechanisms. Overall, these data demonstrate great potential for these VERU-111 analogs as anticancer drug candidates.

Materials and Methods

Cell Culture and Reagents

Human melanoma carcinoma cell lines A375, M14, RPMI-7951, and WM-164 (American Type Culture Collection or ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis MO). Murine melanoma B16F10 cells (ATCC, Manassas, VA, USA) were cultured in Minimum essential medium (Invitrogen, Carlsbad, CA), supplemented with 5% heat-inactivated Hyclone FBS (Thermo Scientific, Rockford, IL), 1 % antibiotic-antimycotic mixture (Sigma-Aldrich, St. Louis MO), 1% Mem-sodium pyruvate (Invitrogen, Carlsbad, CA), 1% Mem-vitamin

(Invitrogen, Carlsbad, CA), L-Glutamine (2mM final concentration) (Invitrogen, Carlsbad, CA), and 1% Mem NEAA (Invitrogen, Carlsbad, CA). Parental prostate cancer PC-3, its paclitaxel-resistant daughter line PC-3/TxR, parental prostate cancer DU-145, and its docetaxel-resistant daughter line DU-145/TxR are gifts from Dr. Evan Keller at the University of Michigan Medical School. PC-3 and DU-145 cell lines were cultured in RPMI 1640 media (Gibco® by Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis MO). Taxane resistant PC-3/TxR and DU-145/TxR cell lines were cultured in the same media and additionally supplemented with 10 nM paclitaxel or docetaxel, respectively. Drugs were not included in the media for PC-3/TxR or DU-145/TxR for at least one week prior to *in vitro* testing. All cell lines were authenticated by ATCC by short tandem repeat profiling. Cultures were maintained to 80-90% confluency at 37 °C in a humidified atmosphere containing 5% CO₂. Compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to make a stock solution of 20 mM. Compound solutions were freshly prepared by diluting stocks with cell culture medium before use.

Cytotoxicity Assays

Logarithmic growth phase cells were seeded in 96-well plates at a concentration of 1,000–5,000 cells per well, depending on growth rate of the cell line. After overnight incubation, the media was replaced, and cells were treated with the test compounds at 10 concentrations ranging from 0.03 nM to 1 μ M plus a vehicle control for 72 h in four replicates. Following treatment, the MTS reagent (Promega, Madison, WI) was added to the cells and incubated in dark at 37 °C for at least 1 h. Absorbance at 490 nm was measured using a plate reader (BioTek Instruments Inc., Winooski, VT). Percentages of cell survival versus drug concentrations were plotted, and the IC₅₀ (concentration that inhibited cell growth by 50% of untreated control) values were obtained by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA). The resistant index (RI) was calculated by dividing the IC₅₀ of the resistant cell line with the IC₅₀ of the parental cell line.

X-Ray Crystallography

Protein Expression and Purification. X-Ray crystallography was performed by Dr. Yuxi Wang (Sichuan University, in China). Porcine brain tubulin (Catalog # T-238P) was obtained from Cytoskeleton, Inc. The stathmin-like domain of RB3(RB3-SLD) was transformed into and overexpressed in *E. coli*. The protein was purified by anion-exchange chromatography and gel filtration chromatography. The peak fractions from gel filtration column were concentrated to 10 mg/mL and stored at -80 °C (51, 154, 185). TTL protein was expressed and purified from *E. coli* expression system as described in the previous reference (186). Briefly, the protein was expressed in *E. coli* using LB, purified through Ni-NTA affinity chromatography and gel filtration chromatography (buffer: Bis-Tris Propane pH 6.5, 200 mM NaCl, 2.5 mM MgCl₂, 5 mM β -Me, 1 %

Glycerol). The peak fractions of the target protein were collected and concentrated to 20 mg/ml and saved at -80 °C. Porcine brain tubulin was supplied at 10 mg/ml in G-PEM (General tubulin buffer: 80 mM PIPES pH 6.9, 2 mM MgCl₂, 0.5 mM EGTA and 1 mM GTP) as a frozen liquid and saved at -80 °C until use.

Crystallization and Crystal Soaking. The process to getting crystals of T2R-TTL followed the previously reported procedure (186, 187). Briefly, the complex containing tubulin (10 mg/ml), TTL (20 mg/ml) and RB3 (10 mg/ml) at the molar ratio of 2:1.3:1.2 (tubulin:RB3:TTL) was incubated on ice with additional 1 mM AMPPCP, 5 mM tyrosinol and 10 mM DTT, and then the final sample was concentrated to 20 mg/ml at 4 °C. Crystallization of T2R-TTL complex was carried out at 20 °C using the sitting drop vapor diffusion method by mixing equal volumes of the protein complex and crystallization buffer containing 6% PEG, 5% glycerol, 0.1 M MES, 30 mM CaCl₂, 30 mM MgCl₂, pH 6.7. Seeding was used to optimizing the crystal conditions. Initial crystals were observed after two days and crystals reached their final size of 200-300 μm within 3-5 days. For crystal soaking, 0.1 μl of the ligand solution (**13f** dissolved in 100% DMSO) was added to the 2 μl crystal-containing drop for 12 h at 20 °C. The best crystals were selected and frozen in liquid nitrogen in the presence of cryoprotectant (crystallization buffer containing 20% glycerol).

X-Ray Data Collection and Structure Determination. Crystals of the T2R-TTL-**13f** complexes were mounted in nylon loops and flash-cooled in a nitrogen stream at 100 K. The diffraction data were collected on beamlines BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF) in Shanghai, China. Data were indexed, integrated and scaled using the HKL2000 program package (188). The structure of T2R-TTL-**13f** complex was solved by molecular replacement using the previously published T2R-TTL structure (PDB ID: 4I55) as the search model. The rotation and translation function searches were performed by the program PHASER. The model was manually built with Coot (190) and then refined using the phenix refine module of the Phenix program (191). The model quality was checked with the PROCHECK program showed good stereochemistry according to the Ramachandran plot. Analysis was determined in coordination with Dr. Stephen White's group (St. Jude Children' Research Hospital).

Tubulin Polymerization Assay

Bovine brain tubulin (3 mg/ml final concentration) (Cytoskeleton, Denver, CO) was mixed with 10 μM of the test compounds and incubated in 100 μL of general tubulin buffer (80 mM PIPES, 2.0 mM MgCl₂, 0.5 mM EGTA, and 1 mM GTP) at pH 6.9. The absorbance of wavelength at 340 nm was monitored every 1 min for 40 min by the SYNERGY 4 Microplate Reader (Bio-Tek Instruments, Winooski, VT). The spectrophotometer was set at 37 °C for tubulin polymerization.

Fluorescence Microscopy

WM-164 cells were seeded and allowed to adhere on glass coverslips in 6 well plates (500,000 cells/well) overnight. Cells were incubated for 18 hrs with 50 nM of **10ab**, **10bb**, or docetaxel. DMSO was added to the cells in equivalent volume as a negative control. Microtubules were visualized with anti- α -tubulin antibody (Thermo Scientific, Rockford, IL) and Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes, Eugene, OR). The coverslips were mounted with Prolong Diamond Antifade mounting media with DAPI (Invitrogen, Eugene, OR) and images acquired with a Zeiss 710 Confocal microscope and Zen imaging software (Zeiss, Thornwood, NY).

Colony Forming Assay

Cells were seeded in triplicate in 6 well plates at a density of 500 cells/well in full cell culture media and incubated at 37 °C overnight. Media was changed and cells were treated with 5 nM of the test compound and incubated for another week. At the end of this period, cells were fixed with chilled methanol (5 min), stained with 0.5% crystal violet solution (4 hrs), rinsed with sterile water, and air-dried overnight. Images were taken UVP ChemiDoc-It imager (Bio-Rad, Hercules, CA) and colonies were quantified with the corresponding VisionWorks software.

Scratch Migration Assay

A375 and RPMI-7951 cells were seeded in 24 well plates in 4 replicates at a concentration of 200,000 cells/well and incubated overnight. This concentration allowed a confluent monolayer to form in the wells. Using a 200 μ L pipette tip, a straight line was scratched through the cell monolayer to remove an area of cells. The plates were then washed several times with media to remove any debris and uprooted cells. Cells were treated with 25 nM of **10ab**, **10bb** or vehicle control. Images were obtained immediately after drug treatment and again after 18-22 hours with Evos Fl Imaging System (LifeTechnologies, Carlsbad, CA). Analysis was performed with ImageJ software (NIH, Bethesda, MD).

Liver Microsomes Stability Assay

Liver microsome assays were performed by Dr. Lei Yang (St. Jude Children's Research Hospital). The NADPH regenerating agent solutions A (catalog#: 451220) and B (catalog#: 451200) and mouse liver microsomes (CD-1, mixture of male, catalog#:452701, and female, catalog#: 452702) were obtained from BD Gentest (Woburn, MA). Liver microsomes stability assay was conducted following literature reports^(238, 239). For each test compound, the mouse liver microsomal solution was prepared by adding 0.058 ml of concentrated mouse liver microsomes (20 mg/ml protein concentration) to 1.756 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing 5 μ L

of 0.5 M EDTA to make a 0.6381 mg/ml (protein) microsomal solution. Each test compound (2.2 μ L of 10 mM DMSO solution) was added directly to 1.79 ml of mouse liver microsomal solution and 90 μ L was transferred to wells in 96-well plates (0, 0.25, 0.5, 1, 2, and 4 h time points each in triplicate). The NADPH regenerating agent was prepared by mixing 0.113 ml of NADPH regenerating agent Solutions A, 0.023 ml of solution B and 0.315 ml of 0.1 M potassium phosphate buffer (pH 7.4) for each tested compound. To each well of the 96-well plate, 22.5 μ L of the NADPH regenerating agent was added to initiate the reaction, and the plate was incubated at 37 °C for each time point (0, 0.25, 0.5, 1, 2, and 4 h time points each in triplicate). The reaction was quenched by adding 225 μ L of cold acetonitrile containing warfarin (4 mg/ml) as internal control to each well. All of the plates were centrifuged at 4000 rpm for 20 min and the supernatants (100 μ L) were transferred to another 96-well plates for analysis on UPLC–MS (Waters Acquity UPLC linked to Waters Acquity Photodiode Array Detector and Waters Acquity Single Quadrupole Mass Detector) on Acquity UPLC BEH C18 1.7 mm (2.1x50 mm) column by running 90–5% gradient for water (+0.1% formic acid) and acetonitrile (+0.1% formic acid) in 2 minutes. The area under the single ion recording (SIR) channel for the test compound divided by the area under the SIR for internal control at 0 time concentration was considered as 100% to calculate remaining concentration at each time point. The terminal phase rate constant (k_e) was estimated by linear regression of logarithmic transformed concentration versus the data, $k_e = \text{slope} \times (-\ln 10)$. The half-life $t_{1/2}$ was calculated as $\ln 2/k_e$. The intrinsic clearance ($CL_{int,app}$) = $(0.693/\text{in vitro } t_{1/2}) \times (1\text{ml incubation volume}/0.5\text{ mg of microsomal protein}) \times (45\text{ mg microsomal protein/gram of liver}) \times (55\text{ g of liver/kg body weight})$.

In Vivo Experiments

All animal experiments were performed in accordance with the NIH animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center (UTHSC, Memphis, TN).

Xenograft Models. Nude mice, 6–8 weeks old, were purchased from Harlan/Evigo. Logarithmic growth phase A375 or PC-3/TxR (5×10^7 cells per ml) cells were prepared in phenyl red-free, FBS-free cell culture media and mixed at a 1:1 ratio with Matrigel. Tumors were established by injecting 100 μ L of cell suspension subcutaneously in the dorsal flank of each mouse (2.5×10^6 cells). After 2 weeks, mice were randomly divided into control or treatment groups ($n=8$ for A375 model of **13f**, $n=10$ for A375 model of **10bb**, and $n=8$ for PC-3/TxR model of **10bb**). Test compounds were dissolved in a 1:1 ratio of PEG300:PBS solution to produce desired concentrations. 100 μ L of the drug solution corresponding to 15 or 30 mg/kg doses were administered via i.p. injection once daily for the duration of the study. The control group was given only a vehicle solution of the same preparation and injected at the same frequency.

Tumor volume was measured three times a week with a caliper and calculated by using the formula $a \times b^2 \times 0.5$, where a and b represented the larger and smaller diameters, respectively. Tumor growth inhibition (TGI) at the conclusion of the experiments was

calculated as $100 - 100 \times ((T - T_0)/(C - C_0))$, where T , T_0 , C and C_0 are the mean tumor volume for the specific group on the last day of treatment, mean tumor volume of the same group on the first day of treatment, mean tumor volume for the vehicle control group on the last day of treatment and mean tumor volume for the vehicle control group on the first day of treatment, respectively (100). Animal activity and body weight were monitored during the entire experiment period to assess potential acute toxicity. At the end of the experiment, mice were sacrificed and the tumors and tissues were dissected out and fixed in 10% buffered formalin phosphate solution for more than 1 week before pathology staining analysis.

Lung Metastasis Model. C57BL/6 mice from Charles River Laboratories International, Inc., age 7–8 weeks old, were used to study the inhibition effect of **10bb** on lung metastasis of melanoma cells. B16F10 melanoma cells growing in a logarithmic growth phase were suspended in the conditioned media at a density of 1×10^6 per ml. Each mouse was injected with 100 μ L (1×10^5 cells) via the lateral tail vein. The treatment started on the third day after the inoculation to ensure the initiation of metastasis before beginning treatment. **10bb** (30 mg/kg) was formulated as described above. All the treatment solutions were kept in the same volume (100 μ L) and administered via i.p. injection for 2 weeks, 5 days a week. Vehicle control group was treated by i.p. injection with 100 μ L of PEG300 and PBS in a 1:1 ratio. Mice were sacrificed after 15 days after the initiation of the experiment, and the lungs were separated, expanded and preserved in 10% neutral buffered formalin. The number of lung metastasis nodules was recorded. Major organs were also preserved in 10% neutral formalin buffer for subsequent examination of potential toxicities. Animal activity and body weight were monitored during the entire experiment period to assess acute toxicity.

Results and Discussion

SAR Investigation

VERU-111 Analogs of 3-Indolyis. To compare the cytotoxicity of VERU-111 that was reported previously, new VERU-111 analogs were evaluated for their cytotoxic effect in human melanoma cell lines including A375, WM-164, and M14 or RPMI-7951. Colchicine was used as a positive control, and VERU-111 was used as a prototype for comparison. In the first series of compounds developed to optimize the potency and investigate the SAR of VERU-111 analogs, a substitution at the 2-position of the indole ring was introduced (**Table 3-1**). Compared to the prototype VERU-111, analog **10aa** containing a bulky phenyl group on the 2-position of indole moiety showed markedly reduced activity and had IC_{50} s more than 1 μ M against three melanoma cell lines. This indicated that bulky substituent on the indole might not be tolerated. We next compared 6 analogs with a substitution at the 4-position of the indole ring, including **10ab-10ag**. The best inhibitory effect was observed in 4-methyl analog **10ab**, which had IC_{50} s ranging from 1.7-3.2 nM and was ~3-fold more potent than VERU-111. Analog **10ac** had a small

Table 3-1. *In vitro* cytotoxicity of VERU-111 analogs modifying the indole moiety.

Compound scaffold	ID	R	IC ₅₀ ± SEM (nM)		
			A375	M14	WM-164
VERU-111	H	8.1 ± 1.6	5.6 ± 0.9	7.2 ± 0.9
	10aa	2-Ph	>1000	>1000	>1000
	10ab	4-CH ₃	1.8 ± 0.2	1.7 ± 0.2	3.2 ± 0.3
	10ac	4-F	7.2 ± 0.7	8.2 ± 0.9	ND
	10ad	4-Br	13.8 ± 1.7	10.4 ± 1.2	67.1 ± 14.5
	10ae	4-OCH ₃	>1000	>1000	>1000
	10af	4-OBn	>1000	>1000	>1000
	10ag	4-OH	9.2 ± 1.4	8.6 ± 0.8	19.1 ± 1.8
	10ah	5-CH ₃	277.7 ± 36.3	177.4 ± 16.3	552.3 ± 108.3
	10ai	5-F	19.2 ± 1.5	18.0 ± 1.3	80.9 ± 16.6
	10aj	5-Cl	61.8 ± 9.3	64.9 ± 4.3	159.5 ± 24.1
	10ak	5-Br	164.2 ± 16.7	104.3 ± 5.9	441.7 ± 110.0
	10al	5-OBn	>1000	>1000	>1000
	10am	5-OH	>1000	>1000	>1000
	10an	6-CH ₃	21.1 ± 3.9	21.7 ± 4.7	23.7 ± 6.1
	10ao	6-F	17.5 ± 3.0	5.8 ± 0.8	10.3 ± 1.2
	10ap	6-Br	67.8 ± 10.5	41.2 ± 7.5	55.4 ± 7.4
	10aq	6-OCH ₃	793.4 ± 89.9	879.8 ± 88.2	>1000
	10ar	6-OBn	>1000	>1000	>1000
	10as	6-OH	>1000	>1000	>1000
	10at	7-CH ₃	13.1 ± 1.3	10.8 ± 1.0	61.3 ± 11.0
	10au	7-OCH ₃	38.3 ± 3.7	34.1 ± 3.5	115.4 ± 25.0
	10av	7-OBn	>1000	>1000	>1000
	10aw	7-OH	>1000	>1000	>1000
10bb	4-indolyl	3.6 ± 1.0	3.7 ± 0.8	1.6 ± 0.3
	10bc	5-indolyl	8.6 ± 3.3	18.5 ± 3.7	6.1 ± 1.2
	10bd	6-indolyl	8.0 ± 1.0	6.1 ± 1.1	3.8 ± 0.5
	10be	7-indolyl	285.5 ± 78.7	240.6 ± 38.1	435.8 ± 171.3
Colchicine			14.1 ± 2.2	16.6 ± 1.5	10.8 ± 1.9

substituent (4-F) and displayed nearly equipotent antiproliferative activity to that of VERU-111 with its IC₅₀ ranging from 7.2-8.2 nM. In contrast, **10ad**, an analog with a larger halogen 4-bromo, was half as potent as VERU-111, having IC₅₀s ranging from 10.4-67.1 nM. For analogs **10ae** with a 4-methoxy group and **10af** with a 4-benzyloxy group, considerable reductions of activities were observed, demonstrating IC₅₀s of more than 1 μ M. However, hydrogenation to a 4-hydroxy in compound **10ag** rescued activity, with IC₅₀s from 8.6-19.1 nM. 5-position substituted analogs (compounds **10ah-10am**) showed decreased antiproliferative activities in comparison with their corresponding 4-position substituted counterparts. For example, the 5-fluoro analog **10ai** and 5-bromo analog **10ak** were 2-fold and 10-fold less potent than **10ac** and **10ad**, respectively. We also observed that the activity decreased with halogen size, where the order of potency from F > Cl > Br substitutions on **10ai**, **10aj**, and **10ak**, respectively. The activity 5-methyl analog **10ah** possessed approximately 100-fold decreased cytotoxicity compared to **10ab**. The best activity was observed in 5-substitution set was the 5-fluoro analog **10ai**, which had IC₅₀s ranging from 18.0-80.9 nM. Although a bulky benzyloxy led to completely lost antiproliferative activity (analog **10al**), hydrogenation of benzyloxy to hydroxy (analog **10am**) did not contribute to any improvement of activity, unlike what was observed in **10ag**. Next, we compared analogs with a substitution at the 6-position of the indole ring and found analogs with bulky functional groups at this position such as **10aq** and **10ar** also resulted in significant loss of activity, and hydrogenation of the benzyloxy to hydroxy on the 6-position (**10as**) did not provide any significant improvement of activity. Smaller substituents, however, had better cytotoxicity. For example, the best activity from a 6-position substitution was observed in the 6-fluoro analog **10ao**, with IC₅₀s ranging from 5.8-17.5 nM. At the final position for this series of 3-indolyl analogs, 4 compounds were synthesized with substitutions at the 7-indole position (**10at-10aw**). **10at**, containing a small methyl substitution, possessed the most potent inhibitory effect of the 7-indole substitutions, with IC₅₀s ranging from 10.8-61.3 nM. Interestingly, **10au** with a bulky 7-methoxy substituent showed significantly stronger activity (IC₅₀s of 34.1-115.4 nM) than its 4-methoxy and 6-methoxy counterparts (**10ae** and **10ap**). Taken these data together, small substituent (e.g. fluoro and methyl) is preferred to bulky functional groups on the 3-indolyl moiety. By comparing different positions of substituted analogs, it was determined that 4-position is the most amenable to the introduction of a functional group.

VERU-111 Analogs with Indolyl Rotation. Hwang et al. have disclosed a series of indolyl-imidazopyridines that are tubulin inhibitors, and their SAR study revealed that indolyl rotation significantly affected the potency and antiproliferative activities. (143). We hypothesized that this concept would also apply to VERU-111 analogs, considering its structural similarity to indolyl-imidazopyridines. Therefore, we synthesized four VERU-111 analogs **10bb-10be** demonstrate their cytotoxicity in **Table 3-1**. In this series, 5- and 6-indolyl analogs **10bc** and **10bd** showed comparable antiproliferative activities to that of VERU-111 and had IC₅₀s ranging from 6.1-18.5 and 3.8-8.0 nM, respectively, while the 7-indolyl analog **10be** showed a drastic decline in potency. **10bb**, the 4-indolyl analog, demonstrated greater cytotoxicity than VERU-111, with IC₅₀s ranging from 1.6-3.7 nM against the tested cancer cell lines, and was one of the most potent of all the new

analogs. Overall, these data suggest that rotating the indole moiety is feasible to improve the antiproliferative activity of VERU-111.

VERU-111 Analogs with Modified 3,4,5-Trimethoxyphenyl Moiety. Based on our recently reported high resolution crystal structure of DJ101 (**Figure 2-2**) (199), a close analog of VERU-111, we discovered that: (1) only one methoxy of the TMP moiety in DJ101 was involved in the formation of a hydrogen bond interaction with the β -Cys241 residue of tubulin; and (2) there is very limited space around the TMP moiety to accommodate any larger moieties (179). We hypothesized that two of the three methoxys of the TMP moiety could be modified to optimized activity without damaging the interactions to tubulin. We carried out a focused SAR investigation of VERU-111 by modifying the TMP moiety, specifically by linking two adjacent methoxy moieties into a conformationally restricted ring system (**Table 3-2**).

The benzo[4,5]-dioxane analog **13a** lacks the 3-methoxy present in the TMP of VERU-111. It exhibited remarkable loss of cytotoxicity (IC_{50} ranges from 119 to 218 nM). The ring contraction of the six-member benzo[4,5]-dioxane ring to the five-member benzo[4,5]-dioxole ring in analog **13b** demonstrated showed comparable antiproliferative activity to that of **13a**. In contrast to **13c**, the 3-methoxybenzo substituted analog of **13a**, demonstrated significantly increased potency, with IC_{50} values ranging from 11.3 to 29.2 nM. This is consistent with our previous SAR, where we discovered that removing one or more of the methoxy groups in the TMP moiety negatively affects the antiproliferative potency for this scaffold (98). Similarly, introducing the 3-methoxy to the benzo[4,5]-dioxole analog **13b** led to the formation of **13d**, which restored the potency (IC_{50} ranges from 3.5 to 5.6 nM, 30-fold more active than **13b**). Interestingly, this five-membered ring analog was ~4-fold more potent than the six-membered ring analog **13c**. Thus, while the 3-methoxy was crucial for the activities of VERU-111 analogs, the size of the cyclic rings on the phenyl moiety also played an important role (**13c** vs **13d**). Consistent with this, further increasing the ring size to a seven-membered ring resulted in 3-methoxybenzo[4,5]dioxepin analog **13e**, resulted in a moderate decrease in potency (IC_{50} ranging from 32.2 to 47.7 nM). By comparing **13c**, **13d** and **13e**, it was revealed that the activity for the size of this saturated ring is $5 > 6 > 7$.

We next introduced an unsaturation to this ring by synthesizing **13f**, a unique 3-methoxybenzo[4,5]-dioxene analog. Interestingly, **13f** exhibited the most potent antiproliferative activity among the TMP-modified analogs (**13a-13f**), with IC_{50} values ranging from 1.1 to 3.3 nM. To determine the combination modifications to both the TMP and indole moieties on **13f**, we further produced analogs **19** and **25** (**Table 3-2**). While the 4-indolyl analog **19** exhibited ~ 11-fold reduced antiproliferative activity when compared to **13f**, the 4-methyl-3-indolyl analog **25** (IC_{50} values ranging from 6.1 to 8.8 nM) was only slightly less potent than **13f**. Overall, our SAR result show that the TMP moiety in VERU-111 can be modified without negatively impacting antiproliferative activities.

Table 3-2. *In vitro* cytotoxicity of VERU-111 analogs modifying the TMP moiety.

Compound Scaffold	ID	R	IC ₅₀ ± SEM (nM)		
			A375	M14	RPMI-7951
13a		158.7 ± 16.4	118.8 ± 14.3	213.7 ± 17.1
	13b		190.6 ± 16.8	154.6 ± 10.4	235.6 ± 19.3
	13c		21.0 ± 1.6	11.3 ± 0.8	29.2 ± 1.7
	13d		3.5 ± 0.4	5.6 ± 0.6	5.6 ± 0.5
	13e		32.2 ± 3.4	38.2 ± 3.5	47.7 ± 3.9
	13f		1.1 ± 0.1	1.2 ± 0.2	3.3 ± 0.3
19		17.1 ± 1.1	13.8 ± 0.9	34.8 ± 2.2
	25		6.1 ± 0.2	6.1 ± 0.2	8.8 ± 0.5
VERU-111			8.1 ± 1.6	5.6 ± 0.9	7.2 ± 0.9

Lead Compounds Show Improved Potency to Resistant Cell Lines

To expand our assessment of the biological activity of the lead compounds, we chose the most potent compound from each modification series to move forward with for additional analysis. This included **10ab** from the 3-indolylys, **10bb** from indole rotation series, and **10f** from TMP modification series. Since other colchicine binding site inhibitors are reportedly less susceptible to taxane-related drug resistance, we wanted to determine if our analogs would still be efficacious in taxane-resistant models (54, 199, 240). We tested our compounds against paclitaxel in PC-3, paclitaxel-resistant PC-3/TxR, DU-145, and docetaxel-resistant DU-145/TxR prostate cancer cell lines (**Table 3-3**). Paclitaxel was more potent in the parental PC-3 cell lines with an IC_{50} of 1.1 ± 0.2 nM compared to 5.2 ± 0.4 , 89.9 ± 6.3 , and 15.2 ± 1.3 nM for **10ab**, **10bb**, and **13f**, which is not surprising given paclitaxel's potency and clinical success. However, in resistant PC-3/TxR cell lines, paclitaxel had a resistance index of 103.5 and an IC_{50} of 113.9 ± 4.3 nM whereas **10ab**, **10bb**, and **13f** all showed a resistance index of less than 1, meaning they had improved potency against the resistant cell line. Along the same lines, paclitaxel was highly potent against the sensitive DU-145 cell line (1.5 ± 0.2 nM), however, no effect was observed at all against the paclitaxel-treated DU-145/TxR cells at concentrations up to 1 μ M. In contrast, all 3 compounds showed remarkably improved resistance indices, ranging from 1.8-2.2. These data support the continued investigation of this scaffold as an alternative treatment for cancers resistant to taxane treatment.

Molecular Interactions of 10f with Tubulin

To understand the molecular basis for the strong interaction of the most potent TMP-modified analog, **13f**, with tubulin, we obtained the crystal structure of the T2R-TTL (consisting of α/β -tubulin, the stathmin-like domain of RB3, and tubulin tyrosine ligase) (186, 241) in complex with **13f** at a resolution of 2.85 Å (PDB ID: 6D88). As expected, **13f** occupies the colchicine binding site at the interface of the α - and β -tubulin, mostly confined in a deep pocket in β -tubulin opposite the GTP molecule that is bound in a pocket in the α -tubulin (**Figures 3-1A**). There are two α/β -tubulin heterodimers in this complex and both interfaces are occupied by the small molecule. In both heterodimers (Chain A/B and C/D), **13f** forms virtually identical interactions. First, it forms three hydrogen bonds with the surrounding amino acids: an imidazole NH to carbonyl of Thr179 (α -tubulin); an indole NH to Ser178 (α -tubulin) and Asn347 (β -tubulin) via a water molecule; and a carbonyl linker to Asp249 (β -tubulin) (**Figure 3-1B**). Second, the 3-methoxybenzo[4,5]-dioxene moiety is stacked between Cys239 and Leu253 from β -tubulin, the latter making a pi-H interaction with the ring. Finally, the imidazole ring is surrounded by Leu246 and Asn256 from β -tubulin while the indole ring is surrounded by both Lys350 and Met257 from β -tubulin and Val181 from α -tubulin. Colchicine targets the β subunit and keeps the tubulin from adopting a straight conformation, thus inhibiting microtubule assembly, and **13f** also blocks the curved-to-straight conformational change of tubulin by steric clashes with surrounding secondary structure elements (**Figure 3-1C**).

Table 3-3. *In vitro* cytotoxicity of 10ab, 10bb, and 10f against parental and taxane-resistant prostate cancer cell lines.

treatment	IC ₅₀ ± SEM (nM)		^a RI	IC ₅₀ ± SEM (nM)		RI
	PC-3	PC-3/TxR		DU-145	DU-145/TxR	
10ab	5.2 ± 0.4	3.9 ± 0.3	0.8	13.8 ± 1.0	25.2 ± 2.0	1.8
10bb	89.9 ± 6.3	66.9 ± 4.4	0.7	169.1 ± 4.4	379.1 ± 80.0	2.2
10f	15.2 ± 1.3	7.6 ± 0.5	0.5	42.2 ± 3.6	81.5 ± 11.6	1.9
Colchicine	10.4 ± 0.5	37.4 ± 2.2	3.6	35.5 ± 3.5	>1,000	>28
Paclitaxel	1.1 ± 0.2	113.9 ± 4.3	103.5	1.15 ± 0.2	>1,000	>869
Docetaxel	0.5 ± 0.1	27.9 ± 1.1	55.8	0.10 ± 0.01	352.3 ± 118.2	>3,523

^aRI: resistance index is calculated by dividing the IC₅₀ value of compound in resistant cell lines by the IC₅₀ in the corresponding parental cell lines.

Figure 3-1. T2R-TTL in complex with 13f.

A) Surface representation of the **13f**-tubulin complex. Various components are marked with arrows. B) Close up view of first α/β -tubulin heterodimer interface occupied with **13f**. The compound is shown in ball & stick model with orange carbons. Water and Mg are shown in ball model, GTP in thick sticks and amino acids in thin sticks. 2Fo-Fc map of the compound drawn at 1.0 Å. sigma is shown as green mesh. C) Interference of **13f** with the tubulin straight conformation. The close-up view of superimposition of the tubulin-**13f** complex (blue ribbons-orange carbon balls, PDB ID: 6D88) and tubulin as found in straight protofilaments (grey ribbons, PDB ID: IJFF) shows that **13f** binding is not compatible with the straight conformation. Conformational changes of the secondary elements in both subunits upon binding of **13f** are labeled. D) Conformational changes at the colchicine-binding sites between colchicine (PDB: 4O2B), and **13f** (PDB code: 6D88). Superimposition of tubulin bound with **13f** bound (shown in yellow) and with colchicine bound (shown in salmon). Crystallographic data obtained by Yuxi Wang (Sichuan University, China) and interpreted by Dr. Stephen White and Dr. Gyanendra Kumar (St. Jude Children's Research Hospital).

Finally, the superimposition of **13f** and colchicine bound to tubulin demonstrates that it overlaps well with colchicine in the binding pocket and supports a shared mechanism of action between colchicine and **13f** (**Figure 3-1D**).

Inhibition of Tubulin Polymerization and Microtubule Dynamics

To further validate their mechanism of action, we tested the effect of **10ab**, **10bb**, and **10f** on polymerization of purified tubulin in a cell-free assay. 10 μ M concentrations of **10ab**, **10bb**, and **10f** were evaluated against vehicle-only control, colchicine, and docetaxel, which is a well-documented enhancer of polymerization. All three new VERU-111 analogs potently inhibited tubulin polymerization, while the vehicle experienced an initial and rapid increase in polymerization and docetaxel strongly and consistently promoted polymerization (**Figure 3-2A**). Colchicine, which was used as a positive control, showed similar activity as the 3 new compounds in tubulin inhibition.

The effect on microtubule networks was then observed by immunofluorescence via confocal microscopy. Microtubule morphology in WM-164 cells was visualized after 18 hrs of treatment of with 100 nM of **10ab**, **10bb**, docetaxel, or untreated control cells. Unlike the control cells, **10ab** and **10bb** effectively disrupted microtubules, resulting in fragmentation and disassembly (**Figure 3-2B**). Docetaxel demonstrated the opposite effect, causing hyper-polymerization and resulting in the formation of dense, aggregated bundles of microtubules. These mechanistic studies confirm that these compounds maintain a mode of action by inhibiting tubulin assembly and interfering with microtubule dynamics.

Inhibition of Cancer Growth and Migration

The long-term inhibitory growth effects of **10ab** and **10bb** were tested in A375 cells in an anchorage-dependent colony formation assay. While the control cells averaged 199.3 ± 10.7 colonies after 12 days, 5 nM concentrations of **10ab** and **10bb** decreased colony number, resulting in an average of 81.7 ± 7.8 and 128.3 ± 9.1 colonies, respectively (**Figure 3-3A**). These compounds even out-performed colchicine, which averaged 150.0 ± 5.3 colonies. There was a significant reduction in colony number for each compound-treated group, where colchicine, **10bb**, and **10ab** treatment resulted in a 24.7%, 35.6%, and 59.5% decrease, respectively. Tubulin and microtubule function are also implicated in cell migration and motility. Therefore, we assessed the effect of **10ab** and **10bb** in a wound healing assay. After removing a confluent monolayer of adherent A375 or RPMI-7951 melanoma cells, cells were treated for 18-22 hrs with 5 or 25 nM of **10ab**, **10bb**, or colchicine and compared against an untreated control. The total closure was calculated as a percentage of the total wound area after treatment compared to the total area of removed cells immediately prior to treatment. In A375 and RPMI-7951 cell lines, the control cells were able to efficiently migrate into the wound channel, recovering $60.7\% \pm 5.9\%$ and $71.8\% \pm 1.8\%$ of the area, respectively (**Figure 3-3B**). Following 5

Figure 3-2. New VERU-111 analogs inhibit tubulin polymerization.

A) Polymerization of purified tubulin in a cell-free assay. Tubulin (3.33 mg/ml) was exposed to vehicle control or 10 μ M of the compounds (n=2). Absorbance at 340 nm was monitored at 37 $^{\circ}$ C every minute for 40 min. B) Effect on microtubules of WM-164 cells following 18h exposure to 100 nM of **10ab**, **10bb**, docetaxel or vehicle control. Confocal microscopy was utilized to visualize the microtubules following immunofluorescent staining. Microtubules are shown in red and DNA is shown in blue. Scale bar = 20 μ m.

Figure 3-3. Inhibition of colony proliferation and cancer cell migration.

A) A375 colony formation for control or treated cells represented as mean $\pm$ SD (n=3). B) Scratch migration assay for A375 (top) or RPMI-7951 (bottom) cells after treatment with 5 or 25 nM concentrations of **10ab**, **10bb**, colchicine, or untreated control represented as mean $\pm$ SD (n=3). Area of the wound channel was calculated using ImageJ software. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

nM treatment with **10ab**, **10bb** or colchicine, A375 cells only migrated into $49.4\% \pm 1.6\%$, $38.5\% \pm 3.9\%$, and $46.0\% \pm 2.5\%$ of the wound area, respectively. At higher concentrations of 25 nM, a greater inhibition was observed, where cells recovered $25.9\% \pm 2.8\%$, $27.5\% \pm 6.2\%$, and $20.7\% \pm 3.8\%$ for each of the tested compounds ($P < 0.0001$). A significant difference was also observed in RPMI-7951 cells treated at 25 nM of **10ab**, **10bb** or colchicine, leading to wound closure averaging $35.6\% \pm 6.6\%$, $35.0\% \pm 7.8\%$, and $42.8\% \pm 5.8\%$, respectively ($P = 0.0004$). Averaging the results from both cell lines, the average inhibition of migration of cells treated at 25 nM concentrations with **10ab**, **10bb**, and colchicine was 56.1%, 55.3%, and 34.0%. Taken together, these results demonstrate that **10ab** and **10bb** potentially inhibit proliferation and migration *in vitro* at low nanomolar concentrations.

***In Vitro* Metabolic Stability of VERU-111 Analogs**

To determine the most suitable candidates for *in vivo* studies, we examined the *in vitro* metabolic stabilities of the most potent analogs by measuring their half-life upon incubation with mouse, rat and human liver microsomes in the presence of an NADPH regeneration system. The parent compound VERU-111 was used for comparison and Verapamil was used as a positive control. Overall, all three new VERU-111 analogs exhibited moderate to good stabilities in three different microsome species. (**Table 3-4**). Compared to the parental compound, **10ab** showed an improved half-life and clearance in human liver microsomes (6.7 ± 0.8 hrs, 3.1 mL/min/kg), whereas VERU-111 showed a half-life of 5.1 ± 0.3 hrs and a clearance of 4.1 mL/min/kg. **10bb** showed better stability compared to VERU-111 in all 3 species and, compared to **10ab**, demonstrated an almost identical half-life and clearance in human liver microsomes and better metabolic stabilities in mouse and rat liver microsomes. **13f** showed a slightly less stable profile than the other compounds (2.7 ± 0.3 hours and 7.7 mL/min/kg in human liver microsomes) but still displayed greater stabilities than the reference control. Based on the *in vitro* and stability assessment, we selected **10bb** to undergo testing in animal models. Because of its unique modification to the TMP moiety, **13f** was also selected for *in vivo* evaluation to determine if the strong anticancer efficacy demonstrated *in vitro* would also be evident in animal models.

Anticancer Efficacy in Melanoma Mouse Models Without Toxicity.

The antitumor efficacies of **10bb** and **13f** were evaluated in two melanoma xenograft mouse models. Nude mice bearing A375 melanoma tumors were treated every other day via i.p. injection with 15 mg/kg or 30 mg/kg doses of **10bb**, **13f**, paclitaxel, or given a vehicle solution only. The average TGI for the groups treated with 15 mg/kg and 30 mg/kg of **13f** was 54.8% and 73.9%, respectively. These results were similar to the paclitaxel-treated group, which showed an average TGI of 70.7% (**Figure 3-4A**). In a separate *in vivo* study, the group receiving 15 mg/kg of **10bb** achieved an average tumor growth inhibition of 82.3% and the group receiving the higher dose of 30 mg/kg achieved

Table 3-4. *In vitro* metabolic stabilities of compound 10ab, 10bb, and 13f in liver microsomes.

Compound	Metabolic Stability (Human)		Metabolic Stability (Mouse)		Metabolic Stability (Rat)	
	t _{1/2} (hr)	Cl _{int} (mL/min/kg)	t _{1/2} (hr)	Cl _{int} (mL/min/kg)	t _{1/2} (hr)	Cl _{int} (mL/min/kg)
VERU-111	5.13 ± 0.26	4.05	3.76 ± 0.24	15.20	6.74 ± 0.59	6.94
10ab	6.70 ± 0.82	3.10	2.42 ± 0.15	23.60	1.80 ± 0.09	25.98
10bb	6.72 ± 0.74	3.10	5.04 ± 0.30	11.34	29.93 ± 7.82	1.56
13f	2.69 ± 0.26	7.70	1.42 ± 0.14	40.30	3.57 ± 0.43	13.10
Verapamil	1.68 ± 0.16	12.41	0.94 ± 0.06	60.66	1.29 ± 0.09	36.18

Data obtained by Lei Yang (St. Jude Children's Research Hospital).

Figure 3-4. Anticancer efficacy of VERU-111 analogs in melanoma mouse models.

A) **13f** and B) **10bb** treatments in A375 xenograft models in nude mice represented as mean tumor volume $\pm$ SEM. (n=8 and n=10, respectively). Statistical significance for final tumor volumes in both studies was determined by one-way ANOVA analysis followed by Dunnett's multiple comparison test for the treatment group compared with the corresponded results of the control group (* $P < .05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). C) **10bb** inhibits B16F10 lung metastasis in C57BL/6 mice. The graph represents the tumor index for mice (n=10) and error bars represent 95% CIs. An unpaired student's t-test was performed to show statistical significance between the groups ($P = 0.0013$). Mouse body weights are represented as a percent of weight change compared to initial weight $\pm$ SEM for each study.

a TGI of 90.6% (**Figure 3-4B**). Both groups achieved significantly greater tumor growth inhibition than the paclitaxel group, which averaged a TGI of 68.6%. Because it showed the most potent effect on tumor growth inhibition, we investigated the ability of **10bb** to inhibit metastatic potential in a lung metastasis mouse model at 30 mg/kg doses in C57BL/6 mice against a vehicle control group (**Figure 3-4C**.) After inoculation with the melanoma via the lateral tail vein, mice were treated for two weeks with the same dosing regimen. The average tumor index based on lung metastasis nodule size and the number was 13.6 ± 2.9 for the control group and 2.6 ± 0.6 for the **10bb** treatment group. This was significantly less than the control based on an unpaired student's t test ($P = 0.0013$) and represents an 80.9% decrease in metastasis. Mouse weight and activity were monitored and for both xenograft models and the lung metastasis model, and major weight deviations were not observed. Histopathology analysis was performed on major organs collected from both *in vivo* studies to assess for acute toxicities from **10bb** treatment. H&E stained sections revealed no apparent drug-related injury or pathological changes in the cellular structure of the various tissues from both the xenograft mouse model and the experimental lung metastasis mouse model (**Figure 3-5A**). Additionally, *in vitro* pharmacological profiling was utilized to identify undesirable off-target effects and determine the safety profile. **10bb** was evaluated at 1 μ M in the Safety47™ Panel which includes the assessment of the functional response of 47 major physiologically important targets (**Figure 3-5B**). **10bb** only showed positive responses (higher than |70%|) in 2 of the 78 assays, which were norepinephrine transporter (NET) and serotonin receptor 2B (HTR2B) antagonism.

Circumventing Taxane Resistance

Because of its promising *in vivo* antitumor potency and initial findings demonstrating that taxane resistant cells were still highly sensitive to **10bb** treatment, we wanted to confirm that **10bb** could be an effective treatment to taxane-resistant tumors. We previously demonstrated that paclitaxel is an effective treatment against PC-3 xenograft tumors in a nude mouse model but was ineffective against the taxane resistant PC-3/TxR xenograft tumors (previously shown in **Figure 2-8A**). We hypothesized that **10bb** would show high efficacy in the resistant xenograft model and that paclitaxel would fail, providing support for the development of **10bb** in resistant chemotypes. Therefore, we proceeded to test **10bb** at the same dosing concentration and frequency as in the previous models (30mg/kg, every other day via i.p. injection) in comparison to paclitaxel at 15 mg/kg, a dose that was shown to be highly effective against non-resistant cancer xenograft models. Paclitaxel showed a marked decline in anticancer capabilities, yielding a TGI of only 37.8% and a modest reduction in tumor weight of only 34.5% (**Figure 3-6A-C**). In contrast, treatment with **10bb** caused a significant reduction in tumor growth by 83.8% and decreased average tumor weight by 62.8%. ANOVA analysis ($P < 0.0001$) followed by Dunnett's multiple comparison test confirmed a significant decrease in final tumor volume and weight ($P < 0.0001$) for the **10bb** treated groups without causing significant loss of body weight or animal activity (**Figure 3-6D**). This further validates **10bb** as an alternative treatment to taxane-resistant cancers.

Figure 3-5. Toxicity and safety assessment for 10bb.

A) Pathological sections of major tissues (heart, kidney, liver, lung, and spleen) were obtained from the A375 xenograft (top) and B16F10 lung metastasis (bottom) studies. Organs were H&E stained, and representative images of the tissues were captured. B) *In vitro* pharmacological profiling of **10bb** to assess potential off-target effects to major targets at 1 μ M concentrations using the Safety47™ Panel (n=2). Graph represents mean % response $\pm$ range. Values in between -70% and +70% (indicated with dashed lines) are considered insignificant.

Figure 3-6. Antitumor efficacy of 10bb in a taxane-resistant xenograft model.
A) Tumor volumes for the PC-3/TxR xenograft model shown as mean volume $\pm$ SEM (n=8). B) Final tumor weights for PC-3/TxR mice. Error bars represent 95% CIs. Statistical significance for final tumor volumes and weights were determined by one-way ANOVA analysis followed by Dunnett's multiple comparison test (* $P < .05$, **** $P < 0.0001$). C) Representative images of resected tumors at the end of the study. D) Mouse body weights represented as mean weight change as a percentage compared to initial weight $\pm$ SEM.

Conclusions

In this research, an extensive SAR investigation of VERU-111 focusing on the modification of the indole moiety is reported, revealing that **10ab** and **10bb** are more potent derivatives than prototype VERU-111. We also conducted SAR on the TMP modification series. While modifying the TMP moiety of colchicine binding site inhibitors is usually unsuccessful in improving antiproliferative activities, our results showed that isosteric (conformationally restricted) replacement of the TMP is feasible to significantly increase the potency in this scaffold. We identified the analog **13f**, containing a unique 3-methoxybenzo[4,5]-dioxene moiety, that demonstrated more potent antiproliferative activity than VERU-111. Additionally, compared to paclitaxel, these lead compounds showed significantly improved resistance indexes against taxane-resistance prostate cancer cell lines, suggesting their potential against drug-resistant phenotypes.

The X-ray crystal structure of tubulin in complex with **13f**, the visualization of disrupted microtubule networks of cells after treatment with **10ab** and **10bb**, and the potent inhibition of tubulin polymerization by all 3 compounds in a tubulin assay all support that these compounds have the same mechanism of action as colchicine. The crystallographic analysis is particularly valuable because it revealed that the interactions between **13f** and tubulin are centered on a hydrogen-bonding network, which provides potential avenues for future modifications to improve potency.

10bb and **13f** were selected for *in vivo* efficacy studies and drastically inhibited tumor growth in melanoma xenografts. **10bb** also reduced lung metastasis without causing signs of toxicity. Finally, **10bb** treatment was effective in a PC-3/TxR prostate cancer model, suggesting its ability to circumvent certain MDR mechanisms that nullify paclitaxel action. In conclusion, we have developed improved analogs of VERU-111 by optimizing substitutions through SAR investigation and demonstrated the feasibility of modifying the TMP moiety for enhanced activity. We confirmed their mechanism of action and *in vivo* potency, supporting their development as potential new candidates targeting the colchicine binding site for cancer treatment.

CHAPTER 4. NOVEL TUBULIN INHIBITORS WITH A HETEROCYCLIC PYRIMIDINE SCAFFOLD SHOW VASCULAR DISRUPTING PROPERTIES³

Introduction

Disrupting tubulin dynamics is a well-validated strategy for anticancer therapy (54, 145, 172, 242-249). The three widely studied binding sites in tubulin are the paclitaxel site, the vinca alkaloid site, and the colchicine site (39, 54). Currently, all FDA approved tubulin inhibitors for cancer treatment target either the taxane site (e.g. paclitaxel, docetaxel) or the vinca alkaloid site (e.g. vinblastine, vincristine). Colchicine binding site agents have advantages over other classes of tubulin inhibitors, including reduced susceptibility to MDR from ABC transporter overexpression and upregulation of the β III tubulin isoform (explained in detail in Chapter 1). However, the potential clinical applications of many colchicine site tubulin inhibitors have been halted due to significant toxicities, low solubility, and low bioavailability (23, 39, 54, 214, 244). There have been extensive research efforts to identify new small molecules capable of acting at the colchicine binding site with improved therapeutic indexes. Additionally, there have been numerous reports that tubulin inhibitors targeting the colchicine binding site exhibit promising vascular disrupting effects in addition to inhibiting tumor cell invasion and metastasis (39, 98, 141, 145, 172, 243, 250). Vascular disrupting agents (VDAs) are known to rapidly disrupt existing tumor vessels resulting in prompt vascular collapse and tumor cell death (39, 40, 251). Thus, there has been substantial interest in this class of tubulin inhibitors, since newer generations of tubulin inhibitors can be developed that can overcome the limitations of the existing tubulin inhibitors by evading MDR and reducing toxicity (39, 40).

A number of small molecule vascular disrupting agents targeting the colchicine binding site are currently in clinical development as anticancer agents (**Figure 4-1**, compounds **1a-1c**) (39, 40). Out these VDAs, Azixa/Verubulin (**1a**) and its derivatives have drawn considerable attention in the recent years (101). Azixa emerged a few years ago as a very potent tubulin polymerization inhibitor, and it has been observed to act as a VDA by introducing rapid shut down of tumor blood flow and consequently inhibits the tumor growth (39, 40, 101, 102). Azixa has demonstrated low nanomolar potency against diverse tumor models, including melanoma, brain cancer, ovarian cancer, small cell lung cancer, and prostate cancer (102, 252, 253). Recently, Mueller et al. reported an indole derivative of Azixa/Verubulin (**1d**) that induced significant vascular disruption in the 1411 HP germ cell xenograft tumors, leading to rapid tumor necrosis (102). Gangjee et al. have reported Azixa derivatives (**1e**) that are dual inhibitors of tubulin and vascular

³Modified with permission from American Chemical Society. Souvik Banerjee*, Kinsie E. Arnst*, Yuxi Wang*, Gyanendra Kumar, Shanshan Deng, Lei Yang, Guo-bo Li, Jinliang Yang, Stephen W. White, Wei Li, and Duane D. Miller. Heterocyclic-Fused Pyrimidines as Novel Tubulin Polymerization Inhibitors Targeting the Colchicine Binding Site: Structural Basis and Antitumor Efficacy *Journal of Medicinal Chemistry*. 2018; 61 (4), 1704-1718.

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1a
Azixa/Verubulin
(MPC-6827)
Phase-II

1b
Combretastatin A-4 (R = H)
CA-4P (R = PO₃Na₂)
Phase II/III

1c
CKD-516
Phase I

1d

1e

1f

ChemMedChem **2014**, 9, 847-854

ACS Med. Chem. Lett. **2014**, 5, 480-484

J. Med. Chem. **2014**, 57, 1390-1402

Figure 4-1. Recently developed VDAs that target the colchicine binding site.
Three are currently in clinical trials (**1a**, **1b**, and **1c**) and three reported derivatives of Azixa/Verubulin are in pre-clinical development (**1d**, **1e**, and **1f**).

endothelial growth factor receptor-2 (VEGFR2) (242). These dual actions produced significant reduction in tumor size and disruption of vascularity in three mouse xenograft models.

However, most of the quinazoline analogs reported by Xie et al. have shown low metabolic stability in human liver microsomes ($t_{1/2} \leq 10$ minutes) and substantial *in vivo* toxicity, with MTDs less than 4 mg/kg (145). Although Azixa and many of its derivatives are highly effective as tubulin inhibitors and VDAs, none of them have successfully made it through clinical trials. One of major concerns limiting the clinical progression of Azixa is the cardiovascular toxicities that it demonstrated in Phase I and Phase II studies (254). The identified cardiovascular toxicities include grade 1-3 hypertension, atrial fibrillation, grade 3-4 myocardial infarction, grade 4 non-ST-elevation myocardial infarction, and grade 3 cerebral haemorrhage (254, 255). Additionally, while Azixa demonstrates excellent potency against cancer cells, it also shows potential high toxicity to normal tissues.

To the improve the therapeutic index of tubulin inhibitors based on the scaffolds of Azixa or its reported analogs, we set the goal to design new Azixa analogs with: (a) equal or higher potency than existing Azixa analogs; (b) substantially reduced toxicities as tested in mouse models; (c) significantly improved metabolic stability; and (d) high efficacy against clinically relevant MDR models. We selected our starting point based on the **1f** scaffold reported by Lee and Xie et al. because of its reduced conformational flexibility compared with all other Azixa analogs (145). We revealed the structural modifications from lead compound **1f** that yielded highly potent heterocyclic pyrimidines and their comprehensive *in vitro* and *in vivo* anticancer activities. Lead compounds **4a**, **6a**, **5a**, and **5b** were selected out of the series as the molecules with the greatest potential and underwent additional biological investigation. To confirm the direct binding of these new analogs to tubulin and determine their molecular interactions, we obtained the high-resolution crystal structures of **4a**, and **6a** in complex with tubulin protein. To the best of our knowledge, this is the was the first X-ray crystal structure reported for Azixa analogs bound to tubulin. Importantly, contrary to all previously published molecular modelling studies for Azixa or its analogs, these high-resolution X-ray crystal structures reveal a different actual binding pose, along with significant conformational changes, in the colchicine binding pocket in tubulin (102, 145, 167, 250, 256). Results from this study provide important structural basis for the molecular interactions and enable future structure-based optimization for the general Azixa scaffold. Additional mechanistic studies of **4a**, **6a**, **5a** and **5b** reveal that they share the same mechanism of action as colchicine as tubulin polymerization inhibitors that arrest cells in the G₂/M phase of the cell cycle, interfere with cellular functions such as migration and proliferation, and enhance apoptotic cascades. **4a** was evaluated in two animal models against melanoma and paclitaxel-resistant tumors, emphasizing the potential of this scaffold to overcome multidrug resistance for cancer therapy. Finally, we confirmed ability of **4a** to elicit vascular disrupting effects within tumors and against endothelial capillary-like network formation.

Materials and Methods

Cell Culture and Reagents

Human melanoma cell lines (A375, M14, RPMI-7951, WM-164, SK-MEL-1, M14/MDR1) and breast cancer cell lines (MDA-MB-453, MDA-MB-231, MDA-MB-468) (American Type Culture Collection or ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis MO). Human lung cancer cell lines (A549, H460, H1299) American Type Culture Collection or ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Gibco by Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) FBS and 1% antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis MO). Parental prostate cancer PC-3, its paclitaxel-resistant daughter line PC-3/TxR, parental prostate cancer DU-145, and its docetaxel-resistant daughter line DU-145/TxR were gifts from Dr. Evan Keller at the University of Michigan Medical School. PC-3 and DU-145 cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic/antimycotic mixture. Taxane-resistant PC-3/TxR and DU-145/TxR cell lines were cultured in the same medium and additionally supplemented with 10 nM paclitaxel or docetaxel, respectively. Paclitaxel or docetaxel was not included in the medium for PC-3/TxR or DU-145/TxR for at least 1 week prior to *in vitro* and *in vivo* testing. All cell lines were authenticated by ATCC by short tandem repeat profiling. Cultures were maintained to 80–90% confluency at 37 °C in a humidified atmosphere containing 5% CO₂. Compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to make a stock solution of 20 mM. Compound solutions were freshly prepared by diluting stocks with cell culture medium before use.

Cytotoxicity Assays

Cells were seeded in 96-well plates at a concentration of 2000–5000 cells per well, depending on growth rate of the cell line. After overnight incubation, the medium was replaced, and cells were treated with the test compounds at 10 concentrations ranging from 0.03 nM to 1 μ M plus a vehicle (DMSO) control for 72 hrs in four replicates. Following treatment, the MTS reagent (Promega, Madison, WI) was added to the cells and incubated in dark at 37 °C for at least 1 hr. Absorbance at 490 nm was measured using a plate reader (BioTek Instruments Inc., Winooski, VT). IC₅₀ values were calculated by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA).

Microsomal Stability Assay

Liver microsome assays were performed by Dr. Lei Yang (St. Jude Children's Research Hospital). For each test compound, the human liver microsomal solution was prepared by adding 0.058 mL of concentrated human liver microsomes (20 mg/mL protein concentration) to 1.756 mL of 0.1 M potassium phosphate buffer (pH 7.4) containing 5 μ L of 0.5 M EDTA to make a 0.6381 mg/mL (protein) microsomal solution. Each test compound (2.2 μ L of 10 mM DMSO solution) was added directly to 1.79 mL of human liver microsomal solution, and 90 μ L was transferred to wells in 96-well plates (0, 0.25, 0.5, 1, 2, and 4 h time points each in triplicate). The NADPH regenerating agent was prepared by mixing 0.113 mL of NADPH regenerating agent solution A, 0.023 mL of solution B, and 0.315 mL of 0.1 M potassium phosphate buffer (pH 7.4) for each tested compound. To each well of the 96-well plate, 22.5 μ L of the NADPH regenerating agent was added to initiate the reaction, and the plate was incubated at 37 °C for each time point. The reaction was quenched by adding 225 μ L of cold acetonitrile containing warfarin (4 μ g/mL) as internal control to each well. All of the plates were centrifuged at 4000 rpm for 20 min, and the supernatants (100 μ L) were transferred to another 96-well plate for analysis on UPLC–MS (Waters Acquity UPLC linked to Waters Acquity photodiode array detector and Waters Acquity single quadrupole mass detector) on Acquity UPLC BEH C18 1.7 μ m (2.1 mm $\times$ 50 mm) column by running 90–5% gradient for water (+0.1% formic acid) and acetonitrile (+0.1% formic acid). The area under the single ion recording (SIR) channel for the test compound divided by the area under the SIR for internal control at 0 time concentration was considered as 100% to calculate remaining concentration at other time points. The terminal phase rate constant (k_e) was estimated by linear regression of logarithmic transformed concentration versus the data, where k_e = slope $\times$ ($-\ln 10$). The half-life $t_{1/2}$ was calculated as $\ln 2/k_e$.

Tubulin Polymerization Assay

The polymerization reaction contains 100 μ L of volume of Bovine brain tubulin (3 mg/mL) (Cytoskeleton, Denver, CO) and 10 μ M of the test compounds in general tubulin buffer (80 mM PIPES, 2.0 mM MgCl₂, 0.5 mM EGTA, and 1 mM GTP) at pH 6.9. The absorbance of wavelength at 340 nm was monitored every 1 min for 50 min by the SYNERGY 4 Microplate Reader (Bio-Tek Instruments, Winooski, VT). BioTek Gen5 data analysis software was used to calculate the $V_{\max}$ values. The spectrophotometer was set at 37 °C for tubulin polymerization.

Confocal Microscopy

WM-164 cells were seeded on glass coverslips in six-well plates (125 000 cells/well) and incubated overnight. Cells were treated with 100 nM compounds or equivalent vehicle (DMSO) control for 18 h. Microtubules were visualized after incubating with anti- α -tubulin antibody (Thermo Scientific, Rockford, IL) and Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes, Eugene, OR). The coverslips were

mounted with Prolong Diamond Antifade mounting medium with DAPI (Invitrogen, Eugene, OR) and images acquired with a Zeiss 710 confocal microscope and Zen imaging software (Zeiss, Thornwood, NY).

X-Ray Crystallography Method

Protein Expression and Purification. X-Ray crystallography was performed by Dr. Yuxi Wang (Sichuan University, in China). The stathmin-like domain of RB3(RB3-SLD) was overexpressed in *E. coli* and purified by anion-exchange chromatography and gel filtration chromatography. The final sample was concentrated to 10 mg/mL and stored at -80 °C. The TTL protein was also expressed and purified from an *E. coli* expression system as described previously (Section 2.2.7-2.2.8.) The final sample was concentrated to 20 mg/ml and saved at -80 °C. The purity of RB3 and TTL were assessed by SDS-PAGE. Porcine brain tubulin (Catalog # T-238P, Cytoskeleton, Inc.) was supplied at 10 mg/ml in G-PEM (General tubulin buffer: 80 mM PIPES pH 6.9, 2 mM MgCl₂, 0.5 mM EGTA and 1 mM GTP) as a frozen liquid and saved at -80 °C until use.

Crystallization and Crystal Soaking. Crystals of T2R-TTL were obtained by vapor diffusion using the sitting-drop method following published protocols. Briefly, the protein solution containing tubulin (10 mg/ml), TTL (20 mg/ml) and RB3 (6 mg/ml) at the molar ratio of 2 : 1.3 : 1.2 (tubulin:RB3:TTL) was incubated on ice, supplemented with 1 mM AMPPCP, 5 mM tyrosinol and 10 mM DTT, and then concentrated to 20 mg/ml at 4 °C. Crystallization drops contained equal volumes (1.0 µl) of the T2R-TTL protein solution and the precipitant solution (6% PEG, 5% glycerol, 0.1 M MES, 30 mM CaCl₂, 30 mM MgCl₂, pH 6.7) and were incubated at 20 °C. The seeding method was used to obtain single crystals. Crystals appeared after two days and grew to 200-300 µm within 3-5 days. Compounds were soaked into the crystals to obtain the complex structures. Each compound (**4a** and **6a**) was dissolved in DMSO at 10 mM concentration and 0.1 µl was added to the mother liquor at 20 °C for 12 h. The crystals were cryoprotected in 20% glycerol (30 mM MgCl₂, 30 mM CaCl₂, 0.1 M MES, 20% glycerol, pH 6.7) and flash-frozen at 100 K for synchrotron X-ray data collection.

X-Ray Data Collection and Structure Determination. Crystals of the T2R-TTL-ligand complexes were mounted in nylon loops and maintained at 100 K in a nitrogen stream. Diffraction data were collected on beamlines BL17U1 and BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF) in Shanghai, China. Data were indexed, integrated and scaled using HKL2000. Initial phases were determined by molecular replacement using the previously published T2R-TTL structure (PDB ID: 4I55) as a template. The initial model was completed manually using Coot and iteratively refined with the phenix refine module of the Phenix program (191). The final quality of the structure was checked and validated using the PDB validation server. Analysis was determined in coordination with Dr. Stephen White's group (St. Jude Children's Research Hospital).

Colony Assay

To test the effects of clonogenic potential and proliferation, A375 cells were seeded in 6 well plates at a density of 500 cells/well (n=4). After overnight incubation in at 37 °C cells were treated with the compound or media only control and incubated for 10 days. Cells were then fixed with methanol and stained with 0.5% crystal violet. Images were taken, and colony area was quantified with ImageJ software (NIH, Bethesda, MD).

Endothelial Cell Tube Formation Assay

Matrigel (Corning, Manassas, VA) was thawed on ice overnight then diluted with serum-free media for a final concentration of 10 mg/mL. Matrigel (50 μ L) was plated in 96 well plates and incubated at 37 °C for 1 hr. Low passage number HUVECs (<5) in logarithmic growth phase were trypsinized and suspended in endothelial cell growth media. Cells (1.5×10^4 suspended in 50 μ L) were plated on the Matrigel plates in quadruplicate containing the desired drug concentrations. Images were captured after 4 hr incubation with Evos Fl Imaging System (LifeTechnologies, Carlsbad, CA). Analysis was performed with angiogenesis tool plug-in with ImageJ software (NIH, Bethesda, MD).

Wound Healing Assay

A375 and RPMI-7951 cells were seeded in 12-well plates (200 000 cells/well) in replicates of 3 and incubated overnight. Using a 200 μ L pipet tip, a straight line was scratched through the cell monolayer to remove an area of cells, and cells were washed several times to remove any debris and uprooted cells. Medium was replaced containing 5 or 25 nM of **4a** or colchicine. Control wells received medium without drug. Images were obtained at 0 h and 18 h (RPMI-7951 cells) and 22 h (A375 cells) with Evos Fl imaging system (Life Technologies, Carlsbad, CA). Analysis of the scratch area was performed with ImageJ software (NIH, Bethesda, MD).

For real time cell migration analysis, A75 or M14 cells were seeded 96-well Image-Lock plates (Essen bioscience, Ann Arbor, MI) at a density of 30,000 cells/well and incubated overnight. The WoundMaker™ tool (Essen bioscience, Ann Arbor, MI) was used to create uniform identical scratches in all the wells of a 96-well plate. After creating the wound, the medium was aspirated, and the wells were washed twice with fresh medium to remove any cell that had been uprooted from the scratched area. 100 μ L of medium containing the indicated concentrations of the **5a** or colchicine were then added to each well (n=4). The plate was fitted into the IncuCyte Imaging System chamber, and images of the cell migration were recorded every 2 hours for a total duration of 24 hours. The affiliated IncuCyte ZOOM™ live cell imaging software calculated scratch closure in real time and determined the relative wound density within the scratch-channel area at each time point. The relative wound density was calculated as

the ratio of the occupied area compared to the total area of the initial scratched area. Rate of change was calculated as % change in relative wound density/ hour.

Cell Cycle Analysis

To determine cell cycle distributions in the G₂ and M phases, 1×10^6 cells A375 cells plated in 10 cm tissue culture-treated dishes, incubated overnight, and then serum-starved for 24 hours to synchronize. They were then treated for 24 hrs with 10 or 50 nM of drug in full media. Cells were harvested with trypsin, fixed with 4% paraformaldehyde, and stained using P-Histone H3 rabbit antibody (Cell Signaling Technologies, Danvers, MA) and Alexafluor-488 secondary antibody AlexaFluor® 488 IgG (Invitrogen, Eugene, OR) followed by stained using PI/RNase solution (Sigma, Sigma-Aldrich, St. Louis MO) for 30 minutes at room temperature and protected from light. Data was collected with Bio-Rad ZE5 flow cytometer (Bio-Rad, Hercules, CA) and the acquisition software used was Everest v2.1.07, plotting at least 20,000 events per sample. Analysis was performed using FlowJo v10.3.

Apoptosis Assay

A375 cells were seeded, treated, and harvested in the same manner as described above. Apoptosis analysis was performed using the Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, MA) as per manufactures instructions utilizing fluorochrome-conjugated annexin V and propidium iodide solution. Data was collected with Bio-Rad ZE5 flow cytometer (Bio-Rad, Hercules, CA) and the acquisition software used was Everest v2.1.07, plotting at least 15,000 events per sample. Analysis was performed using FlowJo v10.3.

Western Blot

1×10^6 cells A375 or M14 cells were seeded in 10 cm tissue culture-treated dishes, incubated overnight, then treated for 24 hours with indicated compounds and concentrations of 0, 15, 40, or 100 nM. Harvested cells were collected in RIPA lysis buffer including protease inhibitors and sonicated. The lysates were centrifuged at $13,000 \times g$ for 15 min at 4 °C. The concentration of total protein was measured using the BCA assay method and absorbance was read at 562 nm on a plate reader (BioTek Instruments Inc., Winooski, VT)(n=2). Protein samples were separated with 15% SDS-PAGE and transferred onto the methanol-activated PVDF membranes. Primary antibodies PARP, c-PARP, Akt, p-Akt, PH3, and GAPDH and secondary anti-rabbit and anti-mouse were all obtained from Cell Signaling Technologies (Danvers, MA.) Images were obtained using the ChemiDoc-It2 Imaging System (Bio-Rad, Hercules, CA).

***In Vivo* Xenograft Studies**

All animal experiments were performed in accordance with the NIH animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center (UTHSC, Memphis, TN). Nude mice, 6–8 weeks old, were purchased from Envigo. We first estimated the acute MTD for **4a** and **6a** formulated in PEG300. The MTD following a single dose was estimated to be at least 100 mg/kg for **4a** and at least 60 mg/kg for **6a**. To ensure an adequate safety margin for the xenograft study, the maximum dose was scaled down to 30 mg/kg for **4a** and 15 mg/kg for **6a**.

Logarithmic growth phase A375, PC-3, or PC-3/TxR was prepared in phenyl red-free, FBS-free medium and mixed with Matrigel immediately before injecting into mice. Tumors were established by injecting 100 μ L of this mixture subcutaneously in the dorsal flank of each mouse (2×10^6 A375 cells or 2.5×10^6 prostate cancer cells). After tumor volumes reached approximately 100 mm³ mice were randomized into control or treatment groups ($n = 8$ for A375 and PC-3 model, $n = 7$ for PC-3/TxR xenograft model). **4a**, **6a**, or paclitaxel was dissolved in a 2:1 ratio of PEG300/PBS solution to produce desired concentrations. The vehicle control solution was formulated with equal parts PEG300 and PBS only. 100 μ L of the drug treatment or vehicle control was administered via i.p. injection every other day for 2–3 weeks.

Tumor volume was measured three times a week with a caliper and calculated by using the formula $a \times b^2 \times 0.5$, where a and b represented the larger and smaller diameters, respectively. Tumor growth inhibition (TGI) at the conclusion of the experiments was calculated as $100 - 100 \times ((T - T_0)/(C - C_0))$, where T , T_0 , C , and C_0 are the mean tumor volume for the specific group on the last day of treatment, mean tumor volume of the same group on the first day of treatment, mean tumor volume for the vehicle control group on the last day of treatment, and mean tumor volume for the vehicle control group on the first day of treatment, respectively. Animal activity and body weights were monitored during the entire experiment period to assess potential acute toxicity. At the end of the experiment, mice were sacrificed and the tumors were weighed. Tumors and tissues were dissected out and fixed in 10% buffered formalin phosphate solution prior to pathology staining analysis.

Histology and Immunohistochemistry

The fixed tumor xenograft tissues were embedded in paraffin. Serial sections were obtained and stained with hematoxylin and eosin (H&E) and immunohistochemistry. IHC staining was performed with rabbit anti-cleaved caspase 3 antibody (Cell Signaling Technology Inc., Danvers, MA) and rabbit anti-CD31 (Cell Signaling Technology Inc., Danvers, MA) following ABC-DAB methods. Antigen retrieval was performed with H-3300 antigen unmasking solution (Vector Laboratories, Burlingame, CA). Slides were scanned, and representative images were obtained at 100 $\times$ magnification with Aperio ImageScope (Lecia Biosystems Inc., Buffalo Grove, IL).

Statistical Analysis and Calculations

Data was analyzed using Prism Software 5.0 (GraphPad Software, Inc., San Diego, CA). Data was provided as mean $\pm$ SD unless otherwise indicated. Non-linear regression of transformed data was used to calculate IC₅₀ values for cytotoxicity assays. Log P was calculated using Schrödinger Molecular Modeling Suite (Schrödinger LLC, New York). The statistical significance ($P < 0.05$) was calculated by one-way ANOVA followed by Dunnett's multiple comparison test, comparing each treated group at the final time point to the control group for the migration, colony, capillary-like formation *in vitro* assays, and the tumor weight and final tumor volume *in vivo* studies.

Results and Discussion

Drug Design, Potency, and Metabolic Stability

We designated a four-ring system similar to **1f**, and the starting scaffold (ring A in red, ring B in purple, ring C in green, and ring D in pink) is shown at the top of **Table 4-1**. First, we designed and synthesized a series of seven heteropyrimidine derivatives with a diverse A/B ring. These include pyridopyrimidines (**4a** and **4b**), furopyrimidine (**6a**), thiophenopyrimidine (**6b**), N-Methyl-pyrazolopyrimidine (**8a**), N-methyl-purine (**10**), and 1H-pyrazolopyrimidine derivatives (**14**). The two pyridopyrimidines, **4a** and **4b**, showed exceptional IC₅₀ values against melanoma cell lines (IC₅₀ $\sim$ 6 nM) and **4b** (IC₅₀ $\sim$ 4 nM) relative to the lead **1d** (IC₅₀ $\sim$ 2 nM). These compounds also showed increased water solubility (**1d**; logP = 4.5, **4a**; logP = 3.7, **4b**; logP = 4.0). The furopyrimidine compound, **6a**, also was highly active against the 3 tested cancer cell lines, with an average IC₅₀ of 3.9 nM.

Next, we synthesized three C ring modified derivatives, including a keto-pyridopyrimidine (**16**), alcohol-pyridopyrimidine (**17**) and a morpholino-pyridopyrimidine (**19**). While the alcohol-pyridopyrimidine showed moderate potency, with an average IC₅₀ of 45.5 nM, the other modifications were detrimental to activity (**Table 4-1**).

Then, we investigated a series of modifications that involved opening the C ring, and increasing the number of methoxy substituents on ring D, since many reported tubulin inhibitors, including colchicine, contain trimethoxy groups. Dimethoxy (**21a**), trimethoxy (**21b**) and dioxymethylene (**23**) and naphthyridin (**25**) analogs were synthesized. The dimethoxy compound (**21a**) was found to possess moderate activity with an IC₅₀ value 42 nM, however, the more sterically hindered trimethoxy compound (**21b**), the dioxymethylene compound (**23**) was and the naphthyridin compound (**25**) had reduced or abolished activity against cancer cells (**Table 4-1**).

Table 4-1. Cytotoxic effects heterocyclic-fused pyrimidine analogs against malignant melanoma cancer cell lines and log P values.

	ID	Structure	IC ₅₀ ± SEM (nM)				^a Log P
			A375	M14	RPMI-7951	Average
A/B ring	4a		5.6 ± 1.6	5.5 ± 1.0	8.2 ± 1.5	6.4	3.7
	4b		4.0 ± 0.2	4.2 ± 0.8	4.4 ± 0.6	4.2	4
	6a		3.2 ± 0.5	3.3 ± 0.4	5.1 ± 0.9	3.9	3.9
	6b		49.0 ± 3.3	52.6 ± 5.6	91.3 ± 9.8	64.3	4.8
	8a		> 1000	> 1000	> 1000	> 1000	3.8
	8b		16.1 ± 1.4	18.8 ± 1.0	17.6 ± 1.1	17.5	2.8
	10		39.6 ± 2.9	41.2 ± 3.2	58.9 ± 3.1	46.6	3.4
	14		> 1000	> 1000	> 1000	> 1000	2.9
C ring	16		> 1000	> 1000	> 1000	> 1000	2.5
	17		36.1 ± 2.8	52.6 ± 5.6	41.9 ± 7.5	43.5	2.9
	19		223.3 ± 22.2	268.9 ± 17.0	404.5 ± 49.9	298.9	3.2
C/D ring	21a		39.0 ± 4.3	39.5 ± 3.0	47.5 ± 4.4	42	3.4
	21b		> 1000	> 1000	> 1000	> 1000	3.5
	23		161.7 ± 14.0	164.1 ± 12.4	178.9 ± 21.4	168.2	2.8
	25		> 1000	> 1000	> 1000	> 1000	4.5
colch			6.4 ± 0.3	6.5 ± 0.4	6.6 ± 0.5	6.5	3.5

^aLog P was calculated using Schrödinger Molecular Modeling Suite.

Finally, we designed new pyridopyrimidine analogs with a dihydroquinoxalinone head group, as compared to the tetrahydroquinoline head group in **4a** analogs. We hypothesize that switching to the dihydroquinoxalinone head group would make the phenyl ring of the head group electron deficient, leading to a more inert and metabolically stable methoxy group. Additionally, incrementing hydrogen bonds with the tubulin binding site should render stronger binding and lead to improved anticancer efficacy. We synthesized two different analogs, **5a** and **5b**, for this series and tested them for their *in vitro* activity. In addition to melanoma cell lines tested for the previous compounds, **5a** and **5b** were tested in additional melanoma cell lines including the P-gp overexpressing M14/MDR1 cell line, as well as other cancer cell types including prostate, lung, and breast cancers. (Table 4-2). We found that both compounds were highly potent, particularly **5b**, which demonstrated greater cytotoxic activity than colchicine against all tested cell lines, with IC₅₀s ranging from 1.2-10.3 nM. It is also notable that the P-gp overexpressing M14/MDR1 cell line was significantly less sensitive to colchicine treatment than the parental M14 cell line, whereas **5b** was nearly equipotent against the resistant cell line (RI=1.25). Similarly, PC-3/TxR cells were not resistant to treatment with **5a** or **5b** but were about ~7-times more resistant to colchicine treatment.

Because we wanted to see if the modifications that improved the potency of the analogs would also improve metabolic stability, we measured the half-life and clearance of compounds **4a**, **6a**, **5a**, and **5b** in human, mouse, and rat liver microsomes (Table 4-3). The modification of the A ring to a pyridopyrimidine or furopyrimidine (**4a** and **6a**) resulted in significant improvement in metabolic stability. In human liver microsomes, **4a** demonstrated a half-life of 84 mins, comparable to Azixa (79 mins), whereas **1d** previously reported a half-life of only 10 minutes (145). We also found that **5b** had improved metabolic stability, with a half-life in human liver microsomes 5.54 ± 0.32 hrs, and **5a** showed even greater improvement, with a half-life greater than 8 hours. Based on these initial findings of our compounds on cell viability, metabolic stability, and water solubility, the most promising compounds, **4a**, **6a**, **5a** and **5b** were selected to undergo additional biological analysis (Structures shown in Figure 4-2A).

Mechanism of Action via the Colchicine Domain

To experimentally validate whether the newly designed analogs preserve their mechanisms of action as tubulin polymerization inhibitors, we evaluated the lead compounds (**4a**, **6a**, **5a**, and **5b**) in a cell-free microtubule polymerization assay (Figure 4-2B). The greatest maximal velocity of tubulin polymerization was demonstrated by paclitaxel, a potent enhancer to tubulin polymerization. The vehicle control treated group also displayed robust polymerization. **4a**, **6a**, and **5b** potently inhibited the tubulin polymerization, while colchicine and **5a** suppressed tubulin polymerization altogether. It is interesting to note that while **5b** had stronger efficacy against cancer cell lines, **5a** demonstrated greater inhibitory action on tubulin polymerization.

Next, we investigated the effect of drug treatment on microtubule networks *in vitro* via confocal immunofluorescent microscopy. WM-164 melanoma cells were treated

Table 4-2. Cytotoxic effects of 5a and 5b against melanoma, prostate, lung, and breast cancer cell lines.

Cancer Type	Cell Line	IC ₅₀ ± SEM (nM)		
		5a	5b	Colchicine
Melanoma	A375	14.9 ± 1.3	4.1 ± 0.2	11.7 ± 0.2
	RPMI-7951	20.6 ± 4.7	3.3 ± 0.5	16.8 ± 3.1
	WM-164	50.3 ± 5.6	4.9 ± 0.2	14.4 ± 2.9
	SK-MEL-1	44.0 ± 12.4	2.6 ± 0.3	4.1 ± 0.6
	M14	58.7 ± 4.8	8.2 ± 0.5	21.7 ± 1.3
	M14/MDR1	258. ± 49.7	10.3 ± 0.3	~800
Prostate	PC-3	6.8 ± 0.6	4.4 ± 0.3	5.3 ± 1.5
	PC-3/TxR	7.7 ± 0.4	4.3 ± 0.1	35.4 ± 4.2
	DU-145	17.7 ± 1.8	6.2 ± 0.5	47.5 ± 11.7
	DU-145/TxR	65.8 ± 5.8	7.7 ± 0.3	846.1 ± 270.6
Lung	A549	182.6 ± 14.3	6.9 ± 0.5	75.8 ± 6.6
	H460	169.8 ± 32.4	5.2 ± 0.7	27.6 ± 6.2
	H1299	7.1 ± 0.8	2.5 ± 0.5	13.6 ± 2.5
Breast	MDA-MB-453	6.2 ± 0.9	1.2 ± 0.4	3.9 ± 0.9
	MDA-MB-231	7.7 ± 0.5	3.5 ± 0.7	11.1 ± 0.6
	MDA-MB-468	8.8 ± 3.2	6.1 ± 2.1	6.2 ± 0.8

Table 4-3. *In vitro* metabolic stability of 4a, 6a, 5a and 5b in liver microsomes.

Compound	Metabolic Stability (Human)		Metabolic Stability (Mouse)		Metabolic Stability (Rat)	
	$t_{1/2}$ (hr)	Cl_{int} (mL/min/kg)	$t_{1/2}$ (hr)	Cl_{int} (mL/min/kg)	$t_{1/2}$ (hr)	Cl_{int} (mL/min/kg)
Azixa	1.32 ± 0.10	15.80	0.06 ± 0.003	987.69	0.81 ± 0.07	57.53
4a	1.39 ± 0.08	15.0	0.15 ± 0.01	379.6	0.74 ± 0.06	63.0
6a	0.32 ± 0.02	65.3	0.08 ± 0.003	748.2	0.09 ± 0.01	511.2
5a	36.00 ± 19.13	0.58	16.90 ± 2.79	3.38	ND	ND
5b	5.54 ± 0.32	3.75	1.02 ± 0.03	56.12	5.07 ± 0.20	9.23
Verapamil	1.40 ± 0.10	14.82	1.51 ± 0.12	37.74	3.39 ± 0.24	13.80

Data obtained by Lei Yang (St. Jude Children's Research Hospital).

Figure 4-2. New analogs potentially inhibit tubulin polymerization.

A) Structures of lead compounds **4a**, **6a**, **5a**, and **5b**. B) Polymerization of purified tubulin in a cell-free assay. Tubulin (3.33 mg/ml) was exposed to vehicle control or compounds at the indicated concentrations (n=2). Absorbance at 340 nm was monitored at 37°C every minute for 50 min.

with 100 nM of compound **4a**, **6a**, **5a**, **5b**, docetaxel, or left untreated. The immunofluorescent images obtained visually demonstrate how the microtubules are affected by stabilizing or destabilizing agents (**Figure 4-3A**). The control cells display fibrous microtubules extending throughout the cell, providing cellular shape and structure. The newly synthesized lead compounds all induced extensive fragmentation of the microtubules, decreased polymeric tubulin filaments, and increased cytoplasmic soluble tubulin. Docetaxel treatment caused the opposite action on microtubules, and dense tubulin accretion, representative of aggressive polymerization, can be observed. These results clearly demonstrate that the Azixa analogs exhibit characteristics of tubulin polymerization inhibitors and have a mechanism of action that is opposite of stabilizing agents.

Unlike the paclitaxel or vinblastine binding sites, the colchicine binding site in tubulin can accommodate diverse scaffolds of ligand structures which do not seem to have apparent similarities (192). A seemingly minor change to a potent colchicine site ligand could significantly compromise its binding and diminish antiproliferative potency (144). The high flexibility of the loop α -T5 (Ala174-Val182 loop) in the α -tubulin monomer and the loop β -T7 (Phe242-Asp249 loop) in the β -tubulin monomer are partially responsible for the unique characteristics at the colchicine binding site. However, this flexibility is also responsible for significant challenges in using molecular modelling to predict correct binding poses of one ligand scaffold with existing crystal structures of different ligand scaffolds. This proves an even greater obstacle for the Azixa scaffold because of the structural similarity of the upper and lower part of the molecule separated by the middle nitrogen atom.

Therefore, to unambiguously determine the real binding modes of these analogs to tubulin and to correctly determine the detailed molecule interactions, we determined the co-crystal structures of tubulin-RB3_SLD-TTL protein, in complex with two of the most potent analogs, **4a**, and, **6a** (**Figure 4-3B**). In the un-liganded complex, the β -T7 loop has a 'closed' conformation and it interacts with the α -T5 loop from α -tubulin (PDB ID: 4I55). These loops move apart as colchicine binds at the interface and occupies a pocket in β -tubulin (**Figure 4-3C**). This loop opens to make a pocket in β -tubulin to accommodate colchicine (PDB ID: 4O2B, violet color).

In case of our compounds, the α -T5 loop in α -tubulin remains in place as seen in case of un-liganded complex, but β -T7 loop of β -tubulin opens as seen in case of colchicine bound structure, although the two conformations of the loop are slightly different. Both compounds bind at the site created as a result of loop opening, with ring A and B occupying the pocket that contains the TMP moiety of colchicine, sandwiched between the side chains of Cys239, Leu253 and Ala314 of β -tubulin. Ring C and D are facing α -T5 loop of α -tubulin, sandwiched between side chains of Asn256 and Lys350 of β -tubulin (**Figure 4-3D** and **E**). The chlorine attached to ring B interacts with the hydrophobic side chain of Leu240 from β -tubulin. The D-ring with its methoxy group also makes hydrophobic interactions with the side chain of Met257 from β -tubulin. There is a water-mediated hydrogen bond between the 1-position N atom of ring B and main

Figure 4-3. Compounds destabilize microtubules and target the colchicine binding site.

(A) Microtubules of WM-164 cells following 18 hr treatment with 100 nM of **5a**, **5b**, **4a**, **6a**, compared to docetaxel or untreated control, visualized by α tubulin primary and AlexaFluor 647 secondary antibody via confocal microscopy. B) X-ray crystal structures of the tubulin-RB3-SLD-TTL complex with the ligand. C) Superimposed un-liganded (grey), colchicine bound (violet) and **4a** bound (gold) tubulin-RB3-SLD-TTL complex showing the different loop conformations resulting from binding of ligands. D) High resolution X-ray crystal structures of tubulin protein in complex with **4a** (resolution 2.3 Å), and E) **6a** (resolution 2.7 Å). The α -tubulin monomer is shown in green and the β -tubulin monomer is shown in cyan. Crystallographic data obtained by Yuxi Wang (Sichuan University, China) and interpreted by Dr. Stephen White and Dr. Gyanendra Kumar (St. Jude Children's Research Hospital).

chain -NH of Cys239. Additionally, the main chain C=O of Val236 is also within hydrogen bonding distance of this water molecule. These crystal structures not only provide a rationale for the affinity of these compounds, they also provide opportunities for improving these compounds further. Surprisingly, the actual binding poses for these Azixa derivatives determined by this X-ray crystallography study showed different binding pose than previously published molecular modelling predictions of Azixa and its derivatives, using available X-ray crystal structures containing colchicine (PDB ID: 4O2B) or its close analog DAMA-colchicine (PDB ID: 1SA0)(102, 145, 167, 250, 256). In those molecular modelling predictions, the pyrimidine ring was oriented towards the α -monomer, and the 6-methoxyphenyl moiety overlapped the trimethoxyl moiety in colchicine, which is oriented towards the β -monomer. In contrast, the high-resolution X-ray crystal structures revealed that the 6-methoxyphenyl moiety is actually oriented towards the α -monomer, instead of the β -monomer. These two dramatically different binding poses would guide structural optimizations for the Azixa analogs in very different directions. Thus, the current X-ray findings provide, for the first time, insight into the true molecular interactions of Azixa analogs with the colchicine binding site of tubulin and will help future X-ray crystallography-guided synthesis of more efficient derivatives. It also underscores the complications associated with reliability of using molecular modelling and the superiority of definitive X-ray crystallographic analysis.

Interference with Cellular Functions

To investigate the interference of cellular functions inflicted on cancer cells *in vitro* by the selected compounds, we next tested their actions against anchorage-dependent colony formation and proliferation. A375 cells were challenged with long-term exposure to **5a**, **5b**, and colchicine at concentrations ranging from 0.25 nM to 2 nM, which are below their determined IC₅₀s. **5b** was able to reduce colony number proportionally to the drug concentration (**Figure 4-4A**). **5a** and colchicine showed a nominal decrease in total colony number at the lower concentrations compared to the control, and there was not a significant difference between the counted colony numbers for the lower or higher concentrations within a treatment group. However, based on the overall colony area coverage, there was a significant difference for all treated groups compared to the control, as well as a trend within groups receiving different concentrations of the same compound. This may indicate that **5a** and colchicine can inhibit proliferation at low concentrations but do not have as great of an effect on the clonogenic potential, while **5b**, the most potent compound, was able to inhibit the clonogenicity and cellular propagation.

Since microtubule targeting agents can interfere with microtubule networks to inhibit cell proliferation, migration, expansion and consequently cause morphological changes to endothelial morphology, we wanted to determine if **5a** and **5b** would exhibit similar actions against HUVECs *in vitro* (222). The capillary-like tubular network collapse of HUVECs grown on Matrigel was measured as total tube length and number of formed junctions (**Figure 4-4B**). **5a** decreased total tube length by 31.9%, 54.0% and

Figure 4-4. Compounds reduce formation of cancer cell colonies and HUVEC tube cell networks.

A) Colony formation assay of A375 cells treated with **5a**, **5b** or colchicine at concentrations from 0 to 2 nM (n=3). Colony assays were treated for 10 days in 6-well plates and quantified as total number of colonies and colony surface area $\pm$ SD as determined by VisionWorks software. B) Representative images of HUVEC capillary formation on Matrigel taken after 4 hr incubation with indicated concentrations of **5a**, **5b**, or colchicine. Quantification of total tube length and junction number was performed with ImageJ analysis and expressed as mean $\pm$ SD (n=4). Statistical analysis for both assays was performed using one-way ANOVA followed by Dunnett's multiple comparison test of treatment groups to the untreated control. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

47.4% at 25, 50, and 100 nM concentrations, respectively. Even at the highest concentration, colchicine only reduced overall network length by 28.1%. **5b** showed reductions in total length between 49.5% and 52.8% at all concentrations. Junctions were also quantified and provide more information on the extent of angiogenic inhibition. **5a** showed a linear trend, decreasing junction number by 58.2% at the lowest concentration and up to 77.6% at the highest concentration. All tested **5b** concentrations reduced the total number of junctions formed by at least 74.4%. Colchicine, on the other hand, was only able to decrease the junction number by 45.4% at the highest concentration of 100 nM. Statistical analysis was determined by comparing each treated group to the untreated control group. Both **5a** and **5b** were more effective than colchicine by both measures (network length and number of junctions). Because of the significant inhibition achieved by **5b** at the lowest concentration of 25 nM and the concentration-dependent effect observed by **5a**, these results show that the compounds impair formation and structure of the capillary-like networks formed by HUVECs cultured on a Matrigel basement membrane matrix. This offers further support that **5a** and **5b** may have additional actions as VDAs/anti-angiogenic agents.

Drugs that target microtubules have also been reported to interfere with cell migration and motility at low concentrations. We tested the ability of **4a** to decrease cell migration compared to colchicine and untreated control cells in a wound healing assay (**Figure 4-5A**). The control A375 and RPMI-7951 cells that were not treated showed an average wound closure of $60.7\% \pm 3.5\%$ and $71.8\% \pm 1.8\%$, respectively. At 5 nM concentrations, **4a** caused less A375 and RPMI-7951 cells to migrate into the wound, closing the gap by $36.4\% \pm 2.5\%$ and $46.4\% \pm 2.0\%$. At higher concentrations of 25 nM, even greater migration inhibition was observed by the cells, where cells migrated into only $16.9\% \pm 1.7\%$ and $25.5\% \pm 5.8\%$ of the wound area for A375 and RPMI-7951, respectively. A significant decrease in wound closure was observed by cells treated with **4a** by both concentrations and against both cell lines compared to the control. Colchicine showed less inhibition than **4a**, where the A375 wound channel was closed by $46.0\% \pm 1.4\%$ and $20.7\% \pm 2.2\%$ when treated with 5 nM and 25 nM of the drug, respectively. The RPMI-7951 cells treated with 25 nM of colchicine migrated into $42.8\% \pm 5.8\%$, and a significant decrease in migration was not observed when treated with 5 nM of colchicine. **5b** was also tested in scratch migration assay against A375 and M14 melanoma cells in a time-dependent and concentration-dependent manner (**Figure 4-5B**). The wound density covered by the migrating cells was measured in real-time compared to the starting wound area, and rate of change was calculated by the change in relative wound density % per hour. For A375 cells, there was an obvious reduction in migration for the **5b** treated cells at concentrations as low as 2.5 nM compared to the control. Treatments with **5b** at 5 and 10 nM concentrations showed similar inhibitory effects but to a greater degree. Colchicine did not show a concentration-dependent inhibition against A375 cells from 2.5- 10 nM concentrations, though it still slowed cell migration more than the untreated cells. The M14 cells demonstrate different results. Treatment with colchicine was not effective at hindering cell migrations at concentrations up to 10 nM. While little efficacy was apparent for **5b** at 2.5 nM concentrations against this cell line, it induced concentration-dependent action against cell migration at 5 and 10 nM.

Figure 4-5. Compounds inhibit melanoma cell migration.

A) Scratch migration assay of A375 (top) and RPMI-7951 (bottom) cell lines (n=4). Cells were treated with **4a** or colchicine at 5 or 25 nM for 24 hours and migration is presented as mean percent of the area closed $\pm$ SD. ImageJ software was used to calculate scratch area. Scale bar = 1000 μ m. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test, comparing each treatment group to the control group. (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). B) Wound density for A375 (left panels) and M14 (right panels) after treatment with **5b** or colchicine at indicated concentrations (n=4). Real-time analysis was performed with Incucyte live cell image system every 2 hours for 24 hours. The top graph shows % relative wound density $\pm$ SD and the bottom graphs show rate of change represented as change in relative wound density per hour $\pm$ SD.

concentrations. At the highest concentration, cell death became apparent beginning around 18 hours, as evidenced by the decrease in wound density and increase in rate of change during this time.

Compounds Induce G₂/M Phase Cell Cycle Arrest and Apoptosis

A common feature of tubulin inhibitors targeting the colchicine binding site is that they arrest cells in the G₂/M phase of the cell cycle (100). To determine whether **5a** and **5b** would arrest cancer cells in a similar fashion, we carried out cell cycle analysis utilizing flow cytometry to see if there was a dose-dependent effect. After syncing A375 the cells by serum-starvation, they were treated with a low or high concentration (10 or 50 nM) of **5a**, **5b**, or colchicine for 24 hrs (**Figure. 4-6A**). Compared to untreated control cells, where 11.7% of the population were indicated to be in the G₂/M phase of the cell cycle after 24 hours after synchronization, **5a**, **5b**, and colchicine all increased cell distribution at 50 nM concentrations, arresting 15.9%, 38.9% and 31.6% of the cells in this phase, respectively. At lower concentrations of 10 nM, only **5b** arrested cells in the G₂/M phase significantly more than the control cells, showing 35.4% of the population arrested in this phase. Even though **5a** and colchicine did not dramatically induce arrest at 10 nM, it is clear from the cell cycle distribution that the G₂/M phase arrest is concentration-dependent.

We also wanted to confirm that **5a** and **5b** treatment would lead to apoptotic cell death. Therefore, we quantified the percentage cells that were undergoing early stage apoptotic events (Annexin V positive stained cells) and late stage apoptotic events (Annexin V and propidium iodide positive stained cells) compared to synchronized A375 cells (**Figure 4-6B**). Untreated cells only showed 4.6% of the population undergoing apoptosis, while 50 nM of colchicine treatment led apoptosis for 33.7% of cells after 24 hrs. There was a modest increase in apoptotic events for **5a** treated cells at 50 nM concentrations, where 7.2% of cells were apoptotic. 10 nM treatment of **5b** caused a significant increase in the cells undergoing apoptosis, where 19.1% of the cell population was apoptotic. At higher concentrations of 50 nM, there was not a proportional increase in cell death initiation (20.7% apoptotic). However, these results correlate with the trend we observed from the cell cycle analysis, where the higher concentration (50 nM) of **5b** yielded only a slight increase in the percentage of cells arrested in the G₂/M compared to cells treated at lower (10 nM) concentration. We concluded that 24 hr treatment of **5b** with these concentrations (10 and 50 nM) will demonstrate similar efficacy, and either lower concentrations or time-dependent analysis would be needed to demonstrate a more palpable trend. Targeted cleavage of the Poly(ADP-ribose) polymerase (PARP), a nuclear protein, is another signature biomarker in the apoptotic process (257). We investigated total vs cleaved PARP in A375 and M14 cells after treatment with our compounds to determine if the results would correlate with our previous findings (**Figure 4-6C**). In both cell lines, no cleaved PARP was evident from the control cell lysates, but **5a** treatment exhibited concentration-dependent PARP cleavage from 15-100 nM after 24 hrs. **5b**

Figure 4-6. Compounds induce G₂/M phase arrest and apoptotic events.

A) Representative flow cytometry diagram of cell cycle distribution. Synchronized A375 cells were treated with 10 or 50 nM of **5a**, **5b**, or colchicine for 24 hours. B) Apoptosis analysis under the same conditions and determined by Annexin V and PI staining, where Q1 represents early state apoptosis and Q2 is late state apoptotic cells. C) Western blot analysis for protein levels of A375 (top) and M14 (bottom) cell lines after 24 hr treatment with 15, 40, or 100 nM concentrations of **5a**, **5b**, or colchicine. Apoptotic proteins (total and cleaved PARP), cell survival proteins (total and phosphorylated Akt), and cell cycle dependent protein phospho-histone3 are shown.

treatment showed strong expression of the cleaved PARP protein at all concentrations in the cell lysates, whereas colchicine-treated cells showed similar cleaved PARP levels to **5b** at the highest concentration, but lower levels than **5b** at 15 nM.

The phosphatidylinositol-3-kinase serine/protein kinase B (Akt) signaling pathway plays an important role in regulating cell survival, and constitutive activation of this pathway is commonly implicated in a variety of cancers, including melanomas (258, 259). The phosphorylation of Akt at Thr³⁰⁸ and Ser⁴⁷³ leads to its full activation, and elevated expression levels of phospho-Akt (p-Akt) is correlated with melanoma pathogenesis and progression, and it is inversely related to patient survival (260). We found that treatment with **5a** or **5b** in melanoma A375 cells reduced p-Akt expression levels. **5a** caused the greatest reduction at 100 nM concentrations, and **5b** was equally effective at concentrations as low as 15 nM.

Finally, we looked at phospho-histone H3 levels, which distinguish cells between the G₂ and M phase of the cell cycle, and its expression is negligible or absent in other stages (261). In M14 melanoma cells, **5a** treatment caused only a slight increase in phospho-histone H3 levels compared to the control cells. On the other hand, significant protein expression was observed in the **5b** and colchicine treated cells. This corroborates the cell cycle analysis findings, which showed relatively weaker induction of cell cycle arrest for the **5a** treated cells compared to the profound effects observed from the **5b** treated cells. These *in vitro* studies verify that our compounds, particularly **5b**, induce apoptosis and arrest melanoma cells in the G₂/M phase of the cell cycle in a similar manner as colchicine.

***In Vivo* Antitumor Efficacy**

We first determined the MTD in mice for compounds **4a** and **6a** (prior to full xenograft studies) and found that i.p. administration of 50 mg/kg (**4a**) or 30 mg/kg (**6a**) for 5 consecutive days was well-tolerated in mice and no apparent acute toxicities were observed. This is in contrast with Azixa and its reported analogs, where 1~4 mg/kg doses by i.v. injection were generally lethal for mice (145, 252, 254, 255, 262). We did consider though, that plasma concentration following systemic administration by intravenous injection does not necessarily proportionally reflect the drug levels achieved following intraperitoneal intervention. Therefore, we also tested the MTD for Azixa formulated at 10 mg/kg via i.p. injection, and while we did not have an Azixa-treated comparison group in our subsequent xenograft study, we did find that this was not a tolerable dose for the mice. **4a** and **6a** have comparable *in vitro* potency to Azixa and demonstrated higher MTDs, suggesting that our analogs may have an improved therapeutic index over Azixa with this route of administration.

We next investigated the antitumor effects of these compounds in an A375 xenograft model in nude mice, following our previously reported protocols. Once tumors developed, mice were randomized and treated by i.p. injection for two weeks with compound **4a**, **6a**, paclitaxel, or a vehicle solution. Tumor growth inhibition (TGI) was

calculated for the treatment groups compared to the vehicle control group (**Figure 4-7A and B**). **4a** treatment caused 57.1% and 72.3% TGIs for 15 mg/kg and 30 mg/kg treatment groups, respectively. 15 mg/kg doses of **6a** was also able to inhibit tumor growth by 66.5%. The group receiving 15 mg/kg doses of paclitaxel was used as a positive control and resulted in an overall TGI of 76.5%. Final tumor weights were also recorded prior to IHC analysis and reiterated the effect of **4a** and **6a** treatment on tumor inhibition. We found that 15mg/kg treatments with **4a** and **6a** reduced tumor weight by ~45%, and higher doses of 30 mg/kg of **4a** or paclitaxel treatment caused ~60% reduction compared to the control group (**Figure 4-7C**). Abnormal deviations in animal body weight indicative of acute toxicities were not observed (**Figure 4-7D**). One-way ANOVA followed by Dunnett's multiple comparison test demonstrated that each of the treatment groups caused a significant reduction in tumor size compared to the control group, yielding P values < 0.001 .

After tumors were fixed, histological analysis was performed and IHC staining revealed that there was an increase in the number of cells undergoing apoptosis for the groups receiving treatment with **4a**, **6a**, or paclitaxel (**Figure 4-7E**). Furthermore, CD31 staining showed that tumors from the treated groups displayed less overall microvessel density and exhibited morphological changes in the vessel structure. Although comparable *in vivo* testing for **5a** and **5b** is still underway, the initial *in vitro* results showing elevated apoptosis by flow cytometry, increased apoptotic activity indicated by cleaved PARP, and endothelial network disturbance of HUVECs closely shadows the *in vivo* results for **4a** and **6a**. Future testing in animal models with **5a** and **5b** is warranted to confirm their efficacy in biologically relevant models and compare their anticancer efficacy to their Azixa analog counterparts and other tubulin inhibitors.

Compound 4a Overcomes Taxane Resistance

To determine if **4a** is capable of escaping resistance associated with taxanes, we first tested the potency in parental prostate cancers PC-3 and DU-145, as well as paclitaxel resistant PC-3/TxR and docetaxel resistant DU-145/TxR cell lines (**Figure 4-8A**). **4a** was the most potent against PC-3/TxR cancer cells, with an IC_{50} of 8.7 ± 0.9 nM and a resistance index of 0.7. Colchicine treatment to PC-3/TxR cells revealed a resistant index of 3.5, and PC-3/TxR cells were even less susceptible to paclitaxel and docetaxel treatment ($RI > 30$). We then proceeded to determine the anticancer action of **4a** in a PC-3/TxR xenograft model in nude mice. To prove the efficacy of paclitaxel against non-resistant tumors, we conducted a study in parallel in a PC-3 xenograft model and confirmed that the non-resistant tumors were highly prone to paclitaxel treatment (**Figure 4-8B**). However, when paclitaxel was administered to mice bearing paclitaxel resistant tumors, there was no difference in tumor volumes compared to group that was administered only the vehicle solution (**Figure 4-8C and D**). Additionally, mouse weight began to drop moderately after 2 weeks of treatment, possibly as a result of toxicity (**Figure 4-8G**). On the other hand, treatment with 30 mg/kg of **4a** at the same frequency resulted in a significant decrease in tumor volume ($p < 0.01$), with a calculated TGI of

Figure 4-7. Inhibition of tumor growth *in vivo*.

A) A375 xenograft model in nude mice for **4a**, **6a**, and paclitaxel treatment groups. Graph represents mean tumor volume $\pm$ SEM (n=8). B) Individual tumor final volumes represented as mean tumor volume for each group $\pm$ 95% confidence interval. C) Tumor weights $\pm$ SEM. D) Mouse body weights represented as a % change of the initial weight $\pm$ SEM values. Statistical significance was determined by Dunnett's multiple comparison test for the treatment groups compared with the vehicle control group (**** $P < 0.0001$, *** $P < 0.001$). E) Representative images of tumor samples for treatment groups by H&E and IHC staining. Compared to the control, treatment groups displayed elevated cleaved caspase-3 expression indicative of apoptosis (middle column), and decreased density of CD31-stained microvessels (right column).

Figure 4-8. 4a overcomes taxane resistance and demonstrates antitumor efficacy.

A) Cytotoxicity of **4a** against parental and taxane-resistant prostate cancer cells lines. Curve was fitted by nonlinear regression to determine IC₅₀ values (n=4). B) Prostate cancer xenograft models in nude mice for PC-3 tumors and C) paclitaxel-resistant PC-3/TxR tumors represented as mean tumor volume $\pm$ SEM (n=7). D) Individual PC-3/TxR tumor final volumes. Bar graph represents the mean tumor volume for each group $\pm$ SEM. E) PC-3/TxR tumor weights $\pm$ SEM. F) Representative images of PC-3/TxR tumors. G) Mouse body weights in the PC-3/TxR xenograft model represented as mean body weight change as a percentage compared to initial weight $\pm$ SEM values. Statistical significance was determined by ANOVA analysis followed by Dunnett's multiple comparison test for final tumor volumes and weights compared to the corresponding control group (**** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$).

55.6%. There was also a considerable difference in tumor weight for the **4a** treated group ($p < 0.05$) and observable differences in tumor size (**Figure 4-7** and **F**). One-way ANOVA showed statistical significance between groups based on final tumor volume ($p = 0.0007$) and weight ($p = 0.002$).

In this paclitaxel-resistant PC-3/TxR prostate cancer cell line model, more than 200 genes are upregulated in addition to P-gp overexpression, representing a large number of potentially clinically relevant taxane-resistant mechanisms (174, 175). The efficacy of compound **4a** in suppressing tumor growth in this highly aggressive, tumor model suggests that these new analogs have the potential to overcome resistance mechanisms employed by MDR tumors. This is also consistent with the general properties of Azixa or its related analogs targeting the colchicine binding site in tubulin (101, 242, 252).

Conclusions

In summary, we have reported the design and potency of new conformationally restricted Azixa analogs with a heteropyrimidine scaffold as well as dihydroquin-oxalinone pyridopyrimidine analogs. Biological assays indicated that two of the most potent compounds of the series, **4a** and **6a**, had IC₅₀ values in the low nanomolar range against a panel of metastatic melanoma cell lines, similar to that of colchicine and existing Azixa analogs. However, **4a** and **6a** showed significantly improved metabolic stability in human liver microsomes than similar conformationally restricted Azixa analogs (quinazoline analogs) reported by Xie et al. The **5a** and **5b** analogs showed even greater improvement in metabolic stability and demonstrated strong anticancer potency in a multitude of cancer types, including melanoma, prostate, lung, and breast cancer, as well as including P-gp overexpressing M14/MDR1 cells and taxane resistant PC-3/TxR and DU-145/TxR cell lines.

These new analogs inhibit tubulin polymerization, promote microtubule destabilization, and directly target colchicine binding site, which was confirmed with high-resolution crystal structures for representative compounds **4a**, and **6a**, in complex with tubulin. Binding poses for these compounds revealed by the X-ray crystal structures were dramatically different than binding conformations predicted from molecular modeling, and this underscores the complexity and flexibility of the colchicine binding site. The ability of the compounds to impede cellular activities was demonstrated through a variety of mechanisms including migration, clonogenicity, endothelial cell network formation. They also share trademark characteristics with other potent antimitotic agents such as inducing G₂/M phase cell cycle arrest and promoting apoptosis. *In vivo*, **4a** and **6a** significantly reduced tumor growth in a xenograft model in nude mice challenged with A375 melanoma at the respective doses of 30 mg/kg and 15 mg/kg after 2 weeks of treatment. Subsequent immunohistochemistry analysis of the tumors also indicated that treatment with **4a** and **6a** caused elevated apoptosis and extensive vascular disruption within the tumors. Finally, compound **4a** demonstrated the ability to overcome paclitaxel resistance *in vitro* and *in vivo* (30 mg/kg), substantiating the efficacy of colchicine

binding site inhibitors against resistant cancers. These studies demonstrate the potential of **4a**, **6a**, **5a**, and **5b** as anticancer agents, provide proof-of-concept for their mechanisms of action, and reveal the structural basis to support the continued development for these lead compounds as tubulin inhibiting and vascular disrupting chemotherapeutic agents.

CHAPTER 5. INTRODUCTION TO TARGETING THE INHIBITOR OF APOPTOSIS PROTEINS⁴

Inhibitor of Apoptosis Proteins

Apoptosis is a critical component of cell regulation, homeostasis, and programmed cell death. While cell death can proceed through several pathways such as autophagy or necroptosis, apoptosis is perhaps the best-understood and primary pathway through which programmed cell death is carried out. Dysregulation of apoptosis is observed in many human diseases and is one of the hallmarks of cancers (263, 264). In principle, evasion of programmed cell death is the result of failure to propagate apoptosis and caspase activation pathways (265). Inhibitor of apoptosis proteins (IAPs) are up-regulated in many types of cancer and contribute to tumor progression, treatment resistance, and poor prognosis. They suppress apoptosis induced by an array of stimuli, including death receptor activation, growth factor withdrawal, ionizing radiation, viral infection, endoplasmic reticulum stress, and genotoxic damage (266, 267). IAPs also promote cell survival through regulation of signaling pathways. While many traditional treatment approaches such as radiation and chemotherapy seek to treat cancer by inducing apoptosis, cancer cells often develop resistance to these treatment strategies, particularly when IAP's are upregulated. Targeting these apoptotic regulators and suppressing their inhibitory function to promote cell death is an attractive anticancer strategy (264, 268-270). Substantial progress has been made in the development small molecules that are capable of penetrating the cell membrane and potent enough to restore apoptotic function via IAP suppression. IAP inhibitors may induce cell death and tumor regression as single agents by direct IAP interaction or synergize with additional cytotoxic agents to sensitize cancer cells to apoptosis and overcome drug resistance. Increased knowledge and greater insight of the biological mechanisms and structure of IAPs has laid the groundwork for the targeting strategy of small-molecule IAP inhibitors and has greatly contributed toward their translation and incorporation into clinical practice.

Structure of IAP Proteins

IAP proteins are a family of endogenous anti-apoptotic proteins that hinder apoptosis by interfering with caspase activation. In addition to their roles in apoptosis, they can also influence non-apoptotic processes such as cell differentiation, invasion, migration, and metastasis (271-273). The first IAP family member was identified during a genetic screening of SF21 baculoviruses, where a 1.6kB gene encoding a 31kDa anti-apoptotic protein with a zinc finger-like motif was discovered (274). To date, there are eight mammalian Baculoviral IAP repeat-containing (BIRC) protein family members:

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neuronal IAP (NIAP, BIRC1), cellular IAP1 (cIAP1, BIRC2), cellular IAP2 (cIAP2, BIRC3), X chromosome-linked IAP (XIAP, BIRC4), survivin (BIRC5), apollon (BRUCE, BIRC6), melanoma IAP (ML-IAP, livin, BIRC7), and IAP-like protein 2 (ILP-2, BIRC8) (275) (Fig. 1). Among these, XIAP, cIAP1, cIAP2, and ML-IAP are known to participate in the inhibition of apoptosis through caspase inhibition and cell signaling (276, 277).

Proteins in this family are characterized by at least one (but up to three) baculoviral IAP repeat (BIR) domains in the N-terminal portion of the protein. The BIR domains are highly conserved, comprised of ~80 amino acids, and contain histidine and cysteine residues that coordinate a zinc ion. In the C-terminus they may also contain a Really Interesting New Gene (RING) domain with E3-ligase activity, caspase activation recruitment domain (CARD), a ubiquitin-conjugating (UBC) domain, or a ubiquitin-associated (UBA) domain (276)(**Figure 5-1**). Only X-linked IAP has been shown to potentially inhibit apoptosis through caspase interaction and suppression. The BIR domains are largely responsible for binding to caspases, whereas RING domain-containing IAPs can regulate apoptosis through their E3 ligase activity (278, 279). Dimerization of the RING domain potentiates their E3 ubiquitin ligase activity, which induces IAP-mediated ubiquitylation by recruiting E2 ubiquitin-conjugating enzymes and facilitating the transfer of ubiquitin to target proteins and binding partners (280, 281). The function of the CARD domain in cIAP1 and cIAP2 is largely unknown, though it has been indicated to play regulatory role in auto-inhibition of E3 ligase activation by preventing RING dimerization (282). The UBA domain enables IAP proteins to bind to mono-ubiquitin as well as Lys 63- and Lys 48-linked poly-ubiquitin chains and is implicated in cell survival and oncogenesis (283). Survivin is the smallest member of the IAP family, containing a single BIR domain, but plays a versatile role in a multitude of cellular processes. While its function is still somewhat controversial, it influences cell death by both anti-apoptotic and pro-proliferation mechanisms. The direct interaction of survivin with caspases is unclear, but it can interact with apoptotic proteins to induce caspase-independent cell death (284, 285).

IAP Proteins Regulate Cell Death Pathways

All apoptotic signaling converges upon the activation of caspases, making their activity a crucial determinant in programmed cell death (286). Caspases, or cysteine-dependent aspartate specific proteases, are a family of cysteine proteases that cleave target proteins after specific aspartic residues (287). They are synthesized as inactive zymogens and remain latent until activated by cell death stimuli. Apoptotic caspases are further classified as initiator or executioner caspases according to their mechanism of action and manner of activation. Initiator caspase-8 and caspase-9 are activated by dimerization and responsible for activating downstream executioner caspase-3, caspase-6 and caspase-7 by cleaving their catalytic domain (288, 289). Executioner caspases catalyze a proteolytic cascade by cleaving cellular substrates that promote the ordered

Figure 5-1. Domains of mammalian IAP proteins

The domain organization for the 8 identified mammalian IAP proteins is shown. The defining characteristic is the presence of one or three baculoviral IAP repeat domains. These domains are required for the majority of protein-protein interactions with caspases or other apoptotic proteins. Several IAPs (XIAP, cIAP1, cIAP2, ML-IAP, and ILP-2) contain an E3 ubiquitin ligase zinc-finger at the carboxy-terminus. Both cellular inhibitors of apoptosis contain a caspase recruitment domain in the region between the BIR and the RING domains. Ubiquitin-associated (UBA) domains of XIAP, cIAP1, cIAP2, and ILP-2 are capable of binding to ubiquitin chains, and apollon contains a ubiquitin-conjugating (UBC) E2 domain. IAPs with roles in mitosis (survivin and apollon) have a slightly longer, type 2 BIR domain compared with the other IAPs.

disassembly and destruction of the cell. Caspases propagate apoptosis through two major pathways: the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway (**Figure 5-2**).

Intrinsic and Extrinsic Pathways. The mitochondrial pathway is activated by a vast array of non-receptor-mediated intracellular stress signals and pro-apoptotic stimuli such as cellular injury by toxins, viral infections, radiation, free-radicals, hypoxia or DNA damage. These stimuli cause permeability changes in the mitochondrial outer membrane, a process which is tightly regulated by several BCL-2 family proteins, and results in the loss of transmembrane potential (290, 291). Upon activation, BCL-2 family proteins BAK and BAX form pores in the outer mitochondrial membrane and direct the release of cytochrome *c* from the intermembrane space to the cytosol. Cytosolic cytochrome *c* then binds to apoptotic protease-activating factor 1 (Apaf-1) to form a complex called the apoptosome (292, 293). The apoptosome recruits and processes procaspase-9 into active caspase-9, forming a holoenzyme complex (294). Caspase-9 then cleaves and activates executioner caspase-3, which in turn targets downstream substrates to induce the final stages of apoptosis and cell death (295). Concurrent with cytochrome *c* efflux from the mitochondria, SMAC is also introduced into the cytosol and neutralizes the inhibitory effects IAP proteins (296). SMAC and its functions will be covered in greater a detail.

The extrinsic apoptosis pathway is initiated by the binding of death ligands such as FasL/CD95 ligand, TNF-related apoptosis-inducing ligand (TRAIL), or tumor necrosis factor α (TNF α), to their respective cell death receptors, Fas/CD95, DR4 or DR5, and TNFR1. Binding of death receptors ligands such as TRAIL or FasL stimulates receptor oligomerization and leads to the recruitment of the adaptor protein Fas-associated death domain (FADD/Mort-1) and procaspase-8. This forms the death-inducing signaling complex (DISC), which processes and activates caspase-8. Alternatively, the binding of TNF α with TNFR1 stimulates the formation of two complexes (297-299). Firstly, complex I is assembled at the membrane in conjunction with TNFR1 and consists of TNF receptor-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein 1 (RIP1). Complex I can translocate into the cytosol and recruit FADD and procaspase-8 form complex II assembly. Complex I internalization can be inhibited by cIAP1/2-mediated ubiquitylation of RIP1, which consequently suppresses apoptotic signaling (300, 301). As with DISC, complex II generates active caspase-8, which can then cleave and activate caspases-3 and caspase-7. Additionally, caspase-8 can advance apoptosis by cleaving and activating Bid, a pro-apoptotic BCL-2 family protein that is critical in pathway convergence (302, 303). The activated form of Bid, truncated Bid (tBid), migrates to the mitochondria and leads to activation of BAX and BAK, mitochondria membrane permeabilization, cytochrome *c* release, and induction of the intrinsic pathway (304-306). Executioner caspases-3 and caspase-7 elicit downstream apoptotic protein activation, including the nuclear enzyme poly ADP ribose polymerase (PARP), ultimately resulting in DNA fragmentation, degradation of nuclear and cytoskeletal proteins, formation of apoptotic bodies, expression of ligands for phagocytic uptake and cell death (307, 308).

Figure 5-2. Cell death pathways.

The extrinsic (death receptor) pathway is activated by the binding of cell death ligands to their respective receptors, which trigger the formation of signaling complexes that activate caspase-8. Caspase-8 can either process Bid to truncated Bid (tBid), which stimulates the activation of the mitochondrial pathway, or it can directly interact with and activate downstream caspases. The mitochondrial pathway is stimulated by a variety of death stimuli and results in the release of SMAC and cytochrome c from the mitochondria through mediator proteins BAK and BAX. Cytochrome c release leads to the activation of initiator caspase-9 and then subsequent executioner caspases, whereas SMAC promotes apoptosis by inhibiting XIAP. cIAP proteins can block the formation of complex II by ubiquitylation of RIP1.

A third minor pathway involves granzyme B, a granule-associated serine protease found in cytotoxic T-cells and natural killer cells. It can initiate cell death by caspase-3 activation, Bid cleavage, and digestion of regulatory proteins (309, 310).

Mechanisms of Action of Different IAP Proteins. XIAP is probably the best-categorized IAP in terms of its role in apoptotic regulation and can potently suppress apoptosis through direct binding of caspase-3, caspase-7, and caspase-9 (311). The linker region between the BIR1 and BIR2 domain of XIAP is essential for caspase-3 and caspase-7 inhibition and competitively binds at the active site. Inhibition of caspase-3 requires only the linker region, whereas caspase-7 requires binding of both the linker region and the subsequent binding of the XIAP BIR2 domain (312-314). The BIR3 domain of XIAP inhibits caspase-9 by binding to its monomeric, inactive form. This is achieved by forming a heterodimer with procaspase-9, which prevents its dimerization and activation (315, 316). Another mechanism by which XIAP regulates apoptotic function involves the E3 ubiquitin-ligase activity of the RING domain. Studies have shown that removal of the RING domain resulted in an increase in caspase-3 activation, accelerated rate of apoptosis, and enhanced organism survival in a *Eμ-Myc* mouse lymphoma model (317). This underscores the importance of the ubiquitin-ligase function of XIAP for the inhibition of caspases and tumorigenicity. In addition to caspase inhibition, XIAP proteins participate in cell signaling and NF-κB activation. One surface of the BIR1 domain of XIAP binds with the N-terminal domain of the TGF-β activated kinase 1 (TAK1) binding protein, TAB1, while the opposite surface is responsible for BIR1 dimerization. This interaction and dimerization propagates XIAP-induced TAK1 activation and stimulates NF-κB signaling (316, 318, 319). Altogether, inhibiting caspases through direct interaction is the primary mechanism by which XIAP suppresses apoptosis, but it can also influence cell survival through cell signaling pathways.

IAPs do not all obstruct apoptosis in the same manner. Weak inhibitors of caspases, such as ML-IAP, influence cell survival by binding and sequestering SMAC, an endogenous inhibitor of IAP proteins. In the absence of SMAC, XIAP is uninhibited and may more freely promote cell survival (320, 321). On the other hand, while cIAPs can participate in caspase binding, they mainly regulate apoptosis via the E3 ubiquitin ligase activity of their RING domain (322, 323). They are capable of inducing both cross-ubiquitylation of a multitude of substrates as well as auto-ubiquitylation and subsequent degradation (281, 324, 325). For example, cIAP1 can promote ubiquitylation of caspase-3 and caspase-7 and can also mediate ubiquitylation of other IAP proteins including cIAP2 and XIAP (326). In the death receptor pathway, cIAPs impede apoptosis by maintaining constitutive ubiquitylation of RIP1, preventing its association with FADD activation of caspase-8 (327). Unlike XIAP, the BIR1 domains of cIAP1 and cIAP2 interact with TRAF2 and regulate intracellular signal transduction of TNF receptors (328, 329). cIAPs are also implicated in MAPK signaling and regulate TNFα-mediated NF-κB activation (330, 331).

The mechanism of action of survivin regarding the inhibition of apoptosis is not as clearly defined and further complicated by its involvement in other cellular processes, particularly cell division. There is support for a variety of mechanisms by which survivin

regulates apoptosis, including direct binding to effector caspases (332), interacting with SMAC and releasing bound IAPs (333, 334), reducing DNA fragmentation by decreasing levels of mitochondrial apoptosis-inducing factor (AIF) in the nucleus (335), and forming a complex with cofactor hepatitis B X-interacting protein (HBXIP), which binds to procaspase-9 and obstructs subsequent processing (336). Survivin also cooperates with XIAP to inhibit caspase activity by forming an XIAP-survivin complex that increases XIAP stability and protects it against ubiquitylation and proteasomal degradation (337). Survivin is prognostically important because of its minimal expression in normal differentiated adult cells compared to its elevated levels of expression in a wide range of cancers (338). It has been suggested that this could serve as diagnostic tool and biomarker for detection of malignancy (339).

IAP Proteins Regulate Cell Signaling Pathways

Nuclear factor- κ B (NF- κ B) signaling mediates many facets of biological activity including inflammation, proliferation, and survival. Its activation stimulates the expression of many proteins that promote cell cycle progression, resist apoptosis, and facilitate cell adhesion, angiogenesis, and metastasis. It is not surprising that NF- κ B is strongly correlated with carcinogenesis and enables malignant transformation and tumor progression (340). As mentioned previously, IAPs, especially cIAP1 and cIAP2, play a critical role in apoptotic suppression by ubiquitylation of NF- κ B protein substrates and mediating cell signaling. cIAPs positively regulate the canonical NF- κ B pathway and negatively regulate non-canonical NF- κ B signaling (341) (**Figure 5-3**).

The canonical pathway is initiated by the binding of TNF α to TNFR1, followed by the recruitment of TRADD and RIP1. TRADD then engages TRAF2 and cIAP1/cIAP2, which are bound to TRAF2 through their BIR1 domain. cIAPs induce E3 ligase-mediated poly-ubiquitylation of RIP1 (300, 327) to generate a binding platform for the induction and activation of the inhibitor of κ B kinase (IKK) complex, composed of IKK α , IKK β , and NF- κ B essential modulator (NEMO). Additionally, the TAK1-TAB1 complex and the linear ubiquitin chain assembly complex (LUBAC) are recruited to the binding platform. The activated IKK complex phosphorylates and degrades the inhibitor of NF- κ B (I κ B), causing the release of its p50 and RelA dimers (342). p50 and the transcription factor RelA then translocate into the nucleus to activate transcription of targeted genes and NF- κ B signaling (343). Therefore, the presence of cIAP1/cIAP2 promotes cell survival through activation of the NF- κ B pathway by ubiquitylation of RIP1 (344).

In the non-canonical pathway (also referred to as the alternative pathway), constitutive degradation of NF- κ B inducing kinase (NIK) by cIAP1/2 in a complex with TRAF3/TRAF2 represses the activation of NF- κ B signaling (324). In the absence of activation signals, TRAF3 recruits TRAF2-bound-cIAP proteins to NIK in the cytoplasm. cIAP-mediated ubiquitylation of NIK causes its subsequent destabilization and degradation, which prevents activation of IKK α proteins downstream. When the TNF-related weak inducer of apoptosis (TWEAK) ligand binds to the FN14 receptor, it

Figure 5-3. NF- κ B signaling pathways.

cIAP proteins are positive regulators in the canonical pathway and negative regulators in the non-canonical signaling. In the canonical pathway, ubiquitination of RIP1 forms a binding platform for the IKK complex (composed of NEMO , $\text{IKK}\alpha$, and $\text{IKK}\beta$), the TAB1-TAK1 complex, and the linear ubiquitin chain assembly complex (LUBAC). Activated $\text{IKK}\beta$ leads to proteasomal degradation of inhibitor of NF- κ B ($\text{I}\kappa\text{B}$) and subsequent translocation of p50/RelA into the nucleus to activate of target genes. In the non-canonical pathway, constitutive degradation of NIK by cIAP proteins represses NF- κ B signaling. Degradation of cIAP proteins (either initiated by binding of ligands such as TWEAK to FN14 or by IAP antagonists), allows NIK to stabilize and accumulate. This leads to phosphorylation of $\text{IKK}\alpha$, processing of p100 to p52 , and the translocation of the p52/RelB dimer into the nucleus to activate NF- κ B signaling.

engages the TRAF proteins and induces lysosomal degradation of cIAP1-TRAF2 (345). Alternatively, IAP antagonists may induce rapid proteasomal degradation of cIAPs. The absence of cIAP proteins allows for stabilization and accumulation of NIK, which can then phosphorylate IKK α (346). Activated IKK α causes a chain of events resulting in the dissociation of p100 from p52, its dimerization with RelB, and transcription of NF- κ B target genes (347). Thus, the E3 ubiquitin ligase function of cIAPs and their ability to bind to TRAF2 are essential elements in the regulation of NF- κ B pathways and promotion of cell survival.

IAPs also play a role in the other signaling pathways such as Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), TGF- β , Myc, and PI3K/Akt (348). In the Myc pathway, cIAP1 catalyzes ubiquitylation and degradation of a cellular antagonist of Myc and promotes cellular proliferation (349). cIAPs also orchestrate NOD-induced signaling by poly-ubiquitylation of RIP2, which leads to the activation of TAK1 and MAPK signaling (350). In addition to its role in TAK1-mediated NF- κ B signaling, XIAP can serve as a cofactor to several TGF- β proteins and potentiate TGF- β signaling (351). Also, XIAP, NAIP, and ML-IAP can activate the JNK1 pathway through their interactions TAK1 (352). This data further demonstrates the anti-apoptotic functions of IAPs by activation and regulation of pro-survival pathways.

IAP Proteins in Cancer

IAPs are aberrantly expressed in a multitude of human cancer, and the elevated levels have been linked with tumor progression and aggressiveness, treatment resistance, and poor prognosis. For example, high nuclear XIAP expression in breast carcinoma patients significantly increases risk of relapse (353) and shortened overall survival (354). XIAP expression is also indicative of poor prognosis in colorectal cancer (355), bladder cancer (356, 357), chronic lymphocytic leukemia (358), and numerous other cancers (359). XIAP expression also increased with advanced tumor stage and was correlated with tumor aggression in clear-cell renal carcinomas (360, 361) and metastatic melanomas (362). While XIAP expression levels in gastric adenocarcinomas were not significant independently, disturbances in the balance of XIAP to XIAP-associated factor 1 (XAF1), particularly increased ratios of XIAP:XAF1, were substantial determining factors in metastasis and patient survival (363, 364). Similarly, its overexpression has been linked to chemoresistance in ovarian carcinoma cells after cisplatin treatment (365) and XAF1 expression was drastically lower than XIAP in malignant versus non-malignant tumors (366). In hepatocellular carcinoma, elevated XIAP expression increased the risk of relapse after total liver resection and orthotopic liver transplantation, promoted metastasis, increased CDK4/Cyclin D1 expression, and corresponded with poor overall prognosis (97, 367, 368).

IAP expression is not necessarily an indicator of unfavorable outcome in every case. Studies have indicated there is no correlation between XIAP, cIAP1, or cIAP2 expression and predictive response to chemotherapy in non-small cell lung cancer (NSCLC) patients (369, 370) nor is XIAP expression pertinent to disease stage and

patient survival in cervical carcinoma (371). The relevance of XIAP expression in acute myeloid leukemia is somewhat ambiguous, as contradicting results have been reported (372). Some studies indicated no prognostic relevance of XIAP or survivin levels (373), though numerous other studies demonstrated lower XIAP expression was in accordance with longer survival and remission duration (374-376). While elevated expression of several IAP proteins is evident in prostate cancer, XIAP expression has been reported to actually correlate with lower incidences of reoccurrence (377, 378). Nevertheless, targeting specific IAPs in relevant subgroups of cancer patients still warrants investigation, and additional studies are needed to define these parameters and identify prognostic biomarkers.

Genetic aberrations contribute to overexpression of IAPs and resistance to apoptosis. Amplification of 11q21-11q23, which contains the genes encoding cIAP1 and cIAP2, occurs frequently in a multitude of cancers including cervical carcinomas (379), esophageal carcinomas (380), liver cancer (381), glioblastomas (382), lung carcinomas (383), and renal cell carcinomas (270). The amplification of 11q22, specifically cIAP1, increased the risk of radiotherapy resistance in cervical cancer patients (379) and related to carcinogenesis in esophageal cancer patients (380). Additional proof demonstrating that cIAPs are oncogenic drivers is evidenced by the amplification of chromosome 9A1, the syntenic region the human chromosome 11q22, in murine osteosarcoma and hepatocellular cancer models (381, 384).

Another genetic error affiliated with cIAP expression and its oncogenic potential involves translocation events in mucosa-associated lymphoid tissue (MALT) lymphomas. The t(1;14)(p22;q32) and t(14;18)(q32;q21) affect the regulation of Bcl10 and Malt1 genes and promote NF- κ B signaling (385). The t(11;18)(q21;q21) is the most frequently genetic abnormality associated with MALT lymphoma and is found in about half of all low-grade MALT lymphomas (386). This translocation fuses the protein 1 (MALT1) gene on chromosome 18 with the cIAP2 gene on chromosome 11 (341, 387, 388). The anti-apoptotic cIAP2-MALT1 fusion protein, containing the N-terminal BIR domains of cIAP2 (but not the RING domain) and the caspase-like domain in C terminal of MALT1, forces constitutive activation of both pathways of NF- κ B signaling and promotes tumorigenesis (389-392). Several mechanisms have been proposed for activation of canonical signaling, including IKK stimulation by cleavage of A20, a negative regulator of NF- κ B, binding to TRAF2 and/or TRAF6, and enhancement by Bcl10 interaction with the fusion protein (393). Whereas potent activation has been attributed to the functionality of both domains in the fusion protein for canonical signaling, the loss of E3 ubiquitin ligase function in the RING-deficient cIAP2-MALT1 fusion protein is sufficient to induce non-canonical signaling through NIK stabilization (394). In addition to their oncogenic properties, cIAPs also play a tumor-suppressing role because of their ability to both positively and negatively regulate NF- κ B signaling. For instance, in multiple myeloma, genetic deletion of cIAP1/2 results in aberrant non-canonical pathway stimulation (395-397). Though cIAPs can act as tumor suppressors or oncogenes, dysregulation of cIAP expression in human malignancies always results in elevated NF- κ B signaling, leads to cell survival, and enhances tumorigenesis (270).

Survivin expression is elevated in most hematologic malignancies, and is associated with chemotherapy resistance, relapse, and lower patient survival. In most tumor types, it is a significant independent prognostic indicator of unfavorable outcome, some exceptions being gastric cancer, non-small-cell lung cancer and osteosarcoma (398). Discrepancies in prognostic value may occur due to differing functions of splice variants, detection methods, or subcellular localization (nuclear or cytoplasmic). Survivin regulates multiple pro-survival processes, especially in the mitotic stage, such as cytoprotection, cell cycle regulation, and evasion of cell death. Because of its ubiquitous expression in cancers and undetectable levels in most transformed cells, it is an attractive therapeutic target. However, there is recent evidence that it is also expressed in primitive hematopoietic cells, progenitor cells, T lymphocytes, polymorphonuclear neutrophils, and vascular endothelial cells, and is necessary for their proliferation and normal cell function (398). The pathways through which survivin regulates proliferation of tumor cells and normal cells and the overlap between the two needs to be further defined, though it seems that mitochondrial survivin is exclusive to tumor transformation (399). Clarification of its differential expression, propagation, and function in malignant and housekeeping regulation, especially in hematopoietic and immune systems, is needed to maximize therapeutic benefit and minimize toxicity in anticancer strategies targeting survivin. Similar to survivin, ML-IAP is not expressed in differentiated adult tissue but is preferentially and highly expressed in cancers such as melanoma and renal cell carcinoma (400, 401). High ML-IAP expression is indicative of poor prognosis in neuroblastoma patients (402) and correlated with tumor stages and pathogenesis of esophageal carcinoma (403). Nuclear expression in patients high-grade central osteosarcoma patients dramatically patient shortened life-span compared to those with only cytoplasmic expression (404) but was a favorable independent predictor of cancer-specific survival and progression-free survival in RCC patients (405). Its expression in childhood acute lymphoblastic leukemia (ALL) was associated with sensitivity to chemotherapy and positive prognosis (406), but found to be a negatively correlated with survival in adult ALL (407). Another study showed that ML-IAP expression was not detectable in malignant mesothelioma, even when other IAPs were found (408). The inconsistency with the correlation between ML-IAP expression and prognosis can probably be attributed to its dual functionality. It has been redefined as a regulator of apoptosis rather than anti-apoptotic protein due to the discovery that its different isoforms promote opposite effects on cell survival. Not only do its alpha and beta isoforms contribute differently to tumorigenicity, its anti-apoptotic role is reversed by cleavage to its truncated (tLivin) form by a strong apoptotic stimulus (409, 410). Cleavage of Livin- β potently suppresses tumor proliferation, and the balance between the full-length and cleaved protein, subsequent processing of tLivin, and its subcellular localization all influence its regulation of apoptosis (411).

SMAC: The Endogenous Inhibitor of IAP Proteins

SMAC, also referred to as DIABLO (direct IAP-binding protein with low pI), is an endogenous antagonist of IAP proteins. It promotes caspase activation in the mitochondrial pathway by binding to IAPs, particularly XIAP (412, 413). preSMAC is

translated in the cytosol and then transported into the mitochondrial intermembrane space to undergo processing, where it remains until activated by apoptotic stimuli (414). The mature form of SMAC is generated from the monomeric SMAC precursor molecule by proteolytic cleavage of the 55 amino acid residue sequence in the N-terminus and undergoes subsequent homodimerization formed through extensive hydrophobic interactions (296, 414). SMAC efflux into the cytosol is also mediated by BCL-2 family proteins but is a caspase-catalyzed event that occurs downstream of cytochrome *c* release, whereas cytochrome *c* release is largely independent of caspase activity (415).

The mature form of SMAC has only four amino acid residues (Ala-Val-Pro-Ile) located in the amino terminal that make up the IAP binding motif responsible for the interaction with IAPs (296, 416-418). This AVPI tetra-peptide is able to bind to the BIR2 and BIR3 domains of XIAP, but not BIR1 (296, 419). SMAC suppresses XIAP-mediated inhibition of apoptosis by binding to the BIR3 domain of XIAP and disrupting its interaction with the ATPF tetra-peptide binding motif in the linker region of caspase-9 (420). SMAC simultaneously binds to the BIR2 domain of XIAP, reduces exposure of the linker region in XIAP through steric hindrance, and relieves executioner caspase inhibition (421-423). The presence of both BIR2 and BIR3 domains of XIAP is required for effective and efficient antagonism by the SMAC dimer. Also, while not directly interacting with XIAP BIR1, SMAC antagonizes the XIAP-TAB1 interaction via steric exclusion when it concurrently binds to the BIR2 and BIR3 domain of XIAP and therefore prevents XIAP-induced NF- κ B activation (319).

Another important method by which SMAC antagonizes IAPs is by promoting proteasomal degradation of cIAP proteins. The AVPI binding motif of SMAC interacts with the BIR domains of cIAP1 and cIAP2 and induces RING domain-mediated auto-ubiquitylation (324, 424). Overall, SMAC is an effective antagonist against several critical IAP proteins and capable of promoting apoptosis, thereby supporting the rationale for designing small-molecule drugs mimicking SMAC for anticancer therapy.

Targeting IAP Proteins for Cancer Therapy

In the past decade, great strides have been taken in implementing inhibitors of IAPs to restore apoptotic function in laboratory and clinical settings. Some strategies to inhibit IAPs target mRNA and down-regulate protein levels with antisense oligonucleotides, ribozymes, or small-interfering RNAs (425-427). Another approach utilizes antigen-based vaccinations to target IAPs and promote immune-mediated tumor destruction (428-431). One of the most appealing strategies for targeting IAPs has been with the use of small-molecule IAP antagonists, and several of these compounds have reached clinical trials (341, 432, 433). Two classes of small-molecule IAP inhibitors are SMAC mimetics and survivin-specific antagonists. There have been other small-molecule XIAP antagonizing compounds identified, such as embelin, a naturally occurring compound that comes from a Japanese *Ardisia* herb (434). However, its poor solubility has prevented further preclinical development (435). Other screening strategies have led to the discovery of polyphenylurea-based antagonists 1396-12 and 1396-34 (436) and

aryl sulfonamide analogs including TWX006 and TWX024 (437), which can block (or reverse) XIAP-mediated caspase-3 inhibition. But again, these compounds also have not undergone additional clinical development. Therefore, the scope of this review will be limited to the design, development, and therapeutic potential small-molecule SMAC mimetics and survivin inhibitors, focusing on a selection of compounds that are undergoing clinical advancement.

SMAC Mimetics: Design and Mechanism of Action

The design of SMAC mimetics was initially based on the crystal structure (416) and NMR (418) of SMAC in complex with the BIR3 domain of XIAP. These structural studies revealed that the AVPI binding region of SMAC recognizes and binds to a surface groove on BIR3 domain of XIAP. The exact mode of binding at each peptide and structural basis for the design of SMAC mimetics was previously described by Sun et al. (438). To summarize, the methyl group on the first alanine fits into a hydrophobic pocket in the surface groove of the XIAP BIR3 domain and the free amino group forms hydrogen bonds with Glu314 and Gln319 in BIR3. The carbonyl backbone can participate in hydrogen bonding to Trp323 in BIR3. In the second position, valine's amino and carbonyl groups can form optimal hydrogen bonds with Thr308. In the third position, proline participates in van der Waals interactions with Trp323 and Tyr324. In the final position of the AVPI tetra-peptide, the amino group of isoleucine forms a hydrogen bond with Gly306. The side chain of isoleucine fits into a hydrophobic pocket formed by the side chains of Leu292 and Val298, as well as the hydrophobic portion of Lys297 and Lys299 (438) (**Figure 5-4**). Based on these monumental structural findings revealing the binding interactions between XIAP and SMAC, both peptidic and non-peptidic SMAC mimetics have been developed.

Through biochemical analysis of peptide variations and modifications, the minimum sequence requirements to still achieve potent binding were determined, as well as favorable and undesirable substitutions of SMAC-based peptides (439, 440). In the first position, the alanine residue cannot tolerate replacement with another amino acid without significantly reducing its binding affinity but demonstrated comparable binding affinity when it was replaced by 2-aminobutyric acid, indicating that a methyl or ethyl side chain is ideal in the first position for activity. The second position is flexible to replacement by an array of amino acids or residues, however replacement with proline, aspartic acid, or valine is not favorable. The replacement of the cyclic proline with a 4 or 6-member ring decreases binding affinity, but activity can be enhanced if a hydrophobic group is attached to the proline ring. A hydrophobic substitution in the fourth position is desirable, with phenylalanine showing the greatest affinity, whereas polar molecules obliterate activity (438). While SMAC-based peptides could attain potent binding affinities to XIAP, they were not cell-permeable without a carrier to facilitate delivery into the cell. Extensive chemical modifications had to be made for generation of the first SMAC-based peptidomimetic that demonstrated cell-permeability and XIAP antagonism (441). It was also established that demethylation at position 1 reduced activity, an L-

Figure 5-4. Crystal structure of the SMAC in complex with XIAP.

Detailed interactions of the AVPI binding motif of SMAC in complex with the BIR3 domain of XIAP (Protein Data Bank access code: IG73). Hydrogen bonds for Ala1, Val2, and Ile4 are shown in yellow. Van der Waals interactions are between Pro3 and the side chains of Trp323 and Tyr324. A hydrophobic pocket is formed by the Leu292 and Val298 side chains and the hydrophobic portion of Lys297 and Lys299 side chains.

configuration was required for position 2, adding hydrophobic groups to position 3 increases affinity, and the position 4 favors hydrophobic entities, particularly phenylalanine and phenylglycine (418, 438, 441). These compounds provided the first proof-of concept for small-molecule SMAC peptidomimetics as potential anticancer agents and served as prototypes for small-molecule SMAC mimetics. Another significant improvement to the design of these compounds was incorporation of a conformationally constrained scaffold, which improved affinity and permeability (442-444).

To date, there are two types of SMAC mimetics: monovalent compounds designed to mimic a single IAP binding site of SMAC and bivalent compounds containing two SMAC mimetics tethered together by a chemical linker. Because mature SMAC can form an elongated homodimer that interacts with both BIR2 and BIR3 domains, it was reasoned that bivalent, non-peptidic SMAC mimetics resembling the SMAC dimer should be more potent and effective in promoting apoptosis by concurrently interacting with both domains to restore caspase activity. The first bivalent molecules that were discovered demonstrated that bivalent compounds did achieve greater affinity than their monomeric analogs, leading to this new category of IAP inhibitors (268). In addition to increased affinity, bivalent SMAC mimetics are remarkably more potent in inducing apoptosis in tumor cells, with some studies suggesting by a factor of 100-1000 fold (438). The significant increase in potency is partially due to their ability to promote cIAP dimerization, auto-ubiquitylation, and proteasomal degradation, thereby inducing the autocrine/paracrine TNF α signaling loop and NF- κ B signaling (324, 445). However, it has been suggested that the more profound effect on potency is due to the ability of bivalent compounds to engage in binding multiple domains of XIAP, resulting in activation of executioner caspase-3 and caspase-7 in addition to caspase-9 (341, 446).

Though SMAC mimetics were designed around the SMAC-BIR3 interaction of XIAP, they can also interact with the BIR domains of cIAP1, cIAP2 and ML-IAP. The inactivated cIAP1 is conformationally constructed such that its RING domain is sequestered within its monomeric structure and unable to dimerize. IAP antagonists preferentially bind to the BIR3 domain and cause conformational changes that induce dimerization of the RING domain and generate the E3 ligase function (447). The BIR3, UBA, and CARD domains of cIAP1 are inhibitory elements to RING dimerization but can be overcome by SMAC mimetics (448). Upon binding, cIAP1 undergoes rapid auto-ubiquitylation and depletion, whereas the cIAP2 dimer has a higher intrinsic E3 ligase activity, is more stable, and less influenced by SMAC mimetics. Elevated E3 ligase activity causes cIAP-mediated ubiquitylation and activation of RIP1 and promotes canonical NF- κ B signaling pathway. This activation is merely transient, however, since cIAPs are subsequently degraded. Since cIAPs negatively regulate the non-canonical pathway by consistent ubiquitylation of NIK, cIAP inhibitors activate this pathway by inducing cIAP degradation. Additionally, the combination of TNF α production and the loss of cIAPs lead to RIP1/FADD association, triggering caspase-8 activation and extrinsic apoptosis (300, 324, 445). Therefore, inhibitors of IAP proteins are capable of reversing the pro-survival action of cIAP proteins by triggering E3 ligase mediated-cIAP

degradation, inducing non-canonical TNF α production, and stimulating extrinsic TNFR1-mediated apoptosis.

IAP inhibitors were initially designed to be non-specific and able to bind to different families of IAP proteins. However, to understand the divergent apoptotic mechanisms of different IAPs, cIAP-specific inhibitors were developed. Selective targeting of cIAP by antagonists demonstrated that inhibition of cIAP alone can induce cell death in tumor cells and did not require binding to the XIAP BIR3 domain (449). Nevertheless, molecules that simultaneously target both cIAP and XIAP proteins are more potent and can more efficiently induce cell death than by cIAP-only antagonism (450). Site-specific SMAC mimetics that bind to a single BIR domain have also been developed. BIR2-selective SMAC mimetics have demonstrated the ability to sensitize TRAIL-resistant breast cancer cells (451) and TRAIL-resistant prostate cancer cells (452) to apoptosis *in vitro* but were not toxic as single agents. Discovery of BIR2-specific benzazepinones has provided useful insights to the apoptotic events that differ mechanistically from pan-IAP inhibitors, but initially had shortcomings that limited their clinical utility (453). Optimization of these compounds provided further proof-of-concept of their ability to promote apoptosis in xenograft models by caspase-3 and caspase-7 activation without inducing TNF α release (454). Still, it is suggested that BIR2 and BIR3 should instantaneously be targeted for optimal activation and potency (341, 359). Several SMAC mimetics showing strong IAP inhibition and favorable toxicity profiles have moved into clinical trials and are being evaluated as single agents and in combination therapy. The chemical structure, clinical stage, and developer for the following selection of compounds can be found in **Figure 5-5A**.

Clinical Development of SMAC Mimetics

GDC-0152 and GDC-0917/CUDC-427. The first SMAC mimetic to enter clinical trials was designed by Genentech and was one of a series of monovalent compounds developed to mimic the AVPI binding motif. Initial studies have shown GDC-0152 to be a potent antagonist of cIAP1/2, ML-IAP, and XIAP (455). Using a peptidomimetic approach, each position was assessed for its overall contribution to binding affinity and evaluated to see if substitutions could be made to improve its binding. For example, it was learned that while the P1 alanine was buried within a hydrophobic pocket of the BIR domain, positions 2-4 were more amenable to substitution. GDC-0152 demonstrated a K_i binding affinity of <50nM, and induced auto-ubiquitylation and proteasomal degradation of cIAP1 in melanoma cells. GDC-0152 promoted cell death in MDA-MB-231 breast cancer cells without affecting human mammary epithelial cells in cell viability assays. Additionally, GDC-0152 inhibited tumor growth in MDA-MB-231 xenograft models. A phase 1a first-in-human standard dose-escalation study revealed linear pharmacokinetics with moderate clearance and volume of distribution (455). Another study showed that GDC-0152 was able to induce apoptosis through the down-regulation of IAPs and inhibit growth of K562 and HL60 leukemia cells in a dose- and time-dependent manner (456). Its toxicity and efficacy are driven by TNF α levels as a result of increased NF- κ B transcriptional activity (457).

Figure 5-5. Preclinical and clinical development of small-molecule SMAC mimetics and survivin inhibitors

A) SMAC mimetics in the preclinical stage include SM-164 (University of Michigan) and BV6 (Genentech). Phase I SMAC mimetics include SM-406/AT-406/Debio 1143 (University of Michigan/Ascenta Therapeutics/Debiopharm), GDC-0152 (Genentech), GDC-0917/CUDC427 (Genentech/Curis), and AEG40826/HGS1029 (Aegera Therapeutic) (not shown). Compounds advancing to Phase II clinical trials include LCL161 (Novartis), and Birinapant/TL32711 (TetraLogic Pharmaceuticals). B) Survivin inhibitors in preclinical stages include FL118 (Canget BioTekpharma) and Shepherdin (University of Massachusetts). Phase II inhibitors include YM155 (Astellas) and AICAR (PeriCor Therapeutics).

Also developed by Genentech, GDC-0917 (CUDC-427) is an orally bioavailable, second-generation IAP antagonists. While much of the assessment of this drug was built on its predecessor, GDC-0152, a combination of mathematical modeling, simulation techniques, and *in-vivo* pharmacokinetic studies demonstrate its potential as an anticancer agent. GDC-0917 binds with affinities similar to that of GDC-0152 and has a more robust effect MDA-MB-231 breast cancer cells while still exhibiting little toxicity to normal human mammary epithelial cells. Favorable pharmacokinetic properties in terms of hepatic clearance, volume, and half-life have been reported (458). One of its main advantages is that it can be administered orally unlike its predecessor, which was given by IV every 2 weeks. Human simulations to determine ED₅₀ and ED₉₀ are expected to be 72mg and 660mg assuming a 5 mg starting dose. These expectations were based on MDA-MB-231 xenograft models and were predicted to be reached in a time frame of about 1-2 years (458). Curis licensed GDC-0917 from Genentech, and it has since been renamed CUDC-427. Phase 1 trials in patients with advanced or refractory solid tumors or lymphoma are ongoing.

LCL161. Novartis is responsible for the development of the monovalent, orally bioavailable SMAC mimetic LCL161. It targets cIAP1 to induce apoptotic activity through E3 ubiquitylation ligases, thereby promoting TNF α secretion and NF- κ B pathway activation (459). Cleavage of caspase-3 was confirmed in mouse xenograft models. Further evaluation in TNF α mRNA indicated a strong correlation between TNF α expression and LCL161 dose, where the greatest inhibitory growth response was presented by tumors with the highest basal TNF α levels. LCL161 has been investigated as both a single agent and in combination and has produced mixed results. Studies done by Pediatric Preclinical Testing Program did not show significant LCL161 activity as a single agent in SCID mice xenograft models and pediatric cell lines, which they concluded was due to LCL161 being ineffective in a majority of cell lines (460). This remains somewhat controversial, as previous reports had indicated efficacy in multiple tumor models and cell lines (461). Another study concluded that paclitaxel sensitized hepatocellular carcinoma cells to LCL161, but there was significant inhibition of off-target proteins such as pERK and pJNK, suggesting apoptosis resulted from toxicity as opposed to direct action of LCL161-mediated IAP inhibition (462). Additionally, the anticancer mechanism was mainly by anti-proliferative effects instead of apoptotic stimulation. In a phase 1 first-in-human study of LCL161 as a single agent, it was determined that a dose of 1800 mg was above what would be required to achieve target inhibition. However, because of the lack of efficacy, sensitivity to LCL161 as single agent was not conclusive. Adverse effects included cytokine release syndrome consistent with robust NF- κ B activity (461). Finding an appropriate level of inflammatory cytokine production may ultimately be the key to success if the primary function of the drug involves NF- κ B pathway activation and TNF α production (463). In a phase 1b study, increasing doses of LCL161 were combined with paclitaxel in patients with advanced tumors and was well tolerated with manageable toxicities, predominantly neutropenia, fatigue, and neuropathy (464). Altogether, these results suggest that LCL161 is a promising anticancer agent, particularly in combination therapy, and warrants further investigation to optimize its therapeutic value.

SM-406/At-406/Debio 1143. SM-406, renamed AT-406 for Ascenta Therapeutics, is a small-molecule, non-peptidic SMAC mimetic capable of binding XIAP, cIAP1 and cIAP2 (465). As it was previously discovered that designing SMAC mimetics that are conformationally constrained improved their binding affinity, the design of these molecule incorporated a rigid scaffold (442, 443, 446). Preclinical trials show good antagonists activity of XIAP at the BIR3 domain and restored caspase-9 activity, induced rapid cIAP degradation, and increased cell death in a time-and-dose dependent manner in MDA-MB-231 cells (465). SM-406 effectively inhibited tumor growth in MDA-MB-231 xenograft models with minimal toxicity and achieved complete tumor inhibition at 100 mg/kg oral dosing. AT-406 was further evaluated as a single agent and in combination therapy with carboplatin, a first line platinum therapy (466). As a single agent, AT-406 induced apoptosis in 3 out of 5 carboplatin resistant cancer cell lines. The primary function of AT-406 is down-regulating XIAP protein levels, which have greater expression in chemoresistant ovarian cancer cells compared to normal ovary cells. These provide additional evidence that reduction of cIAP1 alone is not sufficient to induce apoptosis. AT-406 also showed the ability to sensitize resistant cells to carboplatin *in vitro* and *in vivo*, as indicated by prolonged mouse survival (466). Phase 1 trials have been completed for Debio 1143 (AT-406) for patients with advanced solid tumors and lymphomas and phase II studies are ongoing.

SM-122 and SM-164. SM-122 is a monovalent, non-peptidic mimetic of SMAC and SM-164 is a bivalent analog created from two SM-122 analogs tethered together by a chemical linker (467). The bivalent properties of SM-164 allow for binding to BIR2 and BIR3 domains of XIAP simultaneously. SM-122 and SM-164 both bind to cIAP1 and the BIR3 domain of cIAP2 and exhibit *Ki* values ranging between .31 and 2.7 nmol/L, where SM-122 showed a slightly greater affinity. SM-164 was 300 times more potent than SM-122 in binding to XIAP and 1000 times more potent in inducing apoptosis. These results clearly demonstrate the impact of XIAP antagonism on apoptosis (467). It was then shown to effectively promote cell death in MDA-MB-231 tumor tissues of mouse models at a 5mg/kg dose. Within an hour, there was a significant decrease in cIAP1 levels, and by 3 hours, activation of caspase-8, caspase-9 and caspase-3 and PARP cleavage was observed. Damage was limited to the tumor tissue and normal, even highly proliferative tissues were unaffected. More impressively, tumor growth was reduced by 65% without causing toxicity or weight loss by day 36. In order to achieve stronger apoptotic activation, SM-164 has also been tested in combination with TRAIL (468). While TRAIL demonstrates low cytotoxicity and is well tolerated, its applications have previously been limited (469, 470). SM-164 was highly synergistic with TRAIL in 12 out of 19 TRAIL-resistant and TRAIL-sensitive cancer cell lines of breast, prostate, and colon tumor types. While these two compounds failed to achieve appreciable antitumor activity as single agents, they reduced tumor volume by an average of 80% after two weeks of combination treatment in a 2LMP xenograft model. The synergy between TRAIL and SMAC mimetics relies on the interaction of procaspase-8 with RIP1 and enhanced by the ablation of cIAP1 and XIAP (468). In a separate combination study with doxorubicin, SM-164 sensitized hepatocellular carcinoma cells to TNF α -related apoptosis and suppressed activation of Akt *in vitro* (471). These preclinical data provide support for the potential of combination therapy with SM-164 for a variety of cancers.

Birinapant. Birinapant (TL32711), by Tetralogic Pharmaceuticals, is a bisindole-based bivalent SMAC mimetic that binds with high affinity to the BIR3 domains of XIAP, cIAP1, cIAP2, and the single binding domain of ML-IAP (472, 473). Though its first-generation analog had a greater binding potency to the BIR3 domains of XIAP and cIAP2, birinapant exhibited greater tolerability in an MDA-MB-231 xenograft model and demonstrated dose-dependent tumor regression. The improvements are attributed to decreased potency against cIAP2, lower affinity for XIAP BIR3 and activation of caspase-dependent apoptosis while avoiding inhibition of XIAP-dependent NF- κ B signaling (473). Potent activation of apoptosis was evidenced by cleavage of PARP, a substrate of caspase-3, in MDA-MB-231 cells 72 hours post treatment. Subsequent corresponding studies showed that birinapant induced rapid degradation of TRAF-bound cIAP1 and cIAP2 to inhibit TNF α -mediated NF- κ B activation and promoted cell death as a single agent (472). Birinapant also promoted activation of caspase-8 via DISC complex formation in birinapant-sensitive cells following TNF stimulation and was active in approximately 60% of a panel of 111 cell lines tested as a single agent or in combination with TNF α and TRAIL. *In vivo* studies revealed observable antitumor activity after 5 treatments of 30mg/kg in approximately one third of the 50 patient-derived xenotransplant models tested and potentiated the cytotoxicity of certain chemotherapeutic agents including SN-38 (the active metabolite of irinotecan), gemcitabine, and 5-azacytidine. Consistent with these findings, an aggressive, inflammatory breast cancer model increased apoptotic activity in TRAIL-insensitive ErbB2-overexpressing cells and significantly increased potency of TRAIL-induced cell death in aggressive TRAIL-sensitive tumors (474). In combination with TNF α , it inhibited proliferation in melanoma cells with acquired resistance to BRAF (475). A Phase I first-in-man study of adult patients with advanced solid tumors and lymphoma showed that it was well tolerated when administered intravenously with no dose-limiting toxicities (476). Birinapant has been evaluated in combination with multiple chemotherapies including liposomal, doxorubicin, gemcitabine, docetaxel, irinotecan, and paclitaxel at standard dosing and demonstrated excellent tolerability (477). Phase 2 studies of birinapant in combination with irinotecan in colorectal patients showed an overall clinical benefit rate of 57%, supporting the hypothesis of synergy between birinapant and irinotecan as a TNF α -inducing agent (478). Clinical investigations of birinapant in patients with solid tumors and hematological malignancies are ongoing.

BV6. BV6, another compound by Genentech, is a bivalent SMAC mimetic that is able to engage in binding of both cIAP and XIAP to produce apoptotic activation. Like other SMAC mimetics, BV6 effectually diminishes the pro-survival effects cIAP1 and cIAP2 through induced auto-ubiquitylation and degradation and it inhibits XIAP through antagonism of caspase binding (324). This study made profound contributions to the understanding of IAP antagonist-induced cell death dependent on NF- κ B signaling. BV6 is capable of sensitizing several cell lines to radiation-induced apoptosis of non-small cell lung carcinoma *in vitro*, including the SMAC mimetic resistant cell line, H460 (479). Another study investigated whether BV6 could sensitize tumor cells to non-specific cytokine-induced killer cells (480). While it was shown that BV6 did sensitize hematologic and solid malignancies to CIK cells, it also promoted apoptosis in the CIK

cells themselves as well as other peripheral mononuclear cells (PMNCs). Despite its effect on CIK cells, the fact that it still sensitized non-malignant cells such as PMNCs toward CIK cell-mediated killing limits its potential in combination with CIK immune-based therapies. On the other hand, BV6 has been shown to interact synergistically with glucocorticoids such as dexamethasone to enhance glucocorticoid-triggered cell death in pediatric acute lymphoblastic leukemia (481). Additional trials with BV6 in combination therapy are warranted.

AEG40826/HSG1029. AEG40826, renamed HSG1029, is a bivalent SMAC mimetic developed by Aegera Therapeutics and licensed by Human Genome Sciences for clinical development. The results of phase 1 HGS1029 trials indicate a favorable safety profile with IV administration and good tolerability in patients with advanced solid malignancies. However, dose limiting toxicities such as dose-dependent short-lived lymphocytopenia and possibly a transient neutrophilia in some patients were observed (482). As expected, HGS1029 stimulated decrease in cIAP1 and up-regulation of monocyte chemoattractant protein 1 levels. No structural data has been published.

Small-Molecule Survivin Inhibitors

Though survivin is reported to inhibit apoptosis, the exact mechanism by which it does so remains elusive. This is further complicated by the myriad of cell cycle processes in which it is involved, including cellular homeostasis, cell division, intracellular signaling and apoptosis (338). Several strategies that selectively target survivin are under development, but there are only a handful that do so by targeting the protein with small molecules. Even so, these small molecules do not seek to inhibit survivin by forcing suppression by direct binding but rather target promoter regions or disrupt protein-protein interactions in survivin complexes (270, 339, 427). The chemical structure, clinical stage, and developer for the following selection of compounds can be found in **Figure 5-5B**.

Clinical Development of Survivin Inhibitors

YM155. YM155, or sepantronium bromide, is a small imidazolium-based molecule that was identified by high-throughput screening (HTS) of chemical libraries of compounds that diminished survivin expression in a survivin promoter-luciferase assay. YM155, unlike SMAC mimetics, suppresses survivin expression by interfering with the promoter transcription region (483). The molecular mechanism by which YM155 inhibits survivin is at least in part by dissociation and subcellular relocation of transcription factors such as Sp1 from its DNA binding sites and acts in cell cycle-independent manner without G₁ cell arrests (484). Preclinical studies of YM155 demonstrated inhibition of cell growth in a broad spectrum of cancer cell lines including non-Hodgkin's lymphoma, hormone-refractory prostate cancer, ovarian cancer, sarcoma, non-small-cell lung cancer, breast cancer, leukemia and melanoma (485). Additionally, YM155 was able to produce antitumor activity in p53-defective human tumor xenograft models. Because abnormal p-53 expression is associated with increased survivin expression, this could provide an advantage over current therapies. In phase 1 trials, it

was shown to be well tolerated in patients with advanced solid tumors or hematologic malignancies (485). Phase 2 studies have been less encouraging though: while severe side effects were uncommon, it only achieved modest efficacy at best as a single agent or in combination therapy (372, 486, 487). YM155 is a substrate for p-glycoprotein-mediated transport, making it susceptible to multidrug resistance and therefore limiting its therapeutic applications (488).

FL118. FL118 is a small-molecule identified through HTS of compound libraries. It not only inhibits survivin promoter activity, but also inhibits anti-apoptotic Mcl-1, XIAP and cIAP2 expression without disrupting control genes (489). FL118 had superior antitumor activity in mouse xenograft models when compared with YM155 and acts in a p53-independent manner. It was even able to completely eradicate colon and head-and-neck tumors in a mouse model. Furthermore, it is suggested that FL118 is not a substrate for the ABCG2 pump and could potential bypass multiple treatment-resistant mechanisms (490). Its clinical potential has been further realized by improvements on its formulation and route of administration (491). Based on these findings, it is a favorable candidate for clinical trials.

Shepherdin and AICAR. Shepherdin is a small-molecule peptidomimetic that was designed to interfere with the interaction between survivin and Heat-shock protein90 (Hsp90) (492, 493). Hsp90 is a molecular chaperone protein that binds to and stabilizes survivin. Disruption of the Hsp90-survivin complex leads to survivin degradation and mitochondrial-dependent apoptosis (494). Within the BIR1 domain, residues Lys79 – Leu87 are essential for the association of survivin with Hsp90. Shepherdin exploits that interaction and binds extensively to the ATP pocket of Hsp90 to destabilizes survivin and induce tumor cell death by both apoptotic and non-apoptotic mechanisms (492). Not only does it disrupt the Hsp90-survivin interface, is acts as a global inhibitor of Hsp90 function by competition with ATP. Systemic administration of shepherdin *in vivo* was found to be well-tolerated and inhibited human tumor growth in mice without significant toxicity. It also achieved anticancer activity *in vivo* in an acute myeloid leukemia xenograft model as determined by loss of expression of survivin and Akt without causing toxicity to normal tissue (495). Clinical evaluation of shepherdin in patients has not yet been reported. Other small molecules targeting of Hsp90 include nonpeptidic 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR). Other studies yielded similar results, indicating that destabilization of Hsp90 led to apoptosis in multiple tumor cell lines while not affecting proliferation of normal human fibroblasts (493). However, because AICAR is a potent agonist of AMP-activated protein kinase (AMPK), deleterious side effects such as significant drop in plasma potassium levels have been reported (496). Further studies are needed to address concerns associated with survivin antagonism.

Challenges for Clinical Development

Though IAP antagonists have demonstrated antitumor efficacy in a variety of cancer types, there are still challenges that need to be addressed in order to incorporate

them into practical anticancer regimens. One potential issue observed is due to the potent activation of cIAP-dependent NF- κ B signaling. While up-regulation of this pathway and its targeted genes such as TNF α are responsible for the TNF α -mediated apoptosis, they can also pose the threat of provoking toxic side effects associated with stimulation of inflammatory cytokine production (463). Some IAP antagonists may therefore cause adverse side effects such as Cytokine Release Syndrome, exemplified by LCL161 in a phase 1 dose escalation study (461). Because it is the same pathway that both drives apoptosis and causes unintended side effects associated with inflammatory cytokines, additional studies are warranted to optimize a therapeutic window to effectively inhibit antitumor activity and decrease risk of cytotoxicity. Other issues correlated with elevated NF- κ B signaling include increased osteoclastogenesis and osteoporosis development as a result of NIK stabilization (497).

Another challenge encountered is determining suitable biomarkers in patient population to assess susceptibility to IAP antagonist treatment. Preclinical and clinical studies have identified several biomarkers for potential response, including serum cytokines and cIAP levels (498). However, SMAC mimetics may induce cIAP degradation at non-toxic concentrations, in non-tumor tissues, and in non-response malignancies, so cIAP loss would not be indicative of therapeutic relevance (341). Baseline TNF α levels may serve as more suitable biomarkers and additionally may induce non-apoptotic forms of cell death, such as by necroptosis (499, 500). However, it is still unknown if there are other cytokines or apoptotic markers that may have served as a more reliable source for predicting therapeutic efficacy (463). Furthermore, IAP expression is not always directly correlated with adverse prognosis. For example, XIAP is elevated in prostate cancer, but recent studies indicate that elevated levels decrease the risk of tumor recurrence compared to subjects with lower levels (378). Studies on patients with advanced non-small cell lung cancer indicate no relation between XIAP expression and clinical outcome (501). Additional studies are required to determine indicators of potential efficacy to IAP antagonist treatment. Caution should be exercised with therapies targeting XIAP concomitantly with death receptors. The corresponding increased rate of apoptosis can become deleterious for type II cells, such as hepatocytes, pancreatic beta cells, and lead to liver toxicity (502).

Small-molecule survivin antagonists may also produce deleterious effects. Because it is a chromosomal passenger protein complex that participates in multiple facets of cell division, survivin antagonism can cause generation of polyploidy cells and are more susceptible to mutations, genetic variation and instability. This may result in increased tumor aggression and resistance to treatment (270, 503). Moreover, although survivin is not expressed in adult differentiated cells, it is still expressed in proliferating cells, such as in the immune system. Loss of survivin can affect T-cell maturation and proliferation (504). Also, because of the dynamic role of survivin in numerous biological processes including mitosis, apoptosis, cell cycle regulation, and autophagy as well as its interaction with numerous endogenous molecules, it is difficult to evaluate the efficacy and target specificity of its clinical candidates by any high throughput assay. These complexities also obscure the therapeutic significance of small-molecule survivin antagonists (338, 505).

Another crucial question lies in the synergism between IAP inhibitors in combination with other agents to achieve cell death. As single agents, SMAC mimetics trigger apoptosis in only a small subset of cancer cell lines. In combinations with death receptor ligands such as TNF α , TRAIL, and CD95, IAP antagonists have a greater capacity to elicit potent activation of apoptosis in a variety of cell lines (506). The answer may lie in determining which combination regimen produces the most robust effect on tumor regression without toxicity to healthy tissues. Additionally, IAPs can prime cancer cells toward apoptosis by lowering the apoptotic threshold and rendering them vulnerable to a variety of cytotoxic factors including chemotherapeutic agents (507). The combination of IAP antagonists with chemotherapeutics, radiation therapy, and kinase inhibitors is being explored but is still largely unknown.

Summary

In summary, IAPs inhibit cell death pathways, promote pro-survival signaling, and are up-regulated in many types of cancer, making them desirable molecular targets for anticancer therapy. Significant progress has been made in developing IAP inhibitors, particularly with small molecules, and these drugs represent a promising strategy for inducing cell death in human diseases and malignancies. SMAC mimetics have shown the ability to interact with XIAP and suppress their inhibitory action on caspases as well as the ability to bind cIAPs and produce apoptotic effects by regulating NF- κ B signaling and extrinsic cell death pathways. Other small-molecule inhibitors also aim to suppress the anti-apoptotic effects of survivin but must overcome additional complexities due to its multifaceted role in many cellular processes. While a handful of small molecules have progressed into clinical trials, there are still many challenges that need to be addressed such as off-target toxicities associated with increased NF- κ B signaling, identifying appropriate biomarkers that correlate with elicited response, and developing synergistic combination therapies, especially to avoid development of multiple drug resistance. Overall, small-molecule IAP antagonists have great potential as anticancer drugs as single agents or in combination with existing therapies.

Hypothesis for Novel Survivin Inhibitors

In Chapter 6, we investigate a series of new survivin inhibitors modified from the UC-112 scaffold, which was previously identified through virtual screening. UC-112 demonstrated favorable properties over the most widely studied survivin inhibitor YM155 in that it was shown to be highly effective against drug efflux pump mediated resistance (508). SAR analysis of a predecessor drug, MX-106, revealed pertinent structural information for optimizing the UC-112 scaffold and suggested that a hydrophobic substituent on the benzene is amenable to activity. We therefore hypothesized that further modification to the benzyloxy moiety in MX-106 would yield potent analogs and that these analogs would maintain activity against P-gp

overexpressing cell lines, selectively inhibit survivin over other IAP family members, and demonstrate strong tumor growth inhibition against xenograft tumors in mice.

CHAPTER 6. INDOLE-BASED UC-112 ANALOGS AS POTENT AND SELECTIVE SURVIVIN INHIBITORS⁵

Introduction

Survivin is the smallest member in the IAP protein. Its expression is very low in healthy tissues and is highly expressed in tumors (509). The overexpression of survivin in tumor cells has been positively correlated with their development of multidrug resistance and radiation resistance (510, 511). For example, it is well established that cancer cells with high expression of survivin (e.g., prostate, ovarian and colorectal cancer cells) develop resistance to cisplatin and vincristine much easier than tumors cells with low expression of survivin (512). Survivin is therefore considered as a cancer specific biomarker and an attractive therapeutic target for the development of anticancer therapy (339) (513).

Several reported strategies have been applied to block the anti-apoptotic ability of survivin. These strategies include but are not limited to introducing recombinant cell-permeable dominant-negative survivin protein (514, 515), obstructing protein translation using antisense oligonucleotides (516), and developing small-molecule based survivin antagonists (517, 518). The crystal structure of human survivin was obtained in the early of this century, revealing its unusual bow tie-shape dimer structure (519). However, targeting survivin using small-molecule survivin inhibitors has been proven to be challenging because no experimentally validated binding pocket in survivin has been identified yet. The difficulty in developing a survivin specific small-molecule inhibitor also lies in the fact that survivin interacts with many other proteins to mediate apoptosis and mitosis, such as p53, STAT3, caspases and elements in the notch signaling pathway (520-523). A number of scaffolds have been reported in the literature to significantly suppress the expression of survivin and some have entered clinical trials for cancer treatment (483, 489, 505, 508, 524-529). However, none of these molecules have gained FDA approval. The most widely studied survivin inhibitor is YM155 which was initially discovered by Astellas Pharma in 2007. YM155 has an IC₅₀ in the sub-nanomolar range against several types of cancer cell lines (484). It inhibits survivin expression by inhibiting survivin promotor activity instead of directly degrading survivin protein. In clinical trials, YM155 was evaluated as monotherapy or combinational forms for patient with different cancer types, including blood cancer and solid tumors (530). Unfortunately, YM155 was withdrawn in phase II clinical trials due to its low ability to degrade survivin and high systemic toxicity.

We have recently discovered UC-112 through virtual screening followed by biological validation (**Figure 6-1**)(508). UC-112 is a potent survivin inhibitor that

⁵Modified with permission from Elsevier. Qinghui Wang*, Kinsie Arnst*, Yi Xue*, Zi-Ning Lei, Zhe-Sheng Chen, Duane D. Miller, Wei Li. Synthesis and biological evaluation of indole-based UC-112 analogs as potent and selective survivin inhibitors. *European Journal of Medicinal Chemistry*. 2018; 149:211-224.

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UC-112 (R=H)
MX-106 (R=i-Pr)

Figure 6-1. Structure of UC-112 and MX-106.

selectively degrades survivin protein via the proteasome pathway, activating caspases 3/7 and 9, and thus leads to cancer cell apoptosis. Unlike YM155 which is highly susceptible to P-gp mediated drug efflux pump mediated drug resistance, UC-112 was shown to be effective against P-gp overexpressing multidrug-resistant cancer cell lines. Subsequent structural modifications of UC-112 results in MX-106 (**Figure 6-1**), which showed improvements in suppressing survivin expression both *in vitro* and *in vivo* (531). According to the SAR analyses, the 8-hydroxyquinoline and the pyrrolidine in UC-112 are essential for maximum activity; hydrophobic substituent on the benzene is beneficial to activity. With this observation, we created analogs with modifications to the benzyloxy moiety in UC-112 as well as conformational restricted analogs formed by reducing the flexibility of the benzyloxy moiety in this scaffold to improve the activity. In this chapter, we reveal our results from modifying the benzyloxy moiety in the UC-112 scaffold and the biological investigation that followed. Thirty-three UC-112 analogs were synthesized and evaluated for their initial potency activities and lead compounds were further evaluated for their selective survivin inhibition and *in vivo* efficacy.

Materials and Methods

Cell Culture and Reagents

Human melanoma A375, M14, WM-164, RPMI-7951, and M14/MDR1 cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA), and cultured in DMEM media (Mediatech, Inc., Manassas, VA) at 37 °C in a humidified atmosphere containing 5% CO₂. The culture media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic-antimycotic mixture (Sigma-Aldrich, St. Louis, MO). Compounds were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) to make a stock solution of 10 mM. Compound solutions were freshly prepared by diluting stocks with cell culture medium before use (final solution contained less than 0.5% DMSO). 5000 cells in logarithm growing phase were seeded overnight into each well of a 96-well plate. Then the cells were continuously incubated for 48 h with sequential diluted compound solution (100 µM to 3 nM, 100 µL per well) in cell culture medium. The cell viability was determined in MTS assay and IC₅₀ was calculated (n = 4), following similar procedures as described previously (100, 143, 508, 531). Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and trypsin 0.25% were purchased from Hyclone (GE Healthcare Life Science, Pittsburgh, PA). Phosphate buffered saline (PBS) was purchased from Invitrogen GIBCO (Grand Island, NY). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St. Louis, MO). The P-glycoprotein (P-gp) overexpressing KB-C2 cell line was established from a parental human epidermoid carcinoma cell line KB-3-1, by a step-wise selection of KB-3-1 in increasing concentrations of colchicine up to 2 µg/mL (532). SW620/Ad300, which is also a P-gp overexpressing drug resistant cell line, was established by stepwise exposure of the parental human colon cancer cell line SW620 to increasing concentrations of doxorubicin up to 300 ng/mL(533). The KB-3-1

and KB-C2 cell lines were generously provided by Dr. Shin-Ichi Akiyama (Kagoshima University, Japan), and the SW620 and SW620/Ad300 cell lines were kindly provided by Dr. Susan E. Bates (Columbia University, NY, USA) and Dr. Robert W. Robey (NIH, MD, USA). All the cell lines were grown in DMEM supplemented with 10% FBS and 100 unit/mL penicillin/streptomycin in a humidified incubator containing 5% CO₂ at 37 °C.

Cytotoxicity Assays

A375, M14, WM-164, RPMI-7951, and M14/MDR1 were seeded in 96-well plates at a concentration of 1,000–5,000 cells per well, depending on growth rate of the cell line. After overnight incubation, the media was replaced, and cells were treated with the test compounds at 10 concentrations ranging from 0.03 nM to 1 μ M plus a vehicle control for 72 h in four replicates. Following treatment, the MTS reagent (Promega, Madison, WI) was added to the cells and incubated in dark at 37°C for at least 1 h. Absorbance at 490 nm was measured using a plate reader (DYNEX Technologies, Chantilly VA). Percentages of cell survival versus drug concentrations were plotted, and the IC₅₀ (concentration that inhibited cell growth by 50% of untreated control) values were obtained by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA).

Cytotoxicity against P-gp overexpressing cell lines by the MTT assay was obtained by Zi-Ning Lei (St. John's University). Cells were seeded in 96-well plates at 5000 cells/well (KB-3-1 or KB-C2 cells) or at 7,000 cells/well (SW620 or SW620/Ad300 cells) in 180 μ L completed medium and cultured overnight. Then various concentrations of the compounds (20 μ L) were added to the designated wells. After 72 hr continuous drug incubation, 20 μ L of MTT reagent (4 mg/mL) was added to each well and the plates were incubated at 37 °C for 4 hr. Subsequently, the medium was removed and 100 μ L of DMSO were added to dissolve the formazan crystals in each well. The absorbance was determined at 570 nm by the accuSkan™ GO UV/Vis Microplate Spectrophotometer (Fisher Sci., Fair Lawn, NJ). The IC₅₀ values of each compound on each cell line were calculated from the survival curves to represent the cytotoxicity of the compounds. The fold of drug resistance was calculated by dividing the IC₅₀ of the P-gp overexpressing cells by that of the parental cells. Two known P-gp substrates, YM155 and paclitaxel, were used as positive controls for P-gp overexpressing cell lines. On the other hand, cisplatin, which is not a substrate of P-gp, was used as negative control.

Liver Microsomes Stability Assay

Liver microsome assays were performed by Dr. Lei Yang (St. Jude Children's Research Hospital). NADPH regenerating agent solutions A (catalog#: 451220) and B (catalog#: 451200) and mouse liver microsomes (CD-1, mixture of male, catalog#:452701, and female, catalog#: 452702) were obtained from BD Gentest (Woburn, MA). Liver microsomes stability assay was conducted following literature

reports (238, 239). For each test compound, the mouse liver microsomal solution was prepared by adding 0.058 mL of concentrated mouse liver microsomes (20 mg/mL protein concentration) to 1.756 mL of 0.1 M potassium phosphate buffer (pH 7.4) containing 5 μ L of 0.5 M EDTA to make a 0.6381 mg/mL (protein) microsomal solution. Each test compound (2.2 μ L of 10 mM DMSO solution) was added directly to 1.79 mL of mouse liver microsomal solution and 90 μ L was transferred to wells in 96-well plates (0, 0.25, 0.5, 1, 2, and 4 h time points each in triplicate). The NADPH regenerating agent was prepared by mixing 0.113 mL of NADPH regenerating agent Solutions A, 0.023 mL of solution B and 0.315 mL of 0.1 M potassium phosphate buffer (pH 7.4) for each tested compound. To each well of the 96-well plate, 22.5 μ L of the NADPH regenerating agent was added to initiate the reaction, and the plate was incubated at 37 °C for each time point (0, 0.25, 0.5, 1, 2, and 4 h time points each in triplicate). The reaction was quenched by adding 225 μ L of cold acetonitrile containing warfarin (4 mg/mL) as internal control to each well. All of the plates were centrifuged at 4,000 rpm for 20 min and the supernatants (100 μ L) were transferred to another 96-well plates for analysis on UPLC–MS (Waters Acquity UPLC linked to Waters Acquity Photodiode Array Detector and Waters Acquity Single Quadrupole Mass Detector) on Acquity UPLC BEH C18 1.7 mm (2.1x50 mm) column by running 90–5% gradient for water (+0.1% formic acid) and acetonitrile (+0.1% formic acid) in 2 min. The area under the single ion recording (SIR) channel for the test compound divided by the area under the SIR for internal control at 0 time concentration was considered as 100% to calculate remaining concentration at each time point. The terminal phase rate constant (k_e) was estimated by linear regression of logarithmic transformed concentration versus the data, where $k_e = \text{slope} \times (-\ln 10)$. The half-life $t_{1/2}$ was calculated as $\ln 2/k_e$. The intrinsic clearance ($CL_{\text{int,app}} = (0.693/\text{in vitro } t_{1/2}) \times (1 \text{ mL incubation volume}/0.5 \text{ mg of microsomal protein}) \times (45 \text{ mg microsomal protein/gram of liver}) \times (55 \text{ g of liver/kg body weight})$).

Western Blot

To determine the change of protein levels of survivin and closely related IAPs, lysates of A375 or M14 melanoma cells treated by the compound solution for 24 h were used for western blotting analysis and determined by Dr. Yi Xue (UTHSC). Primary rabbit antibodies against IAP proteins including survivin (#2808), XIAP (#2045), cIAP1 (#7065), Livin (#5471), Cleaved PARP (#9185) and the loading control protein GAPDH (HRP Conjugate) (#3683) were purchased from Cell Signaling Technology, Inc. (Danvers, MA) and used according to manufacture instructions as reported previously.

Molecular Modeling

Molecular modeling data was provided by Dr. Wei Li (UTHSC). The molecular docking studies were conducted in Schrodinger Molecular Modeling Suite 2014 (Schrodinger Inc., Portland, OR) following previously described procedures (508, 531). Ligand was prepared to generate various conformation before being docked into the SMAC AVPI binding site of a human survivin crystal structure (Protein Data Bank entry:

3UIH). Prior to molecular dynamic calculation, the docking to minimize the energy of potential ligand binding poses was performed. Results were visualized using the Maestro interface of the Schrodinger software.

***In Vivo* Xenograft Model**

All animal experiments were performed in accordance with the NIH animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center. Nude mice, 6–8 weeks old, were purchased from Envigo (Indianapolis, IN).

Logarithmic growth phase A375 cells (5×10^7 cells per mL) were prepared in phenyl red-free, FBS-free media and mixed with Matrigel immediately before injecting into mice. Tumors were established by injecting 100 μ L of this mixture subcutaneously in the dorsal flank of each mouse (2.5×10^6 cells). After tumor volumes reached approximately 150 mm³ mice were randomized into control or treatment groups (n=7~8). **10f** or paclitaxel was dissolved in a 1:1 ratio of PEG300: PBS solution to produce desired concentrations. The vehicle control solution was formulated with equal parts PEG300 and PBS only. 100 μ L of the drug treatment or vehicle control was administered via i.p. injection every other day two weeks.

Tumor volume was measured three times a week with a caliper and calculated by using the formula $a \times b^2 \times 0.5$, where a and b represented the larger and smaller diameters, respectively. Tumor growth inhibition (TGI) at the conclusion of the experiments was calculated as $100 - 100 \times ((T - T_0)/(C - C_0))$, where T, T₀, C and C₀ are the mean tumor volume for the specific group on the last day of treatment, mean tumor volume of the same group on the first day of treatment, mean tumor volume for the vehicle control group on the last day of treatment and mean tumor volume for the vehicle control group on the first day of treatment, respectively. Animal activity and body weights were monitored during the entire experiment period to assess potential acute toxicity. At the end of the experiment, mice were sacrificed and the tumors were weighed. Tumors and tissues were dissected out and preserved in 10% buffered formalin phosphate solution.

Results and Discussion

UC-112 Analogs with Modification of the Benzyloxy Moiety

Our preliminary SAR investigation of UC-112 emphasized the replacement of the benzyloxy with other substructures and has led to the syntheses of analogs **2a-2d**, **6a-6f**, **8** and **10a** (Table 6-1). Compared to the reference compound MX-106, **2a** (5-fluoroindazole analog), **2b** (benzotriazole analog) and **2c** (4-chloropurine analog) exhibited 3~5-fold reduced activities. Replacement of the benzyloxy with the other

Table 6-1. *In vitro* cytotoxic effects of UC-112 analogs with modification to the benzyloxy moiety.

IC ₅₀ ± SEM (μM)					^a RI
	A375	WM-164	RPMI-7951	M14	M14/MDR1	
2a	3.6 ± 0.2	8.6 ± 1.1	11.1 ± 0.7	5.4 ± 0.3	7.0 ± 0.1	1.3
2b	1.9 ± 0.1	>30.0	6.5 ± 0.2	2.3 ± 0.1	3.1 ± 0.1	1.3
2c	3.9 ± 0.1	56.4 ± 1.1	17.4 ± 1.2	4.1 ± 0.2	7.9 ± 0.1	1.9
2d	14.1 ± 0.7	33.9 ± 1.1	21.0 ± 0.4	11.5 ± 0.4	14.3 ± 0.2	1.2
6a	>30.0	13.0 ± 0.5	>30.0	>30.0	>30.0	^b ND
6b	4.7 ± 0.2	3.7 ± 0.3	26.2 ± 6.2	5.1 ± 0.1	10.7 ± 1.2	2.1
6c	3.5 ± 0.3	2.8 ± 0.2	18.4 ± 0.3	8.6 ± 0.2	7.4 ± 1.2	0.9
6d	1.5 ± 0.1	1.6 ± 0.1	12.9 ± 0.2	>30.0	12.2 ± 0.2	ND
6e	6.0 ± 1.3	24.9 ± 1.0	25.3 ± 2.4	6.7 ± 0.3	12.2 ± 0.5	1.8
6f	2.9 ± 0.15	ND	6.8 ± 0.6	2.4 ± 0.1	3.3 ± 0.2	1.4
8	>30.0	20.18 ± 0.49	17.3 ± 0.3	>30.0	8.7 ± 0.2	ND
10a	0.8 ± 0.1	0.9 ± 0.1	4.4 ± 0.3	0.8 ± 0.1	2.1 ± 0.1	2.6
UC-112	1.9 ± 0.6	ND	ND	2.1 ± 0.4	3.2 ± 0.5	1.5
MX-106	0.9 ± 0.1	1.1 ± 0.2	ND	0.8 ± 0.2	1.8 ± 0.4	2.3

^aRI: Resistance index is calculated by dividing the IC₅₀ values on multidrug-resistant cell line by IC₅₀ by the matching parental cell line.

^bND: Not Determined.

moiety as shown in compounds **2d** resulted in significant reduction of inhibitory effects (> 3-fold). Replacing the benzyloxy with phenyl acetamides (analogs **6a-6d**) was detrimental to the antiproliferative activities. Shortening the linkage by one methylene in **6b** and **6d** resulted in analogs **6e-6f** and was revealed to not affect the activities. The introduction of a benzylurea (analog **8**) dramatically attenuated the antiproliferative activity. Among all analogs in this series, non-substituted indole analog **10a**, exhibited the most potent activity and was comparable to that of MX-106, having an average IC₅₀ of 0.9 μ M.

UC-112 Analogs with Substitutions on the Indole Moiety

Because the indole analog **10a** had an equipotency to that of MX-106, we subsequently focused our effort to investigate the substitutional effect on the indole ring. Analogs **10b-10p** with mono-substituent and analogs **10q-10v** with di-substituents were then synthesized (Table 6-2). For the 2-position substituted analogs, **10b** with a methyl group and **10c** with an ethyl group showed slightly reduced activities compared to **10a**. Introducing substituents to the 4-position on the indole diminished the antiproliferative activity, which was demonstrated by analogs **10d** and **10e**. For analogs that had substituents on the 5-position of indole, comparable activities to that of **10a** were observed, for example, compounds **10f**, **10g** and **10h** had IC₅₀ values ranging from 0.7 to 1.1 μ M; analog **10i** that had bulky ester functional group showed ~2-fold reduced activity in comparison with MX-106. In the series of analogs with substituents on the 6-position of the indole (**10j-10n**), they were generally equipotent to MX-106 and **10a**, having IC₅₀ values as low as 0.7 μ M. Analogs having substituents on the 7-position of indole were slightly less potent than corresponding 5- or 6-position substituted counterparts. All the six di-substituted analogs did not show any improvement of activity compared to their mono-substituted counterparts. **10s-10v** were designed based on the most potent mono-substituted analogs **10f**, **10k**, and **10n**. Incorporation of a secondary substituent to 5-fluoroindole, 6-chloro, or 6-methoxyindole generally was not beneficial to activity; for example, **10s** and **10u** showed reduced cytotoxicities compared to **10f** and had IC₅₀ values of >1.5 μ M; the 3-cyano-6-methoxy indole analog **10t** was less potent than the mono-substituted counterpart **10n**; 5,6-dichloroindole analog **10v** showed cytotoxicity comparable to that of 5-chloroindole analog **10k**.

Potency Against P-gp Overexpressing Cell Lines

P-gp belongs to the family of ABC transporters and is encoded by the ABCB1 gene. P-gp is responsible for the decline of concentrations of extensive anticancer drugs in multidrug resistant cells. Therefore, the ability to overcome P-gp mediated drug-resistance is a favorable property for drug candidates. In addition to the M14 melanoma cell line, its P-gp overexpressed daughter line M14/MDR1 was also tested to evaluate the abilities of new analogs to overcome P-gp mediated drug-resistance (Table 6-2). According to assay result, more than twelve of the twenty-two indole analogs exhibited

Table 6-2. *In vitro* cytotoxic effects of UC-112 analogs with mono- and di-substituents on the indole moiety.

Structure	ID	R	IC ₅₀ ± SEM (μM)					^a RI
			A375	WM-164	RPMI-7951	M14	M14/MDR1
Mono-substituent	10a	H	0.8 ± 0.1	0.9 ± 0.1	4.4 ± 0.3	0.8 ± 0.1	2.1 ± 0.1	2.6
	10b	2-Me	2.2 ± 0.2	7.5 ± 1.2	2.2 ± 0.2	1.4 ± 0.2	0.7 ± 0.1	0.5
	10c	2-Et	2.0 ± 0.1	8.9 ± 1.4	3.2 ± 0.4	1.5 ± 0.2	1.1 ± 0.1	0.7
	10d	4-F	1.6 ± 0.3	6.5 ± 0.6	2.2 ± 0.2	1.2 ± 0.2	0.8 ± 0.1	0.7
	10e	4-NO ₂	1.8 ± 0.1	7.0 ± 0.3	0.6 ± 0.1	2.1 ± 0.1	0.6 ± 0.1	0.3
	10f	5-F	0.7 ± 0.2	0.7 ± 0.2	^b ND	1.9 ± 0.4	ND	ND
	10g	5-CN	1.0 ± 0.1	1.1 ± 0.1	ND	5.1 ± 0.3	ND	ND
	10h	5-NO ₂	0.7 ± 0.1	0.8 ± 0.1	3.7 ± 0.3	ND	2.2 ± 0.1	ND
	10i	5-COOEt	1.9 ± 0.2	4.7 ± 0.6	1.5 ± 0.1	2.4 ± 0.2	0.8 ± 0.1	0.3
	10j	6-F	0.9 ± 0.1	0.7 ± 0.1	2.7 ± 0.5	1.3 ± 0.1	1.3 ± 0.1	1.0
	10k	6-Cl	0.9 ± 0.4	0.9 ± 0.4	7.7 ± 0.5	0.8 ± 0.4	3.8 ± 0.1	4.8
	10l	6-Br	0.9 ± 0.1	1.4 ± 0.2	5.3 ± 0.6	3.5 ± 0.3	0.7 ± 0.1	0.2
	10m	6-NO ₂	0.9 ± 0.1	0.7 ± 0.1	4.3 ± 0.7	2.8 ± 0.2	2.8 ± 0.2	1.0
	10n	6-OMe	0.8 ± 0.2	0.7 ± 0.1	6.2 ± 0.4	0.9 ± 0.4	2.8 ± 0.1	3.1
	10o	7-F	1.7 ± 0.2	1.9 ± 0.2	ND	2.6 ± 0.4	ND	ND
Di-substituent	10p	7-Me	2.0 ± 0.2	11.5 ± 0.8	2.6 ± 0.2	1.4 ± 0.1	0.9 ± 0.1	0.6
	10q	2,3-(Me) ₂	1.9 ± 0.2	7.1 ± 0.8	8.2 ± 0.6	1.5 ± 0.2	1.0 ± 0.1	0.7
	10r	2-Me-5-OMe	1.9 ± 0.2	4.6 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.0 ± 0.1	0.5
	10s	2-COOEt-5-F	2.0 ± 0.2	8.5 ± 0.8	3.6 ± 0.4	1.3 ± 0.1	1.0 ± 0.1	0.7
	10t	3-CN-6-OMe	1.7 ± 0.1	16.7 ± 0.5	3.7 ± 0.1	1.6 ± 0.1	1.9 ± 0.1	1.2
	10u	5-F-6-Cl	2.0 ± 0.2	4.5 ± 0.2	1.7 ± 0.1	2.0 ± 0.2	1.0 ± 0.1	0.5
	10v	5,6-(Cl) ₂	0.7 ± 0.1	0.9 ± 0.1	9.3 ± 1.1	8.5 ± 0.2	2.2 ± 0.1	0.3

^aRI: Resistance index is calculated by dividing IC₅₀ values on multidrug-resistant cell line M14/MDR1 by IC₅₀ values on the matching sensitive parental cell line M14.

^bND: Not Determined.

more potent inhibitory effects against P-gp overexpressing M14/MDR1 cell line than the parental drug sensitive M14 cell line and had resistance indexes pf less than 1, indicating that new indole analogs had significant ability to circumvent drug-resistance mediated by P-gp overexpression. It is worth noting that five out of the six di-substituted indole analogs have RIs less than 1, suggesting that a secondary substituent on the indole is beneficial for overcoming drug-resistance.

In addition to melanoma cell lines, cervical and colorectal carcinoma cell lines were also tested to confirm the capabilities of the new analogs to circumvent P-gp mediated drug resistance (**Table 6-3**). The small-molecular survivin inhibitor YM-155 showed remarkable cytotoxicity against KB-3-1 and SW620 cell lines with IC₅₀ values of 4.9 nM and 3.9 nM, respectively. YM155, however, displayed significantly reduced activities against the corresponding P-gp overexpressing cell lines (KB-C2 and SW620/Ad300) and had IC₅₀ values of more than 20 μ M. Similarly, paclitaxel, a known substrate of P-gp, was not effective against P-gp overexpressing cell lines. In contrast, our indole analogs **10f**, **10h**, **10k**, **10n** and the previously reported MX-106 showed stronger inhibitory effects against P-gp overexpressing cell lines (KB-C2 and SW620/Ad300) than their corresponding parent cell lines (KB-3-1 and SW620). While the IC₅₀ values range from 1.28 to 1.71 μ M in the non-resistant KB-3-1 cell line for **10f**, **10h**, **10k** and **10n**, their potency against the P-gp overexpressing KB-C2 cell line significantly increased (IC₅₀ values range from 0.17 to 0.44 μ M). Similarly, **10f**, **10h**, **10k** and **10n** had IC₅₀ values ranging from 0.16 to 0.31 μ M against non-resistant SW620 cell line while their activities in KB-C2 cell line were 3-6 folds more potent with IC₅₀ values ranging from 0.027 to 0.1 μ M. Collectively, these new indole analogs of UC-112 can show potential to reverse drug-resistance mediated by P-gp overexpression.

***In Vitro* Metabolic Stability Study**

Prior to *in vivo* study, the *in vitro* metabolic stabilities of analogs **10f**, **10h**, **10k** and **10n** were examined by measuring their half-life upon incubation with mouse, rat, and human liver microsomes in the presence of an NADPH regeneration system (**Table 6-4**). All compounds possessed acceptable stability profile in three microsome species. They were more stable in human microsome than in mouse and rat microsomes. Among the four analogs, **10f** with a 5-fluoro was the most stable against mouse and human microsomes.

UC-112 Analogs Maintain Selective Inhibition for Survivin among IAPs

To determine whether our new UC-112 analogs maintains their selective inhibition of survivin as the prototype UC-112 does, we measured protein levels of several IAPs including cIAP1, XIAP, Livin, and survivin after treatment with **10f**, **10h**, **10k** and **10n** in A375 cells (**Figure 6-2**). All four compounds strongly inhibited survivin expression in A375 cell line in a concentration-dependent manner, while the other IAP

Table 6-3. *In vitro* cytotoxic effects UC-112 analogs against P-gp overexpressing cervical cancer and colorectal cell lines.

Compound	IC ₅₀ ± SD (μM)		^a RI	IC ₅₀ ± SD (μM)		RI
	KB-3-1	KB-C2		SW620	SW620/ Ad300	
10f	1.36 ± 0.19	0.17 ± 0.09	0.13	0.16 ± 0.04	0.027± 0.006	0.17
10h	1.39 ± 0.41	0.18 ± 0.05	0.13	0.19 ± 0.09	0.06 ± 0.02	0.32
10k	1.71 ± 0.22	0.25 ± 0.05	0.15	0.24 ± 0.09	0.05 ± 0.03	0.21
10n	1.28 ± 0.25	0.44 ± 0.28	0.34	0.31 ± 0.05	0.10 ± 0.04	0.32
MX-106	1.25 ± 0.10	0.33 ± 0.12	0.26	0.15 ± 0.01	0.03± 0.01	0.20
YM155	0.0049 ± 0.0005	37.30± 3.97	7612.24	0.0039 ± 0.0056	23.37 ± 2.37	5992.3
Paclitaxel	0.0005 ± 0.0001	0.25 ± 0.01	500.00	0.030 ± 0.001	1.97 ± 0.36	65.67
Doxorubicin	0.37 ± 0.07	1.69 ± 0.44	4.57	0.21 ± 0.03	15.24 ± 2.89	72.57
Cisplatin	1.15 ± 0.13	1.73 ± 0.11	1.50	1.79 ± 0.06	5.12 ± 0.65	2.86

Data obtained by Zi-Ning Lei (St. John's University).

^aRI: Resistance index is calculated by dividing IC₅₀ values for multidrug-resistant cell lines by IC₅₀ values for the matching sensitive parental cell lines.

Table 6-4. *In vitro* metabolic stability of UC-112 analogs in liver microsomes.

Compound	Metabolic Stability (Human)		Metabolic Stability (Mouse)		Metabolic Stability (Rat)	
	t _{1/2} (hr)	Cl _{int} (mL/min/kg)	t _{1/2} (hr)	Cl _{int} (mL/min/kg)	t _{1/2} (hr)	Cl _{int} (mL/min/kg)
10f	4.59 ± 0.39	4.5	1.36 ± 0.06	42.1	1.29 ± 0.03	4.5
10h	2.16 ± 0.19	9.6	1.17 ± 0.06	48.7	1.20 ± 0.05	39.0
10k	3.50 ± 0.36	5.9	1.20 ± 0.07	47.8	1.62 ± 0.10	29.0
10n	2.80 ± 0.18	7.4	0.88 ± 0.06	65.1	1.26 ± 0.06	37.0

Data obtained by Lei Yang (St. Jude Children's Research Hospital).

Figure 6-2. UC-112 analogs selectively downregulate survivin.

Western blot analysis A375 cells treated with 1, 3, or 10 μ M of **10f**, **10h**, **10k** and **10n** for 24 h. Protein levels of various IAP proteins show that UC-112 analogs selectively target survivin over other IAPs (cIAP1, XIAP, and Livin) and increase apoptotic protein cleaved-PARP. Western blot was performed by Dr. Yi Xue (UTHSC).

family proteins were not affected. This evidence indicates high selectivity of survivin inhibition among the IAP proteins. Consistent with survivin inhibition, all four analogs effectively induced cancer cell apoptosis, as indicated by the elevated level of cleaved PARP.

Molecular Modeling of Lead Compound

To explain the observed strong potency of **10f**, a molecular modeling study was developed using the complex of human survivin-SMAC AVPI (PDB entry: 3UIH) (**Figure 6-3A and B**). **10f** formed appealing hydrogen bonding and π - π stacking interactions with the survivin protein BIR domain: (1) two hydrogen bonding interactions between the 8-hydroxyquinoline of **10f** and residues Glu76 and Lys79; (2) two hydrogen bonding interactions between the pyrrolidine of **10f** and residue Asp71; (3) π - π stacking interaction between the 8-hydroxyquinoline of **10f** and residue His80; (4) π - π stacking interaction between the 5-F indole of **10f** and residue His80; (5) π -cation interaction between the pyrrolidine of **10f** and residue Trp67 (red line in **Figure 6-3B**). The molecular modeling result suggested that substitute on the 7-position of the indole ring will introduce the repulse between the 8-hydroxyquinoline and indole ring and further lead to attenuated π - π stacking interactions mentioned above, for example, **10p** and **10q** exhibited significantly decreased potency in comparison with **10a**.

In Vivo Antitumor Efficacy Assessment

As one of the most potent indole analog, **10f** not only exhibited the most favorable stability against mouse and human liver microsomes but also significantly inhibited the survivin expression at a low concentration; therefore, **10f** was selected for further evaluating antitumor efficacy *in vivo*. An A375 melanoma xenograft model in nude mice was used. Tumors were implanted by inoculating mice with A375 human melanoma cancer cells subcutaneously in their hindflank. After the development of viable tumors, mice were treated every other day for two weeks by i.p. injection with 20 mg/kg **10f**, 15 mg/kg paclitaxel, or vehicle solution only. After 15 days of treatment, the groups receiving 20 mg/kg **10f** had significantly smaller tumor volumes than the vehicle control group with a calculated TGI of 68.6% (**Figure 6-4A**). This was very similar to the paclitaxel treated group which averaged a TGI of 70.6% compared to the vehicle control group. One-way ANOVA followed by Dunnett's multiple comparison test also revealed that there was a significant reduction in final tumor volume ($P < 0.0001$) for all treatment groups compared to the vehicle control group. These results are in accord with the tumor final weights, where the average tumor weight for the control group was 2.08 ± 0.32 grams, and **10f** and paclitaxel groups averaged 0.85 ± 0.26 grams and 0.78 ± 0.12 grams, respectively (**Figure 6-4B**). Statistically analysis was performed the same and yielded an overall P value of 0.0012, and a significant difference ($P < 0.01$) for each treated group compared to the control group. Mouse body weight was measured, and animal activity was monitored throughout the experiment, and no significant deviations in animal weight or behavior were observed (**Figure 6-4C**).

Figure 6-3. Potential binding mode of 10f to survivin.

A) The best docking pose of **10f** binding in survivin (survivin PDB: 3UIH), shown with electron potential color-coded survivin surface (red: electron negative potential; blue: electron positive potential). Interactions between **10f** and nearby residues in survivin are shown. B) Types of interactions are shown with color-coded lines between **10f** with residues in survivin protein in this 2D interaction map. Molecular modeling generated by Dr. Wei Li (UTHSC).

Figure 6-4. 10f inhibits tumor growth *in vivo*.

A) Average tumor volumes $\pm$ SEM in an A375 xenograft model in nude mice (n=7-8). B) Individual tumor weights. Long line represents mean and error bars represent SEM. Statistical significance was determined by one-way ANOVA ($P=0.0012$) analysis followed by Dunnett's multiple comparison test for each treatment group compared to the control group (** $P < 0.01$). C) Average mouse body weights $\pm$ SEM. Graph represents percent change in body weight compared to the starting weight.

Conclusions

In this report, thirty-three new analogs of UC-112 were synthesized and their SAR was investigated. The results showed that most indole analogs exhibited potencies stronger than or comparable to UC-112 and MX-106. The most potent activities were observed in **10f**, **10h**, **10k** and **10n**. Compared to YM155, a widely studied survivin promoter inhibitor, our new indole analogs of UC-112 showed stronger activities against P-gp overexpressing cancer cell lines and parental cancer cell lines. Mechanistic studies revealed that new indole analogs of UC-112 selectively suppressed the expression of the survivin protein in a dose-dependent manner without affecting other members of the IAP family. In an *in vivo* xenograft model in nude mice, **10f** exhibited significant tumor growth inhibition without signs of toxicity. These results support conducting detailed SAR investigation of novel scaffolds to develop optimized analogs and ultimately led to the discovery of **10f**, a potent and selective survivin inhibitor with strong anticancer action.

CHAPTER 7. SUMMARY AND FUTURE DIRECTIONS FOR TUBULIN INHIBITORS AND SURVIVIN INHIBITORS

In the tubulin project, we investigated several scaffolds of small molecules that specifically target the colchicine binding site on tubulin. Colchicine binding site agents, unlike vinca alkaloids or taxanes, have been reported to circumvent several mechanisms of multidrug resistance, particularly resistance mediated by the overexpression of P-gp and other drug efflux pumps. Therefore, we had the overall goal to develop compounds that would show broad anticancer effects, demonstrate improved IC₅₀s, and disrupt microtubule networks and mitotic spindle formation. We also aimed to generate analogs that would optimally target the colchicine binding site and would thus maintain a low resistance index against ABC transporter overexpressing cell lines and show significant efficacy against taxane-resistant tumors in animal models.

In Chapter 2, we hypothesized that our lead indolyl-imidazopyridines, DJ95 and DJ101, would show strong potency to a variety of cell lines including those exhibiting MDR. This was confirmed by their cytotoxicity against our expanded melanoma cell panel and against the NCI-60 cell lines. DJ101 showed the greatest activity against melanomas, with IC₅₀s below 10 nM. Both DJ101 and DJ95 showed low resistance factors to taxane-resistant prostate cancer cell lines, especially when compared to other tubulin inhibitors including colchicine, paclitaxel, and docetaxel. DJ95 was also evaluated against gene-transfected cell lines overexpressing ABCB1, ABCC1, and ABCG2, and additionally against drug-selected MDR cell lines. MDR cell lines were more receptive to DJ95 treatment than to colchicine, vincristine, and paclitaxel. To test our hypothesis regarding their effects on microtubule networks, we demonstrated their strong destabilizing action and disruption to mitotic spindles via confocal microscopy, followed by X-ray crystallographic analysis detailing their verified interaction at the colchicine domain. Finally, we hypothesized that the compounds would show potent anticancer activity in animal models. Both DJ95 and DJ101 demonstrated tumor growth inhibition in melanoma xenografts. DJ101 presented superior activity, where 30 mg/kg doses inhibited tumor growth by 92.8%. In a subsequent metastatic melanoma xenograft model, treatment with DJ101 using the same dosing regimen drastically reduced pulmonary metastatic nodules by 83.8% compared to the control group. Finally, DJ101 treatments potently inhibited the growth of taxane-resistant prostate cancer tumors, confirming its activity *in vivo* and against MDR-presenting cancers. Therefore, we confirmed the hypothesis for this series of agents, with DJ101 showing the greatest anticancer activity.

In Chapter 3, we set out to test the assumption that the indole moiety and 3,4,5-trimethoxyphenyl moiety of VERU-111 could be optimized to generate analogs with lower IC₅₀s, greater activity against tubulin polymerization, and *in vivo* efficacy in mouse models. We identified the best candidate from each series of modifications: indole substitution (**10ab**), indolyl rotation (**10bb**) and TMP modification (**13f**). We then demonstrated their high efficacy against taxane-resistant cell lines and validated their mechanism of action through their ability to potently prohibit polymerization of purified

tubulin protein. Their destabilization action was further established with confocal microscopy, and their activity against microtubules resulted in fragmentation and decreased polymeric tubulin. Even with its unique allosteric replacement of the critical TMP moiety, **13f** still showed direct interactions with the colchicine binding site by X-ray crystallography. Finally, based on their potency and metabolic stabilities in liver microsomes, we tested **10bb** and **13f** in melanoma xenograft models, and 30 mg/kg treatments suppressed tumor growth by 73.9% and 90.6%, respectively. **10bb** also effectively inhibited metastasis and taxane-resistant tumor proliferation in two additional animal models without showing signs of toxicity. The pharmacological profile of **10bb** did not indicate significant interactions with physiologically important off-targets based on the Safety Screen panel, nor did the toxicological analysis of major organs (heart, liver, kidney, lungs, and spleen) from the drug-treated xenograft model groups exhibit apparent drug-related injury to the tissues. Therefore, we identified improved analogs of VERU-111, and the most promising compound, **10bb**, showed significant *in vivo* activity against MDR tumors, inhibited the development of metastases, and improved the therapeutic window.

In Chapter 4, we hypothesized we could design Azixa analogs that had improved metabolic stabilities, could induce vascular disrupting effects, interfere with cellular processes through tubulin inhibition, promote G₂/M phase mitotic arrest, and finally, demonstrate potent antitumor effects. First, we selected **4a**, **6a**, **5a**, and **5b** from a series of heteropyrimidines based on their exceptional potencies in initial cell viability assays. **5a** and **5b** were tested in additional cancer types, including malignant melanomas, prostate, lung, and breast cancer cell lines as well as several drug-resistant sublines. **5b** outperformed colchicine in every instance and revealed that it was a poor substrate of P-gp. Compared to Azixa, **4a**, **5a**, and **5b** all showed better metabolic stabilities in human liver microsomes and were substantially better than other reported Azixa analogs. All four compounds showed robust destabilizing activity based on tubulin polymerization and microtubule morphology, and two compounds (**4a** and **6a**) had confirmed binding to the colchicine site at the interface of the α and β tubulin subunit, all supporting their action as colchicine binding site inhibitors. **5a** and **5b** hindered cell migration, inhibited colony proliferation, induced G₂/M phase cell cycle arrest and apoptosis in a concentration-dependent manner, and disturbed HUVEC capillary-like formation *in vitro*. **4a** and **6a** were assessed *in vivo* and inhibited melanoma tumor growth by 72.3% and 76.5%, respectively, following 30 mg/kg treatments, and **4a** treatment also reduced taxane-resistant prostate cancer tumor volume by 55.6%. Tumor sections from the treated groups showed greatly diminished overall microvessel formation and morphologically distortion vasculature, and additionally revealed elevated levels of apoptosis compared to the control group. These *in vivo* results of **4a** and **6a** correlate with the *in vitro* data demonstrated by **5a** and **5b**, ultimately confirming our hypothesis and supporting their action as both tubulin inhibitors and as vascular disrupting agents capable of inducing strong anticancer action against clinically significant drug-resistant models.

In conclusion, from our overall evaluation of tubulin inhibitors, we optimized several scaffolds of colchicine binding site agents and generated lead analogs with improved potency, metabolic stability, and antitumor efficacy. After confirming their

cytotoxic effects against a variety of cell lines, lead compounds underwent additional biological analysis to confirm their mechanisms of action. We determined that they shared common characteristics with other colchicine binding site agents including; inhibiting tubulin polymerization, destabilizing microtubules and mitotic spindles, directly interacting with the colchicine binding site, inducing G₂/M phase cell cycle arrest, and hindering migration. Furthermore, several of the compounds were evaluated in ABC transporter overexpressing cell lines, drug-selected resistant cell lines, and taxane-resistant xenograft models, and showed improved efficacy. Two of the compounds were able to potently suppress the establishment of pulmonary metastases of melanoma *in vivo*, supporting their use for metastatic intervention. We also investigated the vascular disrupting potential of some of the compounds and presented their capacity to inhibit endothelial cell network formation and diminish intra-tumor vasculature. Pharmacological profile screenings revealed that the compounds minimally interact with off-target receptors, transporters, ion channels, kinases, and enzymes, which may reduce the attrition risk at later stages in the drug development process. Overall, we identified promising candidates, including indolyl-imidazopyridines (DJ95 and DJ101), VERU-111 analogs (**10ab**, **10bb**, and **13f**), and heterocyclic pyrimidines (**4a**, **6a**, **5a**, and **5b**), confirmed the mechanism of action as colchicine binding site agents, and demonstrated their anticancer potential, particularly against MDR cell lines and tumor models.

To effectively escape the shortcomings of current anticancer drugs, newer generations of tubulin inhibitors should exploit the colchicine binding site to decrease the susceptibility of transporter-mediated efflux and resistance to the β III tubulin isoform. Additionally, drugs targeting this site often are effective as vascular disrupting agents and can accommodate a large range of structurally diverse scaffolds. Innovative strategies for microtubule targeting agents should capitalize on combination therapies to reduce the emergence of multidrug resistance. Other issues limiting the clinical potential of microtubule targeting agents are poor solubility, low metabolic stability, and dose-limiting neural and cardiotoxicities. Several techniques have been employed to improve drug properties and limit toxicity such as delivery via nanoparticle formulations and antibody-drug conjugation. Finally, the emergence of dual kinase tubulin inhibitors provides several benefits for this class of drug, as these dual inhibitors may improve the therapeutic window and reduce the risk of drug resistance development by targeting multiple pathways. While the scaffolds of the tubulin compounds we investigated were quite diverse in structure, their common feature was that they all target the colchicine binding site and were highly effective against multiple mechanisms of resistance. We believe that of the investigated compounds, DJ101 is one of the strongest candidates to avidly push forward with characterization. Additional models could be used to test its efficacy, such as patient derived xenograft models, and further characterization of the biological events that ensue for it to maintain efficacy against MDR phenotypes will reveal the compound's clinical potential. Ultimately, our studies collectively support the continued development of the investigated scaffolds as anticancer therapeutics, especially as an alternative treatment to taxane-resistant cancers.

In the survivin project, we explored analogs of UC-112, an IAP inhibitor selective to survivin. We hypothesized that by modifying the benzyloxy moiety, we could generate

analogs with improved potency against P-gp overexpressing cell lines, specifically target survivin with high selectivity, and show *in vivo* anticancer efficacy. After attempts to modify the benzyloxy with numerous other substructures, we determined that the non-substituted indole analog had the most comparable potency to the parent compound. From there, we shifted our focus to determining the best substitution on the indole ring and identified **10f** (containing a fluorine at the 5-position of indole). **10f**, the most promising candidate, demonstrated exceptional potency against parental and resistant cancer cell lines and selectively suppressed the expression of the survivin protein in a dose-dependent manner without affecting other members of the IAP family. Finally, **10f** treatment of 20 mg/kg significantly inhibited tumor volume in a xenograft model by 68.6%, which was equally as effective as paclitaxel treatment (TGI of 70.6%), without causing toxicity to the animals. Therefore, our hypothesis regarding UC-112 modification, survivin selectivity, and anticancer action was confirmed.

Identifying appropriate biomarkers that correlate with elicited response and developing synergistic combination therapies, especially to avoid development of multiple drug resistance, could prove to be invaluable strategies to maximize clinical efficacy for IAP inhibiting agents in general and particularly for survivin inhibitors. Because dynamicity of survivin and its implicated in many critical biological processes including mitosis, apoptosis, cell cycle regulation, and autophagy, it is important to evaluate the anticancer efficacy of survivin antagonism against the potential dysfunction of other cell regulating mechanisms that may provoke deleterious side effects. Overall, our results validated the in-depth investigation for this novel survivin inhibitor scaffold, and additional biochemical and mechanistic characterization is warranted in the future.

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APPENDIX. DOSE RESPONSE CURVES FROM NCI-60 CELL LINES

Figure A-1. Dose-response curves of DJ101 against the NCI-60 cell line panel. Percent growth of NCI-60 cell lines treated with DJ101 in 5- dose assay as determined by the National Cancer Institute Developmental Therapeutics Program (NCI-DTP).

Figure A-2. Dose-response curves of DJ95 against the NCI-60 cell line panel. Percent growth of NCI-60 cell lines treated with DJ95 in one-dose (top) and 5-dose (bottom) assays as determined by the NCI-DTP.

VITA

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