

Small-molecule inhibitors of the cell cycle: an overview

Craig M. Crews^{1*}, and J. Brad Shotwell²

¹Department of Molecular, Cellular, and Developmental Biology,
Department of Chemistry, Department of Pharmacology,

²Department of Chemistry,
Department of Molecular, Cellular, and Developmental Biology,
Yale University, New Haven, CT 06520, USA.

* To whom correspondence should be addressed

Potent and selective small-molecule mediated inhibition of the cell's replication machinery remains a principal aim in the development of novel therapeutics and biological probes. Recent efforts have identified small molecules capable of arresting the cell cycle via specific interaction with a variety of intracellular protein targets. Advances in combinatorial and diversity oriented synthetic methods, coupled with a continued effort to identify sources of bioactive natural products, promise to contribute to the growing library of small-molecule inhibitors of the cell cycle.

INTRODUCTION

Dividing cells are regularly faced with the prodigious task of accurate and swift replication of the complete genome. Errors made during this process can be devastating for generations of daughter cells to come, and hence the cell cycle is under strict regulatory control. The myriad of proteins necessary for the seamless duplication of a cell's genetic material represent vulnerable targets for small molecules. Bioactive small molecule inhibitors of the cell's replication machinery are currently being exploited as cellular probes and novel therapeutics.

The cell cycle continuum is divided into four distinct phases (G_1 , S, G_2 , M), each of which is regulated by a specific class of enzymes and regulatory proteins. A number of "checkpoints" monitor factors including environmental clues and genomic integrity and control progression to the next phase of the cell cycle. Once triggered to enter the cell cycle, G_1 -specific genes for cell cycle progression are transcribed and translated in preparation for the period of DNA synthesis. Upon duplication of the entire genome in S phase, the cell enters G_2 . The cell next divides its chromosomes between the two daughter cells, as chromosome segregation and cellular division occur in the short span of the M phase.

INHIBITORS OF ESSENTIAL PROTEIN KINASES

Small Molecule Inhibitors of the Cyclin-Dependent Kinases (CDK).

No group of regulatory proteins is as intimately coupled to cell cycle progression as the CDKs (1-8). Catalytically inactive CDKs require association with their regulatory partners, the cyclins, for activation. Different CDKs are active periodically throughout the cell cycle and are responsible for driving the cell from one phase to the next. CDK activity is tightly controlled *via*: (1) association with cyclins, (2) synthesis and proteolysis of the CDKs themselves (3) posttranslational modification, and (4) interaction with a num-

ber of natural kinase inhibitors (CDIs). (9) At different points throughout the cell cycle, different cyclin proteins are rapidly degraded, resulting in a loss of activity for their CDK partners. This loss of CDK activity, in turn, allows transit from one phase of the cell cycle to the next. CDKs are targets of checkpoints that control entry into the next phase of the cell cycle. In addition, a number of external stresses can lead to CDI expression and to subsequent cell cycle arrest. The three major CDI families include p21^{CIP/WAF}, p27^{KIP}, and p16^{INK4a} (10-12).

With nearly 850 active kinases in the human body, all sharing a substantial degree of active-site structural homology, the development of small-molecule, ATP-competitive inhibitors of the various CDKs is a daunting task (3). Although the fine-tuning necessary to generate truly specific control over the cell cycle *via* selective inhibition of various essential protein kinases has not yet been realized, a growing library of structure/activity data coupled with x-ray crystallographic analysis of small molecules bound to CDK targets promises to hasten efforts toward rational design of specific CDK inhibitors. Crystallographic structures for staurosporine, olomoucine, flavopiridol, roscovitine, purvalanol A, and indirubin-3'-monoxime bound to their CDK effectors show that all fill the ATP binding site with similar key hydrogen-bonding interactions. However additional contacts are made outside of this binding pocket, where various protein kinases show a lesser degree of homology (*vide infra*) (13-19).

Purine analogs including olomoucine, roscovitine, and the purvalanols represent a group of relatively specific CDK inhibitors that have proven useful as probes for basic cell biology and as lead compounds for antiproliferative therapeutics. Olomoucine arose from a purine screen originating from the modest CDK1 inhibitory profile of 6-dimethylaminopurine (5). It represents a relatively selective kinase inhibitor, potently inhibiting CDK1, 2, and 5 but relatively inert towards CDK4 and 6. These properties manifest them-

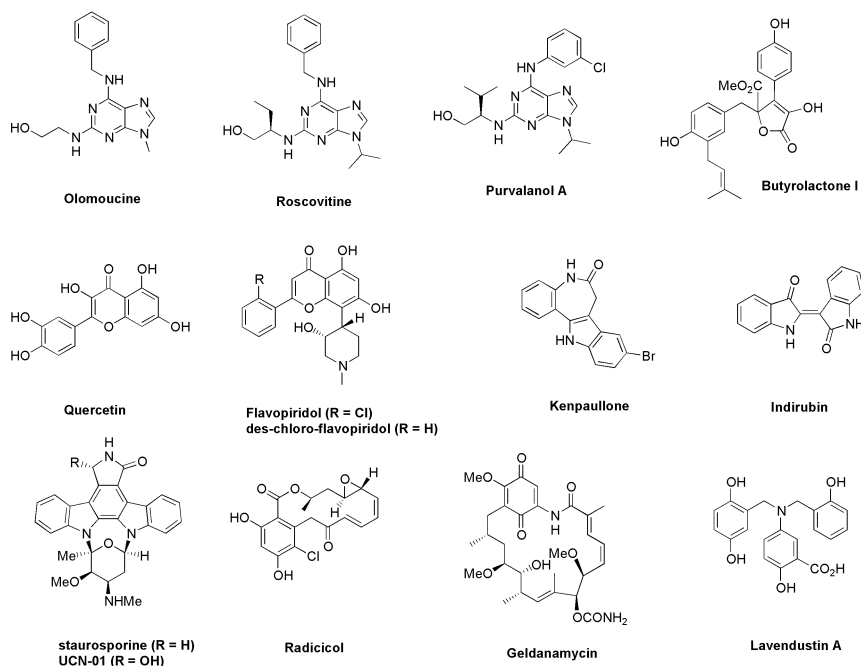


Figure 1. Inhibitors of Protein Kinases.

selves in dose-dependent inhibition of the G_1/S and $G_2/M/G_1$ transitions, arresting a variety of cell types in the G_1 and G_2 phases (17, 20). Although olomoucine binds to the ATP binding pocket of its various CDK targets, its N6 substituent makes contact outside the conserved ATP binding region, likely accounting for its selectivity (15, 21).

The development of CDK inhibitors based on the flavanoid core arose from the observation that the naturally occurring flavanoid quercetin shows a wide range of antiproliferative effects (3). Flavopiridol arose from the exploration of the chemical space surrounding the flavone core and has been the focus of extensive clinical studies (22, 23). In general, flavopiridol causes G_1 or G_2 arrest *via* the collective inhibition of CDK4, CDK2, CDK1, and PKC (23-25). Crystallographic analysis of des-chloro-flavopiridol reveals the phenyl moiety makes contact outside the ATP binding pocket, potentially accounting for its modest selectivity.

The National Cancer Institute's computer-based algorithm COMPARE was employed to predict molecules which would possess flavopiridol-like antiproliferative properties. This analysis identified a novel class of inhibitors, the paullones (26). Kenpaullone inhibits CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, and CDK5/p25 (IC_{50} 's in 0.5-10 μ M range) while remaining relatively inert toward CDK4/cyclin D activity. This kinase inhibitory profile ultimately leads to arrest in G_1 . Modeling studies pre-

dict that kenpaullone also occupies the ATP binding site, but makes contacts with residues outside the pocket as well. The same work suggests that kenpaullone may bind with an orientation unlike its purine and flavone counterparts, leaving active-site contacts vacant, which could be utilized to introduce further specific interactions. Kenpaullone could serve as a lead in the development of more specific CDK inhibitors, which take advantage of unused active-site chemical space.

The indigo isomer indirubin, one of the major active agents of Danggui Longhui Wan (a complex mixture of herbs used to treat myelocytic leukemia), manifests some of its biological properties through ATP-competitive inhibition of cyclin dependent kinases. Indirubin inhibits all CDKs with roughly equivalent potencies (IC_{50} 's = 10 μ M) leading to cell cycle arrest in late G_1 and/or G_2/M . X-ray crystallographic analysis of indirubin-5-sulfonate with CDK2 has revealed that its binding to the ATP-active site is analogous to that of roscovitine and olomoucine (19).

Butyrolactone was identified from a screen for inhibitors of CDC2/cyclin B (27, 28). It induces G_2/M arrest by blocking histone phosphorylation in a concentration dependent manner *via* the inhibition of CDK2 and CDK1. Interestingly, butyrolactone is relatively inert towards CDK4, MAPK, PKC, and protein tyrosine kinases, although it too binds competitively with the ATP binding site. For a detailed review of CDK inhibitors, see chapter 24.

Inhibitors of Protein Kinase C.

Protein Kinase C (PKC) plays an important role in cell proliferation and differentiation, albeit well upstream of the level of CDKs, *via* its role in the kinase cascade initiated by hormones at various cell surface receptors. Staurosporine is a relatively non-specific natural product PKC inhibitor, which also shows inhibition of CDK2/cyclin A and CDK4/cyclin D (27-31). Its crystal structure complexed with CDK2 shows key hydrogen bonds between the aglycone and the ATP site analogous to those made by ATP itself. Staurosporine has been observed to arrest normal cells in G₁ while being relatively ineffective at halting tumor cell progression through the cell cycle (32, 33), suggesting it could be useful in protecting normal cells from drugs eliciting cytotoxic effects on proliferating cells (34, 35). UCN-01, a naturally occurring staurosporine congener, has also been observed to arrest cells at the G₁/S transition *via* the nonselective inhibition of PKC, CDK2, CDK1, and Raf-1 kinase (36). The non-specificity of this class coupled with the distance between PKC and cell cycle regulatory proteins has led to a relatively poorly understood mode of action for these molecules.

Inhibitors of Protein Tyrosine Kinases.

The realization that plasma-membrane bound protein tyrosine kinases are constitutively active in many cancers has generated considerable interest in the identification of small molecule inhibitors of this class of kinases. Many cyclins are under growth factor control (PDGF, EGF, NFG) during some stage of the cell cycle. For example, cyclin D is translated in response to PDGF or EGF signaling *via* the MAP-kinase cascade during early G₁, its upregulation being the key factor in CDK1/CDK2 activation by CDK4 (37). Specific inhibition of protein tyrosine kinases could offer a novel inroad to the arrest of cellular proliferation in cells undergoing overstimulation by growth hormones or malfunctioning G-protein receptors.

A number of small molecules exhibiting potent inhibition of tyrosine kinases have also been observed to arrest cells in various stages of the cell cycle. Lavendustin A has been identified as a potent inhibitor of several protein tyrosine kinases including epidermal growth factor receptor (EGFR) PTK and the non-receptor-PTK, Syk (38, 39). However, the link between lavendustin's antiproliferative properties and its PTK inhibitory profile is questionable. Lavendustin A has also been shown to be a moderately effective inhibitor of tubulin polymerization, and consequently may be acting as a cytotoxic agent *via* this mechanism.

Radicicol is a topologically intriguing macrocycle which exhibits a wide range of biological properties including antifungal, antibiotic, and anti-angiogenic activities (40-43). While being relatively inactive against a wide range of Ser/Thr kinases (PKC, PKA) of the mitogen activated pathway, radicol remains a potent inhibitor of the receptor tyrosine kinase p60^{v-src}, subsequently arresting cells in G₁ and G₂. Radicol appears to share a molecular mode of action with the

ansamycin class of antibiotics (including geldanamycin). Ansamycin was also originally thought to be a direct tyrosine kinase inhibitor, but ultimately it was found to mitigate its activity *via* the HSP90 molecular chaperone (44-47). Radicol, too, appears to owe some of its anti-proliferative properties to a disruption of HSP90 mediated protein kinase activation. Many aspects of the molecular mode of action of radicol still remain poorly understood. The use of numerous biotinylated radicol analogs has served to confirm HSP90 as an intracellular target, but also implicate ATP citrate lyase (ACL) as a possible secondary effector (48). Through competition studies of various analogs it has been demonstrated that a unique portion of the radicol macrocycle binds each intracellular target (*i.e.* the α , β -enone appears to be required for ACL binding whereas the unsubstituted phenolic moiety appears to be more important to its HSP binding profile). Due to radicol's multitude of biological properties and several apparent intracellular targets, the elucidation of details relevant to the mechanism of radicol's biological profile still remains an active area of study.

INHIBITORS OF PROTEIN SYNTHESIS AND DEGRADATION

Inhibitors of the 26S Proteasome.

Normal cell proliferation involves a continued cycling of proteins. Although lysosomal degradation was once believed to be the principal role by which all unwanted or misfolded proteins were degraded, the proteasome is now recognized as the undisputed master regulator of intracellular proteolysis. Comprising up to 1% of intracellular protein, the proteasome is a 700 kDa, cylindrical-shaped, multicatalytic protease complex composed of 28 subunits organized into four rings. The protease activities of the proteasome are generally divided into 3 major classes (trypsin-like activity, chymotrypsin-like activity, and peptidyl-glutamyl peptide hydrolyzing (PGPH) activity or caspase-like), and appear to function independently, reducing most proteins to 6-8 amino acid peptides. Proteins are targeted for proteolysis *via* conjugation to the 76 amino acid polypeptide ubiquitin (Ub) *via* a multi-step process involving a series of Ub ligases which: activate the Ub monomer (E1), recognize the protein targeted for degradation (E3), and transfer multiple Ub monomers to lysine residues on the targeted protein (E2). Interest in proteasomal function and inhibition has grown as the proteasome has been implicated as a key player in a number of important cellular processes including apoptosis, cell differentiation, MHC class I antigen presentation, NF- κ B activation, tumor suppression, and cell division (49-52).

The proteasome plays key positive and negative roles in normal cell proliferation. Although CDK levels are maintained relatively constant throughout the cell cycle, cyclin and CDI abundance is intimately coupled to ubiquitination and proteolysis. The list of proteins essential for normal cell cycle progression which are regulated by the proteasome is extensive. Proteasome mediated degradation of p27^{KIP1} and cyclin A is essen-

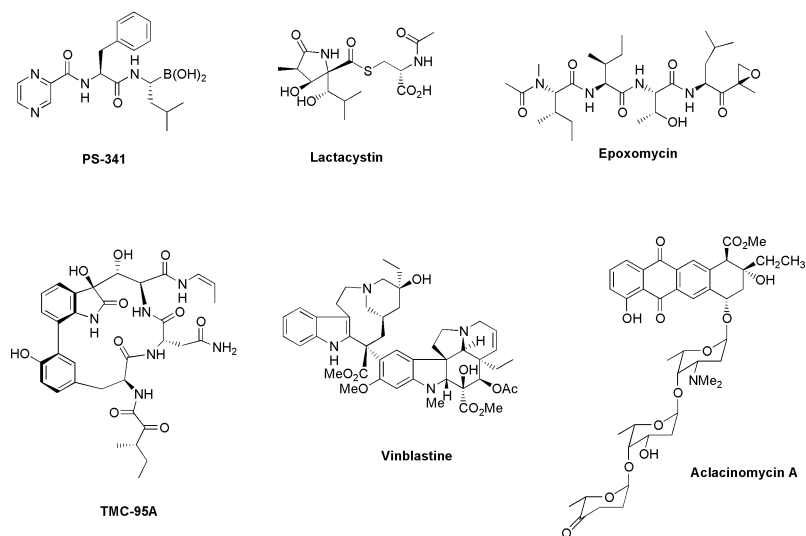


Figure 2. Inhibitors of protein degradation.

tial for progression through the G_1/S checkpoint, and increased p27^{KIP1} degradation has been associated with uncontrolled growth (53-57). The potent CDI p21^{CDP1}, which is translationally upregulated during G_1 and ultimately responsible for G_1 arrest, is also ubiquitinated and degraded prior to entry into the S phase (58). The M/G_1 interface is characterized by recognition of cyclin B by a specific E3 ligase, which mediates its proteasomal down-regulation prior to entry into interphase. Cyclins E and D are also ubiquitinated and degraded either free or bound to their respective CDKs. With such an impressive and therapeutically relevant target profile, the proteasome itself is increasingly becoming the focus of a number of classes of specific inhibitors including: peptide aldehydes, peptide vinyl sulfones, peptide boronic acids, glyoxals, and α,β -epoxyketone containing peptides.

Lactacystin, originally characterized as a microbial metabolite that induced neural outgrowth in neuroblastoma cells (59, 60) was later found to block cell proliferation (61). Using a [³H] lactacystin analog, Schreiber and coworkers demonstrated that lactacystin and its related *clasto*- β -lactone covalently bind to the N-terminal threonine of the 20S proteasome subunit X/MB1 (59, 61, 62). Like the peptide aldehydes and vinyl sulfones, lactacystin is a relatively nonspecific protease inhibitor, also showing significant inhibition of peptidyl peptidase II and cathepsin A (60, 63-65).

Recently, more selective inhibitors of the proteasome have been developed. Peptide boronic acids, like PS-341, were first recognized as excellent serine protease inhibitors (64, 65). Later, PS-341 was identified as a potent and specific inhibitor of the proteasome (0.62 nM) as compared to a variety of other proteases (cathepsin G, 630 nM, thrombin, 13,000 nM, chymotrypsin, 320 nM). PS-341's potency toward serine

proteases has been rationalized in terms of the excellent electronic overlap of the active-site serine oxygen lone pairs with the vacant *p*-orbital present on the boronic acid moiety. It is likely that an analogous situation exists with the essential N-terminal threonine present in the 26S proteasome. Further, this accounts for the relatively modest inhibition of cysteine proteases by PS-341 (*i.e.* orbital size differences between sulfur and boron would make overlap much less favored) (66, 67).

Epoxomicin, a member of the α,β -epoxy ketone class of proteasome inhibitors, was isolated from an actinomycete strain and found to exhibit *in vivo* anti-tumor activity against B16 melanoma (68). Early structure activity studies suggested covalent modification of its cellular effector, as the epoxide moiety was found to be essential for the observed activity (69). The design and synthesis of a biotinylated analog hastened the identification of the epoxomicin cellular effectors, implicating the proteasomal subunits Z, MECL1, LMP7, and X (52). The extremely high proteasomal specificity of this molecule (epoxomicin has no observable inhibitory profile against papain, chymotrypsin, trypsin, cathepsin B, or calpain) has been further optimized and investigated using combinatorial methods (70). X-ray crystallographic analysis has demonstrated that the covalent adduct between epoxomicin and the 20S proteasome manifests itself in the form of morpholino ring formation between the amino terminal threonine and the epoxomicin pharmacophore. Thus, apparently epoxomicin, like PS-341, draws its specificity from the uniqueness of the proteasomal N-terminal threonine, as common proteases lacking an N-terminal nucleophilic residue cannot form stable covalent morpholino adducts with epoxomicin (71, 72).

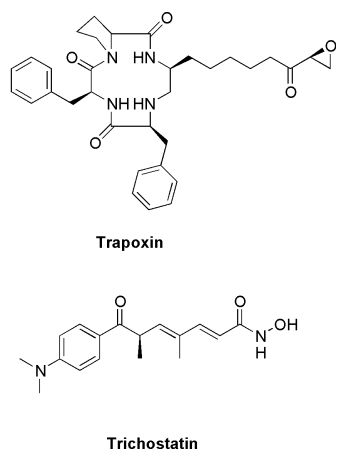


Figure 3. Inhibitors of Histone Deacetylase Activity.

A topologically striking atropo-isomeric macrocyclic structure coupled with potent and reversible inhibition of the chymotrypsin-like (5.4 nM), trypsin-like (200 nM), and PGPH (60 nM) activities of the 20S proteasome have led to a great deal of current biological and synthetic interest in TMC-95A (71-76). Crystallographic work shows that TMC-95A is not covalently bound when complexed with the yeast proteasome core particle. Furthermore, TMC-95A shows no inhibition of calpain, cathepsin, or trypsin. TMC-95A could foster a new era of designed inhibitors of the proteasome, affording exquisite control over the cell cycle.

Interestingly, a group of previously well-investigated anti-mitotic agents thought to elicit their cellular responses solely *via* interaction with cytoskeletal processes have subsequently been shown to be modest inhibitors of proteasomal function. The anti-mitotic agent aclacinomycin A is the first described non-peptide inhibitor of the proteasome, showing discrete selectivity for the chymotrypsin-like activity of the 20S proteasome (77, 78). Vinblastine, well-characterized for its ability to disrupt microtubule stabilization (79-81) has also been implicated as operating *via* the proteasome, showing reversible inhibition of the chymotrypsin-like, trypsin-like, and PGPH activities of the 20S proteasome (82). The implications of these observations have not yet been fully realized, but will likely serve to further demonstrate the significance of the proteasome in normal cell cycle progression. For a detailed review of proteasome inhibitors, see chapter 44.

Inhibitors of Histone Deacetylase Activity.

Methylation, acetylation, phosphorylation, or ubiquitination of histones induce conformational changes in the associated DNA segment which serve to modulate transcription factor recognition of various DNA promoter sequences. Underacetylated portions of the genome are generally transcriptionally underutilized, suggesting the covalent modification of histones is a means by which protein transcription is reg-

ulated. Trichostatin, a small molecule inhibitor of histone deacetylase, arrests cells in G₁ and G₂ and leads to increased cellular levels of cyclin A and p21^{CIP/Waf} (83). The small molecule trapoxin is also a potent inhibitor of histone deacetylase. [³H] trapoxin has been shown to co-purify with histone deacetylase activity and titrate competitively with trichostatin, suggesting these molecules share a molecular mode of action. A chemical genetic approach toward molecular target identification using trapoxin-derived molecular probes implicate RbAp48 as the intracellular target, a 50kDa protein known to bind to pRb (84). Histone deacetylase inhibition is currently considered a potential anticancer therapeutic strategy (85), apparently operating *via* the upregulation of p21 and/or interference with some cell-cycle checkpoint relating to acetylation of histones surrounding important cell cycle genes (84). For a detailed review, see chapter 27.

MOLECULES WHICH INTERFERE WITH CYTOSKELETAL PROCESSES

Microtubules, which show great instability/activity during mitosis, are the target of a class of small molecules which arrest cells in the M phase. The elongation and contraction of microtubules searching for kinetochores typifies mitotic behavior. Either the stabilization or destabilization of microtubules at this critical stage can serve to hasten cell cycle arrest. The classic microtubule binding molecule colchicine, which led to the discovery of tubulin itself, depolymerizes cytosolic microtubules at higher concentrations, while at lower concentrations it inhibits such depolymerization (both arrest the cell cycle). Amazingly, epothilone A/B, discodermolide, and taxol all compete for binding of [³H] colchicine to its microtubule target, and induce cell cycle arrest *via* the stabilization of tubulin in an apparently analogous manner. Nuclear magnetic resonance conformation work has suggested that these seemingly topologically distinct molecules may share a common pharmacophore, and synthetic analogs incorporating salient features of the class collectively also show similar biological properties (86). For a detailed review of microtubules inhibitors, see chapter 32.

A number of small molecules mitigate cell cycle arrest *via* interaction with actin. For detailed review see chapter 52. Latrunculin A/B, jasplakinolide, phalloidin, and mycalolide B are representative of unique classes of natural products which bind G-actin and subsequently depolymerize actin filaments (87, 88). The dolastatins are an emerging class of actin-binding molecules which arrest cells in G₂/M *via* a major reorganization of the actin filament network. Early work with this class indicated that the dolastatins do not compete with vinblastine for binding to unpolymerized tubulin, and dolastatin treated cells appear phenotypically very different to those treated with jasplakinolide (87-89). Recent studies demonstrate dolastatin 11 does not compete for actin binding with fluorescent phalloidin, suggesting that the growing number of natural dolastatin analogs may function *via* a mechanism unique among actin-binding small molecules (90, 91).

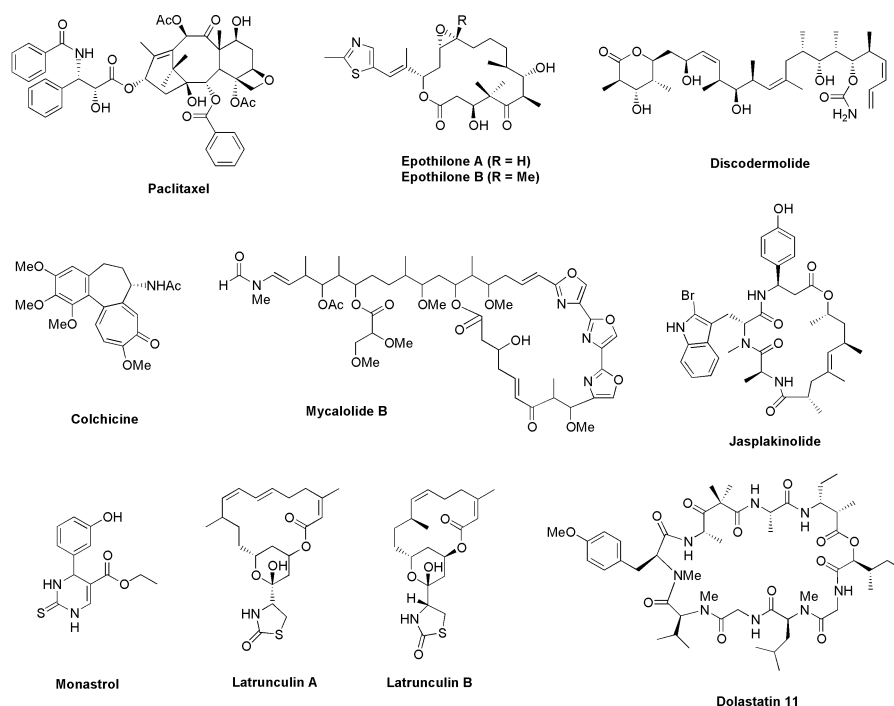


Figure 4. Molecules which interfere with cytoskeletal processes.

Direct binding to actin and tubulin does not represent exclusive means of disrupting the cytoskeletal processes necessary for mitotic progression. Monastrol was identified from a chemical genetic screening strategy directed at the identification of anti-mitotic small molecules with unique binding proteins (92). Named for the phenotypic mono-astral microtubule array and surrounding chromosomes observed during M phase arrest, monastrol has been found to bind specifically to the mitotic kinesin Eg5 (implicated in spindle bipolarity). The usefulness of monastrol as a molecule probe is noteworthy as it apparently has a high specificity (being relatively inert toward other conventional kinesins) and has no effect on kinesin localization. These properties make it unique among antiproliferative agents acting *via* the inhibition of cytoskeletal processes (93, 94).

INHIBITORS OF THE ATPASE PUMPS

Small molecules, which trigger a programmed exit from the cell cycle *via* apoptosis, have immense clinical potential in the treatment of cancers. Recently, a growing class of macrolide antibiotics, known to be inhibitors of the F_0F_1 ATPase or V-ATPase pumps, have shown exquisite cytotoxic selectivity against a variety of cancer cell lines. The recently isolated apoptolidin (95) was found to induce apoptosis in cells transformed with the oncogene E1A while exhibiting no effect on normal cells. A combination of pharmacological assays and structural analogies has recently demonstrated that apoptolidin shares a F_0F_1 ATPase

inhibitory profile with oligomycin, cytovaricin, and ossamycin. While the precise mechanism for the induction of apoptosis by these molecules remains elusive, the current model recognizes some interplay between the mitochondrial apoptotic machinery and a shift from aerobic to anaerobic carbon metabolism. Tumor cells exhibiting anaerobic carbon metabolism in the presence of oxygen are generally resistant to apoptolidin induced apoptosis, but have been found to be sensitized by co-treatment with the small molecules oxamate and 2-deoxyglucose, both of which increase cellular dependence on mitochondrial derived aerobic energy (95-98).

Vacuolar ATPases are present in a number of intracellular compartments including lysosomes, endosomes, and the Golgi. Although the macrolide natural product bafilomycin shows some structural homology to apoptolidin and cytovaricin, it has a distinct selectivity for the inhibition of V-ATPase pumps in addition to inhibiting acidification and protein degradation in the lysosomes. Bafilomycin provokes apoptosis at nanomolar concentrations in a variety of cell types, in addition to eliciting a response in several oligomycin-resistant cell lines (WEHI-231, B lymphoma line) (99). Further study of this macrolide class of selectively cytotoxic agents shows promise in the development of new therapies and of a better understanding of the role of apoptosis in tumor proliferation.

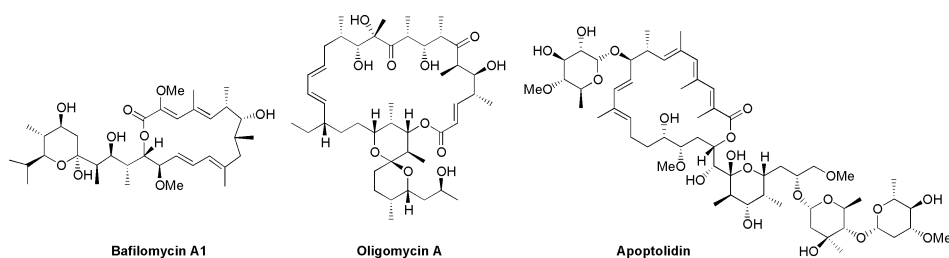


Figure 5. Molecules Which Inhibit ATPase Pumps

CONCLUSIONS

Advances in combinatorial and diversity oriented synthetic methods, the continued search for biologically relevant natural products, and the development of chemical genetics approaches to small molecule target identification assures that potent and specific inhibitors of the cell cycle will continue to be developed. Small molecules have played an essential role in our emerging understanding of the cell cycle, and promise to maintain an unparalleled importance in the development of new therapeutic treatments and further biological discovery.

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