Current Drug Targets, 2006, *7*, 305-326 305

Antimitotic Agents of Natural Origin

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Abstract: Antimitotic agents have been the most successful pharmacological agents for the treatment of cancer. The term "antimitotic agent" has traditionally been synonymous with tubulin-targeting compounds, but as a consequence of the large number of new compounds and mechanisms that have been identified recently, a much broader definition is currently needed. This review attempts to provide a broad overview of compounds and their cognate protein targets which result in a block in mitosis. Focus has been placed on agents that act directly on the mitotic machinery rather than on targets further upstream such as growth factor receptors.

INTRODUCTION

Nature has proven itself to be the richest source of biologically active compounds as millions of years of evolution have resulted in selection compounds with greatest propensity to modulate biological targets [1, 2]. Natural products have been the source of inspiration and challenge to chemists who attempt to create new compounds that mimic and alter their biological activities. With the art of isolation, structure elucidation and synthesis of natural products becoming increasingly routine, chemists can use natural products and their analogs to create new pharmacological agents. Despite major scientific and technological progress in combinatorial chemistry and structure-based design, natural products remain an important source of instruction on how to create compounds that can modulate new biological targets.

Mitosis is a controlled series of events in which identical copies of the genome are moved to the two poles of a mitotic spindle that eventually become nuclei of the resulting daughter cells. Since uncontrolled and rapid cell division is a hallmark of cancer, understanding the molecular mechanisms underlying mitosis is key to understanding how various natural product antimitotic agents function. Whereas the vinca alkaloids were originally identified as compounds that arrested cells in mitosis with aberrant mitotic spindles [3], compounds such as colchicine had been known to possess antimitotic activity for decades [4]. In the 1960s, vincristine and vinblastine were introduced as cancer drugs and display remarkable efficacy in the treatment of testicular cancer, Hodgkin's lymphoma and acute lymphocytic leukemia. The importance of natural products as a source of drugs was highlighted by discovery of taxol, a tubulin "stabilizing" drug. Paclitaxel was originally isolated from the bark of the Pacific yew tree *Taxus brevifolia* [5]. Even with a promising clinical profile, further progress was delayed because of its limited supply from natural sources. This problem was solved by development of a semi-synthetic analog (docetaxel) which is derived from an abundant source, the leaves of *Taxus baccata* [6].

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Despite the success of the tubulin-targeting agents, the therapeutic potential of this class of compound is limited by the fact that many rapidly dividing cell types are required for normal physiological function. In normal cells, microtubules are responsible for a variety of functions like cell shape, cell motility, signal transduction, intracellular transport and muscle contractions. Many tubulin-targetting drugs are known to cause neurotoxicity through their ability to interfere with the function of axonal microtubules that mediate neuronal vesicle transport. Another drawback of tubulin-targeting drugs is their lack in efficacy in many settings. For example, although paclitaxel is one of the most broadly active anti-tumor agents, the majority of patients with advanced disease do not enter long-term remission. The resistance to the drugs is developed because of drug efflux pumps and alterations in tubulin functions. The vinca alkaloids and taxanes are both good substrates for the 170 kDa P-glycoprotein (Pgp) efflux pump encoded by the multidrug resistance *mdr1* gene [7, 8]. This review attempts to cover the most promising antimitotic molecular targets along with the prominent inhibitors in each class.

CLASSIFICATION

The tubulin-interacting class of antimitotic agents can be classified by their influence on the tubulin polymerization and by their binding site on tubulin. One class of compounds promotes microtubule polymerization and stabilizes microtubules (e.g. taxoids, epothilones, and discodermolide). Another class of compounds acts on microtubules by inhibiting polymerization (e.g. vinca alkaloids, colchicines, cryptophycins, halichondrins, and estramustine). Microtubule disruptors are further sub-divided on the basis of their binding site on tubulin (Colchicine binding site and Vinca binding site). Non-tubulin targeting agents can be divided into many subclasses with many more being identified with powerful new techniques such as RNAi knockdown.

Microtubules are long cylindrical polymers composed of tubulin monomers (and tubulin) that display highly dynamic properties. Many important cellular events such as progression of cell cycle, intracellular material transport and cellular motion are associated with microtubule dynamics. Therefore, the dynamic behaviors of microtubules are tightly controlled by their associated molecules inside cells.

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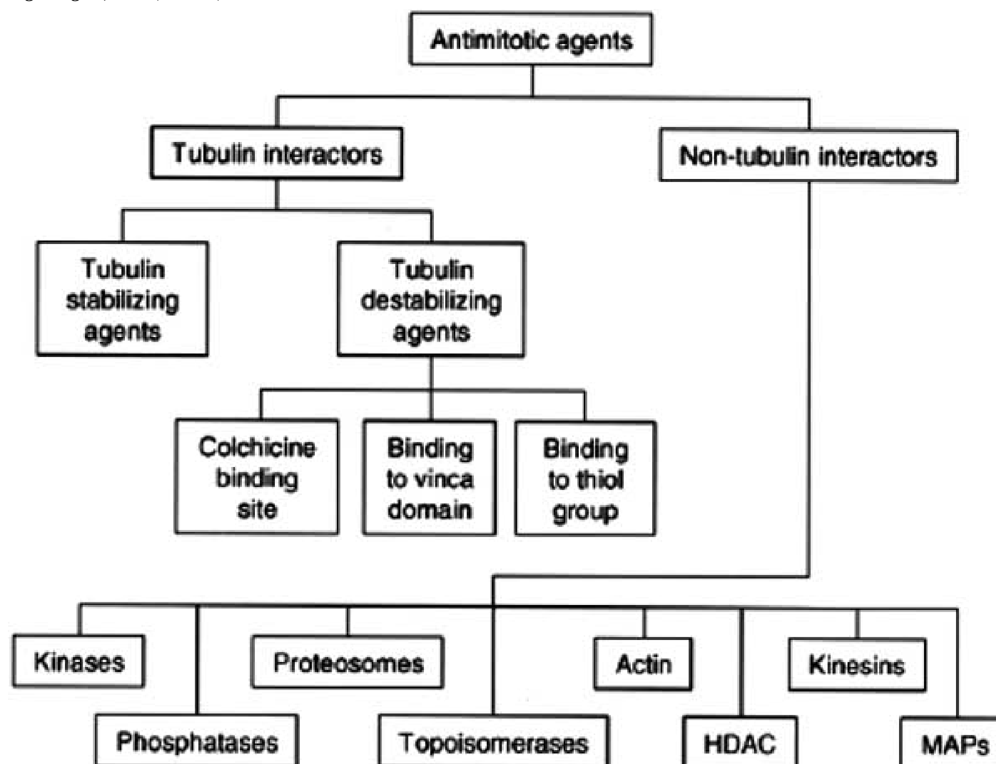


Fig. (1). Classification of antimitotic agents.

MICROTUBULE STABILIZING DRUGS

Paclitaxel and Docetaxel

Paclitaxel (Taxol^{TM}) (**1**), isolated from the stem bark of the Pacific yew tree *Taxus brevifolia* [5], possesses an unusual diterpenoid ring structure called a taxane. It was found to have potent antimitotic and antileukemic activities but its therapeutic potential was limited by poor solubility and low natural availability. Paclitaxel blocks mitosis by stabilizing tubulin polymers and thereby inhibiting disassembly of microtubules [9]. The efficacy and therapeutic window obtained using Paclitaxel has made it an attractive alternative to radiotherapy and surgery for the treatment of a variety of cancers. In fact, since receiving FDA approval for the treatment of ovarian (1989) and breast cancers (1992), Paclitaxel has been ranked the best-selling anticancer drug [10].

Docetaxel (Taxotere^{TM}) (**2**) was discovered as a semi-synthetic analog of paclitaxel [11], and showed similar *in vitro* activity in cellular growth and tubulin polymerization assays. Docetaxel has a number of properties that may make it a superior therapeutic relative to Paclitaxel: it is 5 times more potent as a cytotoxic agent on taxol-resistant cells [12], it exhibits a longer retention time in cancer cells [13], and it exhibits better pharmacokinetic behavior. Docetaxel is in clinical phase testing in patients with a variety of cancers including breast, prostate, ovarian, non-small cell lung cancers.

Epothilones

Epothilones are a family of 16-membered macrolides isolated from soil bacteria. Two important prototypes, Epothilone A (**3**) and B (**4**), were isolated from myxobacterium *Sorangium cellulosum* [14] and exhibit both antifungal and cytotoxic activities. Like paclitaxel, epothilones were shown to stabilize tubulin polymers leading to mitotic arrest and cell death [15]. Epothilone A and B bind competitively with paclitaxel to tubulin suggesting that they share a common binding site. Epothilones exhibit potent cytotoxic effects against numerous cancer cell lines including both P-glycoprotein (pgp) expressing multiple drug resistant (MDR) cells and paclitaxel-resistant cells with mutations in -tubulin [16-19]. Their potent cytotoxicity, higher water-solubility than paclitaxel [20] and almost unlimited availability by bacterial fermentation prompted intense interest in epothilones. It has also been shown that epothilone-treated cells do not develop drug-resistance even after long-term treatment [21]. Phase II trials of epothilone B (EPO-906^{TM}) have been completed for colorectal, ovarian, and kidney cancers.

BMS-247550 (Ixabepilone^{TM}) (**5**), a lactam analog of epothilone B, emerged as an alternative to epothilone A and B which both exhibit poor metabolic stability and pharmacokinetics. BMS-247550 induces tubulin polymerization *in vitro* with activity equipotent to that of paclitaxel and epothilone B. Like epothilone B, BMS-247550 retains its antineoplastic activity in cancer cells that are resistant to paclitaxel. Clinical phase II trials reported that BMS-247550 is active in breast, gastric, lung, and prostate cancers [22].

Epothilone D (**6**, deoxyepothilone B, Kosan-862^{TM}) is a naturally occurring analog of epothilone B, with an internal olefin replacing the epoxide ring. Cellular studies of various epothilone analogues showed that cells are least likely to become resistant to epothilone D. Epothilone D inhibits the proliferation of malignant glioma cell lines at low nanomolar

levels and leads to tubulin damage [23]. Epothilone D is efficacious in human colon carcinoma HCT-116 and human leukemia K562 xenografts in which paclitaxel shows marginal activity [24]. Phase II trials of epothilone D were initiated in 2003 for colorectal, non-small cell lung cancer and metastatic breast cancer.

(+)-Discodermolide

Discodermolide (**7**), a polyhydroxylated lactone structure, was isolated from the marine sponge *Discodermia dissoluta* [25], and originally found to have immunosuppressive activity. Later it was discovered that discodermolide is microtubule-stabilizing agent that competes for the binding of paclitaxel to the tubulin polymer. Discodermolide induces microtubule rearrangement in breast carcinoma cells at 10 nM (100 times greater potency than paclitaxel) and induces assembly of purified tubulin *in vitro* with an of EC_{50} of 2.3 μM (paclitaxel EC_{50} = 32 μM) [26]. It also potently inhibits the growth of paclitaxel-resistant, multidrug-resistant carcinoma cell lines [27]. (+)-Discodermolide is now in Phase I clinical trials as a potential drug for solid tumors [28].

TUBULIN DESTABILIZING AGENTS

Another major class of tubulin interacting agents are the inhibitors of tubulin polymerization. Although this general class of compounds has similar mechanism of action, they do not all bind to the same site on tubulin.

COLCHICINE BINDING SITE

Colchicine

Colchicine (**8**) is an alkaloid obtained from plant species "*Colchicium autumnale*". It was first isolated as the active ingredient of *Colchicum* in 1820, but its antimitotic activity was linked to tubulin interaction more than a century later. Today colchicine is used for the treatment of a variety of ailments such as gout, Mediterranean fever, and liver cirrhosis [29]. Colchicine and its analogs have been extensively studied for cancer therapy. Unfortunately, it lacks anticancer efficacy *in vivo* at its maximum tolerated dose (MTD). Even the more potent analogs of colchicine such as thiocolchicine (**9**) retains the toxicity associated with colchicine [30]. Despite lack of clinical success, colchicine has been extensively studied for its mode of action.

Colchicine binds to soluble tubulin leading to the formation of a tubulin-colchicine complex. These complexes then undergo co-polymerization into microtubule ends with the majority of the tubulin molecules being unaffected. The microtubule ends have the ability to polymerize further, but tubulin-colchicine complexes induce a conformation which slows new tubulin addition, probably by adopting a conformation which weakens the lateral bonds at the microtubule end. This process retards and eventually causes the microtubule spindle to disassemble because of the structural instability during the metaphase of mitosis [31].

Combrestatin A4

The combrestatins are a class of antimitotic agents which are isolated from the bark of the willow tree *Combretum caffrum.* Combrestatin A4 (**10**), the most potent and simplest compound of this class, is known to bind to the colchicine site and displays an IC_{50} of 0.53 μM-1.2 μM in tubulin polymerization assays. Several prodrugs were investigated to improve the solubility and pharmacokinetics of combrestatin A4 which led to the development of disodium phosphate analog CA-4P (**11**). CA-4P is currently in phase I/II clinical trials for the treatment of solid tumors including breast, lungs, colon and liver tumors.

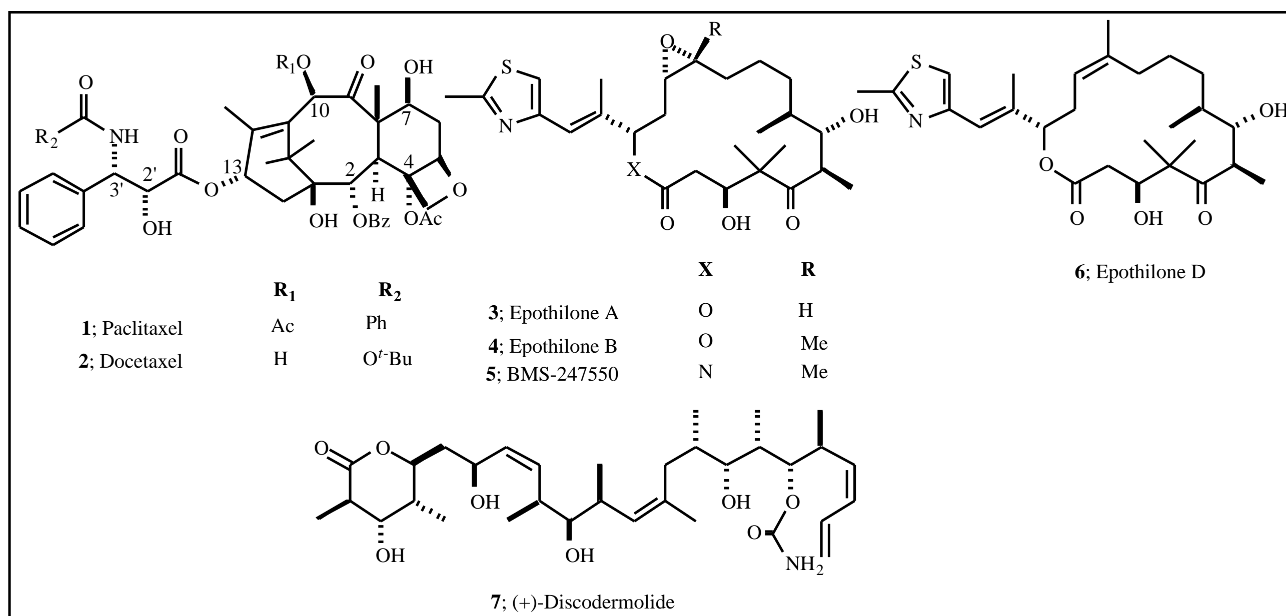


Fig. (2). Tubulin stabilizing agents.

The excellent *in vitro* potency and simple structure of combrestatin has stimulated the synthesis of a large number of analogs. Similar to colchicine, the 3,4,5-trimethoxyphenyl group is essential for its antimitotic activity. The *cis*-double bond serves as a linker and brings the trimethoxyphenyl (A ring) and the other aryl group (B ring) together in a way that mimics the colchicine pharmacophore. It is therefore not surprising that both the *trans* and alkyne analog of CA-4 are very weak inhibitors of tubulin polymerization [32-35].

Podophyllotoxin

Podophyllotoxin (**12**) is a lignan that was isolated from the roots of *Podophyllum peltphylum*. Apart from its known antimitotic properties, it is also used for the treatment of liver sclerosis and rheumatism. Studies have shown that the inhibitory effects of podophyllotoxin are competitive in nature with colchicine. Podophyllotoxin has been demonstrated to inhibit the hydrolysis of GTP by tubulin. Extensive SAR studies have led to the development of two semi synthetic derivatives: etoposide (**13**) and teniposide (**14**) which are more potent and less toxic. However, these compounds have been shown to act *via* additional mechanisms such as inhibition of topoisomerase II. Podophyllotoxin and its analogs have been extensively reviewed recently [36].

Halichondrin B

Halichondrin B (**15**) is a large polyether macrolide which was isolated from the marine sponge *Halichondria okadai*. It was found to be highly cytotoxic against L1210 murine leukemia cells with an IC_{50} of 0.3 nM. Further studies proved

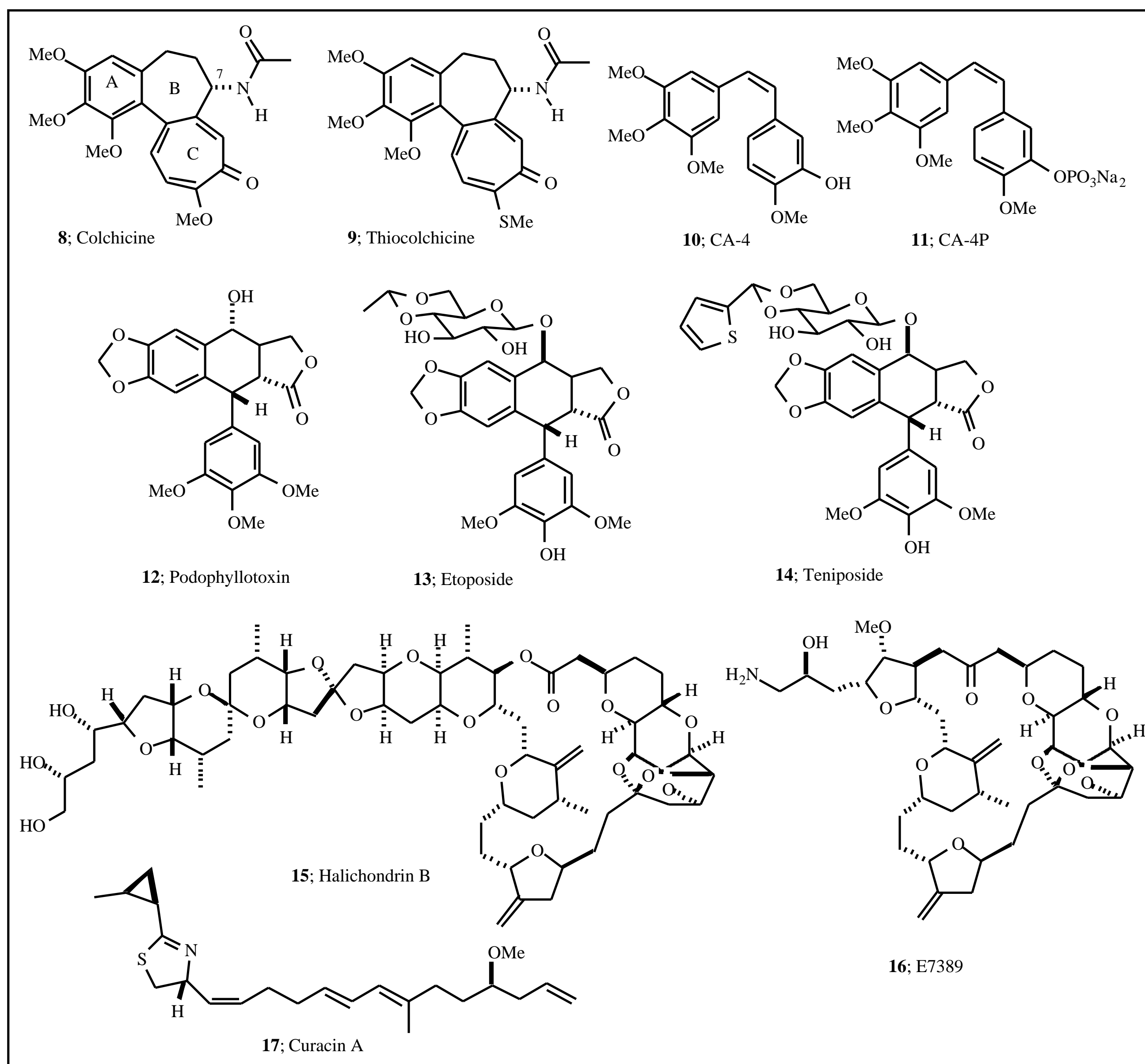


Fig. (3). Tubulin destabilizing agents: colchicine site.

that it functions as a non-competitive inhibitor of vinblastine to tubulin. Despite the excellent potency of the compound, further efforts to develop it as a drug were stifled by the limited supply and complexity of the structure. Synthesis of the halichondrin B and its analogs led to the discovery and development of E7389 (**16**; a macrocyclic ketone analog) with IC_{50} values ranging from 0.09-9.5 nM against various cell lines. E7389 is currently in phase II clinical trials for non-small-cell lung carcinoma (NSCLC) [37, 38].

Curacin A

Curacin A (**17**) is a marine natural product first isolated by Gerwick and co-workers from the blue–green cyanobacterium *Lyngbya majuscule*. It was initially found to be highly cytotoxic against brine shrimp with an LC_{50} of 3 ng/mL. Upon further testing on various cancer cell lines, it was found to be active against breast, colon and renal cancer cell lines. Curacin A was found to accumulate Chinese hamster ovary cells at the G2/M phase suggesting that it may function as a tubulin binder. It was later demonstrated that curacin binds to the colchicine site of tubulin in 1:1 ratio with an association constant 3400 times greater than tubulin for itself. Curacin A inhibits tubulin polymerization with an IC_{50} value of 0.72 μM [39-41]. The thiozaline ring and conjugated double bonds of Curacin A are susceptible to oxidation which limits the potential of the compound to become a drug. Extensive SAR studies by Wipf and co-workers have provided new leads with favorable physiochemical properties, however the studies are in the preliminary stages [42].

VINCA BINDING DOMAIN

The name "vinca binding domain" was coined when vinca alkaloids were found to bind to tubulin at a site distinct from that occupied by colchicine. Compounds that bind to the vinca domain usually function as rapid, reversible and temperature-independent inhibitors of tubulin assembly. Based on the experimental evidence, it has been suggested that there are two distinct sites on tubulin; hence the name "vinca domain" has been coined to exemplify the uncertainness to specify the exact position on tubulin.

Vinca Alkaloids

The Vinca alkaloids are derived from the periwinkle plant, *Catharanthus roseus* G. Don (frequently known as *Vinca rosea* Linn). The *Vinca* alkaloids are dimeric compounds in which indole and dihydroindole nuclei are fused together with other complex ring systems. The first natural compounds to be identified are vinblastine (**18**) and vincristine (**19**) which differs only in the presence of a formyl or methyl group in the vindoline moiety. Extensive modifications were made to both the velbanamine (catharanthine) and vindoline moieties, which led to the semi synthetic derivatives such as vinorelbine (**20**) and vinflunine (**21**). This modest difference in structure, which does not alter in any fundamental way the mechanism of action, does significantly alter the clinical spectrum of efficacy and toxicity of these agents.

Vinflunine (**21**), a difluorinated derivative of vinorelbine, is the most recent vinca alkaloid to have progressed into clinical trials [43, 44]. However, the effects of vinflunine and vinorelbine on microtubule dynamics differ from those observed with vinblastine [45]. It was observed that the potency of vinflunine as an inducer of MDR *in vitro* (A549 lung carcinoma cells) and *in vivo* (P388 leukemia cells) is lower relative to the parent compound vinorelbine [46]. However, when compared with vinorelbine, the *in vivo* activity of vinflunine against several tumor xenografts proved to be superior [47]. The superior *in vivo* activities of vinflunine relative to other vinca alkaloids, together with its reduced potential to induce MDR make vinflunine a promising candidate for further clinical profiling.

The antineoplastic activity of the *Vinca* alkaloids is usually attributed to their ability to disrupt microtubules, causing dissolution of mitotic spindles and metaphase arrest in dividing cells [3, 48-52]. However, disruption of microtubules can also lead to toxicity in nonmitotic cells. Although the *Vinca* alkaloids are classified as mitotic inhibitors, their antineoplastic activity in the clinical treatment of cancer probably arises from suppression of microtubule dynamics as well as from disruption of the cell cycle and induction of programmed cell death [53, 54].

Dolastatin-10

Dolastatin-10 (**22**) and its related congener Dolastatin-15 (**23**) are polypeptide natural products which were isolated from marine sea hare *Dolabella auricularia*. Both of these compounds are more potent than vinblastine in tubulin polymerization assays, although dolastatin-10 is 9 times more potent than its closely related analog. This family of compounds acts as non-competitive inhibitors of vinblastine binding to tubulin indicating that there are distinct ways to bind to the vinca domain [55, 56].

Rhizoxin

Rhizoxin (**24**) is an antitumor antibiotic isolated as a toxin from the culture broth of *Rhizopus chinensis* which is a potent pathogen of rice seedling blight. Rhizoxin was observed to be a competitive inhibitor of the binding of both vincristine as well as ansamitocin P-3 (**25**), a maytansine analog (**26**) to tubulin. Rhizoxin inhibits the polymerization of purified tubulin with an IC_{50} of 6.8 µM. Studies have shown that the interaction of tubulin with rhizoxin and maytansine are similar in that they both prevent formation of an intrachain cross-link in -tubulin by *N,N'*-ethylenebis (iodoacetamide), whereas vinblastine is only partially effective. Rhizoxin was effective against human and murine tumor cells resistant to vincristine and adriamycin when compared to maytansine (**25**) both *in vitro* and *in vivo*. A maximum 60% increase in life span was obtained in mice inoculated with P388 leukemia resistant to vincristine. However, Rhizoxin showed greater cytotoxicity in cultured tumor cells than did vincristine. Rhizoxin failed phase II clinical trails due to lack of efficacy likely resulting from the rapid elimination from the plasma [57-59].

Spongistatins

The spongistatins are a group of complex macrocyclic lactones isolated from the sponges *Spirastrella spinispirulifera* and *Hyrtios erecta*. Spongistatin 1 (**27**) is a potent inhibitor of the binding of vinblastine and GTP to tubulin, but

has no effect on the binding of colchicine to tubulin. Spongistatin 1 (**27**) has exhibited potent activity against leukemic (IC_{50} = 20 pM against L1210 murine leukemia cells) and a variety of solid tumor cell lines including melanoma, and colon cancers. Extensive modeling studies revealed a novel region on -tubulin that could accommodate the large molecular volume of spongistatin 1. Even though the spongistatins are structurally complex, the total synthesis and SAR studies of spongistatin 1 has led to a structurally simpler, rationally designed spiroketal subunit of the spongistatins, Spiket P (**28**). Spiket P (**28**) causes tubulin depolymerization in cell-free turbidity assays and exhibited potent cytotoxic activity against cancer cells [60, 61].

DRUGS BINDING TO SULFHYDRYL GROUPS

The thiol groups on cysteine residues of tubulin are highly reactive species. Tubulin has 20 cysteine residues, of which twelve are in -tubulin and eight are in -tubulin [62, 63]. The drugs which bind to these residues often possess Michael acceptor functionalities which react with the nucleophilic thiols. Though not fully understood, this covalent interaction appears to prevent the formation of stable microtubules, and thus prevents cell division.

Calvatic Acid

Calvatic acid (**29**) is a known antibiotic and cytostatic agent isolated from *Calvatica liacina* [64, 65]. The cytostatic properties arise from the prevention of microtubule assembly. In the presence of cysteine, the inhibition of assembly is prevented which suggests that tubulin sulfhydryl groups are the biological targets for the compound. Calvatic acid also prevents the binding of colchicine to tubulin through a mechanism that involves a structural alteration of the protein structure, rather than by direct competitive binding to the colchicine binding site [66].

Cytochalasin A

Cytochalasin A (**30**) is a fungal metabolite that inhibits the assembly of microtubules *in vitro* and prevents the binding of colchicine to tubulin. Studies have shown that cytochalsin A forms a covalent adduct with tubulin sulfhydryl groups in a manner that results in blockage of microtubule assembly [67]. Further research has shown that ethyl acetylacrylate**,** an analog of the reactive portion (Michael acceptor) of cytochalasin A (**30**), also reacts with tubulin sulfhydryls in a manner akin to the parent compound.

KINASES

Protein phosphorylation serves as the reversible signal that allows information to be relayed within cells. It has been estimated that close to 10,000 proteins in humans undergo reversible phosphorylation [68]. A change in phosphorylation status can stimulate or inhibit a protein's activity, induce conformational changes, assemble or disassemble protein complexes, and alter cellular localization [69]. More than 98% of all protein phosphorylation occurs on the hydroxyl of serine or threonine while the remaining 2% occurs on tyrosine.

The timing of the eukaryotic cell cycle is coordinated by successive waves of phosphorylations performed by the cyclin-dependent family of protein kinases (CDKs). CDKs are also intricately involved in a wide range of specialized cellular processes such as meiosis, apoptosis, transcription, differentiation, and neuronal signaling [70, 71]. Due to the pivotal roles that CDKs play in regulating the cell cycle, their activity is tightly regulated by a number of mechanisms including phosphorylation, intracellular localization, and activation (cyclins) and inhibition by protein partners ($p21^{Waf}$, $p27^{Kip}$). While the genome of the unicellular yeast *S. cerevisae* encodes only a single CDK subunit (Cdc28), mammalian cells contain nine CDKs (CDK1-9) and 12 cyclins. CDKs have received considerable attention as targets for inhibitor development due to periodic CDK activation being necessary for uncontrolled cellular proliferation. In addition, there is considerable evidence that many of the components of the CDK regulatory network are themselves the targets of mutations (CDK4, $p16^{INK4a}$), amplifications (CDK4, CDK2), overexpression (Cdk6, CyclinD1, CyclinD2, CyclinD3, CyclinE1, CyclinA1, Cdc25A/B), or deletions ($p16^{INK4a}$, $p15^{INK4b}$, pRb) in a host of human neoplasms. Most CDK inhibitor discovery efforts have focused on small molecule ATP-site directed compounds because this mode of inhibition was demonstrated to be feasible with the discovery of compounds such as Staurosporine and Olomoucine. At the inception of the efforts in the late 1980s, the general opinion was that high potency kinase inhibitors would be non-selective due to the highly conserved nature of the ATP-binding site. However, the discovery that the trisubstituted purine Olomoucine (**31**) was a moderately potent inhibitor of starfish CDK1/CycB (IC_{50} = 7 μM) without inhibiting a panel of other kinases, provided hope that selectivity for specific kinases could be achieved. Following these discoveries, a variety of natural and synthetic compounds have been characterized as CDK inhibitors.

The trisubstituted class of CDK inhibitors was discovered as derivatives of plant cytokinins such as isopentenyladenine and 6-dimethylaminopurine using a biochemical assay. The first compound that was described in detail was Olomoucine which served as a lead compound for further optimization by medicinal chemical and library-based approaches. The selectivity of Olomoucine for CDKs over a panel of other kinases could be rationalized based on an X-ray co-crystal structure with human CDK2. As expected from the kinetic analysis which revealed Olomoucine to be an ATP-competitive inhibitor, the inhibitor bound to the ATP binding cleft. Interestingly, the purine ring of Olomoucine was rotated relative to ATP such that key H-bonding interactions were made by the 6-NH and N7 to the amino acid that normally H-bonds to the exocyclic amino and N2 of ATP. The N6 benzyl amino group extends into a region of the binding site not occupied by ATP providing a plausible explanation for selectivity. In order to improve the enzymatic potency of Olomoucine, a variety of analogs were prepared that ultimately resulted in the discovery of Roscovitine [72]. Two alterations were made to the structure: the N9 methyl was replaced with isopropyl and a chiral R-ethyl substituent was introduced at C2. The N9 modification resulted in a 10-fold improvement in IC_{50} presumably due to more optimal hydrophobic contacts to the small pocket adjacent to the N9 position. The C2 substitution resulted in a further 3-fold IC_{50} improvement potentially due to more extensive hydrophobic

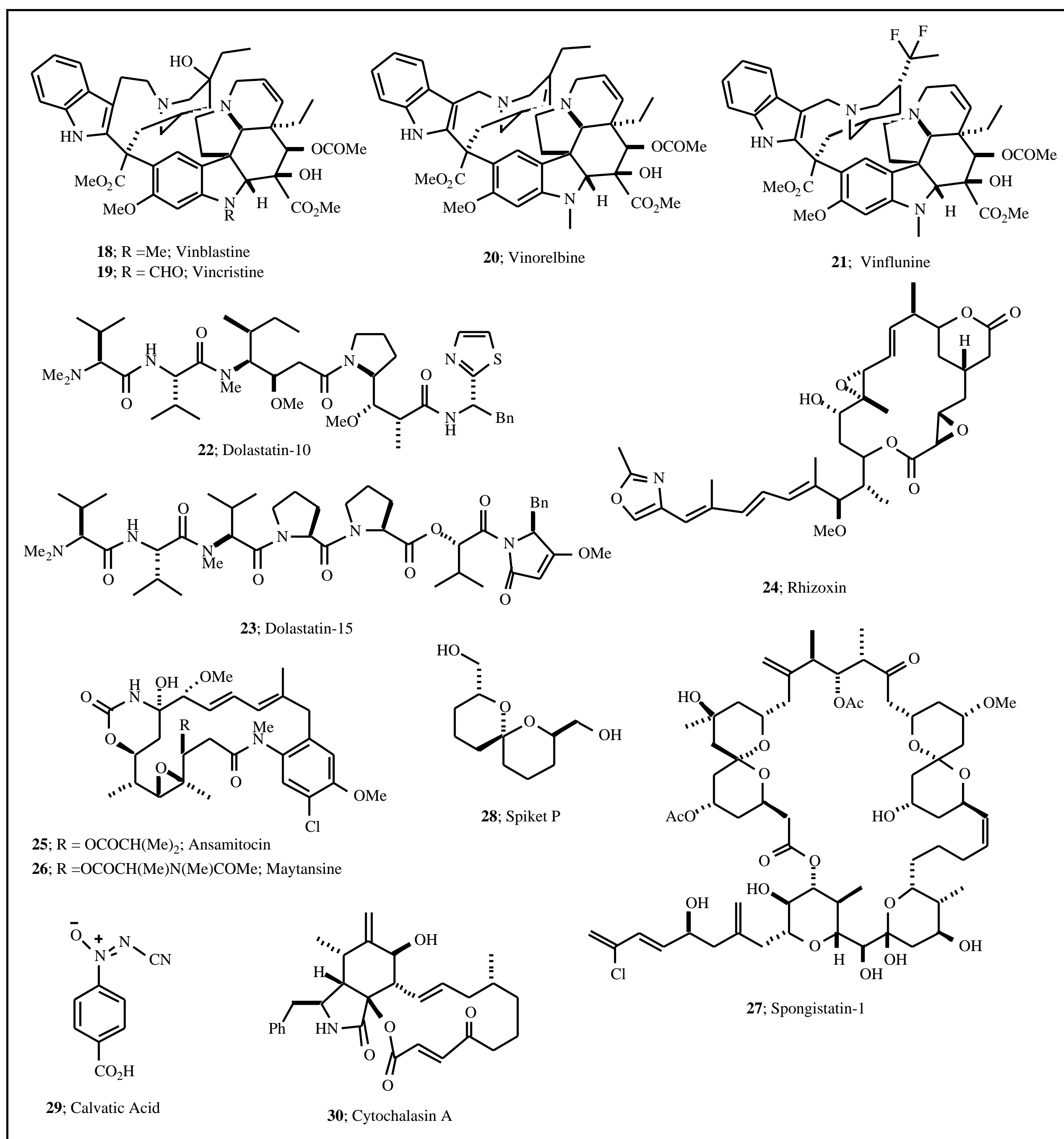


Fig. (4). Inhibitors of Vinca domain and drugs binding to sulfhydryl groups.

contacts and conformational restriction of the hydroxyl which is involved in an H-bond with the protein. A co-crystal structure with Cdk2 confirmed that the binding mode of Roscovitine (**32**) was virtually identical to that observed for Olomoucine [73]. An independent effort to optimize Olomoucine using a combinatorial library approach resulted in the identification of the Purvalanols [74]. Interestingly, the optimal substituents at C2 and N9 were remarkably similar whereas the C6 was substituted with a 3-chloroaniline [75]. The biochemical potency for Purvalanol A (**33**) against Cdk1/B is 1000-fold improved relative to Olomoucine and an exceptionally high degree of kinase selectivity was maintained. Despite excellent biochemical potency, Purvalanol A and Roscovitine typically do not show cellular effects *in vitro* until low micromolar concentrations are reached [76]. For example, Purvalanol A or Aminopurvalanol will induce a reversible accumulation of cells in G2/M phase at concentrations between 1-10 μM and induce apoptosis at concen-

trations higher than 10 μM (study using U937 cells) [75]. Biochemical studies have demonstrated that treatment of cells with Purvalanol A (5 μM) can block retinoblastoma phosphorylation at S780 after a six hour treatment [77]. Antiproliferative effects of the Purvalanols were evaluated against the National Cancer Institute 60 cell-line panel [75]. Purvalanol A exhibited an average GI_{50} of 2.0 μM with some lines showing 20-fold greater sensitivity (e.g. colon KM12 cells GI_{50} = 76 nM). Several studies have seeked to address the molecular basis for differential sensitivity to CDK inhibitors. One study demonstrated that cells arrested in mitosis using Taxol and released into media containing Purvalanol were induced to apoptose much more than cells treated with either drug alone. The proposed mechanism involved blocking CDK1 mediated phosphorylation of survivin - a member of the XIAP class of antiapoptotic proteins [78]. Another recent study has demonstrated that cell lines engineered to overexpress the Myc oncogene are also hypersensitive to treatment with Purvalanol. In addition, a transgenic mouse that over-expresses c-myc selectivity in hepatocytes and spontaneously develops a severe liver cancer were demonstrated to undergo tumor regression following treatment with Purvalanol (20 mpk bid, ip dosing) (Andrei Goga and J. Michael Bishop, personal communication).

Considerable efforts have been made to define the full range of potential protein targets of the Purvalanols in both biochemical and cellular systems. One study used an immobilized Purvalanol derivative to purify a series of kinases from cell lines and mouse tissue extracts by affinity chromatography [79]. Identified targets included Cdk1, Cdk5 (from brain extracts), CK1 (from parasite extracts), Gsk3 & , and Erk 1&2. Follow-up enzymatic studies demonstrated that only Cdk1/CycB and Cdk5/p25 were inhibited with nanomolar IC_{50}s while the other targets were only inhibited in the micromolar range. It was also shown that although Erk 1 and 2 were inhibited with an enzymatic IC_{50} = 3 and 4 μM, cellular measures of Erk 1 and 2 activity were inhibited at similar concentrations [80]. For example, an Elk1-Gal4 luciferase reporter gene expressed in Chinese hamster lung fibroblast cell line CCL39 was inhibited with an EC_{50} = 4 μM while phospho-nucleolin, a marker of CDK1 activity, was inhibited with an EC_{50} = 3 μM. This study suggests that Purvalanol A is capable of blocking the MAP kinase pathway at low micromolar concentrations, a conclusion that would not have been reached from biochemical kinase selectivity assays.

Another series of purine-based CDK inhibitors was discovered where the N9 position is left unsubstituted. Compounds such NU6102 (**34**) bind to CDK2 in a manner distinct from both ATP and the Purvalanols: they form a series of 3 hydrogen bonds from the 2-anilino, N1, and 9-NH.

One of the first natural products to be characterized as a Cdk1 and 2 inhibitor is the Aspergillus derived butyrolactone-I (**38**) [81, 82]. This compound was demonstrated to be an ATP-competitive inhibitor of Cdk1/CycB and inhibits phosphorylation of retinoblastoma protein in cells. Similar to the trisubstituted purines, both G1 and G2/M arrest have been observed with apoptosis induction after longer exposure. A recent study suggests that butyrolactone-I can also inhibit $p21^{WAF1/CIP1}$ expression through a mechanism that may involve cellular targets other than Cdk1 [83]. Unfortunately rather little information is available in regards to the kinase selectivity of this compound and concentrations used in cellular experiments are relatively high (50-100 μM) [82].

Hymenialdisine (**35**) is a marine sponge derived lactam that is a nanomolar enzymatic inhibitor of Cdk1/CycB, Cdk5/p25, Gsk3 , CK1, and Mek1 [84]. Hymenialdisine competes with ATP for binding to these kinases and a co-complex with CDK2 shows that hymenialdisine forms a series of three hydrogen bonds to the Glu81 and Leu83 residues of Cdk2. Follow-up studies have explored the broader potential of the Hymenialdisine scaffold as a kinase inhibitor through the synthesis of diverse analogs, biochemical kinase panels, and affinity chromatography [85]. While these studies were successful in identifying nanomolar to micromolar inhibitors of 11 new targets including p90Rsk, Kdr, c-Kit, Fes, Mapk1, Pak2, Pdk1, Pkctheta, Pkd2, Rsk1, and Sgk, limited progress was made in identifying hymenialdisine analogs with submicromolar cellular potency. Another study has identified that a benzo-fused analog of hymenialdisine is a potent enzymatic inhibitor of the Chk2 checkpoint kinase (IC_{50} = 8 nM) [86].

Flavopiridol (**36**) is a synthetic analog of alkaloid obtained from an Indian plant [87]. Flavopiridol is the first and most thoroughly investigated clinical-stage Cdk inhibitor having been tested in a variety of human cancers including lung, breast, bladder and leukemia [88-92]. The fact that only very modest success has been obtained in the clinic is not surprising as the compound was never optimized for important pharmaceutical properties such as reduced protein binding and pharmacokinetics. Although flavopiridol was initially reported as a cell cycle blocker with potent activity against Cdk1/CycB [93], it was subsequently shown to inhibit Cdk4 and Cdk7 and thereby possesses significant anti-transcriptional activity(recently reviewed [94]). Interestingly, the gene-expression profile resulting from flavopiridol treatment more resembles the profile obtained using general transcription blockers than other Cdk inhibitors such as roscovitine and 9-nitropaullone [95]. Indirubin (**37**) was isolated as the "active" principal of a traditional Chinese anti-leukemia medicine and later shown to be a potent and fairly selective inhibitor of Cdk1 and 2. Although an X-ray co-structure with Cdk2 is available and the cellular effects are consistent with Cdk1/2 inhibition, there have been no further reports on the further development of indirubins.

A variety of synthetic Cdk inhibitors have been developed that share the common feature of recognizing the kinase "hinge" region through a hydrogen bond donating and accepting pair (Fig. **6**). Most were developed based on screening historical pharmaceutical compound collections followed by extensive optimization-based on Cdk1 or 2 guided medicinal chemistry. The most extensively investigated class are the so-called "phenyl-aminopyrimidines" (aka PAPs) such as pyrimidopyridinone (**39**), CGP60474 (**40**) [95], thiazole PAP (**41**) [96, 97], and imidazo [1, 2-b]pyridazine (**42**) [98-100]. Thiazole PAP (**41**) is a pan-CDK inhibitor with greatest biochemical potency against Cdk2/CycE (IC_{50} = 2 nM) and Cdk9/CycT (IC_{50} = 4 nM) while significant activity is also observed against Cdk1/ CycB (IC_{50} = 80 nM), Cdk4/CycD1 (IC_{50} = 53 nM), and

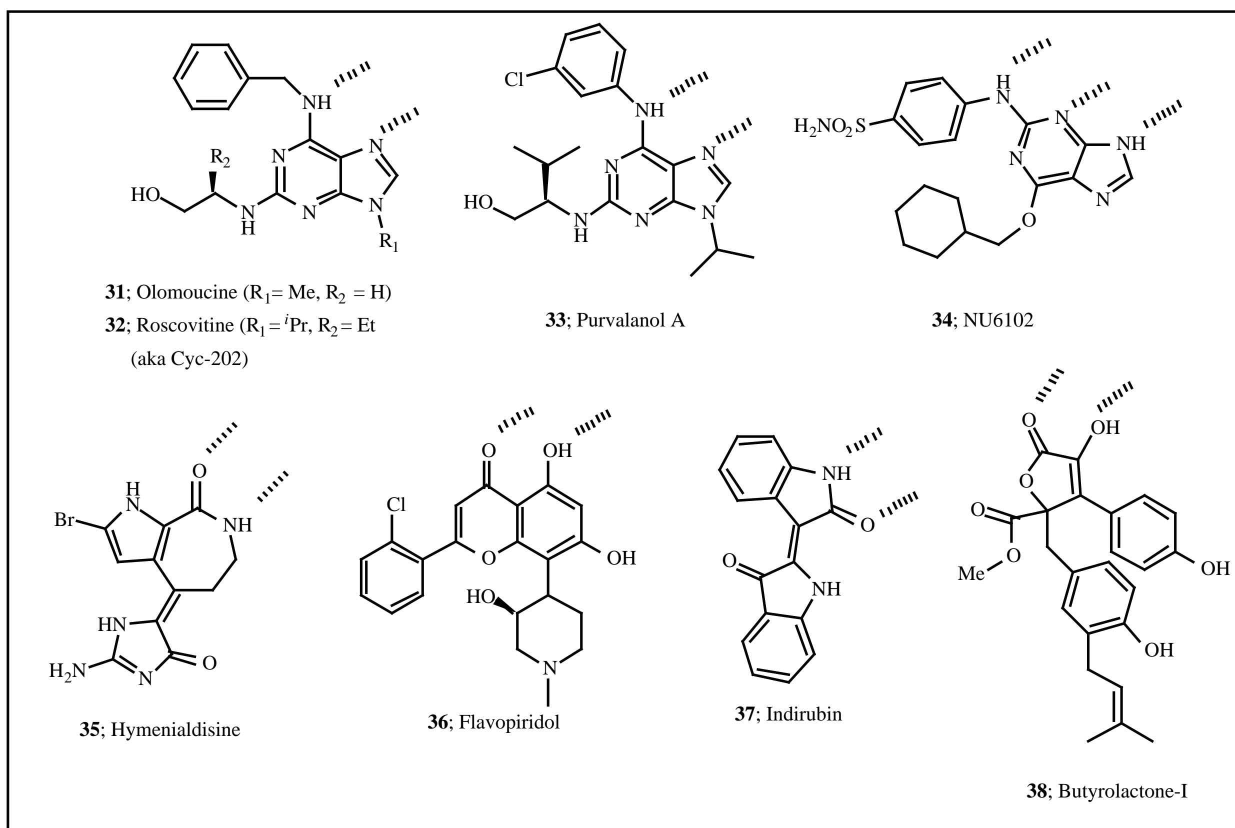


Fig. (5). CDK inhibitors from nature.

Cdk7/CycH (IC_{50} = 70 nM). Against a limited panel of other kinases, potent activity is apparent against Gsk3 (IC_{50} = 20 nM) and tyrosine kinases such as Abl (IC_{50} = 160 nM). In cellular experiments, thiazole PAP **41** (2 μM, 24hrs) was demonstrated to block phosphorylation of Rb at T821 and S249/T252 (Cdk2 and Cdk4 phosphorylation sites) and RNA polymerase II at S2/S5 (possible Cdk7 and Cdk9 sites). The average antiproliferative IC_{50} in a 72 hour MTT assay for thiazole PAP **41** against A549, HT29, SaOS-2 is 300 nM. The greater antiproliferative activity of this compound class versus inhibitors such as Purvalanol A is likely a result from a more broad-based kinase inhibition especially against Cdk7 and Cdk9 which are important for transcription. While many PAPs show poor pharmacokinetic behavior (rapid clearance and/or low oral bioavailabilty), imidazo [1,2-d]pyridazine **42** is exceptional showing a plasma C_{max} = 9 μM, $AUC_{0\text{-}6h}$ = 20 μM.h, $T_{1/2}$ = 3.3 h following a 2 mpk oral dose to mice [99].

Alsterpaullone (**45**) is an optimized analog of a compound that was identified as a Cdk inhibitor based on the similarity of its inhibitory profile against the National Cancer Institute 60-cell line panel to other known Cdk inhibitors [101]. Recently a structure-guided optimization effort resulted in Alsterpaullone derivatives which inhibited Cdk1/CycB and GSK3 in the picomolar range [102].

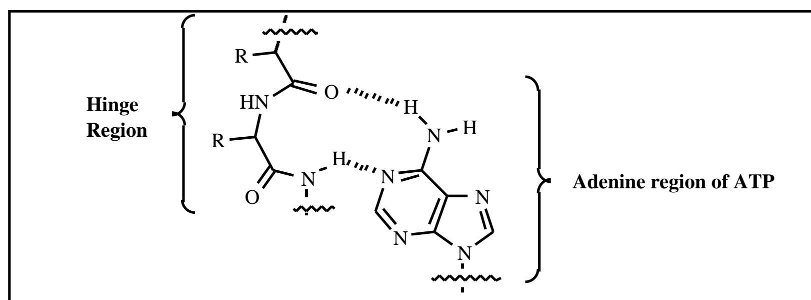


Fig. (6). Contact with kinase hinge region through a hydrogen bond donating and accepting pair.

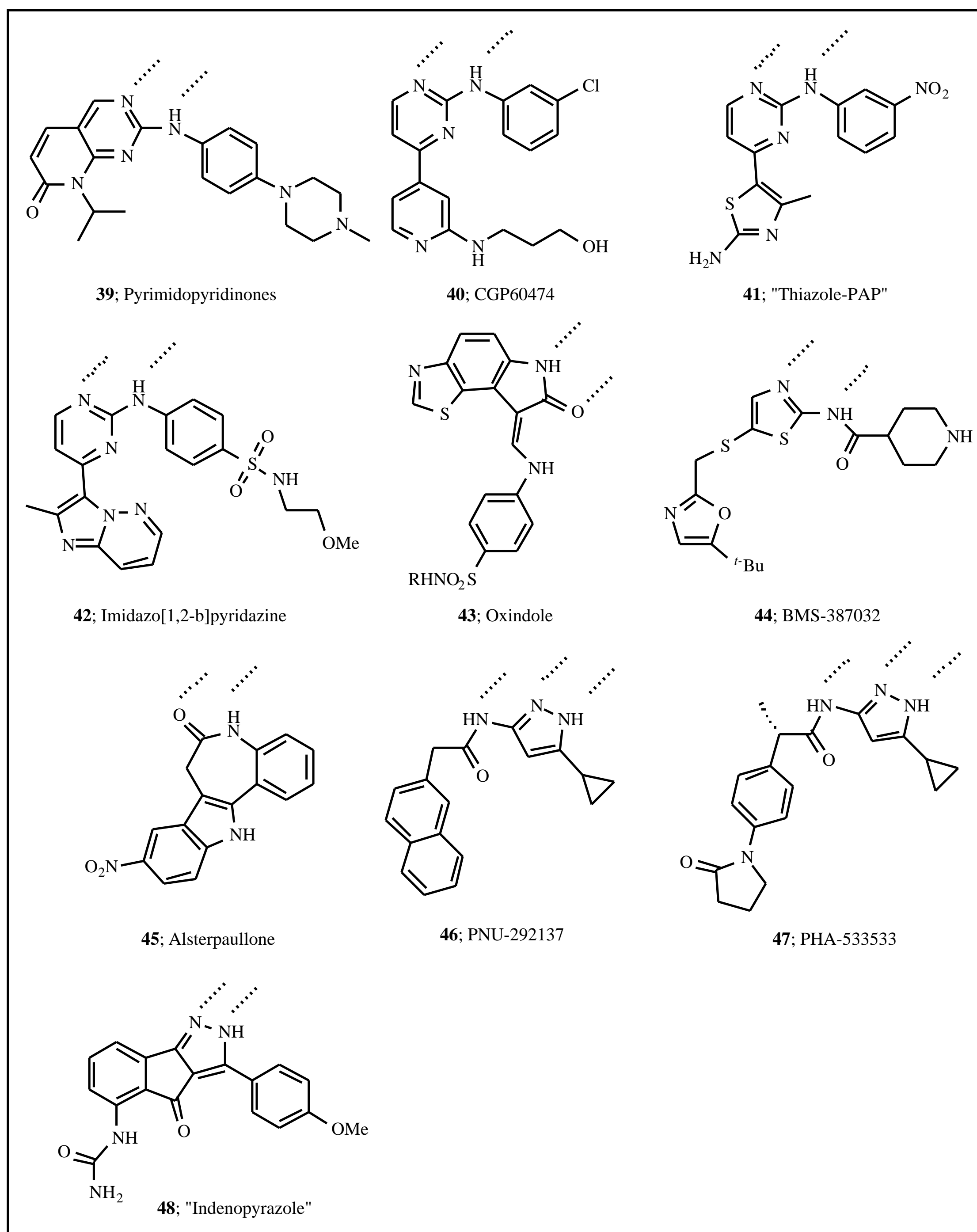


Fig. (7). Synthetic CDK inhibitors.

One of the most clinically advanced Cdk1/CycB inhibitors is the *N*-Acyl-2-aminothiazole BMS-387032 (**44**) [103]. In a biochemical enzyme assay, BMS-387032 showed a Cdk2/CycE IC_{50} = 48 nM and was 10- and 20-fold selective over Cdk1/cycB and CDK4/cycD, respectively and showed selectivity over a small panel of other kinases. The compound inhibited A2780 cell proliferation with an IC_{50} = 95 nM and displayed good pharmacokinetics in multiple species suggesting that the compound could be progressed into clinical testing. In a mouse P388 and A2780 xenograft studies, BMS-387032 dosed at its maximum tolerated dose exhibited a superior efficacy when compared to flavopiridol.

Aurora Kinase Inhibitors

During cell division, the proper segregation of duplicated chromosome is required to insure that full complement of

genetic information is transferred to both daughter cells. The mitotic spindle, composed of tubulin and associated proteins, is the apparatus that accomplishes this vital task. The mitotic spindle emanates from two poles of the microtubule organizing centers (MTOC) and attaches individual tubulin polymers to the kinetochores of each chromosome. The MTOC or centrosome, consisting of a pair of centrioles and pericentriolar material, are responsible for the formation of the spindle poles in animal cells. A loss in the fidelity of the centrosome duplication, separation, spindle formation and segregation can result in the loss of genome integrity and possibly lead to uncontrolled growth and tumorigenesis [104]. In recent years, it has been established that certain families of protein kinases are involved in the proper centrosome and spindle function such as the Nima related kinase (nek) 2, Polo-like kinase (plk) 1, and Aurora kinase [105, 106].

Aurora kinase was first discovered in a yeast screen for mutants that displayed improper ploidy (ipl) following cell division [107]. In Drosophila, mutations in Aurora kinase were later found to prevent centrosome separation resulting in monopolar spindles [108]. There are three known isoforms of Aurora kinase described in mammals: Aurora A, B and C. While Aurora A and B are ubiquitously expression, Aurora C shows predominant expressed in the testis suggesting a possible role in meiosis [109, 110]. Although the kinase catalytic domain of all three Aurora isoforms displays high homology, the cellular kinase localization, regulation and substrate specificity vary. Both Aurora A and B can phosphorylate histone H3 on serine 10 although only Aurora B seems necessary *in vivo* [111]. Aurora A is implicated in centrosome maturation and spindle assembly [112]. Both Aurora A and B have the ability to transform cell lines (NIH3T3 or CHO) that are capable of forming tumors in mice [113-115]. The role of the Aurora kinases in cell cycle and tumorigenesis has made them a potential target for the development of small molecule therapeutics.

Recently four small molecule inhibitors of Aurora kinases have been described: Hesperadin (**49**), an indolinone discovered in a screen for inhibitors of HeLa cell proliferation [116], ZM447439 (**50**), a quinazoline discovered and optimized using a biochemical Aurora A kinase assay [117], VX-680 a trisubstituted pyrimidine (**51**) [118], and a 1, 4, 5, 6-tetrahydropyrrolo [3, 4-c]pyrazole bi-cycle inhibitor [119] (**52**). The cellular effects of ZM447439 (at 2 μM) and Hesperadin (at 50 nM) appear to mimic those induced by siRNA knockdown of Aurora B including inhibition of serine 10 phosphorylation of histone H3, improper chromosome alignment at metaphase, loss of spindle checkpoint and anaphase progression suggesting that compounds are capable of inhibiting Aurora B in cells. VX-680 inhibits all isoforms of Aurora *in vitro* whereas no data is available for the activity of ZM447439 on Aurora C and Hesperadin on Aurora B and C [116-118]. Compound **52** is a potent Aurora inhibitor (Aurora A, -B, and -C enzymatic IC_{50} for A/B/C = 27/135/120 nM), blocks histone H3 phosphorylation at 1 μM and displays antiproliferative effects on various tumor cell lines at concentrations between 50 and 500 nM.

As one of the main goals in developing Aurora kinase inhibitors is to demonstrate their potential to inhibit tumor growth *in vivo* it would be valuable to have highly selective tool compounds. Hesperadin is reported to inhibit Mek1, Mapkap-K1, Ampk, Chk1, Phk, Lck when tested against a panel of 25 kinases at 1 μM (and Cdk1 at slightly higher concentrations). Hesperadin's activity in cellular experiments is apparent at concentrations lower than would be expected from inhibition of Aurora B kinase observed *in vitro* and it is, therefore, not clear that hesperadin's primary cellular target is Aurora B. ZM447439 does not show any effects on centrosome maturation and may not inhibit Aurora A, which is commonly overexpressed in cancer cells. VX-680 is more selective although it also inhibits Flt3 (IC_{50} = 30 nM), Lck (IC_{50} = 80 nM), Itk (IC_{50} = 220 nM) and Src (IC_{50} = 350 nM). ZM447439 has been reported to inhibit Mek1, Src and Lck in a limited panel of 16 kinases. Inhibitor **52** appears to show good selectivity when tested against a limited panel of 25 kinases with only fibroblast growth factor receptor tyrosine kinase 1 (Fgfr1) being inhibited at submicromolar concentrations. Therefore, the activity of these compounds in cellular experiments and *in vivo* may result from significant off-target contributions. Further work, perhaps involving expression of inhibitor resistant alleles of aurora kinases, will be necessary to prove if the cellular effects of these inhibitors are derived primarily through inhibition of Aurora kinase.

PHOSPHATASES

A typical mammalian genome encodes several hundred protein kinases, but has only a few dozen protein phosphatases. Protein phosphatases insure that phosphorylation is reversible in a regulated manner. Protein phosphatases can be grouped into three categories based on whether they act upon phospho-serine and threonine, tyrosine, or all three. Tyrosine phosphatases play a vital role in a number of processes and have been linked to a variety of diseases such as type II diabetes. Unlike their kinase counterparts, development of selective tyrosine phosphatase inhibitors is still at an early stage.

Okadaic Acid

Okadaic acid (OA; **53**) is a frequently used multi-targeted natural product phosphatase inhibitor. The toxin binds in a hydrophobic groove adjacent to the active site of the protein phosphatases and interacts with basic residues within the active site. Okadaic acid potently and specifically inhibits PP1 (IC_{50} of 0.2 nM) and PP2A (IC_{50} of 20 nM). Despite sharing similar protein folding and catalytic mechanisms, PP-2B is inhibited to a much lesser extent (IC_{50} of 5 μM). OA was found to induce a transformed phenotype to cells [120], stimulate DNA synthesis [121] and induce Cdk1/cyclin A and B activity [122-124] thereby promoting premature mitosis [125, 126] and preventing apoptosis [127]. In other settings, OA has been found to reverse the transformed phenotype [128], inhibit Src-mediated transformation [129], induce cell growth arrest by down-regulating Cdk1 expression [130, 131], arrest mitosis [132-135], and induce apoptosis [136, 137].

Fostriecin

Fostriecin (**54**) is the only phosphatase inhibitor that progressed to a phase I clinical trial [138-141]. Fostriecin is a very potent inhibitor of serine/threonine protein phosphatase

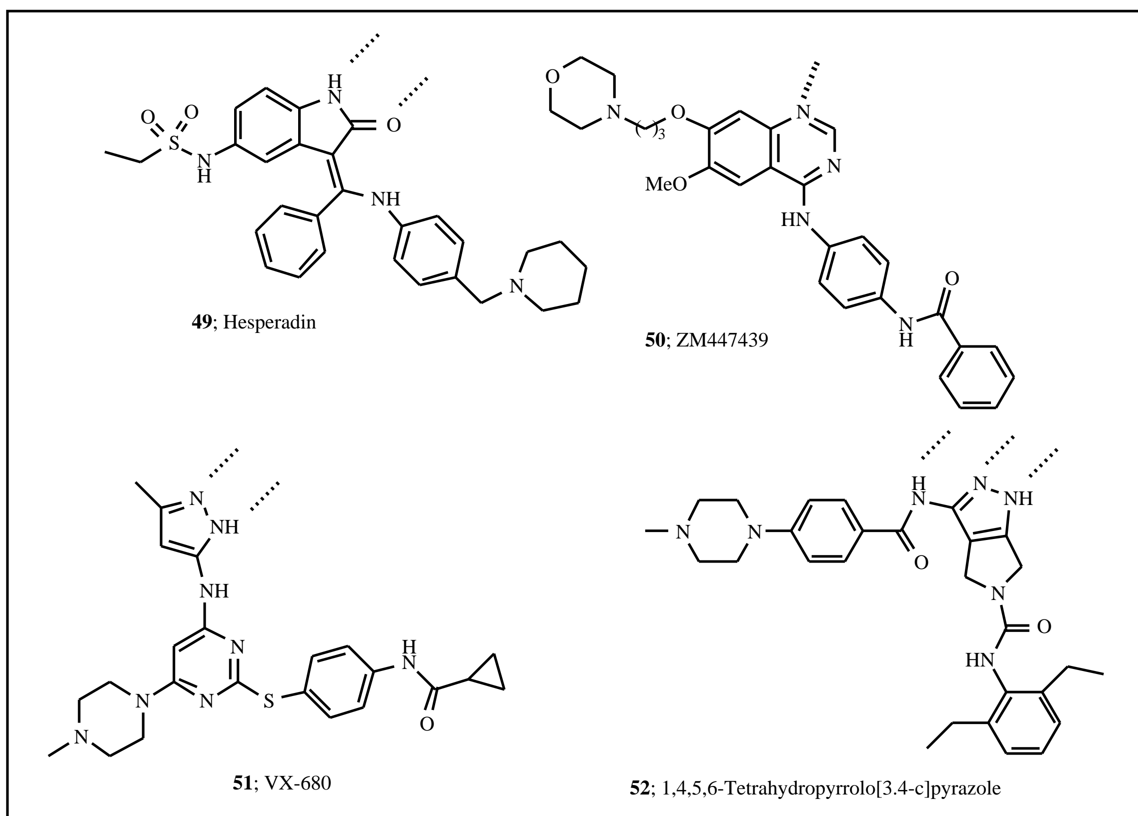


Fig. (8). Aurora kinase inhibitors.

PP2A (IC_{50} = 1 nM) and PP4 (IC_{50} = 40 nM) and a much weaker inhibitor of PP1 (IC_{50} = 100 mM). In contrast, fostriecin has no apparent effect on PP2B [142]. The binding site for fostriecin is different from that of okadaic acid [143]. Highly selective PP inhibitors have so far not been reported. Presumbably due to their lack of specificity, presently known phosphatase inhibitors also have considerable systemic toxic effects such as nausea/vomiting [144-146].

Cdc25 Inhibitors

Cdc25 phosphatases are dual-specificity phosphatases which dephosphorylate phospho-tyrosine, serine, and threonine. Three CDC25 genes, known as CDC25A, B and C, have been found in the human genome [147-150]. Cdc25 inhibition represents a promising pharmacological strategy because they are essential for CDK activation and dysregulation of Cdc25 expression and/or function is observed in a number of cancers and correlates with tumor aggressivity and poor prognosis [151]. Cdc25B has been shown to interact with steroid receptors, p300/CBP and CREB binding proteins independently of its phosphatase activity, thereby acting as a transcriptional co-activator [152]. Cdc25A can also dephosphorylate and activate the Cut homeodomain transcription factor, which then downregulates p21Cip1 expression in S-Phase [153]. These results indicated that Cdc25 phosphatases have other substrates or functions in addition to dephosphorylation of the Cdk/cyclin complexes.

A variety of scaffolds derived from natural products are found to be Cdc25 inhibitors as depicted in Fig. **9** [154-159]. Amongst them, NSC95397 (**56**) is clearly the most potent profiled thus far. However, most of the compounds exhibit modest *in vitro* and in cellular activity and specificity toward the Cdc25 targets. Furthermore, *in vivo* activity in animal xenograft models remains to be demonstrated for a majority of the classes of compounds.

PROTEASOME INHIBITORS

The ubiquitin/proteasome pathway is a major pathway for the degradation of a majority of proteins in eukaryotic cells. Proteins are targeted for proteolysis to an ATP-dependent 2500 kDa proteosome complex by being tagged by a poly-ubiquitin polymer. The barrel-shaped 26S proteosome is composed of the 20S portion which contains the catalytic core and the 19S regulatory subunits which cap the complex. X-ray analysis of 20S proteasome of the archaeon *Thermoplasma acidophilum* showed that 20S proteasome is composed of four stacked rings, with each ring consisting of seven - and -type subunits [160]. Proteolysis occurs within the central chamber at the active sites located at the -subunits, with the side threonine hydroxyls acting as the nucleophile.

A variety of cell cycle proteins (cyclins, Cdk-inhibitors) are subject to degradation by the ubiquitin/proteasome in a temporally controlled fashion [161]. For example, the syn-

thesis of cyclin B drives cells to initiate mitosis [162] while ubiquitin/proteasome-mediated degradation of cyclin B drives the exit from mitosis and reentry into G_1 phase of the next cell cycle [163]. Similarly, synthesis and degradation of cyclin E are essential to late G_1 progression [164] and S phase entry respectively [165].

Pharmacological inhibition of the proteasome results in cell cycle arrest. Proteasome inhibitors are therefore potential candidates as antimitotic drugs. Since the mode of proteasome inhibition was first analyzed with nonspecific peptide inhibitors [166], several natural product inhibitors showing greater specificity have been discovered. Lactacystin originally isolated from *Streptomyces* sp., due to its ability to induce differentiation in neuroblastoma cells [167], is the first identified natural proteasome inhibitor. It is an irreversible specific proteasome inhibitor with no effect on serine or cysteine proteases [168]. Lactacystin is spontaneously lactonized to form -lactone (*clasto*-lactacystin- -lactone, also called omuralide; **60**), which is the cell penetrating active species that covalently modifies the amino terminal threonine of catalytic subunits. Apart from Lactacystin, Salinosporamide (**61**) and Epoxomicin (**62**) are the prominent proteosome inhibitors acquired from nature [169-175].

DNA TOPOISOMERASE

DNA topoisomerase are essential nuclear enzymes that create breaks in DNA, thereby allowing the DNA strands to unravel and separate. Topoisomerases can either sever one (type I) or both (type II) strands of the DNA double helix. Topoisomerase II is the molecular target for the most widely

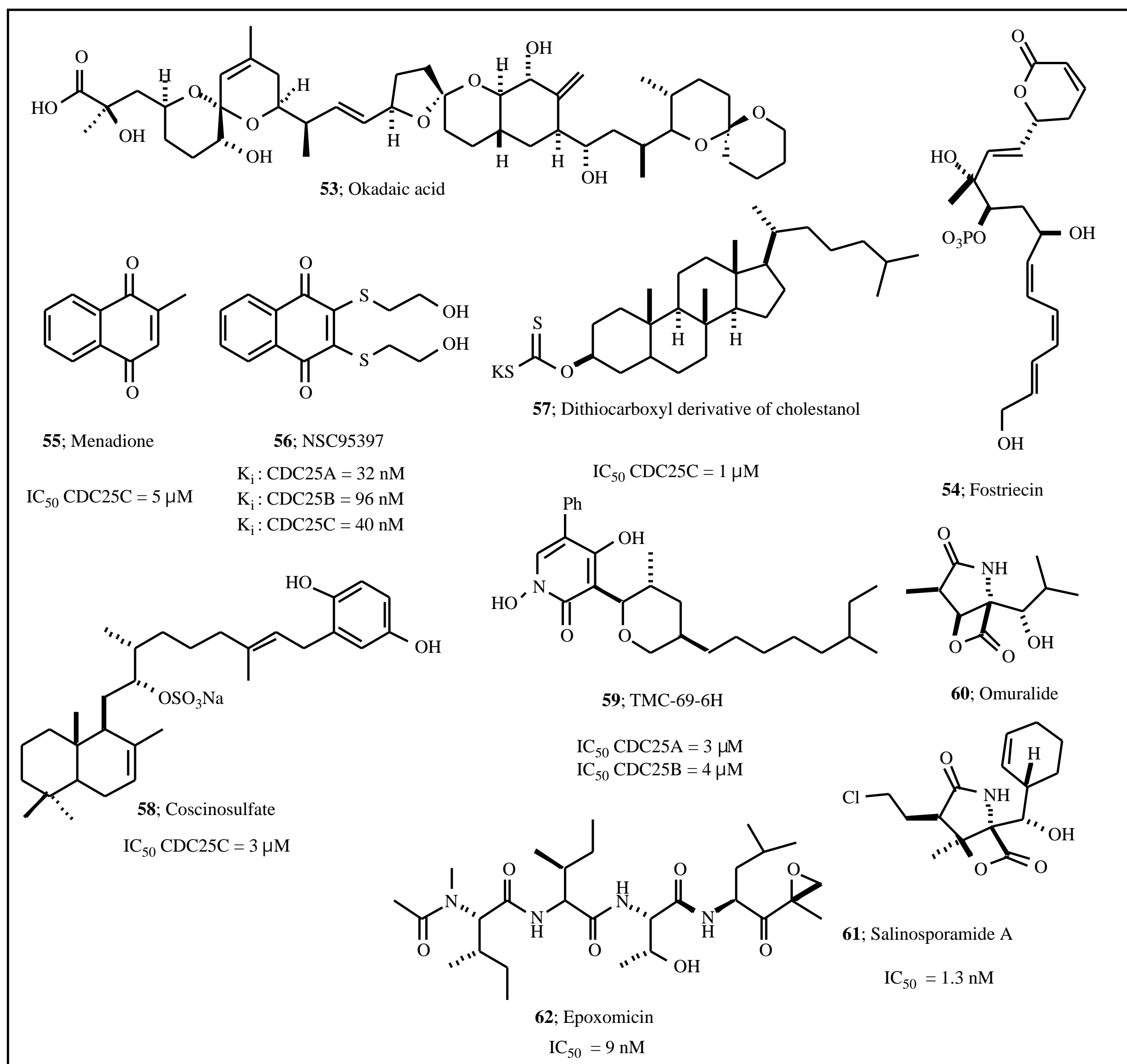


Fig. (9). Phosphatase inhibitors.

used anticancer drugs which act by preventing the enzyme from religating DNA strand breaks. Topoisomerase II inhibition results in the accumulation of double stranded DNA breaks which lead to cell death. Topoisomerase II inhibitors principally affect cell cycle progression during G2/M with little effect on S phase transit, while topoisomerase I inhibitors also have a pronounced influence on S phase progression. Although DNA topoisomerase inhibitors can be considered as classical DNA damaging agents, they are unique in their ability to modulate gene transcription of key regulatory genes such as c-myc and BRCA1. Another major difference between topoisomerase inhibitors and the DNA damaging agents is the active participation of DNA topoisomerases in the recognition and processing of DNA lesions and as mediators of cell cycle arrest [176].

The topoisomerase inhibitors are divided into two types depending on their mode of action. Inhibitors that are able to stabilize the covalent DNA topoisomerase II complex (also known as the cleavable complex) are called topoisomerase II poisons, while agents acting on any of the other steps in the catalytic cycle are called catalytic inhibitors. Catalytic topoisomerase II inhibitors are a diverse group of compounds that might interfere with the binding between DNA and topoisomerase II (aclarubicin and suramin), stabilize noncovalent DNA topoisomerase II complexes (merbarone, ICRF-187 and structurally related bisdioxopiperazine derivatives), or inhibit ATP binding (novobiocin). This article will focus on the inhibitors which are derived from natural products.

Aclarubicin (Aclacinomycin A)

Aclarubicin (**63**) belongs to the anthracycline class of antibiotics which were first isolated from bacterium *Streptomyces species*. It is used clinically in the treatment of acute myelocytic leukemia. Aclarubicin is a potent DNA intercalating agent that prevents the binding of topoisomerase II to DNA. As a result, aclarubicin is antagonistic to classical topoisomerase II poisons, such as etoposide, teniposide, and amsacrine. Later studies have shown that aclarubicin also inhibits topoisomerase I in a concentration-dependent manner. High concentrations of aclarubicin stimulate the formation of covalent DNA topoisomerase I complexes [177, 178]. However, at biologically relevant concentrations, aclarubicin prevents the binding of topoisomerase I to DNA and is therefore antagonistic to camptothecin [179]. Cellular exposure to aclarubicin leads to DNA damage as determined by the single-cell microgel assay (''comet assay''). Aclarubicin induces a concentration dependent growth arrest in the G2 phase of the cell cycle, which can be overcome by checkpoint abrogators.

Novobiocin

Novobiocin (**64**) is an antibiotic obtained from a variety of *Streptomyces niveus* and other *Streptomyces* species and is chiefly effective against staphylococci and other gram-positive organisms. Novobiocin inhibits bacterial gyrase B and mammalian topoisomerase II by blocking the ATP binding site [46, 180]. Novobiocin has been used extensively to modulate the cellular response to both alkylating agents and other topoisomerase inhibitors. Studies have shown that cellular sensitivity to DNA alkylating agents correlates with their levels of topoisomerase II activity [181].

ACTIN BINDING DRUGS

The actin cytoskeleton provides a dynamic intracellular scaffolding system that underlies a myriad of cellular functions such as attachment, motility, intracellular trafficking, cytokinesis, and cellular movements. The actin cytoskeleton is a dynamic network of filamentous actin polymer (F-actin) which is composed of monomeric 43 kDa actin (G-actin). ATP-bound G-actins bind to barbed end of F-actin faster than to pointed-end, whereas ADP-actins which result from hydrolysis inside F-actin are released from pointed-end faster

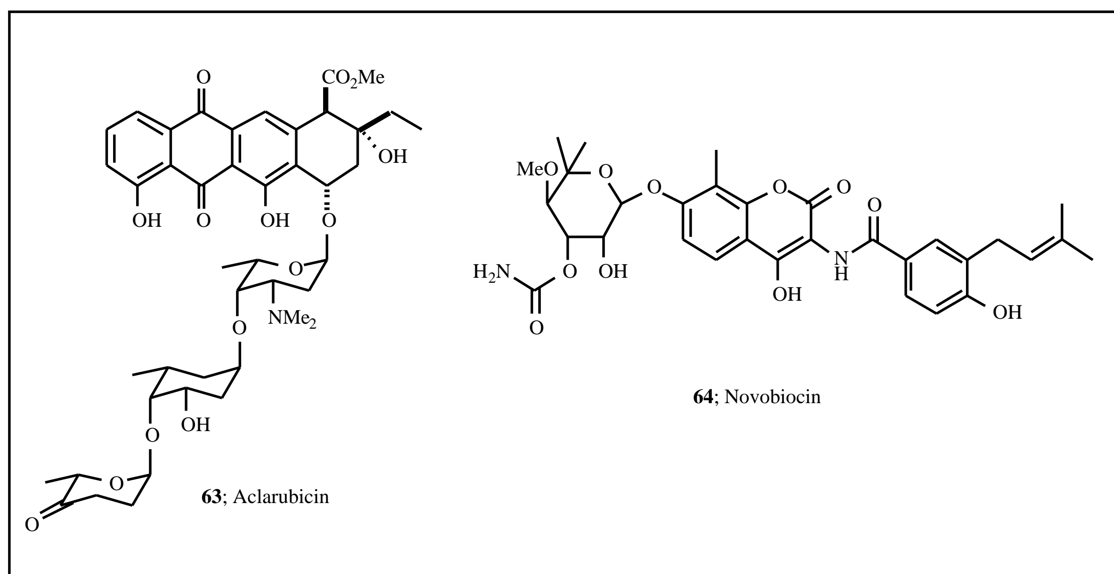


Fig. (10). Topoisomerase inhibitors.

than from barbed end of F-actin. The dynamic equilibrium between F- and G-actins is controlled by various actin binding proteins [182]. The resulting "treadmilling" provides a mechanism by which the actin cytoskeleton can morph depending on cellular needs. For example, actins are key components of the cleavage furrow which progressively deepens until a mother cell completes cytokinesis [183, 184]. Drugs that interfere with actin dynamics clearly have the potential to block the proliferation as well as the motility of cancer cells [185, 186]. There are quite a few members of natural actin-targeting compounds, which can be classified into two categories in analogy to tubulin modulators [187]: those that induce actin polymerization and stabilize filaments and those that disrupt or disassemble actin arrays and destabilize actin filaments. Some of the examples are depicted below [188-206].

HISTONE DEACETYLASE (HDAC)

Histone deacetylase (HDAC) inhibitors target the family of enzymes that catalyze the hydrolysis of acetyl-lysine residues of proteins. The HDAC family consists of eleven enzymes that are intimately involved in the control of gene expression and hence have the potential to be therapeutic targets. Three classes of HDAC enzymes have been identified in humans based on the homology of their catalytic domains to the yeast HDAC enzymes. Class I HDACs (HDAC1, 2, 3, 8 and 11) are homologous to the yeast HDAC Rpd3, Class II HDACs (HDAC4, 5, 6, 7, 9 and 10) are homologous to the yeast HDAC Hda1, and Class III HDACs (SirT1-7) are homologous to yeast Sir2 and are structurally distinct from the other HDACs. Class II HDACs are further divided into two subclasses based upon domain structure: Class IIa consists of HDAC4, HDAC5, HDAC7 and HDAC9, which contain an *N*-terminal non-catalytic protein interaction domain and a C-terminal catalytic domain, while Class IIb consists of HDAC6 and HDAC10, which contain two catalytic domains. The catalytic domains of HDACs are highly conserved and the active site consists of a gently curved tubular pocket with a wider bottom. Acetyl hydrolysis is catalyzed by a charge-relay system consisting of two adjacent histidine residues, two aspartic acid residues and a Zn^{2+} cation.

HDACs are known to mediate the activity of oncogenic transcription factors and have been shown to be overexpressed in a variety of cancers such as gastric and prostate [207-209].

Reduction in the amount of acetylation through the action of HDACs can result in tighter electrostatic association between DNA and nucleosomes which reduces accessibility for transcription factors leading to repressed transcription (Fig.

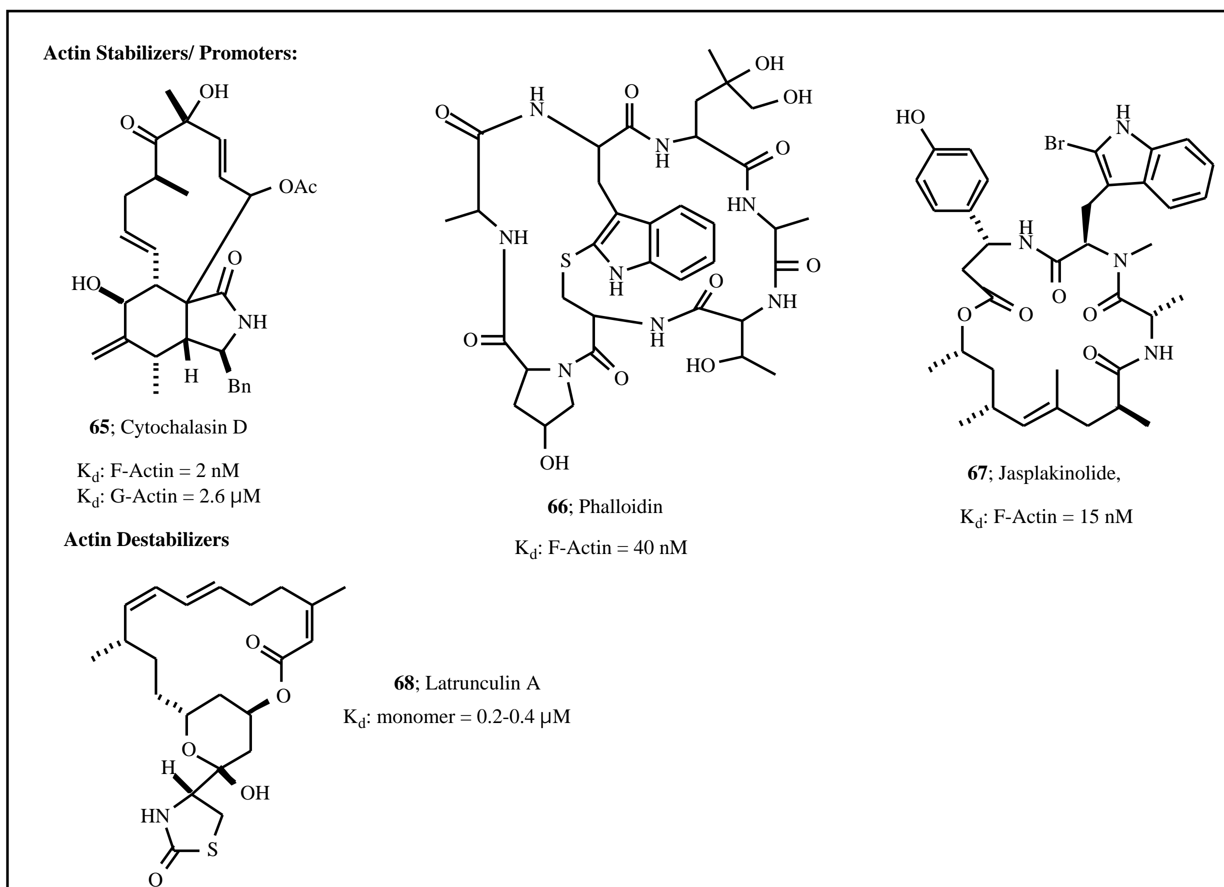


Fig. (11). Inhibitors of actins.

12) [210]. Conversely, blocking histone deacetylation with HDAC inhibitors promotes the accumulation of acetylated histones, which can activate the expression of genes that inhibit transformed cell growth. Although, histone hyperacetylation is induced by HDAC inhibitors in both normal and transformed cells [211, 212], transformed cells appear to be more sensitive to their antiproliferative effects [213].

One such growth suppressing gene upregulated by most HDAC inhibitors is the cell cycle kinase inhibitor $p21^{WAF1}$. Chromatin immunoprecipitation studies have demonstrated that the HDAC inhibitor induced expression of $p21^{WAF1}$ correlates with histone hyperacetylation of its promoter region [214]. Moreover, a link between reduced HDAC activity and increased $p21^{WAF1}$ expression has recently been supported by the finding that proliferation-defective HDAC1-null stem cells display elevated levels of $p21^{WAF1}$ relative to their wild-type counterparts [215, 216].

In addition to their well documented effects on transcription, HDAC inhibitors also affect cell cycle progression by altering the ability of tumor cells to enter and exit mitosis [217, 218]. Histone acetylation is tightly regulated during the cell cycle and may be important for proper deposition of histones during DNA synthesis and chromosome segregation during mitosis. For example, an increase in acetylated histones during S and G2 phase could activate a G2 checkpoint and induce G2 arrest. Loss of this checkpoint, a frequent event in cancer cells, may result in the inability to arrest in G2 phase, leading to aberrant mitosis and induction of apoptosis.

HDAC INHIBITORS

The HDAC inhibitors are classified based on their chemical structure namely: carboxylates, small benzamides, hydroxamates, cyclic peptides and electrophilic ketones. Although these compounds are structurally diverse, they have common pharmacophore that consists of a metal-binding domain, a linker domain and a surface recognition domain. The benzamides, which are in general less potent than the hydroxamates and cyclic peptides, include MS-275 (**69**) and CI-994 (**70**) [219, 220]. These agents typically inhibit HDAC activity at micromolar concentrations. MS-275 is under evaluation in phase II clinical trials and CI-994 has been evaluated in clinical trials.

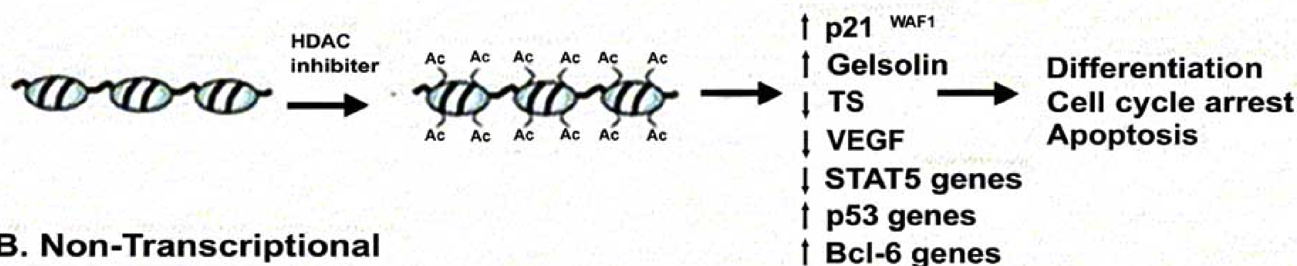
Carboxylates

Butyric acid (BA; **71**) is generated by metabolism of fatty acids and bacterial fermentation of fiber in the colon, and has long been known to be an antiproliferative and differentiating agent [221]. The first report of anticancer activity of **71** on solid tumors in 1933 was followed by the observation that it possesses differentiating activity at high micromolar concentrations on several cancer cell types. It was later proven that **71** is an HDAC inhibitor with activity in millimolar range. Other short-chain fatty acid analogs of BA, such as sodium phenylacetate (**72**), sodium phenylbutyrate (**73**) have also been identified as HDAC inhibitors. Despite poor enzymatic inhibitory activity, a number of carboxylates, including butyric (**71**) and phenylbutyric acid (**73**), are in clinical trials for cancer treatment alone and in combination with other agents. The high concentration of BA and analogs needed for clinical use, coupled with poor bioavailabilty and rapid metabolic degradation, has led to the exploration of prodrugs such as **74** and **75**. Tributyrin (**74**) can deliver 3 equivalents of BA upon hydrolysis. These agents show better absorption and have a more favorable metabolic profile than the corresponding parent compounds. Nonetheless, the clinical utility of modestly active members of the carboxylate class is under investigation and promising results have been obtained [222, 223].

Hydroxamic Acid & Cyclic Peptides

Hydroxamic acid scaffold based molecules were among the first compounds and the largest class of compounds to be identified as histone deacetylase inhibitors. Trichostatin (**76**), a natural product isolated from a *Streptomyces hygroscopicus* strain, was originally identified as a fungistatic an-

A.Transcriptional



B. Non-Transcriptional

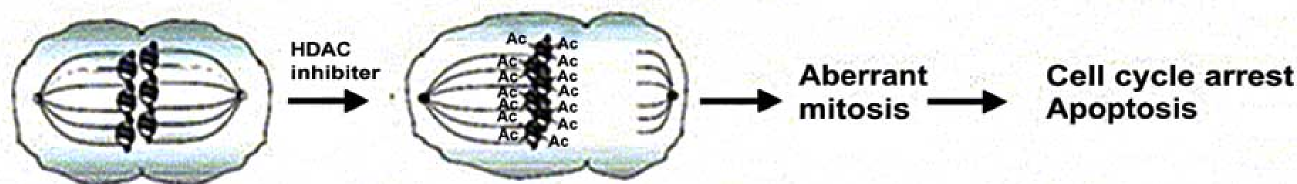


Fig. (12). Mechanism of action for HDAC inhibitors.

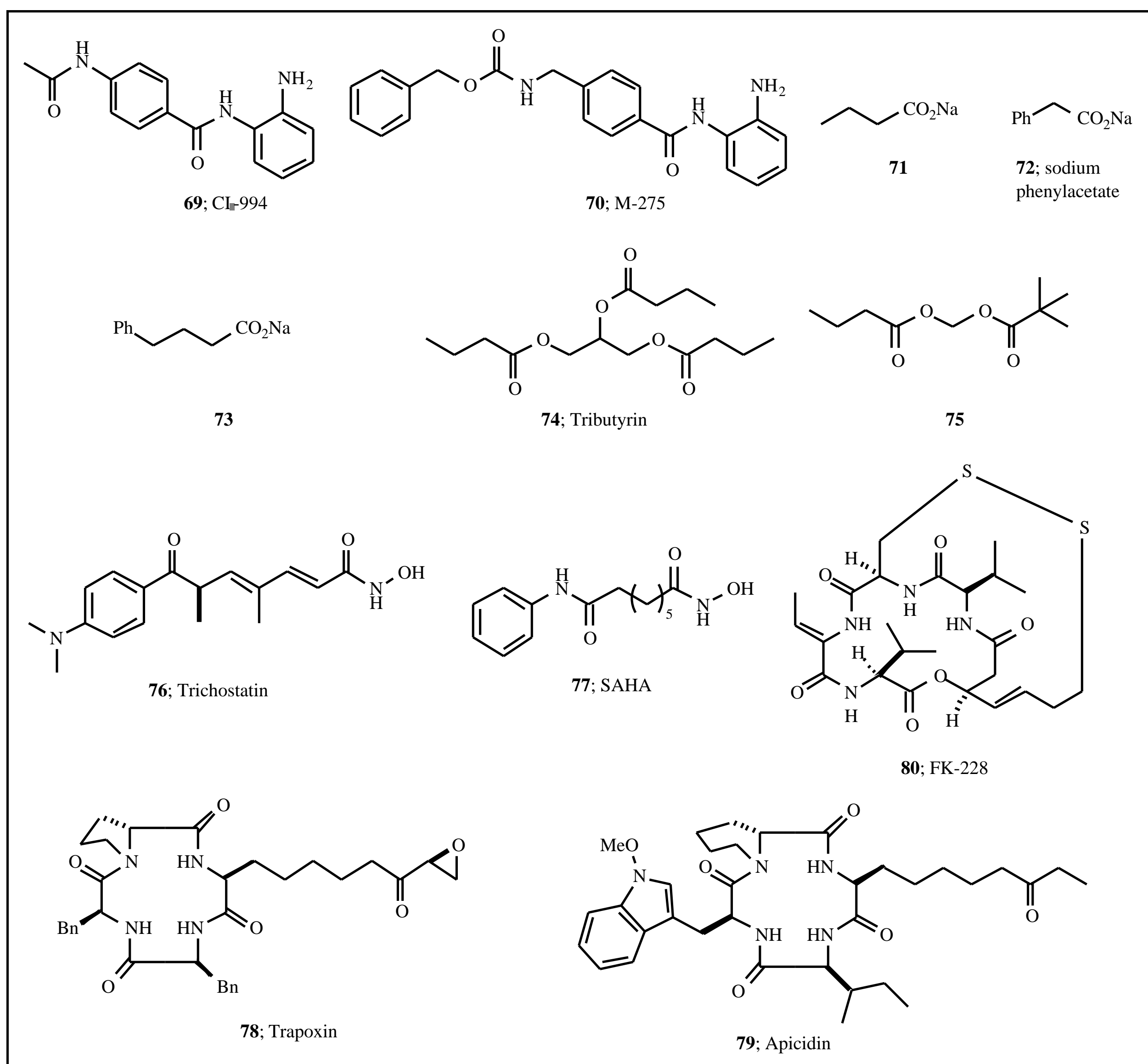


Fig. (13). HDAC inhibitors.

tibiotic in 1976, and its activity was later linked to HDAC inhibition by Yoshida and coworkers in 1990. Extensive SAR studies have led to the development of SAHA (**77**) (IC_{50} = 120 nM), which is currently in phase II clinical trials.

Cyclic peptides constitute the most structurally complex class of HDAC inhibitors and are classified into two subclasses: inhibitors bearing (*S*)-2-amino-9, 10-epoxy-8-oxodecanoic acid (L-Aoe), which contains an epoxy ketone (e.g. Trapoxin; **78**), and inhibitors without the L-Aoe moiety (e.g. Apicidin; **79**). However, the mechanism of action differs between the Aoe-containing inhibitors, which are typically classified as irreversible HDAC inhibitors, and those without the Aoe moiety, which are reversible inhibitors. FK228 (**80**), was discovered from fermentation broth of *Chromobacterium violaceum*. FK228 contains a unique bicyclic structure with four amino acids and a -hydroxyamide moiety, which collectively form a 16-membered lactone with a disulfide bridge. The members of the cyclic peptide class typically possess nanomolar levels of HDAC inhibitory activity. FK228 is currently the only member of the cyclic peptide class under clinical investigation [221].

KINESINS

Kinesins are a super family of motor proteins that couple ATP binding and hydrolysis with force production for unidirectional movement along microtubules. The kinesin super family consists of > 90 members with 30 human kinesins identified so far. Kinesins can be broadly categorized into those involved in transport and those involved in mitosis. The transport kinesins are involved in cytosolic movements and local organization of organelles, while mitotic kinesins are involved in mitotic spindle assembly, chromosome

alignment, segregation and microtubule depolymerization. Eg5 and its human homologue (KSP) are responsible for continuous force production required for maintaining the tension of the bipolar spindle. Inhibition of the motor activity of Eg5 with specific antibodies results in the collapse of bipolar spindles and gives rise to mono polar spindles called monasters. Monastrol (**81**), discovered through a phenotypic screen using mitotic extracts, was reported to be the first small molecule inhibitor of Eg5 [224, 225]**.** This agent was found to inhibit ATPase activity of Eg5 in a non-competitive fashion and had no effect on tubulin polymerization.

Adocia sulfate (AS-2*,* **82**) was isolated from marine sponge *Haliclona* and competitively inhibits interaction of the kinesin motor domain with microtubules at micromolar concentrations [226]. Although AS-2 was apparently specific for kinesins as an enzymatic class, it did not appear to exhibit any selectivity between the three different kinesins surveyed.

MICROTUBULE ASSOCIATED PROTEINS

Microtubule assembly/disassembly is controlled by a number of microtubule-stabilizing and destabilizing proteins that bind along the microtubules [227]. MAPs (microtubule associated proteins) are a group of proteins that stabilize microtubules against disassembly [228-230]. MAPs are targets for protein kinases, including Cdk1, which control changes in microtubule dynamic properties at the G2 to M phase transition of the cell cycle. Phosphorylation of MAPs increases during mitosis and reduces their affinity for microtubules, thereby reducing their ability to promote microtubule polymerization [231]. Major members of Map family are Map1, Map2, Map4 and Tau. Amongst them, Map4 is the most abundant Map in nonneuronal cells that stabilizes microtubules [230]. MAP4 phosphorylation by the Cdk1/CycB complex decreases its microtubule stabilizing ability at mitosis [232].

Tryprostatin-A (**83**) is a secondary metabolite produced by *Aspergillus fumigatus* and is the first natural compound that inhibits microtubule assembly by interfering with the interaction between Maps and the C-terminal domain of tubulin. The inhibition mechanism of tryprostatin-A differs from other well-known microtubule inhibitors, such as colchicine or vinblastine by the fact that it only inhibits Map2-, tau-, and poly-L-lysine-induced tubulin polymerization, but not that induced by paclitaxel or glutamate. In contrast, conventional tubulin inhibitors such as colchicine and vinblastine inhibit tubulin polymerization induced by endogenous maps as well as pharmacological stabilizers. Estramustine Phosphate (**84**) [233], a synthetic antimitotic agent, has been found to alter dynamic stability of MTs by binding to the MAPs, specifically MAP-2. This compound also caused drug induced apoptosis in the human malignant glioma cell line [234].

FUTURE PERSPECTIVES

Rapid cell proliferation of cancerous cells continues to be an attractive phenotype to antagonize with small molecules. Significant progress has been made in the discovery and development of agents that target the core mitotic machinery (tubulin, actin, proteosome, etc). Tremendous advancements have been made in the last decade in understanding the diverse molecular mechanisms underlying neoplastic transformation such as identification of aberrantly activated oncoproteins through point mutations (V599E b-raf in melanoma), chromosomal rearrangements (Bcr-Abl kinase in CML), overexpression (Ras G-protein) and loss of tumor suppressors (PTEN phosphatase). The challenge for the scientific community is to identify which of these primary oncogenic events and their associated downstream consequences can be addressed by small molecule inhibitors. A further challenge is to evolve these pharmacological tool compounds into agents that can be investigated for their

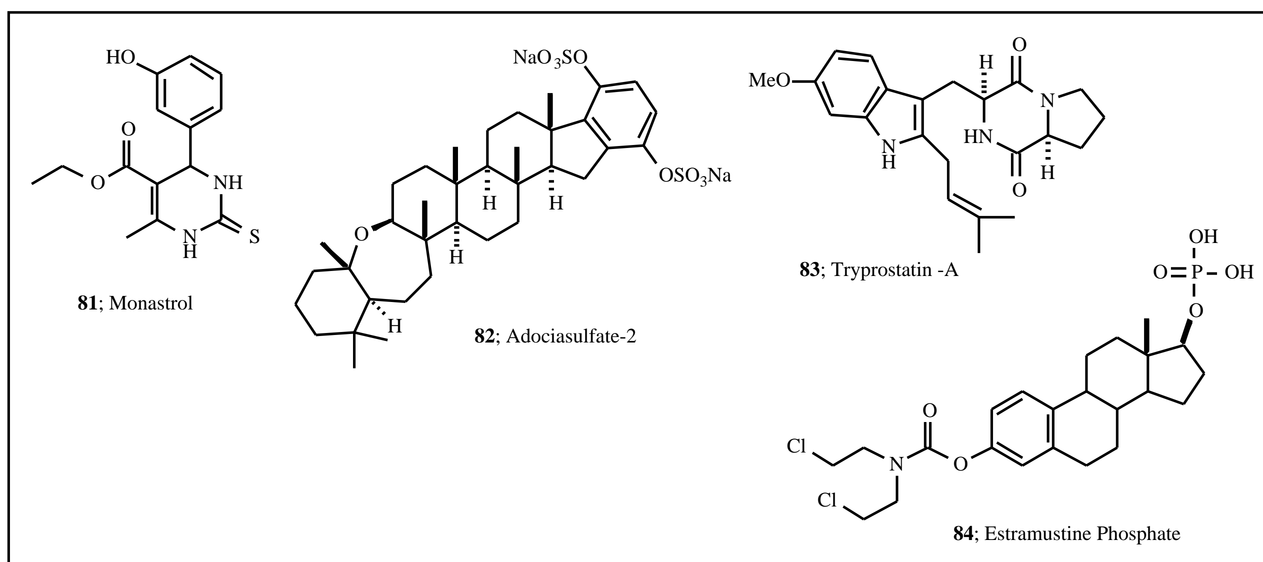


Fig. (14). Inhibitors of kinesins and MAPs.

therapeutic potential in the clinic. Increased access to promising targets through genome-wide siRNA and cDNA screening combined with access to high-throughput screening of small molecule libraries should enable the academic community to identify target-ligand pairs that will hopefully pique the interest of pharmaceutical and biotechnology industry that have the challenge to translate these findings to the clinic.

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Received: September 12, 2005 **Accepted: October 4, 2005**

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