Published in final edited form as:

Curr Med Chem. 2004 July ; 11(13): 1725–1756.

Mechanism Targeted Discovery of Antitumor Marine Natural Products

Dale G. Nagle^{*}, Yu-Dong Zhou, Flor D. Mora, Kaleem A. Mohammed, and Yong-Pil Kim

Department of Pharmacognosy, National Center for Natural Products Research, and Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, Mississippi, 38677-1848, U.S.A

Abstract

Antitumor drug discovery programs aim to identify chemical entities for use in the treatment of cancer. Many strategies have been used to achieve this objective. Natural products have always played a major role in anticancer medicine and the unique metabolites produced by marine organisms have increasingly become major players in antitumor drug discovery. Rapid advances have occurred in the understanding of tumor biology and molecular medicine. New insights into mechanisms responsible for neoplastic disease are significantly changing the general philosophical approach towards cancer treatment. Recently identified molecular targets have created exciting new means for disrupting tumor-specific cell signaling, cell division, energy metabolism, gene expression, drug resistance, and blood supply. Such tumor-specific treatments could someday decrease our reliance on traditional cytotoxicity-based chemotherapy and provide new less toxic treatment options with significantly fewer side effects. Novel molecular targets and state-of-the-art molecular mechanism-based screening methods have revitalized antitumor research and these changes are becoming an ever-increasing component of modern antitumor marine natural products research. This review describes marine natural products identified using tumor-specific mechanism-based assays for regulators of angiogenesis, apoptosis, cell cycle, macromolecule synthesis, mitochondrial respiration, mitosis, multidrug efflux, and signal transduction. Special emphasis is placed on natural products directly discovered using molecular mechanism-based screening.

Keywords

molecular-target; anticancer; mechanism-based assays; screening methods

INTRODUCTION

More than 60% of the currently used cancer chemotherapeutic agents are natural products or small molecules based-upon natural product leads [1]. The first generation of anticancer therapeutic drugs were approved more than 50 years ago. With the exception of a handful of hormone-based therapies, the majority of small molecule-based cancer chemotherapeutic drugs developed over this last half century have been cytotoxic agents. It can be argued that a moderate degree of selectivity has been achieved with nonspecific cytotoxic compounds when used to treat certain forms of leukemia, lymphoma, and other malignancies that result from the proliferation of rapidly dividing cells. However, the lack of “true” tumor cell specificity and the extremely narrow therapeutic margins associated with most cytotoxic

dnagle@olemiss.edu, Telephone: 662-915-7143, FAX: 662-915-6975.

chemotherapeutic agents limit the value of these agents in the treatment of many solid tumors.

It is commonly thought that the reason so many cytotoxic natural products have been discovered from plants, microbes, and marine invertebrates is due to ecological evolutionary pressures that favor the production of cytotoxic chemical defenses [2,3]. While this conclusion may have some validity, likewise it is only logical to conclude many cytotoxic compounds have been identified because of the prevalence of cytotoxicity-based bioassay screening methods among natural products research programs. This trend is especially true in terms of *in vitro* cell-based screening technologies that have been the mainstay of many antitumor drug discovery efforts [4]. Unfortunately, this would suggest that one reason we still depend so heavily on relatively toxic chemotherapeutic agents may be, at least in part, a result of selecting cytotoxicity as the primary anticancer drug target. As a consequence, antitumor drug discovery teams have perhaps relied too heavily upon rather nonselective cytotoxicity-based bioassay screening strategies.

This is not to say that efforts have not been made to identify antitumor compounds with desirable therapeutic margins. For example, screening methods at the National Cancer Institute (NCI-NIH/USA) have been devised to identify antitumor agents with selective cytotoxic activity against tumor cells, relative to normal cells, or specific cytotoxic activity against certain types of tumors, relative to others [5]. The NCI 60-cell line screen COMPARE algorithm of tumor cell cytotoxicity has been used to establish a Pearson correlation coefficient for individual antitumor compounds, that is used to correlate observed tumor cell selectivity and possible mechanism/site of action. By using COMPARE analysis, the NCI has focused on the discovery of new cytotoxic compounds that function through novel mechanisms, distinct from other known antitumor agents. The unique tumor cell *in vitro* cytotoxicity patterns associated with specific crude extracts or purified compounds have been examined and used to provide valuable clues as to the potential mechanisms of action of antitumor natural products and to identify new antitumor molecular targets. Over the past two decades, dramatic progress has been made in understanding tumor cell biology at the molecular level [6–8]. While tumor cell cytotoxicity-based methods have had a certain degree of success, as measured by the discovery of several new antitumor agents, these approaches only indirectly reflect the considerable potential of designing new molecular targeted techniques to specifically target the unique physiological, biochemical, and molecular differences between normal and malignant cells.

Molecular targeted drug discovery and its application to marine natural products are continuously evolving disciplines. Significant changes have occurred, both in terms of specific antitumor molecular targets and the methods used to screen samples. Once the phrase “mechanism-based assays” primarily referred to colorimetric enzyme inhibition assays and other related screens where a biochemical model system could easily be constructed with existing technologies of the time [9,10]. However, many of these mechanism-based assays were only crude representations of actual tumor cell systems and were often plagued by “false positive hits” that resulted from active compounds that were non-specific, too toxic, or did not adequately penetrate cell membranes. In addition, many substances were for all practical purposes, “nuisance” compounds that interfere with the functioning or measurement of these assays. As molecular and cell biology has advanced and become more complex, so have the technologies used to discover new molecular targeted antitumor agents. High-throughput *in vitro* cell-based molecular targeted bioassays and assay systems designed to examine the specific interactions between small molecules and target proteins are now among the most widely used methods in antitumor drug discovery, especially in the highly competitive industrial pharmaceutical arena [7,11]. These modern high-throughput screening programs typically require collaborative research efforts

between chemists, including marine natural products chemists, and molecular or cell biologists and are often a consequence of joint academic and industrial drug discovery programs [12].

Since a considerable portion of mechanism-based antitumor natural products drug discovery is a component of industrial research or part of collaborative academic-industrial programs, much of the research remains proprietary in nature [13]. Therefore, a comprehensive discussion of the results from many molecular targeted antitumor marine natural products programs is not feasible. Rather, this review endeavors to describe examples of published antitumor mechanism-based assays used by various research groups. In addition, this review highlights interesting marine natural products discovered using molecular-targeted assays. However, this review is formatted in terms of several specific antitumor molecular targets that have been the focus of marine natural products-based discovery programs. Due to the interdependent nature of nearly all aspects of tumor cell biology, the organizational format may seem somewhat subjective and perhaps arbitrary. For example, it is nearly impossible to separate discovery methods that focus on interfering with mitosis from those that target the tumor cell cycle. Significant overlap also exists between those agents that regulate the induction of apoptosis that can result from interruption of microtubule integrity or cell cycle checkpoints. However, as often as possible, the methods and results of marine natural products programs are discussed in relation to the specific antitumor targets identified by the originating researchers.

ANGIOGENESIS

Angiogenesis, the formation of new blood vessels from the existing vasculature, is a highly complex, yet tightly controlled process [14]. Angiogenesis is essential for embryogenesis, yet rarely occurs in adults (usually only in the event of tissue repair and remodeling). Pathologic angiogenesis has been implicated in the etiology of a variety of diseases, including solid tumors, ocular degenerative conditions, and inflammatory disorders [reviewed in 15,16]. Tumor-induced angiogenesis is critical for the growth, progression and metastasis of solid tumors. Tumors cannot grow beyond the maximal size of 1–2 mm³ without angiogenesis. High metabolic demands by fast growing tumors and the inadequate capability of often-disorganized tumor vasculature create a positive feedback loop, that promotes a constant pro-angiogenic state in tumors. These newly formed tumor blood vessels provide the cancerous growth with an increased supply of oxygen and nutrients, and a passage for metastasizing tumor cells to enter the bloodstream.

The past three decades have witnessed the conception and development of anti-angiogenic therapeutic options that target the tumor blood supply (vasculature) for the treatment of cancer [reviewed in 17–19]. Anti-angiogenic therapy could offer fewer side effects and reduced development of drug resistance, relative to traditional cytotoxicity-based chemotherapeutic approaches. When combined with current treatment options (surgery, radiation, and chemotherapy), anti-angiogenic therapy could also enhance treatment outcomes. Discovery of angiogenesis inhibitors has targeted multiple aspects of the angiogenic cascade, from inhibiting the functioning of pro-angiogenic factors, to the discovery of enzyme inhibitors for matrix metalloproteinases (MMPs) that break down the extracellular matrix. Encouraging results obtained from animal studies have landed a number of antiangiogenesis agents into clinical trial for the treatment of cancer. According to the U.S. National Cancer Institute (NCI) database, angiogenesis inhibitors that are currently undergoing clinical trial are classified into five functional groups: 1) agents that block matrix breakdown (marimastat, COL-3, and BMS-275291); 2) agents that directly inhibit endothelial cells (thalidomide, squalamine, and 2-methoxyestradiol); 3) agents that block the activation of angiogenesis (SU6668, Neovastat, interferon-alpha, and anti-VEGF

antibodies); 4) agents that inhibit endothelial-specific integrin signaling (Medi-522, and EMD121974); and 5) agents with non-specific mechanisms of action (CAI, Celecoxib, interleukin-12, and IM862) [20].

There have been a number of natural product drug discovery efforts directed at finding novel inhibitors of angiogenesis. Natural products from plants and microorganisms that have shown anti-angiogenic activities *in vivo* have been reviewed by Paper [21]. Herein, we describe the discovery of angiogenesis inhibitors from marine organisms. Some of these compounds have shown activity against a particular *in vitro* target, while others are active against several processes *in vitro* and have been demonstrated to inhibit angiogenesis *in vivo*.

Tumor micro-environmental factors (such as hypoxia) trigger the production and release of angiogenic stimuli by tumor cells. These angiogenic factors increase the permeability of existing vessels and activate endothelial cells. Inhibiting the production/function of angiogenic factors represents a strategy that targets the early steps of the angiogenic cascade. Vascular endothelial growth factor (VEGF) is a key angiogenic factor used by tumors cells to promote angiogenesis [22]. Using a reporter assay for inhibitors of hypoxic activation of VEGF promoter, a new indanone, Fig. (1), from the marine cyanobacterium *Lyngbya majuscula* was found to be active ($IC_{50} = 25 \mu M$) [23]. However, due to the complex multi-level regulation of VEGF gene expression, this compound did not significantly inhibit the hypoxic induction of secreted VEGF protein in tumor cells.

Thymidine phosphorylase is an enzyme that catalyzes the reversible phosphorolysis of thymidine to thymine, and is also known as the angiogenic factor platelet-derived endothelial cell growth factor (PD-ECGF). Inhibitors of thymidine phosphorylase have been shown to inhibit angiogenesis and suppress tumor growth *in vivo* [24]. Using an HPLC-based enzyme assay for inhibitors of thymidine phosphorylase, the active compounds lembahsterol A and lembahsterol B, Fig. (1), were isolated from an Indonesian collection of the sponge *Petrosia strongylata* [25]. These compounds are rather weak inhibitors of thymidine phosphorylase ($IC_{50}s = 41$ and $45 \mu M$, respectively), relative to the synthetic thymidine phosphorylase inhibitor 5-chloro-6-[1-(2-iminopyrrolidinyl) methyl] uracil hydrochloride ($IC_{50} = 12$ nM). However, these marine natural products represent the first non-nucleoside thymidine phosphorylase inhibitors discovered.

Endothelial cells are typically dormant under normal conditions. Upon activation by angiogenic factors released by tumor cells, endothelial cells begin to proliferate and migrate towards the tumor, and form new capillary tubes. Using a bovine aorta endothelial cell-based (BAEC) proliferation assay as the primary assay for discovering inhibitors of endothelial cell proliferation, three active inhibitors were isolated from a Japanese collection of the bryozoan *Watersipora subtorquata* [26]. Bioassay-guided fractionation of the bryozoan acetone extract yielded bryoanthrathiophene, 5,7-dihydroxy-1-methoxycarbonyl-6-oxo-6*H*-anthra[1,9-*bc*]thiophene, and 1,8-dihydroxyanthraquinone. Bryoanthrathiophene, Fig. (1), the most active compound, also reduces BAEC tube formation *in vitro*. However, since the authors describe only the quantity of each compound added to each culture in terms of μmol , no concentration-based potency/efficacy or true IC_{50} was established for these compounds.

Efforts in search of marine extracts and purified compounds that inhibit endothelial capillary tube formation *in vitro* have resulted in the isolation of the known compound (+)-aerophysinin-1 from the sponge *Aplisina aerophoba* [27]. Aerophysinin-1, Fig. (1), is a brominated tyrosine metabolite first isolated from the sponge *Verongia aerophoba* for its antibacterial activity [28]. The antiangiogenic activity of (+)-aerophysinin-1 was further

examined in a series of assays. *In vitro* studies showed that aeroplysinin-1 inhibits endothelial cell growth, induces apoptosis in endothelial cells, and inhibits both endothelial cell migration and invasion. *In vivo* studies demonstrated that aeroplysinin-1, at lower doses (3 nmol/egg), inhibits angiogenesis in the chorioallantoic membrane (CAM) assay. At higher doses (6 nmol/egg), aeroplysinin-1 causes the disorganization of preexisting vessels.

Using a similar *in vitro* endothelial tube formation assay, acalcycixenolide E, Fig. (1) (isolated from a Korean collection of the gorgonian *Acalycigorgia inermis*) was found to be an inhibitor of tube formation at 1 µg/mL (2.5 µM) [29]. In addition, acalcycixenolide E was also shown to inhibit endothelial cell proliferation, migration, and invasion *in vitro*, and angiogenesis in the *in vivo* CAM assay (10 µg/egg).

Breakdown of the basal membrane surrounding capillaries, invasion of the stroma, and formation of a provisional fibrin matrix pave the pathway for activated endothelial cells to migrate toward tumor masses. Metalloproteinases (MMPs) facilitate the breakdown of the extracellular matrix. Therefore, inhibitors of MMPs represent an important class of angiogenesis inhibitors. Fusetani and coworkers at the University of Tokyo recently evaluated extracts of marine invertebrates for MMP inhibitors using a microtiter assay with purified recombinant MMP2 protein and a fluorescent MMP2 substrate. The new alkyl sulfate metabolite sodium 1-(12-hydroxy) octadecanoyl sulfate, Fig. (1), was isolated from a Japanese collection of an unidentified tunicate and found to be a MMP2 inhibitor with an IC₅₀ of 9.0 µg/mL (25 µM) [30]. Analysis of the (*R*)- and (*S*)-MTPA Mosher esters of sodium 1-(12-hydroxy)octadecanoyl sulfate by ¹H NMR indicated that the product isolated was nearly racemic (*R* 55%: *S* 45%). In order to assess if the chirality of the 12-hydroxy moiety plays a role in the bioactivity, chiral sodium 1-(12(*R*)-hydroxy)octadecanoyl sulfate was prepared from 12(*R*)-hydroxystearic acid. However, a synthetically produced chiral compound and the nearly racemic natural product were equally potent. Thus, suggesting that the chirality of the hydroxyl group does not affect the bioactivity.

The modification of cell adhesion, breakdown of extracellular matrix, and the migration of cells are all required for tumor cell invasion, angiogenesis, and metastatic spread. Using a three-step screening assay for inhibitors of tumor cell invasion, motuporamines A, B, C, and an unresolved mixture of motuporamines G, H, and I, were isolated from the marine sponge *Xestospongia exigua* and found to inhibit the invasion of tumor cells into basement membranes [31,32]. The structures of the motuporamines are shown in Fig. (1). The quantitative three-step invasion inhibitor assay (developed by Roberge and coworkers at the University of British Columbia) can be used to specifically screen for compounds that inhibit the invasion of breast carcinoma MDA-MB-231 cells into Matrigel (reconstituted basement membrane) without preventing cell attachment or killing cells [28]. In addition to inhibiting tumor cell invasion, the most potent compound, motuporamine C also inhibits endothelial sprout formation *in vitro* (complete inhibition at 10 µM) and angiogenesis *in vivo* (CAM assay with 30 ng/mL VEGF stimulated angiogenesis, complete inhibition at 10 µM). Structure-activity studies on a series of natural and synthetic motuporamine analogues revealed that the maximum anti-invasion activity of this alkaloid family requires a saturated monocyclic amine with a ring size of 13, 14, or 15, fused to a linear side chain with two basic nitrogen atoms, each separated by three methylene carbons [32].

APOPTOSIS

Apoptosis represents a universal and efficient form of cell death that is executed through a highly ordered intrinsic cellular suicide program. It is important to a variety of normal processes such as fetal development, tissue homeostasis, immune response, aging, etc. Dysregulation of apoptosis (either up- or down-regulation) has been shown to play a role in

about one-half of the major diseases that still lack of adequate therapy or means to prevent [reviewed in 33]. Mutations that cause uncontrolled cell growth and those that lead to insufficient cell death occur commonly in neoplasia and contribute to the etiology of cancer. Inadequate apoptosis has been implicated in a number of conditions that are important to the pathogenesis and progression of tumors, that include: 1) a permissive environment that promotes oncogene activation and tumor suppressor gene inactivation; 2) excessive cell accumulation due to the failure of programmed cell death; 3) tumor cells acquire the ability to evade elimination by the immune system; 4) enhanced tumor cell survival under environmental stresses such as the lack of oxygen (hypoxia) and nutrients; 5) tumor cell survival in a suspended state that is required for metastasis; and 6) resistance to chemotherapy and radiation treatment [reviewed in 34,35].

Intracellular triggers such as DNA damage can activate apoptosis. In addition, apoptosis can also be activated by extracellular cues such as the withdrawal of growth factors, exposure to cytokines, environmental stress (such as treatment with chemotherapeutic agents or radiation), and detachment from the extracellular matrix [34]. Apoptotic cells are characterized by morphological criteria such as the breakdown of the nuclear membrane, chromatin condensation, DNA fragmentation, cell shrinkage and the formation of apoptotic bodies. Caspases are a group of cysteine proteases that play a key role in the regulation and execution of apoptosis [reviewed in 33–36]. These enzymes are initially produced as inactive zymogens. Upon activation by apoptotic signals, the initiator caspases cleave and activate the effector caspases, which then either directly cleave cytoskeletal and nuclear substrates or activate other downstream degradation enzymes. Cellular apoptotic machinery is regulated by a group of proapoptotic and antiapoptotic molecules at nearly every level along the signaling pathway.

Elucidation of the apoptotic pathways and an increased understanding of the importance of apoptosis in the development and progression of cancer have provided the impetus for the development of apoptosis-targeted therapies. Many cytotoxic compounds induce apoptosis. However, the apoptosis-based therapeutic agents currently in pre-clinical development and clinical trial target specifically defined apoptotic pathways [reviewed in 33,37]. Such molecular targeted therapeutic agents are expected to specifically target the tumor cells with minimal toxicity to normal cells and reduced side effects. Very few marine natural products have been identified through direct apoptosis-targeted screening. However, one example is the discovery of orostanol, a sterol derivative isolated from a Japanese collection of the marine sponge *Stelletta hiwasaensis* [38]. Orostanol, Fig. (2), was identified as the active constituent of an extract of *S. hiwasaensis* that induced chromatin condensation and DNA fragmentation in HL-60 cells. Orostanol induced apoptosis at 10 µg/mL, and inhibited cell growth ($IC_{50} = 1.7 \mu M$). However, no detailed biological characterization of orostanol has been reported.

A number of marine natural products that induce apoptosis were first identified as cytotoxic agents, and their apoptotic activity later discovered in secondary pharmacological evaluations. Bryostatin 1, Fig. (2), is a macrocyclic lactone isolated from the marine bryozoan *Bugula neritina* as a potent antineoplastic agent [39]. Considerable research has established bryostatin 1 as a modulator of protein kinase C (PKC) and this compound is currently undergoing clinical trials for cancer [reviewed in 40]. In addition to activating PKC and down-regulating PKC gene expression, bryostatin 1 induces apoptosis and enhances the apoptotic response produced by other drugs. At relatively low concentrations (10^{-11} to 10^{-7} M), bryostatin 1 does not induce apoptosis in the human promyelocytic leukemia HL-60 cells [41]. However, in human intermediate-grade lymphoma WSU-DLCL2 cells, bryostatin 1 (2×10^{-7} M) treatment produces DNA fragmentation and stimulates the formation of apoptotic bodies [42]. Bryostatin 1 has been shown to act

synergistically to induce apoptosis *in vitro* when used in combination with other chemotherapeutic agents such as 1-D-arabinofuranosylcytosine [41,43], vincristine [42], and paclitaxel [44].

Compounds that modify the cellular cytoskeleton can also induce apoptosis. The structures of two examples, the marine natural products isohomohalichondrin B and jasplakinolide are shown in Fig. (2). Isohomohalichondrin B is a polyether macrolide isolated from the New Zealand deep-water sponge *Lissodendoryx* sp. [45]. It showed potent cytotoxic activity against murine leukemia P388 cells *in vitro* (IC₅₀ of 0.18 ng/mL). Analysis using the COMPARE algorithm at NCI revealed a significant correlation between isohomohalichondrin B and tubulin-binding agents. Biochemical studies demonstrated that isohomohalichondrin B prevents tubulin assembly by binding to tubulin and inhibiting GTP binding [46]. Exposure to isohomohalichondrin B at low nanomolar concentrations was shown to result in cell cycle perturbations in a panel of human cancer cell lines [47]. Isohomohalichondrin B (at concentrations up to 2 nM) induced apoptosis (judging by morphological changes and DNA fragmentation) in human leukemic K562 and MOLT-4 cell lines. Jasplakinolide is an antifungal cyclodepsipeptide, first isolated from a Fijian *Jaspis* sp. sponge. [48]. It was later reisolated as a tumor cell cytotoxic component in a Papua New Guinea sponge (*Auletta* cf. *constricta*) extract [49]. At low nanomolar concentrations, jasplakinolide induces actin polymerization [50]. Jasplakinolide (2 µg/mL) induces apoptosis in the human T-cell leukemia Jurkat cells by a caspase-3-like protease pathway [51]. Similar effects were also observed in a panel of transformed murine cell lines. Apoptosis was not observed in jasplakinolide-treated normal (non-transformed) murine thymocytes and spleen T-cells.

Certain protein synthesis inhibitors can also trigger apoptosis. Mycalamide A was first isolated from a New Zealand sponge (*Mycalia* sp.) as an antiviral compound [52]. Mycalamide A inhibited protein synthesis and showed potent cytotoxicity (IC₅₀ < 50 nM) against a panel of murine and human tumor cell lines *in vitro*, and moderate efficacy against tumor xenografts *in vivo* [53]. Using a panel of assays for apoptosis (DNA fragmentation, morphological changes, and externalization of phosphatidylserine), mycalamide A (100 nM) was shown to induce apoptosis in a series of tumor cell lines [54]. Pateamine, another metabolite isolated from the same sponge as a potent cytotoxin (IC₅₀ of 0.15 ng/mL against P388 cells) [55], also exhibited apoptosis inducing property similar to that observed for mycalamide A [54]. The structures of mycalamide and pateamine are shown in Fig. (2).

Compounds that regulate protein kinases and phosphatases can indirectly affect the apoptotic process by modulating signal input/transmission to cell-death pathways. The previously discussed marine compound bryostatin 1 is an example of a protein kinase C modulator that induces apoptosis. Another example is the kinase inhibitor scytonemin. Scytonemin is a UV-absorbing sunscreen pigment produced by cyanobacteria [56]. In a screening effort designed to look for inhibitors of a cell cycle kinase human *polo*-like kinase 1, scytonemin was shown to be active, with an IC₅₀ of 2 µM [57]. Scytonemin also inhibits other cell cycle kinases with similar potency. In human T-cell leukemia Jurkat cells, scytonemin inhibited cell proliferation (IC₅₀ = 7.8 µM) and induced apoptosis in 24% of the cells (at 3 µM). Several marine natural product-derived protein phosphatase inhibitors have also been shown to induce apoptosis in tumor cells. Okadaic acid is a polyether isolated independently from two sponges of the genus *Halichondria* - a Japanese *H. okadae* Kadota and a Caribbean *H. melanodocia* [58]. Okadaic acid is a selective inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A) [reviewed in 59] and has been widely used to probe cellular signaling pathways. Calyculin A is another PP1 and PP2A inhibitor [60], first isolated from the marine sponge *Discodermia calyx* [61]. Okadaic acid was shown to induce morphological changes typical of apoptosis in a panel of mammalian cell lines/primary cells

(rat hepatocytes, human and rat tumor cell lines) at concentrations ranging from 0.1 to 1 μM . [62]. An extensive study conducted in rat promyelocytic leukemia IPC cells revealed that both okadaic acid (1 μM) and calyculin A (< 20 nM) induce apoptosis [63]. The effects of phosphatase inhibitors such as okadaic acid on apoptosis have been well studied. One study revealed that *ras*-mutated tumor cell lines are more sensitive to okadaic acid-induced apoptosis [64]. The structures of scytonemin, okadaic acid, calyculin A are shown in Fig. (3).

Thyrsiferyl 23-acetate, Fig. (4), is a cyclic ether that contains a squalene carbon skeleton. Thyrsiferyl 23-acetate was isolated as a potent cytotoxin (ED_{50} of 0.3 ng/mL against P388 cells) from the marine red alga *Laurencia obtusa* collected in Japan [65]. In serum-deprived Jurkat cells, thyrsiferyl 23-acetate (10 μM) induced chromatin condensation and DNA fragmentation, hallmarks of apoptosis [66]. Although thyrsiferyl 23-acetate has been shown to selectively inhibit serine/threonine phosphoprotein phosphatase 2A (PP2A) [67], its apoptotic activity is not dependent on the inhibition of PP2A [66].

The life or death of a cell is the net outcome achieved by the balance between opposing cell death and cell survival pathways. One critical component of the cellular survival signaling is the phosphoinositide 3-kinase (PI3K)/AKT cascade [reviewed in 68]. Over activation of this survival pathway in tumor cells is an important factor that contributes to treatment resistance. One approach to enhance antitumor treatments is to directly suppress tumor cell survival pathways. Halenaquinone, Fig. (4), is a pentacyclic polyketide first isolated from a marine sponge (*Xestospongia exigua*) collected in Palau [69]. Halenaquinone was later isolated from an Okinawan *X. exigua* and found to induce apoptosis in PC-12 cells (EC_{50} = 10 μM), while the closely related compound xestoquinone was inactive [70]. Derived from a rat pheochromocytoma line, PC-12 cells are tumorigenic and respond to nerve growth factor (NGF) by induction of the neuronal phenotype. Nerve growth factor activates the PI3K/AKT pathway in PC12 cells and the activation of PI3K/Akt is inhibited by halenaquinone (PI3K - IC_{50} = 3 μM) [70]. In a separate study using halenaquinone from a Fijian *X. carbonaria*, halenaquinone was shown to irreversibly inhibit the oncogenic protein tyrosine kinase pp60^{v-src} (IC_{50} = 1.5 μM) [71]. Among the marine pentacyclic polyketides tested, halenaquinol inhibited pp60^{v-src} with similar potency (IC_{50} = 0.55 μM); halenaquinol sulfate (IC_{50} = 28 μM) and xestoquinone (IC_{50} = 60 μM) were less active; and tertahydrohalenaquinone A and B were inactive at the concentrations tested (up to 200 μM). The role(s) of halenaquinone and related compounds as protein tyrosine kinase inhibitors is discussed separately. The v-src human homolog c-src is known to be an activator of AKT [72]. One possible scenario is that compounds such as halenaquinone inhibit the activation of survival kinases such as AKT through multiple pathways.

Other marine natural products have also been shown to induce apoptosis. Ascididemin is a pentacyclic aromatic alkaloid first isolated from the Okinawan tunicate *Didemnum* sp. for its cytotoxic activity (L1210 murine leukemia cells - IC_{50} = 0.39 $\mu\text{g/mL}$) [73]. In addition to its role as a DNA-intercalating agent and DNA topoisomerase II poison, ascididemin also induces apoptosis in HL-60 cells at relatively low concentrations (0.5 μM) [74]. The red bacterial pigment cycloprodigiosin hydrochloride was first isolated from cultures of the marine bacterium *Alteromonas rubra* [75]. Cycloprodigiosin was subsequently isolated from *Pseudoalteromonas denitrificans* cultures and shown to inhibit T-cell proliferation, block H⁺ translocation by H⁺-ATPase, and induce apoptosis in phorbol ester-stimulated Jurkat cells [76]. In HL-60 cells, cycloprodigiosin HCl (300 nM) triggered apoptosis in 20% of the cells and differentiation in 60% of the cells [77]. The ability of cycloprodigiosin HCl to induce apoptosis was also observed in human hepatocellular carcinoma cell lines [78], breast cancer cell lines [79], and colon cancer cell lines [80]. Microcolin A is a lipopeptide first isolated from a Venezuelan cyanobacteria *Lyngbya majuscula* [81]. Microcolin A showed

immunosuppressive activity ($EC_{50} = 1.5 \text{ nM}$) in a mixed lymphocyte response assay and cytotoxicity ($TC_{50} = 22.6 \text{ nM}$) in a lymphocyte viability assay. Further studies revealed that higher concentrations of microcolin A (100 nM) induce apoptosis in murine thymocytes, as demonstrated by DNA fragmentation and the formation of apoptotic bodies [82]. The structures of ascididemin, cycloprodigiosin HCl, and microcolin A are shown in Fig. (4).

CELL CYCLE

As molecular probes of tumor cell biology, natural products and natural product-derived small molecules have contributed significantly to our understanding of eukaryotic cell cycle pathways. Several recent reviews clearly describe the physiological and molecular components of the cell cycle and the role natural products, including marine metabolites, have played in defining the tumor cell cycle process [83–86]. It is not the intention of this review to describe the complex components of the cell cycle or the many pharmacological studies that have observed the effects of known natural products on the cell cycle. Rather, this review highlights the contribution of cell cycle-based methods in the discovery of new marine antitumor natural products. The cell cycle is generally considered to contain four major sequential phases: 1) the G_1 gap phase where the cell prepares for DNA replication; 2) the DNA synthesis and replication stage known as S phase; 3) a second gap phase G_2 where the cell prepares for the division process; and 4) mitosis (M phase) where the cell divides into two daughter cells, each with one copy of the two identical sister chromatids formed in S phase. However, three other cellular states can occur. When deprived of nutrients and growth factors, cells may enter a resting state known as G_0 , where cellular metabolism slows [87]. By removing many of the molecular targets (protein synthesis, DNA replication, and tubulin-assisted process) the prevalence of tumor cells in the G_0 state is thought to “shield” these cells from the effects of many cytotoxic chemotherapeutic agents [88]. In addition, cells can go into a non-dividing differentiated state. Finally, apoptosis or programmed cell death can be considered another cell state that occurs when cells are exposed to certain stresses or specific cellular signals [89]. Research efforts aimed at discovering marine natural products that directly induce tumor cell apoptosis are discussed in a separate “apoptosis” section.

Eukaryotic cell proliferation is a highly synchronized process that involves a coordinated and intricately balanced system of both positive and negative regulatory control mechanisms. These cellular regulatory processes are controlled by signals from inside the cell, such as those produced by modifications in gene expression that respond to the condition of DNA synthesis or damage [90,91]. In addition, hormones, cytokines, and growth factors produced outside the cell also regulate the proliferation of eukaryotic cells [92,93]. The net accumulation of effects that tips this regulatory towards a state of uncontrolled cell growth can result in the formation and progression of malignant cells. This elaborate cellular regulatory system provides many opportunities for abnormal proliferative responses that can lead to oncogenic processes. On the other hand, the many facets that balance this cellular control network provide a vast number of potential molecular targets that can be critical points interrupting the otherwise uncontrolled growth of tumor cells.

Disruption of the tumor cell cycle can be accomplished through a wide variety of different mechanisms [reviewed in 94–95]. Antimitotic agents often arrest the cell cycle in M phase. Inhibitors of topoisomerases usually arrest cells in either S or G_2 phase. Agents that specifically target cell cycle regulatory proteins (such as cyclin-dependent kinases, checkpoint kinases, and dual specificity phosphatases) are known to specifically interrupt normal cell cycle progression in G_1 , S, or G_2 phase. Antimitotics and agents that affect DNA synthesis or repair (i.e. inhibitors of topoisomerases I and II) are presented separately.

However, this section describes mechanism-based marine natural products screening efforts targeting the protein kinases and phosphatases that regulate cell cycle progression.

Cyclin-dependent kinases

Through the phosphorylation of critical cell cycle proteins, cyclin-dependent kinases (CDKs) function as a driving force that promote cellular transition from one phase to the next. The first CDK was identified in yeast and termed Cdc2 (cell division cycle protein 2). Cyclin-dependent kinases are activated by conformational changes that occur when regulatory proteins, known as cyclins, bind to specific binding sites on each CDK. Therefore, changes in the expression of cyclins exert regulatory effects over CDK-mediated protein phosphorylation. Various cyclins (A, B, D, E) activate distinct CDK subtypes that function at different phases of the cell cycle. For example, activation of Cdc2 with cyclin E is associated with progression from G₁ into S phase [95], while cyclin A mediates Cdc2-stimulated G₂/M transition [96,97].

Using a murine Cdc2-mutant cell line (tsFT210), Osada and coworkers at the RIKEN Institute in Japan developed a bioassay system to detect inhibitors of the Cdc2 kinase (and G₂/M-phase transition) from actinomycetes [98,99]. This cell morphology-based assay was validated using staurosporine analogs and sangivamycin as protein kinase inhibitors to affect tsFT210 cell cycle progression and the cells were subjected to flow cytometric analysis to confirm cell cycle stage. Rather than using a purified Cdc2 kinase-based assay, this simple cell-based system could be easily monitored by observing the apparent difference in size between G₂ and G₁ cells. When ran as a primary screen, the Cdc2-mediated inhibition produced by active compounds could then be confirmed in secondary purified enzyme-based systems or by flow cytometry. Osada et al. did not provide the detailed results of their natural products screening efforts. However, two new cell cycle inhibitors tryprostatin A and B (and acetophthalidin) were isolated from the fermentation broth of an *Aspergillus fumigatus* culture, Fig (5). While perhaps not strictly marine, this *A. fumigatus* strain was collected from marine sediments.

A new purine metabolite microxine, Fig. (5), was isolated from a South Australian collection of the marine sponge *Microxina* sp. (class Demospongiae, order Haplosclerida, family Niphatidae) as part of an anti-inflammatory discovery program [100]. Since other purine metabolites have been shown to inhibit Cdc2 kinase, microxine was evaluated for Cdc2 inhibitory activity. In an assay system that measures the phosphorylation of histone H₁ by a purified p34^{cdc2}/cyclin B complex (from starfish oocytes), microxine inhibited Cdc2 kinase (IC₅₀ = 13 μM) [101].

In order to prevent replication of damaged DNA, the G₁/S transition of cell cycle is regulated by p53/p21-mediated control over p16-regulated Cdk4 and Cdk6/cyclin D complexes and the retinoblastoma protein (Rb)-regulated cdk2/cyclin E complex, Fig. (5) [85,94,102]. In addition, G₁ arrest is also mediated through cell cycle checkpoint protein control (discussed separately). The majority of human cancers have been shown to result, at least in part, from defects in the regulation of G₁/S transition pathways [103–106]. Using *in vitro* GST-fusion protein-based kinase assays (with purified CDK-cyclin complexes), researchers at Novartis Pharma AG (Switzerland) screened marine natural products for inhibitors of Cdk4 [107]. Fascaplysin, an antimicrobial red marine pigment (originally isolated by Ireland and coworkers from a Fijian collection of the sponge *Fascaplysinopsis* sp.), was shown to inhibit Cdk4 kinase (Cdk4/cyclin D1 complex, IC₅₀ = 350 nM) [108]. The structure of fascaplysin and other marine natural product isolated using CDK-inhibitor assays are shown in Fig. (5). Many known small molecule CDK inhibitors are relatively non-selective [109–111]. Yet, fascaplysin showed greater than 10-fold selectivity for Cdk4/cyclin D1 (over the Cdk6/cyclin D1 complex) and very little kinase inhibitory activity

against Cdk1 or Cdk2 complexes. Remarkably, fascaplysin was also demonstrated to selectively inhibit the kinase activity of the p16-regulated Cdk4/cyclin D1 complex over other Cdk4/cyclin D complexes.

Another previously isolated sponge metabolite aragusterol A (YTA0040) [112] has been shown to block the G₁/S transition of human tumor cells *in vitro* [113]. Flow cytometric analysis of A549 (human non-small cell lung cancer) cells treated with the antitumor steroid aragusterol A, Fig. (5), showed that these cells were blocked from progression into S phase. Further studies, including Western blot analysis, suggest that aragusterol A functions independently of p53/p21. Aragusterol A is thought to inhibit phosphorylation of Rb (retinoblastoma protein), thereby downregulating the expression and subsequent activation of the Cdk2/cyclin E complex required for entry into S phase of the cell cycle.

Checkpoint Kinases and Dual Specificity Phosphatases

Cell cycle checkpoint proteins control the entry of cells into the transition from one cell cycle phase to the next. This checkpoint control ensures that the cellular machinery is intact and that genetic materials are prepared to successfully complete the next corresponding cell cycle phase. In order to prevent cells from replicating damaged DNA, G₁ checkpoint control is maintained by the p53/p21-mediated cellular arrest system, checkpoint kinase (Chk)-mediated interruption of the cell cycle, and other mechanisms [94,114,115]. In addition to the control exerted over CDKs by their interaction with cyclins, the kinase activity of CDKs is modulated by post-translational phosphorylation [116,117]. For example, the CDK known as Cdc2 is not active until the threonine in the T-loop is phosphorylated [118–120]. Yet, phosphorylation of Thr-14 or Tyr-15 by the kinases Wee1 or Myt1 down regulates the kinase activity of Cdc2 [121,122]. Alternatively, the phosphatase Cdc25 activates the kinase Cdc2 by dephosphorylation the Thr-14 and Tyr-15 residues. When a cell is ready to enter mitosis, a feedback loop is generated between the phosphatase Cdc25 and the activated cyclin B/Cdc2 kinase complex [123]. This positive feedback signal is made possible because this activated cyclin/CDK complex has the capacity to directly activate Cdc25. Inhibition of Cdc25 phosphatase activity would block the transition of cells from G₂ into M phase and produce cellular arrest [124]. Therefore, entry into each stage of the cell cycle can be regulated through activation/deactivation of CDKs via site-specific phosphorylation or dephosphorylation of distinct kinases and phosphatases in each stage of the cell cycle.

A method to screen for inhibitors of Cdc25 protein phosphatase activity was developed at Mitotix Inc. (Cambridge, MA) and used by researchers at Harbor Branch Oceanographic Institution to evaluate marine natural products [125,126]. Gunasekera and coworkers used this purified Cdc25/GST fusion conjugate-based screen to isolate dysidiolide, a new β -alkyl- γ -hydroxybutenolide diterpene, from a collection of the sponge *Dysidea etheria* collected in the Bahamas [126]. The structure and relative configuration of dysidiolide was determined by single-crystal X-ray diffraction, Fig. (6). Dysidiolide showed an IC₅₀ of 9.8 μ M in this Cdc25 inhibition assay. Subsequent evaluation of synthetically produced dysidiolide and related compounds failed to confirm the Cdc25 phosphatase inhibitory activity [127]. However, due to structural ideas generated by the dysidiolide, synthetic manipulations of structurally related cholesteryl acetate and vitamin D₃ analogs have produced several new Cdc25 inhibitors with IC₅₀s in the low μ M range [128–130]. The sesterterpene sulfate sulfircin, Fig. (6), was first isolated from a Bahamian collection of the marine sponge *Ircinia* sp. as a moderately active antifungal agent (*Candida albicans* MIC = 25 μ g/mL) [131]. Sulfircin and a number of analogs were synthesized by researchers at Mitotix and found to inhibit Cdc25 phosphatase (Cdc25a IC₅₀ = 7.8 μ M; *Candida* Cdc25 IC₅₀ = 12.7 μ M) [132]. However, sulfircin appears to be a nonspecific protein phosphatase inhibitor since it also inhibited PTP1B and VHR protein phosphatases at similar concentrations (IC₅₀s = 29.8 and 4.7 μ M, respectively) *in vitro*.

Since few substances were known to inhibit the G₂ checkpoint-mediated transition of tumor cells into M phase, Roberge and coworkers developed a cell-based assay for detecting G₂ checkpoint inhibitors [133]. This ELISA-based high-throughput microtiter assay used MCF-7 human breast carcinoma cells with dominant-negative p53 mutation. Since these MCF-7 mp53 cells have mutated p53, they do not arrest in G₁. The MCF-7 mp53 cells were first arrested in G₂ by DNA damage, induced by γ -irradiation. Following treatment with nocodazole (to induce cellular transition into mitosis) and crude extracts of marine organisms, the release of cells from G₂ into mitosis was measured using an ELISA for phosphorylated nucleolin present in mitotic cells [134]. The percentage of cells entering mitosis corresponds to the amount of phosphorylated nucleolin determined by ELISA. A control for relative cytotoxicity was performed in both MCF-7 and lung adenocarcinoma A549 cells. Over 1,300 extracts of both temperate and tropical sponges and other marine invertebrates were screened for G₂ checkpoint inhibitors using this assay. One of the few known G₂ checkpoint inhibitors, staurosporine (IC₅₀ = 0.2 $\pm$ 0.2 nM) and two semisynthetic analogs (artificially produced oxazolidine and carbamate derivatives) were isolated from a temperate sponge-associated bacterium, Fig. (6). An extract of a Brazilian collection of *Didemnum granulatum* (Urochordata, Ascidiacea) yielded the known compound didemnimide A, several didemnimide analogs, and a novel compound isogranulatimide, Fig. (6). Isogranulatimide, a bright orange compound with some structural similarity to both staurosporines and didemnimides, showed relatively weak G₂ checkpoint inhibitory activity (IC₅₀ = 1.8 $\pm$ 0.2 μ M), relative to staurosporine.

After screening several thousand crude marine invertebrate extracts using the MCF-7 cell-based G₂ checkpoint inhibitor assay, Roberge and coworkers subsequently isolated the marine alkaloid debromohymenialdisine (DBH) and hymenialdisine as active components of Papua New Guinea collections of the sponge *Stylissa flabeliformis* [135]. Although previously isolated over twenty years before from the sponge *Phakellia flabelata*, DBH is structurally dissimilar to any previously isolated G₂ checkpoint inhibitor, Fig. (6) [136]. Hymenialdisine and DBH inhibited G₂ checkpoint-mediated transition into mitosis (IC₅₀s = 6 $\pm$ 3 μ M and 8 $\pm$ 4 μ M, respectively). Previous studies have shown that hymenialdisine is a relatively nonselective inhibitor of protein kinases that include CDKs, glycogen synthase kinase-3 β , and casein kinase 1 *in vitro* and *in vivo* [137]. When evaluated for specific checkpoint kinase inhibitory activity, DBH inhibited checkpoint kinases Chk1 (IC₅₀ = 3 μ M) and Chk2 (IC₅₀ = 3.5 μ M), but did not inhibit several other cell cycle-related protein kinases at the same concentration.

MACROMOLECULE SYNTHESIS

Many marine natural products that target DNA have been discovered as a result of tumor cell cytotoxicity screening (Table 1). The best examples are the antitumor marine natural products known as the ecteinascidins, from the marine tunicate *Ecteinascidia turbinata*. Ecteinascidin 743 (ET-743) inhibits DNA synthesis (therefore cell proliferation) and is in clinical trials for cancer [138,139]. Phase II clinical trials have demonstrated efficacy with ET-743 as a first or second-line treatment of advanced soft-tissue sarcoma [140,141]. ET-743 is an example of potential chemotherapeutic agents targeting DNA synthesis discovered through serendipitous screening. However, mechanism-based assays have also been developed as primary screens for the discovery of new DNA targeting antitumor agents. Three major classes of enzymes - topoisomerases, dehydrogenases, and polymerases, key to the formation of new DNA, constitute a group of important molecular targets for the discovery of new marine natural product-based antitumor agents.

Topoisomerases

The enzymes that control the number and topological conformations of supercoils in DNA are topoisomerases. Two types of topoisomerases have been isolated from both prokaryotes and eukaryotes - Type I (topo I) and Type II topoisomerase (topo II). Because of their role in regulating DNA tertiary structures, topoisomerase are essential to cellular processes such as DNA replication, recombination, and gene transcription. They are the targets for many known compounds that affect DNA replication [142] as well as the targets for the design/discovery of new antineoplastic agents [143,144]. Current clinical usage of the topoisomerase inhibitor topotecan (Hycamtin®) exemplifies the therapeutic potential of topoisomerase inhibitors [145].

Sakagami and coworkers have focused on the identification of topo I inhibitors from marine sources, using a primary bioassay that contains a camptothecin-resistant recombinant human topo I enzyme and the DNA substrate supercoiled plasmid pBR322 [144,146]. Bioassay-guided fractionation has led to the isolation of two ceramide sulfates (topo I - IC_{50} = 0.4 and 0.2 μ M, respectively) from a Japanese collection of the bryozoan *Watersipora cucullata* [147], and a series of related amphimic acids from an Australian sponge of the genus *Amphimedon* (topo I - IC_{50} s range from 0.47 to 6.7 μ M) [146]. Representative structures of marine metabolites found to inhibit topoisomerases are shown in Fig. (7).

By breaking and rejoining double stranded DNA, topo II can change DNA topology and separate two intertwined DNA duplexes during replication. One primary screening assay for topo II inhibitors was designed to take advantage of the difference in sensitivity to topo II inhibitors between a double-strand break repair-deficient Chinese hamster ovary (CHO) cell line xrs-6 and a repair competent CHO cell line BR1 [148,149]. The inability of xrs-6 cells to repair double-strand break results in enhanced cytotoxic sensitivity in the presence of topo II inhibitors, relative to BR1 cells. The effects of extract/compound treatment on xrs-6 and BR1 cell viability were measured using the microtiter MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye] assay, and the ratio of IC_{50} s between these two cell lines calculated. The largest ratios (BR1 IC_{50} /xrs-6 IC_{50}) were observed in the presence of topo II inhibitors [148]. Using this assay, the marine invertebrate pyrroloiminoquinones shown in Fig. (7), makaluvamines (from the sponge *Zyzya fuliginosa*) [150,151] and wakayin (from the ascidian *Clavelina* sp.) [152] were isolated. Subsequently, specific interactions between the makaluvamines and topo II were demonstrated [153].

Inhibitors of topo II can be divided into two categories: DNA intercalators and non-intercalators. Among the intercalators, pyridoacridine alkaloids have been discovered from marine ascidians and sponges, Fig. (7) [154]. The biochemical induction assay (BIA) that detects DNA-interacting compounds was used as the primary assay in the isolation of topo II inhibitory pyridoacridine alkaloids from a Fijian *Cystodytes* sp. Ascidian [155]. The effects of these pyridoacridines on topo II activity were measured in a secondary decatenation inhibition assay [155]. The quantitative microtiter decatenation inhibition assay measures topo II catalyzed decatenation of the trypanosome *Crithidia fasciculata* kinetoplast DNA (kDNA) in the presence of test compounds [156]. Topo II activity correlates positively with the amount of decatenated kDNA. In COMPARE analysis (NCI), compounds with a Pearson correlation coefficient greater than 0.6 often function through a biochemically related mechanism [157]. The pyridoacridine alkaloids were subjected to COMPARE analysis and showed a correlation pattern consistent with topo II inhibitors [158].

Inosine 5'-Phosphate Dehydrogenase (IMPDH)

The enzyme IMPDH catalyzes the NAD-dependent oxidation of inosine 5'-phosphate (IMP) to xanthosine 5'-monophosphate and is the key enzyme in *de novo* GTP biosynthesis [159].

The two substrates of IMPDH bind in an obligate order - IMP precedes NAD, and the products also dissociate in an obligate fashion, with NADH preceding xanthosine 5'-monophosphate. The enzymatic activity of IMPDH can be determined spectrophotometrically by monitoring the formation of NADH at 340 nm. Elevated type-II IMPDH levels have been found in ovarian tumors and leukemia cells [160]. The activity of IMPDH is tightly linked with cell proliferation and the inhibition of IMPDH has anticancer, antiviral, and immunosuppressive effects [161].

Gerwick and coworkers at Oregon State University evaluated over 500 extracts of marine microalgae (primarily cyanobacteria) and macroalgae for their ability to inhibit IMPDH [162]. This assay yielded twenty-four active extracts and resulted in the isolation of the bromophenolic compound isorawsonol ($IC_{50} = 18 \mu M$) from the tropical marine green algae *Avrainvillea rawsonii* [163]. Other marine natural products isolated using this same assay system include pellynic acid ($EC_{50} = 1.03 \mu M$) from the sponge *Pellina triangulata* [164] and bastadin alkaloids (EC_{50} s from 3 to 11 μM) from the Verongid sponges *Ianthella basta* and *I. flabelliformis* [165]. The IMPDH inhibition assay was later modified to control for any IMPDH inhibitory activity that may have been due to trace amounts of elemental sulfur or sulfur compounds. Using the modified assay, bastadins 8 and 10 were found to be the most potent of the bastadin alkaloids. Examples of marine natural products found to inhibit IMPDH are shown in Fig. (8).

DNA polymerase

At least nineteen different DNA polymerases (pols) have been identified in eukaryotic cells [reviewed in 166]. To ensure proper DNA replication and repair, multiple polymerases may be employed for similar functional tasks and one particular polymerase may have multiple functions. Replication of DNA requires pol α , pol δ , and pol ϵ , whereas translesion DNA synthesis requires pol ζ , pol η , pol ι , and pol κ . In many cases, polymerases are multipolypeptide complexes that contain other functional subunits in addition to the polymerizing subunit and often display a proofreading 3'→5' exonuclease activity.

DNA polymerase assays capable of evaluating natural products have been established and optimized for each specific enzyme. Inhibition of each DNA polymerase was confirmed under various conditions with activated calf thymus DNA and/or poly(dA)-oligo(dT) to reveal template-primer-dependent differences. The mode(s) of inhibition was analyzed by Lineweaver-Burk plot, and the K_i is obtained from Dixon plot analysis [167].

Marine natural products have been examined for specific inhibitors of DNA polymerases. Synthetically produced samples of the sulfated glycosylglycerolipid, 1-*O*-(6'-sulfo- α -D-glucopyranosyl)-2,3-di-*O*-phytanyl-*sn*-glycerol (KN-208) [168], a natural product found in the polar lipids from a halophilic archaeobacterium (isolated from an Okinawan sponge), inhibited eukaryotic DNA polymerases. Among the five eukaryotic DNA polymerases (α , β , γ , δ , and ϵ) tested, KN-208 selectively inhibited pol α and β *in vitro*. The sulfated glycolipid KN-208 most strongly inhibited pol β , showing a 10-fold increase in potency for pol β ($K_i = 0.05 \mu M$), relative to the rate constant for pol α ($K_i = 0.5 \mu M$) [167]. The structures of marine natural products found to inhibit DNA polymerases are shown in Fig. (9). Halenaquinol sulfate, a *p*-hydroquinone sulfate obtained from a marine sponge, was shown to preferentially inhibit the α -family of eukaryotic DNA polymerases [169]. Purified metabolites from Okinawan marine sponge- and bivalve-associated microorganisms were screened for inhibitors of DNA polymerase. This afforded the identification of two novel inhibitors of eukaryotic DNA polymerases: sculezonone A and B [170]. Sculezonone-A preferentially inhibited DNA polymerases α , β , and γ (IC_{50} s = 20, 25, and 17 μM , respectively). Sculezonone-B (12-hydroxy-sculezonone A) preferentially inhibited DNA polymerases α and γ (IC_{50} s = 12 and 18.5 μM , respectively). Extracts from cultures of a

Flavobacterium sp. (isolated from a Japanese sample of the marine bivalve *Cristaria plicata*) inhibited DNA polymerase α [171]. Subsequently, the flavocristamides A and B (new 1-deoxyceramide-1-sulfonates) were isolated and shown to inhibit DNA polymerase α , with IC_{50} s of around 20 μ g/mL (about 30 μ M) [171]. Kobayashi, Yoshida and coworkers also isolated the acetylenic fatty-sulfonic acid derivative taurospongins A from an Okinawan collection of the marine sponge *Hippospongia* sp. Taurospongin A was shown to be a relatively non-selective inhibitor of DNA polymerase β , HIV reverse transcriptase, and c-erbB-2 kinase (IC_{50} s = 7.0, 6.5, and 28 μ M, respectively) [172]. Sakaguchi and coworkers also found that the sulfolipid metabolite sulfoquinovosyldiacylglycerol (KM-043), previously isolated from the marine red alga *Gigartina tenella*, inhibited DNA polymerases α and β (IC_{50} s = 0.25 and 3.6 μ M, respectively) [173,174]. The structure of KM-043 is reported (in at least two publications) to contain an all-*trans* version of C_{20:5} fatty acid ester "...3-[5''(E), 8'' (E), 11'' (E), 14'' (E), 17'' (E)-eicosapentaenyl]-syn-glycerol." This seems to be in error, since none of the proton-proton coupling constants reported for the olefinic protons are greater than 12 Hertz. This would indicate that all are "*cis*" and must be all "*Z*."

It is remarkable to note that most of the marine natural products that have been found to inhibit DNA polymerases are anionic detergents (lipid sulfates and sulfonic acids), Fig. (9). In addition, few of the polymerase inhibitors isolated are very potent (few have IC_{50} s < 1 μ M) or show highly selective inhibitory activity towards a specific polymerase. In fact, many of these compounds even inhibit a variety of other unrelated enzymes. While the polymerase inhibitory activity may be attributed to relatively non-selective detergent activity, the marginal specificity of a few of these metabolites to individual polymerases may be associated with competition for DNA-binding domains or other perhaps positively charged binding sites unique to the polymerases.

MITOCHONDRIAL RESPIRATION

Under aerobic conditions, the mitochondrial respiratory chain is the fundamental process that drives ATP synthesis in cells. Alterations in tumor metabolism have been linked to specific differences in the mitochondria of normal and malignant cells [reviewed in 196]. Therefore, tumor mitochondria have recently become an important source of molecular targets for antitumor drug discovery.

Unusual oxidized fatty acid metabolites (oxylipins) have been isolated from the Mediterranean tunicate *Stolonica socialis*. These cyclic peroxides, known as the stolonoxides, display an unusual structure with a 1,2-dioxane ring directly linked to a tetrahydrofuran moiety, Fig. (10) [197,198]. Such an arrangement is closely related to the bis-tetrahydrofuran groups of acetogenins, isolated from members of the Annonaceae (custard-apple family) [199]. Annonaceous acetogenins are known to inhibit mitochondrial respiration [200,201]. Further examination has shown that the stolonoxides also inhibit mitochondrial respiration [202]. Electron transport in the integrated electron transfer chain initiates from complex I (NADH:ubiquinone oxidoreductase) or from complex II (succinate:ubiquinone oxidoreductase) to complex III (ubiquinol:cytochrome C oxidoreductase) by ubiquinone. Electron transfer is then from complex III to complex IV (that contains cytochrome C oxidase) by the protein cytochrome C. The ability of these compounds to inhibit mitochondrial respiration was evaluated in an assay system that used beef heart submitochondrial particles. The methyl esters of stolonoxide A and stolonoxide C completely inhibited cytochrome *c* reductase activity with either NADH or succinate as the electron donor. When assayed using deacyl ubiquinone [203], both compounds strongly reduced the specific activity of succinate-sustained ubiquinone reduction in complex II, and only slightly reduced complex I ubiquinone-sustained NADH oxidation [202]. These data and the cytochrome *c* reductase activity suggest that both stolonoxides behave as specific

inhibitors of complex II and complex III (at submicromolar concentrations), and if sufficient tumor cell selectivity can be established, may be of interest in antitumor research.

MITOSIS

More marine secondary metabolites that interrupt mitotic processes have been characterized than perhaps any other class of antitumor/anticancer marine natural product [204]. One may speculate that there are several reasons for this apparent trend. For example, the production of secondary metabolites by marine bacteria and cyanobacteria that selectively interact with the tubulin of eukaryotic grazers may provide a significant ecological advantage to these prokaryotic microorganisms. Most antimitotic agents arrest tumor cells in M phase and prolonged arrest in mitosis ultimately results in cell death [205]. Therefore, it is also not surprising that many (if not most) antimitotic marine natural products have been discovered as a consequence of years of “mechanism-blind” *in vitro* tumor cell cytotoxicity screening. A detailed description of the mechanism of action studies for marine antimitotic agents is beyond the scope of this article. However, several recent reviews have clearly described much of the current research regarding both terrestrial and marine antimitotic agents [85,204,206,207]. Examples of important antimitotic marine natural products that were first identified through *in vitro* cytotoxicity screening include spongistatin [208–210], halichondrin [211,212], hemiasterlins [213,214], dolastatin 10 [215,216], curacin A [217,218], and eleutherobin [219–221, reviewed in 204]. Antimitotic agents can be generally classified into tubulin interactive agents (those that interfere with the polymerization or depolymerization of tubulin), actin inhibitors (those that interfere with the polymerization or depolymerization of actin), and kinesin inhibitors (those that disrupt the function of kinesin motor proteins). Although the latrunculins (isolated from the Red Sea sponge *Latrunculia magnifica*) have been shown to interfere with actin polymerization [222], mechanism-based screening efforts for antimitotic marine natural products have focused on tubulin and kinesin.

Tubulin

While *in vitro* cytotoxicity screening is still a mainstay of many marine natural product programs, new (often high-throughput) mechanism-based *in vitro* antimitotic assays are now being used to discover natural products that directly interfere with the mitotic processes in tumor cells. For example, Barrows and coworkers have developed a C6 rat glioma cell-based assay in a microtiter plate format and screened marine natural products [223]. This quantitative cell-based antimitotic screen is a more convenient form of a previously described assay that depends on tubulin-dependent morphological changes (sphere formation) in C6 cells following dibutyryl-cAMP treatment [224].

Efforts to directly discover antimitotic marine natural products through mechanism-based high-throughput screening have both yielded new antimitotic agents and helped identify the mechanisms of previously reported cytotoxic compounds, see Fig. (11). Mooberry and coworkers isolated and characterized the microtubule-stabilizing activity of the sponge metabolite laulimalide and its acid-catalyzed rearrangement product isolaulimalide, as a part of a mechanism-based antimitotic screening program of marine invertebrates collected in the Marshall Islands [225, 226]. Interaction between the laulimalides and purified bovine tubulin was determined using the 96-well microtiter-based CytoCYNAMIX Screen (Cytoskeleton, Denver, CO). The laulimalides had previously been isolated and reported as cytotoxic [227–229]. Laulimalide inhibited tubulin polymerization with an $EC_{50} = 4.3 \pm 0.4$ μ M. However, laulimalide exerts an antiproliferative effect on certain tumor cells *in vitro* at concentrations approximately 1000-times lower than the EC_{50} for inhibiting tubulin polymerization. Unfortunately, other than for the evaluation of purified compounds, no

description of the specific “mechanism-based screen” used to evaluate extracts or any general hit rate observed in this antimitotic screening program was provided.

Roberge and coworkers evaluated more than 24,000 extracts of marine organisms, microbes, and plants using a novel ELISA assay for antimitotic compounds [230]. This high-throughput cell-based assay employs a TG-3 monoclonal antibody that recognizes mitotic cells through binding to a mitotically phosphorylated form of nucleolin. A horseradish peroxidase-conjugated secondary antibody was used to detect the TG-3 primary antibody and the peroxidase activity determined using a colorimetric reaction. This ELISA method was simplified by permeabilizing the formaldehyde fixed human breast carcinoma MCF-7 cells directly in the microtiter plates, followed by simultaneous addition of both the primary and secondary antibodies. Over 2,000 crude extracts of marine nudibranchs, soft corals, sponges, and starfish were screened and positive extracts confirmed microscopically. This facilitated the discovery of one new rhizoxin analog from a marine *Pseudomonas* sp. (collected off the coast of British Columbia), and six new eleutherobins from a Dominican collection of the octocoral *Erythropodium* cf. *caribaeorum*. The structures of these tubulin inhibitors are shown in Fig. (11). In addition, several known antimitotic compounds were also identified, one known rhizoxin analog and several known eleutherobins. The new δ -lactone-opened rhizoxin hydroxy-acid analog ($IC_{50} = 8$ nM) was significantly more potent than the known rhizoxin-like metabolite ($IC_{50} = 52$ nM). However, the most potent eleutherobin-like metabolite isolated was the known compound sarcodictyin A ($IC_{50} = 2$ μ M) [231]. Since this assay system is cell-based, it has several advantages over cell-free antimitotic assays. For example, cell-based assays functionally select for natural products with suitable plasma membrane permeability and stability under certain physiological conditions.

Kinesin

Kinesins are a superfamily of more than 100 known motor proteins required to drive microtubule-based transport systems, including those involved in mitotic and meiotic spindle formation, chromosome segregation, and the transport of vesicles and organelles [232]. An assay, designed to detect substances that interfere with microtubule gliding mobility was used to evaluate marine sponge extracts [233]. A fungus-derived (*Thermomyces lanuginosus*) kinesin superfamily protein (T1- γ) was coated onto glass cover slips, supplemented with microtubules, Mg-ATP, sponge extracts, and motility measured by standard methods. An extract from a sponge of the genus *Haliclona* (also known as *Adocia*) disrupted microtubule motility in this assay. Consequently, the active natural product adociasulfate-2, Fig (12), was isolated. Low micromolar concentrations of adociasulfate-2 were shown to interfere with microtubule-stimulated kinesin ATPase activity through an apparent microtubule-like interaction with the motor domain of kinesin.

MULTIDRUG EFFLUX

Multidrug resistance (MDR) is one of the main causes for the failure of chemotherapeutic cancer treatments. Multidrug resistance was first described in 1970 by Biedler and Riehm, based on investigations in resistant cell lines derived from a Chinese hamster lung tissue-derived cell line (DC-3F) and a Chinese hamster fibroblastic cell line (CLM-7) [234]. These resistant cell lines showed cross-resistance between structurally and mechanistically unrelated anticancer drugs. In a study to evaluate the correlation between a 170 kD surface glycoprotein P-glycoprotein (P-gp) and reduced permeability of cultured Chinese hamster ovary (CHO) cells to colchicines, Juliano and Ling found that the relative quantity of surface-labeled P-gp correlated positively with the degree of drug resistance [235]. The P-gp membrane transporter acts as an ATP-dependent drug efflux pump that actively removes a variety of structurally diverse xenobiotics and natural product-based drugs with different

cellular targets and mechanisms of action. The *Cantharanthus roseus* (formerly *Vinca roseus*) alkaloids, epipodophyllotoxins, anthracyclines, actinomycin, taxol, and gramicidin D are all substrates for the P-gp transporters [236,237]. The complementary DNA (cDNA) that encodes P-gp was cloned in 1985 [238]. Based on the coding sequence, the P-gp is a 1280 amino acid protein with two homologous portions of approximately equal length. Each part contains one hydrophobic and one hydrophilic domain. The hydrophilic domains share sequence homologies with peripheral membrane components of bacterial periplasmic transport systems, consistent with the efflux function of the P-gp [239]. The cDNA encoding a second MDR protein, named MRP, was cloned in 1992 [240]. The MRP protein was found to be a member of an adenosine triphosphate (ATP)-binding cassette (ABC) superfamily. Currently, both the MRP and P-gp pumps represent important targets for discovery of anti-MDR compounds. While the list is growing, multidrug resistance is now known to occur via the following distinct mechanisms: 1) accelerated drug removal or secretion; 2) modified detoxification and DNA repair pathways; 3) changes in cellular sites of drug sequestration; 4) decreased drug-target affinity; and 5) synthesis of specific drug inhibitors within cells [241]. Recently, an increased cellular capacity for ceramide glycosylation has been recognized as new mechanism for multidrug resistance [242,243].

Numerous studies have shown that marine organisms represent a vast reservoir of new pharmacological agents with potentially unique modes of action [244–246]. Attempts to overcome MDR have focused on the following two major approaches: 1) the search for clinically useful drugs (such as didemnin B and dehydrothysiferol) that retain relatively good activity on otherwise MDR cells; and 2) the use of resistance modifiers, agents that are able to reduce the degree of resistance in multidrug-resistant cells by interfering with efflux pump function. Accordingly, MDR-related biological screening methods can be divided into two major types: “Type I assays” that compare cytotoxicity in drug-sensitive and drug-resistant cell lines; and “Type II assays” that identify MDR-reversing agents that potentiate the cytotoxicity of antitumor drugs such as vinblastine and adriamycin in resistant tumor cell lines that express MDR.

Investigators at the University of Hawaii have examined cyanobacterial extracts for anti-MDR compounds that function by either of the above two modes. Natural products in the first group are cytotoxic compounds equally efficacious toward drug-sensitive and drug-resistant cell lines (cryptophycins, scytophycins) [247,248]. Compounds in the second group are MDR-reversing agents (tolyporphin, hapalosin) that potentiate the cytotoxic effects of vinblastine and adriamycin in otherwise drug-resistant cells [249,250]. While screening extracts of cyanobacteria, Moore and coworkers reported that about 1% of cyanobacteria extracts exhibited significant MDR-reversing activity. However, most of the compounds discovered by this group, including the promising natural product hapalosin (isolated from *Hapalosiphon welwitchii*) have come from terrestrial cyanobacteria. Yet, several reviews have erroneously grouped these terrestrial cyanobacterial metabolites in with true marine natural products [251,252].

In the early 1990's, researchers at the University of British Columbia reported the evaluation of 15 cytotoxic marine invertebrate samples in drug-sensitive and multidrug-resistant (P-gp +) human breast-tumor cell lines, in order to identify anti-MDR compounds (Table 2). Among the eight samples that exhibited significant dose-dependent cytotoxicity against the drug-sensitive cell lines (ED_{50} s < 25 μ g/mL), five demonstrated similar cytotoxicity against the multidrug-resistant cell lines [253]. Several lamellarins (polyaromatic alkaloids isolated from *Didemnum* spp. tunicates) showed comparable cytotoxicity against both drug-resistant P388 murine leukemia cells and their corresponding drug-sensitive parental cell lines [254]. Studies conducted in multidrug-resistant human colon adenocarcinoma LoVo/Dx cells

revealed that preincubation with lamellarin I, Fig. (13), reverses MDR by directly inhibiting P-gp drug efflux [254].

Williams and Jacobs used a three-day microculture tetrazolium proliferation assay to determine the IC_{50} s for vinblastine, colchicine and adriamycin in the presence and absence of the cyclic octapeptide patellamide D, Fig. (13) [255]. First isolated from the marine tunicate *Lissoclinum patella*, patellamide D was found to be a resistance-modifying agent using the multidrug resistant CEM/VLB₁₀₀ human leukemic cell line [255]. Hamel and co-workers at the NCI used a computer-assisted search for novel compounds with structural homology to colchicine-site inhibitors. They discovered that the marine natural product (+)-discodermolide (previously isolated from the sponge *Discodermia dissolute*) enhanced tubulin nucleation reactions more potently than paclitaxel and inhibited the growth of paclitaxel-resistant cells [256]. The structure of (+)-discodermolide is shown in Fig. (13). Multidrug resistant human colon (SW620AD-300) and ovarian carcinoma (A2780AD) cells that overexpress P-gp have been shown to be 930- and 2800-fold more resistant to paclitaxel, respectively, relative to their non-resistant SW620 (colon) and IA9 (ovarian) parental lines. However, both resistant cell lines retain significant sensitivity to discodermolide. When treated with discodermolide, the resistant colon cell line was only 25-fold more resistant relative to its parental line, and the resistant ovarian cell line was only 89-fold more. Although resistance was still observed in the presence of discodermolide, the relative resistance between the cell lines to discodermolide is only one-thirtieth of that observed with paclitaxel. Additional studies were conducted in two paclitaxel-resistant ovarian tumor cell lines 1A9PTX10 and 1A9PTX22 with altered β -tubulin gene products. These paclitaxel-resistant cell lines retained nearly complete sensitivity to discodermolide [242].

As part of an investigation of marine invertebrate natural products for MDR modulators, Higuchi and co-workers isolated irciniasulphonic acid (ISA), from a Japanese collection of the marine sponge *Ircinia* sp. [258]. Irciniasulphonic acid was determined to be a mixture of three structurally related esters. The ISA mixture and deacyl-ISA, Fig. (13), were found to reverse MDR in P-gp overexpressing KB/VJ300 tumor cells. However, deacyl-ISA is more than 13-times more potent than ISA (IC_{50} s of deacyl-ISA and ISA were 3 μ M and 38 μ M, respectively).

Kobayashi and co-workers used two MDR-resistant cell lines (MDR KB-C2 cells and MDR KB-CV60 cells) and human KB-3-1 epidermoid carcinoma cells (as a non-resistant parental cell line) to evaluate marine natural products for MDR-reversing activity [259,260]. The resistant cell lines were exposed to media that contained either colchicine (MDR KB-C2 cells) or vincristine (MDR KB-CV60 cells). Using this system they isolated the cytotoxic MDR-reversing agent agosterol A, Fig. (13), from a Japanese collection of the marine sponge *Spongia* sp. The MDR-reversing activity and cytotoxicity of agosterol A were measured by means of a 96-well MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye] colorimetric assay. Subsequent mechanism of action studies revealed that agosterol A inhibits MRP1-mediated transport of typical amphipathic substrates, reverses the drug accumulation deficit of MRP1-transfected cells, and reduces intracellular glutathione levels.

The cytotoxic effects of the antitumor compound didemnin B, Fig. (13), (a marine cyclic depsipeptide isolated from the marine tunicates *Trididemnum cyanophorum* and *T. solidum*), were examined in CCRF-CEM human leukemic lymphoblasts and their corresponding MDR-resistant sublines [261,262]. These assay systems measured the fluorescence of living cells simultaneously stained with Hoechst 33342, rhodamine 123 and Nile red by a multi-

parametric multiwavelength microfluorometric approach. It was determined that the cytotoxic activity of didemnin B was not affected by membrane P-gp.

Dehydrothysiferol, Fig. (13), is an unusual antitumor brominated polyether triterpene isolated from the tropical marine alga *Laurencia viridis* (Rhodomelaceae) [263,264]. Dehydrothysiferol was demonstrated cytotoxicity against both drug-sensitive and multidrug resistant human epidermoid cancer cell lines. The IC₅₀ values of doxorubicin and colchicine against the P-gp overexpressing multidrug resistant KB-8-5 cells exceeded the IC₅₀ values obtained from the drug-sensitive KB-3-1 cells by a factor of about 13:1. However, the IC₅₀ value of dehydrothysiferol was about 2.6-times greater in the nonresistant KB-3-1 cells relative to the resistant KB-8-5 cells. Studies conducted in a fluorescence-based efflux system measuring the interference of a test compound with MRP1-mediated drug extrusion suggested that dehydrothysiferol did not inhibit MRP1-mediated drug transport. However, the mechanism of action for dehydrothysiferol has not been determined [265,266].

In resistant tumors, the two known mechanisms for MDR1 activation are constitutive overexpression and induction of gene expression [140,267]. The antitumor agent ET-743, Fig. (13), (currently in phase II clinical trials for the treatment of soft tissue sarcomas) inhibited transcriptional activation of the MDR1 gene by toxins such as histamine deacetylase inhibitors, without affecting basal MDR1 gene transcription [267]. In light of this study, ET-743 may have potential application as an adjuvant in the treatment of resistant tumors.

Intensive screening efforts have led to the discovery of highly promising marine natural product-based anti-MDR compounds that have been evaluated in clinical studies [268,269]. Compounds such as verapamil, dexverapamil and other anti-MDR inhibitors have been introduced into clinical trials, only to be dropped because of toxicity. The toxic effects of some of these agents may result from intrinsic toxicity or may be due to the unforeseen effects of efflux pump inhibition that increase the overall toxicity associated with the modulator. Alternative approaches to overcome the toxicity and rigorous clinical testing of known anti-MDR agents to identify more selective and less toxic drugs, are underway. New *in vitro* models of MDR that closely mimic *in vivo* systems are being developed, based on molecular level protein-drug interactions. Improved models of this highly sought after molecular target may facilitate the discovery of effective anti-MDR drugs from terrestrial and marine natural products [270–272].

SIGNAL TRANSDUCTION

Tyrosine-Specific Protein Kinases

Receptor-tyrosine kinases (protein tyrosine kinases or TKs) are transmembrane receptors for growth factors and other signal transduction proteins. Activation of tyrosine receptor kinases by growth factors and other ligands regulate the expression of downstream target genes and trigger DNA synthesis. Activation of oncogenic TKs can induce a mitogenic response and stimulate tumorigenic growth [273]. With the hope of discovering new antitumor natural products, considerable efforts have been made to identify inhibitors of these signal transduction control/switching molecules. As part of several early NCI-National Cooperative Drug Discovery Groups (NCDDGs), marine natural products researchers began to search for inhibitors of tumor-related TKs in the late 1980's and early 1990's [reviewed in 162,274,275].

Bioassays for TK inhibitors typically measure the incorporation of ³²P from radiolabeled ATP into various peptide substrates. One of the first marine natural products found to inhibit receptor-tyrosine kinases was the tyrosine metabolite (+)-aeroplysinin-1, Fig. (14) [276].

Using a preparation of epidermal growth factor (EGF) receptor-TK from MCF-7 breast tumor cells, (+)-aeropylsinin-1 (isolated from the marine sponge *Verongia aerophoba*) was shown to inhibit protein tyrosine phosphorylation and cause tumor cell death *in vitro* at 0.25–0.5 μM .

As part of an NCDDG anticancer drug discovery program, researchers at the University of California-Santa Cruz and at Syntex Research (Palo Alto, CA) examined marine natural products for new inhibitors of tumor-related TKs [274]. These researchers used a TK assay system with purified pp60^{v-src} (an oncogenic protein tyrosine kinase from Rous sarcoma virus-transformed rat cells) and the substrate protein [Val⁵]-angiotensin II [71]. The polyketide metabolites halenaquinone and halenaquinol, Fig. (14), were isolated from a Fijian collection of a sponge thought to be *Xestospongia carbonaria* and found to inhibit pp60^{v-src} TK activity at relatively low concentrations ($\text{IC}_{50}\text{s} = 1.5$ and $0.55 \mu\text{M}$, respectively). Several structurally related natural products and synthetic quinone analogs were also found to weakly inhibit pp60^{v-src} TK, presumably by acting as Michael acceptors to free sulfhydryl protein binding sites. These quinones also inhibited EGF receptor TK at similar concentrations, suggesting they lack true enzyme-specific selectivity. Further examination of *X. cf. carbonaria* polycyclic quinones found that 14-methoxyhalenaquinone was nearly as potent as halenaquinone in the same pp60^{v-src} TK assay ($\text{IC}_{50} = 5 \mu\text{M}$) [277]. These researchers also examined sponge-derived (*Dysidea* sp.) terpene quinones and hydroquinones and found that the sesquiterpene melemeleone B, Fig. (14), also inhibited pp60^{v-src} TK ($\text{IC}_{50} = 28 \mu\text{M}$) [278]. Using the same bioassay, the known sulfated steroid halistanol trisulfate, Fig. (14), was isolated from two different collections of *Topsentia* sp. sponges and found to inhibit pp60^{v-src} TK activity ($\text{IC}_{50} = 4 \mu\text{M}$) [279]. Acid hydrolysis of halistanol trisulfate yielded the triol derivative halistanol, which was inactive. Similarly, a series of structurally related monosulfated steroids were tested and also shown to be inactive.

In collaboration with Syntex Research, researchers at Oregon State University examined extracts and purified compounds from marine cyanobacteria and algae for inhibitors of pp60^{v-src} TK [reviewed in 162]. Extracts from laboratory cultures of the chrysophyte, *Poterioochromonas malhamensis*, inhibited pp60^{v-src} TK [163]. Malhamensilipin A, Fig. (14), a new C₂₄ hexachlorosulfolipid, was subsequently isolated and found to be a moderately weak TK inhibitor ($\text{IC}_{50} = 35 \mu\text{M}$).

Researchers at the Technical University of Braunschweig in Germany have recently begun screening marine organisms for inhibitors of the lymphocytic p56^{lck} TK (T cell-derived kinase) using a commercial ELISA-based kit (Boehringer Mannheim) [280]. Piceatannol (a natural metabolite of resveratrol that acts as a weak p56^{lck} TK inhibitor, $\text{IC}_{50} = 82 \mu\text{M}$) was used as a positive control in this system. The new terpene quinone styptoquinonic acid and the structurally related known hydroquinone atomaric acid were isolated using this assay, Fig. (14). However, these compounds only inhibit the p56^{lck} TK system at high concentrations. The p56^{lck} TK IC_{50} values of styptoquinonic acid and atomaric acid were 187 and 208 μM , respectively. Further, the effects of these compounds on other enzyme/protein systems were not evaluated in order to establish any evidence for tyrosine kinase selectivity. Bioassay-guided isolation of p56^{lck} TK inhibitory fractions from extracts of a marine sponge-derived (*Callyspongia vaginalis*) culture of the fungus *Ulocladium botrytis* yielded ulocladol, Fig. (15) [281]. The authors reported a 93% inhibition of p56^{lck} TK activity at a single concentration, 0.02 $\mu\text{g/mL}$ (65 nM). This group did not establish either an IC_{50} value for ulocladol or determine if the inhibitory effect was selective to tyrosine kinases. Using the same assay system, these researchers isolated tetramic acid containing alkaloids (ascosalipyrrolidinone A and B), Fig. (15), and several known compounds from extracts of the endophytic obligate marine fungus *Ascochyta salicorniae* [282]. This fungus was

isolated from tissues of the marine green alga *Ulva* sp. Several known compounds were also isolated from the culture extracts, including the known TK inhibitor genistein (presumably from plant derived constituents in the culture media) and 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-*trans*-propenylfuran-3-one, Fig. (15). Since König and coworkers only evaluated the test compounds at two concentrations (40 and 200 µg/mL), no IC₅₀ values were reported for these natural products. However, the p56^{lck} TK IC₅₀ value for ascosalipyrrolidinone A appears to be extremely weak, somewhere between the two test concentrations that correspond to 94 and 468 µM, respectively. The CH₂Cl₂ extract of a Fijian collection of the sponge *Hyrtilos* cf. *erecta* inhibited p56^{lck} TK activity by 80% at 100 µg/mL [283]. Upon further investigation three known compounds with extremely weak inhibitory activity were isolated, Fig. (15). The authors did not report IC₅₀s for these compounds. However, the alkaloid fascaplysin inhibited p56^{lck} TK activity by 90% at the extremely high concentration of 0.7 mM, homofascaplysin A inhibited p56^{lck} TK activity by 56% at 0.3 mM, and the terpene isodehydroluffariellolide inhibited p56^{lck} TK activity by 55% at 0.5 mM. König and coworkers recently isolated the fungus *Drechslera dematiodea* (a known phytopathogen) from the inner tissues of a Mediterranean collection of the red alga *Liagora viscida* [284]. More than a dozen compounds, including 10 new natural products, were isolated from cultures of this fungus. All of these substances were evaluated for p56^{lck} TK inhibitory activity. Several compounds showed extremely weak inhibitory activity at 200 µg/mL. However, only the known terpene quinone metabolite cochlioquinone B, Fig. (15), significantly inhibited (51.9%) p56^{lck} TK activity at the moderately high concentration of 40 µg/mL (85 µM).

Questions associated with the concept of using TK antagonists therapeutically and potential shortcomings recognized with the methods used to identify new TK inhibitors have been described in several recent reports [285–287]. There are a large number of different TK subtypes, of which many occur in both malignant and nonmalignant cells. However, most screening efforts only test marine extracts and purified natural products against a single TK protein (pp60^{v-src} or p56^{lck}). Therefore, it is not necessarily surprising that many discovery efforts have led to the isolation of TK inhibitors, that were subsequently determined to be relatively non-selective and of little therapeutic potential. Since the ultimate goal of these efforts is the discovery of high-affinity TK inhibitors that specifically target only the oncogenic TK proteins, primary screening should perhaps be conducted using a panel of TKs and only inhibitors that show a high degree of specificity should be pursued. The development of the vascular endothelial growth factor receptor (VEGF-R) TK antagonist SU5416 has suggested that inhibition of certain specific TKs can be of therapeutic potential [288]. In addition, a shift in discovery efforts has occurred. Screening efforts now focus on the identification of specific inhibitors of newly identified tumor-specific TKs [289]. Some of these discovery efforts are discussed further in other sections, specific to the cell cycle, angiogenesis, and apoptosis.

EGFR/Ras/Raf/MEK/MAPK Signaling

Proliferation of normal cells is tightly controlled in multicellular organisms by a network of signaling pathways designed to ensure that cells only proliferate at appropriate times. Dysregulation of these signaling mechanisms often leads to the breakdown of normal growth regulation and can result in pathological conditions such as cancer. Cell proliferation and survival are dependent on extracellular stimuli such as growth factors. Epidermal growth factor (EGF) is a growth factor that is required for cell proliferation and differentiation. Binding of EGF to the receptor triggers a chain of events that includes receptor dimerization, activation of receptor tyrosine kinase activity, autophosphorylation, and activation of the classical Ras/Raf/MEK/MAPK signaling cascade [reviewed in 290].

The EGF receptor (EGFR) is a member of the ErbB family that contains an extracellular ligand binding domain, dimerization domain, and intracellular tyrosine kinase domain. Members of the ErbB family include EGFR, ErbB-2/Neu/HER2, ErbB-3, and ErbB-4. The epidermal growth factor receptor is overexpressed in most solid tumors and EGFR overexpression correlates with poor prognosis [reviewed in 291]. Small molecule inhibitors of the EGFR tyrosine kinase activity have been developed and are currently undergoing clinical trials for cancer [reviewed in 287,291]. Most of these inhibitors belong to the chemical class known as quinazolines (ZD1839, OSI774, CI1033, EKB569, and GW2016), while PKI166 is a pyrrolo-pyrimidine. A small number of marine natural products with different chemical structures have been found to inhibit the tyrosine kinase activity of EGFR in pharmacological mechanistic studies, secondary to primary screening efforts [275]. In the course of evaluating marine sponge compounds for selective cytotoxicity, (+)-aeroplysinin-1, Fig. (16), was found to target growth factor dependent tumor cell lines [276]. Secondary pharmacological evaluations revealed that aeroplysinin-1 inhibits the tyrosine kinase activity of EGF receptor present in a purified membrane preparation. Although the IC_{50} of aeroplysinin-1 was not determined, strong inhibition was observed at 0.5 μ M. In secondary pharmacological and mechanistic studies, two sesquiterpenoid quinones isolated from a marine sponge of the family Spongiidae - nakijiquinones C and D selectively inhibited ErbB-2 tyrosine kinase activity (IC_{50} s = 26 and 29 μ M, respectively), relative to their inhibitory effect on EGFR tyrosine kinase (IC_{50} s = 170 and >400 μ M, respectively) [292]. The structures of nakijiquinones C and D are shown in Fig. (16).

In an effort to discover compounds that can disrupt EGF-dependent signaling pathways, a collaborative research team composed of researchers at the University of Utah and Bristol-Myers Squibb Pharmaceutical Research Institute evaluated marine invertebrate extracts for inhibitors of EGF-stimulated cell proliferation and identified naamidine A, Fig. (16), from a Fijian *Leucetta* sp. sponge [293]. In NIH3T3 cells that overexpress the EGF receptor, naamidine A selectively inhibited EGF-stimulated DNA synthesis (IC_{50} = 11.3 μ M), relative to the stimulation produced by insulin (IC_{50} = 242 μ M). In animal studies using A431 squamous carcinoma cells implanted in nude mice, naamidine A (at 25 mg/kg) achieved the active criteria of at least 85% growth inhibition. Initial mechanism of action studies revealed that naamidine A neither interferes the EGF-EGF receptor binding nor inhibits the receptor tyrosine kinase activities, suggesting that naamidine A functions downstream from the activated receptor.

Activation of EGFR leads to the activation of RAS protein, a member of a superfamily of GTP-binding proteins. These RAS proteins control a signaling network composed of multiple effectors with cross-talk, bifurcation, and feedback systems. Mutations that result in constitutively-activated RAS proteins are found in approximately 20% of human cancers [294]. Post-translational modification of Ras protein is required for its biological activity [reviewed in 290]. The cysteine residue of the C-terminus CAAX motif is farnesylated by farnesyltransferase, the -AAX tripeptide is cleaved by a specific endopeptidase, and the carboxyl group of the farnesylated cysteine is then methylated by a specific methyltransferase. The modified Ras protein is then transported and binds to the inner plasma membrane. Inhibitors of farnesyltransferase block the farnesylation of Ras protein and cause the accumulation of biologically inactive Ras protein that remains in the cytosol. Highly effective farnesyltransferase inhibitors have been identified and show great promise in pre-clinical studies. However, similar successes with farnesyltransferase inhibitors have not been achieved in clinical trials [reviewed in 290,295]. This is due to the fact that Ras proteins (K-Ras and N-Ras) can also be geranylgeranylated by geranylgeranyltransferase to overcome the inhibition of farnesyltransferase by farnesyltransferase inhibitors. Human Ras-converting enzyme (hRCE1) is a prenyl protease that recognizes both farnesylated and geranylgeranylated Ras proteins as substrates and removes the AAX tripeptide [296].

Inhibition of Ras proteolysis by hRCE1 represents an attractive target for blocking Ras signaling. Using a fluorescence-based assay for hRCE1 developed by Hollander and coworkers [278], a number of structurally related terpenoids were isolated from the marine sponge *Hippospongia* sp. that inhibit the protease activity of hRCE1 [298]. Barangcadoic acid A, rhopaloic acids A, B, C, and an inseparable mixture of rhopaloic acids D and E, Fig. (17), all showed IC₅₀ values of around 10 µg/mL (~25 µM) in the hRCE1 enzyme assay. These substances also showed tumor cell cytotoxicity, IC₅₀s from about 1–2 µg/mL (~2–5 µM), in a cytotoxicity assay using human colon tumor LoVo cells that contain a constitutively activated K-Ras mutation.

Once in the GTP-bound active state, Ras interacts with Raf kinase and the activated Raf phosphorylates and activates MAPK/ERK kinase 1 (MEK1). Activated MEK1 then phosphorylates and activates mitogen-activated protein kinase (MAPK). MAPK has numerous substrates, both nuclear and cytoplasmic, that modulate cellular functions such as cell-cycle progression and proliferation. In order to identify novel inhibitors of the Ras-MAPK signaling pathway, a high-throughput enzyme-linked immunosorbent assay (ELISA) was developed for the Raf/MEK1/MAPK cascade [299]. A primary assay measured the degree of MAPK phosphorylation (threonine 202 and tyrosine 204) as the end point, while a secondary assay examined Raf and MEK1 selectivity. Several thousand marine sponge extracts were screened in the primary assay for inhibitors of the Raf/MEK1/MAPK cascade, and bioassay-guided isolation led to the identification of eight pyrrole alkaloids from Philippine collections of the sponge *Stylissa massa* [300]. Among the five compounds, Fig. (18), that selectively inhibit MEK1 in the secondary assay, 10*E*-hymenialdisine and 10*Z*-hymenialdisine are most potent, with IC₅₀ values of 6 and 9 nM, respectively. The IC₅₀ values for the less potent compounds 2-bromoaldisine, 10*Z*-debromohymenialdisine, and hymenin are 539, 824, and 1288 nM, respectively. The other three natural products isolated - aldisine, oroidin, and 4,5-dibromopyrrole-2-carbonamide were inactive (IC₅₀s > 2.5 µM in the primary assay). Cell-based assay revealed that human colon tumor LoVo cells (containing the activated K-Ras mutation) are more sensitive to the growth inhibitory effects of 10*E*-hymenialdisine and 10*Z*-hymenialdisine (IC₅₀s = 0.586 and 0.71 µM, respectively), relative to human colon tumor Caco-2 cells (wild type Ras). In Caco-2 cells, the IC₅₀ for 10*E*-hymenialdisine was greater than 3.8 µM and greater than 7.8 µM for 10*Z*-hymenialdisine.

One group of MAPK substrates is the MAPK-activated protein kinases (MAPKAPKs) that include mitogen and stress-activated kinase (MSK) and MAPKAPK-2. Using a scintillation proximity assay (SPA) that measures MSK1 and MAPKAPK-2 activity by the quantity of ³³P incorporated into biotinylated peptide substrates specific for each kinase, four cheilanthane sesterterpenoids were isolated from an Australian collection of the sponge *Ircinia* sp. as inhibitors of MSK1 and MAPKAPK-2 [301]. The four compounds [25-hydroxy-13(24),15,17-cheilanthatrien-19,25-olide; 13,16-epoxy-25-hydroxy-17-cheilanthene-19,25-olide; 25-hydroxy-13(24),17-cheilanthadien-16,19-olide; and 16,25-dihydroxy-13(24),17-cheilanthadien-19,25-olide] all inhibited MSK1 and MAPKAPK-2 with the equal potencies (MSK1 - IC₅₀ = 4 µM; MAPKAPK-2 - IC₅₀ = 90 µM). The structures of these cheilanthane sesterterpenes are shown in Fig. (19).

Protein Kinase C (PKC)

The protein serine/threonine kinase PKC is considered a viable antitumor molecular target due to its involvement in protein synthesis, gene expression, cell proliferation, differentiation, and tumorigenesis. More than a dozen distinct PKC isoenzymes have been identified, many of which are overexpressed in various cancers [reviewed in 302,303]. These PKC isoenzymes are lipid-dependent and most are diacylglycerol-activated mediators of signal transduction. Phorbol esters, teleocidins and several marine natural products such

as bryostatin and aplysiatoxin can activate PKCs and have been used to study their role in cancer [304–307]. Naturally occurring retinoids and the microbial metabolite staurosporine inhibit PKC activity.

As part of the previously discussed NCDDG anticancer drug discovery program that examined marine organisms for inhibitors of tyrosine kinases, researchers at the U.C. Santa Cruz and Syntex Research examined marine natural products for new PKC inhibitors [274]. While the details of the bioassay were not described, it appears this group used purified rat brain PKC- β 1 in a cell-free system that measured ^{32}P incorporation into protein substrates, similar to their TK bioassay. Bioassay-guided fractionation of an active extract from a Papua New Guinea collection of the sponge *Xestospongia* sp. resulted in the isolation of a new polycyclic alkaloid xestocyclamine A, Fig. (20). Xestocyclamine A showed moderately potent PKC- β inhibitory activity ($\text{IC}_{50} = 4 \mu\text{g/mL} = 10 \mu\text{M}$). The crude extract of another Indo-Pacific sponge, *Penares sollasi*, also strongly inhibited PKC- β 1 activity ($\text{IC}_{50} = 0.3 \mu\text{g/mL}$) and did not inhibit pp60^{V-src} TK activity, suggesting that this activity may be relatively selective. Cruz and coworkers subsequently isolated a new azetidine derivative, penazetidine A, Fig. (20) [308]. However, two structurally related azetidines (penaresidins A and B) had previously been isolated from an Okinawan collection of *Penares* sp. sponge by Kobayashi and coworkers [309]. Penazetidine A inhibited PKC- β 1 activity ($\text{IC}_{50} = 1 \mu\text{M}$) and did not inhibit pp60^{V-src} TK. Many TK inhibitors are known to inhibit tyrosine kinases by binding to the ATP-binding site [286]. However, preliminary evidence suggests that penazetidine A may bind to the PKC lipid-binding regulatory site, rather than the less selective kinase ATP-binding site [308].

CONCLUSIONS

Advances in tumor cell biology, molecular biology, and high-throughput screening technologies are rapidly revolutionizing antitumor drug discovery and revitalizing marine natural products research. A transformation has occurred in the selection of antitumor molecular targets and in research focus toward disease-specific mechanism-based screening methods. The reasons for this increased emphasis on molecular targeted antitumor discovery are both philosophical and practical. Practical reasons include the convenience of molecular targeted assays and their suitability for high-throughput screening, required by the pharmaceutical industry. Perhaps more importantly, a philosophical change has occurred in antitumor drug discovery. While still a component of many research programs, anticancer drug discovery is no longer so narrowly focused on tumor cell cytotoxicity, or even tumor-selective cytotoxicity. Tumor-specific molecular mechanisms show promise as new means to provide tumor-selective treatment options. From an increased understanding of tumor cell biology, a new paradigm has arisen shifting treatment strategies toward potentially more disease-specific cancer treatments. New antitumor marine natural products discovery efforts often focus on recently discovered therapeutic targets that have the capacity to arrest tumor cells growth, stimulate tumor cell differentiation, cut off tumor blood supply, prevent metastatic spread, and inhibit the resistance of tumor cells to treatment.

Technical complexities associated with molecular screening methods, such as tumor cell-based reporter assays, often require dedicated facilities and highly trained specialized personnel. This has necessitated increased collaborative efforts between marine natural products chemists and the pharmaceutical industry, government, and other academic researchers. A leading force in the area of molecular targeted antitumor marine natural products discovery in the U.S. has been provided through the establishment of multiple investigator cancer research programs through the U.S. National Cancer Institute [12]. These National Cooperative Drug Discovery Groups (NCDDGs) have served as models to bring natural products chemists and cutting-edge biological scientists together for modern

anticancer drug discovery. While not involved in NCDDG-associated research, it is the opinion of the authors that such collaborative interdisciplinary teams of marine natural products chemists, pharmacologists, molecular biologists, biochemists, and industrial pharmaceutical scientists are helping to define new directions for molecular targeted antitumor research and providing new prospects for future marine natural product-based cancer treatment options.

Acknowledgments

These efforts were supported in part by the DOD/2000-Breast Cancer Research Program DAMD17-01-1-0566 (DGN). The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick, MD 21702-5014 is the awarding and administering acquisition office. The content herein reported does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. Additional support was provided by the USDA/Agricultural Research Service Specific Cooperative Agreement No. 58-6408-2-0009.

ABBREVIATIONS

ATP	Adenosine triphosphate
BAEC	Bovine aorta endothelial cell-based
CAM	Chorioallantoic membrane assay
CDK	Cyclin-dependent kinases
cDNA	Complimentary, DNA
Chk	Checkpoint kinase
CHO	Chinese hamster ovary
COMPARE	Computerized pattern recognition algorithm
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ET-743	Ecteinascidin 743
hRCE1	human Ras-converting enzyme
IMP	Inosine 5'-phosphate
IMPDH	Inosine 5'-phosphate dehydrogenase
ISA	Irciniasulphonic acid
kDNA	kinetoplast DNA
MAPKAPK	MAPK-activated protein kinase
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MMP	Matrix metalloproteinase
MSK	Mitogen and stress-activated kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
NCDDG	National Cooperative Drug Discovery Group
NCI	U.S. National Cancer Institute

PD-ECGF	Platelet-derived endothelial cell growth factor
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
Pol	DNA polymerase
PP	Protein phosphatase
TK	Protein tyrosine kinase
Topo	Topoisomerase
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor

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Fig. 1.
Structures of Antiangiogenic Marine Natural Products.

Fig. 2.
Structures of Marine Natural Products that Induce Apoptosis in Tumor Cells.

Fig. 3.
Structures of Marine Natural Products that Induce Apoptosis in Tumor Cells.

Fig. 4.
Structures of Marine Natural Products that Induce Apoptosis in Tumor Cells.

Fig. 5.
Structures of Marine Natural Products that Inhibit Cell Cycle Cyclin-Dependent Kinases.

Fig. 6.
Structures of Marine Natural Products that Inhibit Cell Cycle Checkpoint Kinases.

Fig. 7.
Structures of Marine Natural Products that Inhibit Topoisomerases.

Fig. 8.
Structures of Marine Natural Products that Inhibit Inosine 5'-phosphate dehydrogenase (IMPDH).

Fig. 9.
Structures of Marine Natural Products that Inhibit DNA Polymerases.

R = H stolonoxide A
R = CH₃ methyl stolonoxide A

R = H stolonoxide C
R = CH₃ methyl stolonoxide C

Fig. 10.
Structures of Marine Natural Products that Inhibit Mitochondrial Respiration.

Fig. 11.
Structures of Antimitotic Marine Natural Products.

Fig. 12.
Structure of the Kinesin Inhibitor Adociasulfate-2.

Fig. 13.
Structures of Marine Natural Products that Inhibit Multidrug Efflux and those that Retain Significant Antitumor Activity in Multidrug Resistant Tumors.

Fig. 14.
Structures of Marine Natural Products that Inhibit Receptor Tyrosine Kinases.

Fig. 15.
Structures of Marine Natural Products that Inhibit Receptor Tyrosine Kinases.

Fig. 16.
Structures of Marine Natural Products that Inhibit Epidermal Growth Factor Signaling.

Fig. 17.
Structures of Marine Natural Products that Inhibit Ras-Converting Enzyme (RCE).

Fig. 18.
Structures of Marine Natural Products that Inhibit MEK1 (MAPK/ERK) and Mitogen-Activated Protein Kinase (MAPK) Signaling.

25-hydroxy-13(24),15,17-cheilanthatrien-19
,25-olide

13,16-epoxy-25-hydroxy-17-cheilanth-19,25-olide

25-hydroxy-13(24),17-cheilanthadien-16,19-olide

16,25-dihydroxy-13(24),17-cheilanthadien-19,25-olide

Fig. 19.
Structures of Marine Natural Products that Inhibit MAPKAPK-2 and Mitogen and Stress-Activated Kinase-1 (MSK1) Signaling.

xestocyclamine A

penazetidine A

Fig. 20.
Structures of Marine Natural Products that Inhibit Protein Kinase C (PKC) Signaling.

Table 1

Selected Marine Natural Products that Target DNA.

Compound	Source	Biological Activity	Potency ^a	Comments/References
ascididemin	ascidian <i>Cystodytes dellechiaiei</i>	Inhibits Topo II	@ 50 μ M	Cytotoxicity-guided isolation: [73] Topo II [74]
bengacarboline	ascidian <i>Didemnum</i> sp.	Inhibits Topo II	@ 32 μ M	Cytotoxic [175]
elenic acid	sponge <i>Plakinastrella</i> sp.	Inhibits Topo II	IC ₅₀ = 0.1 μ g/mL	[176,177]
leptosin M	<i>Leptosphaeria</i> sp. from <i>Sargassum tortile</i>	Inhibits Topo II	IC ₅₀ = 59.1 μ M	Cytotoxic [178]
popolohuanone E	<i>Dysidea</i> sp. sponge	Inhibits Topo II	IC ₅₀ = 400 nM	Cytotoxic [179]
disaccharide S2	coral <i>Galaxea fascicularis</i>	Inhibits Topo I, II	@ 200 μ g/mL	[180]
polysaccharide GA3	dinoflagellate <i>Gymnodinium</i> sp.	Inhibits Topo I	0.05 – 1 μ g/mL	[181]
hexadecadienoic acid (5Z,9Z)	<i>Dicystostelium discoideum</i> slime mold & sponge <i>Chondrilla nucula</i>	Inhibits Topo I	@ 800 μ M	Cytotoxic, isolation: [182,183]
iso and anteiso fatty acids	<i>Streptomyces</i> sp. KM86-9B from a sponge.	Inhibits Topo I	@ ~100 μ g/mL	[184]
manoalide-25 acetal	sponge <i>Hyrtios erecta</i> .	Inhibits Topo I	IC ₅₀ = 25 μ M	Cytotoxic [185]
colopsinol A	dinoflagellates <i>Amphidinium</i> spp.	Inhibits pol α and β	IC ₅₀ s = 13 and 7 μ M, respectively	[186,187]
elenic acid	sponge <i>Plakinastrella</i> sp.	Inhibits pol α and β	IC ₅₀ s = 7.7 and 12.9 μ M, respectively	[188]
kelletinin I	gastropod mollusk <i>Buccinulum corneum</i>	Inhibits DNA pol α , β , and RNA pol	@ 10 – 250 μ M	[189]
sulfolipid KM043	red alga <i>Gigartina tenella</i>	Inhibits DNA pol α and β	@ 5 and 10 μ M, respectively	[173]
sculezonones-A/B	<i>Penicillium</i> sp. from bivalve <i>Mytilus coruscus</i>	Inhibits DNA pol α , β , ϵ , γ	IC ₅₀ s = 12 – 105 μ M	[157]
thiocoraline	Actynomicete <i>Micromonospora marina</i>	Inhibits DNA pol α	@ 0.1 μ M	Isolation: [190]; Bioactivity: [47,191]
ascididemin	<i>Cystodytes dellechiaiei</i>	DNA binding	IC ₅₀ = 0.4 μ M	[192]
dercitin	<i>Dercitus</i> sponge	Intercalates and disrupts DNA synthesis	IC ₅₀ s = 63 – 150 nM	Cytotoxic [193]; DNA binding: [194]
ecteinascidin-743	<i>Ecteinascidia turbinata</i>	Sequence-specific minor groove non-covalent DNA binding	IC ₅₀ = 2 μ M	[139]
mycalamides A,B	<i>Mycale</i> sp. and <i>Theonella</i> sp. sponges	Inhibits protein synthesis	@ 20 nM	[53]
spiroxins A-E	unidentified fungal strain LL-37H248 from a soft coral	Cleaves single-stranded DNA	IC ₅₀ = 0.09 μ g/mL	[195]

^a Some studies report only a concentration required for “inhibition.” Inhibitory concentrations are relative to the quantity of enzyme/substrate used in specific experiments.

Table 2

Multidrug Resistance Screening Systems and Marine Natural Product Inhibitors.

Screen	Cell Line	Compound/Source	Target Protein	Ref.
Type I	Drug sensitive: MCF-7, T-47D, ZR-75-1, MDA-MB-231; Drug-resistant: MCF-7 Ad ^r , MDA-AI ^r ; Primary human breast epithelial cells (HBEC): normal and malignant.	Marine invertebrate samples [crude extracts and secondary metabolites]	None, interfered with P-gp function.	253
	Drug sensitive: human colon adenocarcinoma LoVo, murine leukemia P388; Drug resistant: LoVo/Dx, P388/Shabel.	lamellarin I/tunicates <i>Didemnum</i> spp.	P-gp	254
	Drug sensitive: SW620, IA9; Drug resistant: SW620AD-300, A2780AD, 1A9PTX10, 1A9PTX22.	discodermolide/ <i>Discodermia dissoluta</i>	P-gp	257
	Drug sensitive: CCRF-CEM; Drug resistant: CEM/VLB ₁₀₀ .	didemnin B/ <i>Trididemnum</i> spp.	Not determined.	261, 262
	Drug sensitive: KB-3-1; Drug resistant: KB-8-5, HL60/Adr.	dehydrothysiferol/ <i>Laurencia viridis</i>	Not determined.	265, 266
Type II	Drug sensitive: LoVo, P388; Drug resistant: LoVo/Dx, P388/Shabel.	lamellarin I/tunicates <i>Didemnum</i> spp.	P-gp	254
	Drug sensitive: CCRF-CEM; Drug resistant: CEM/VLB ₁₀₀ .	patellamide D/tunicate <i>Lissoclinum patella</i>	P-gp	255
	Drug sensitive: KB; Drug resistant: KB/VJ300.	irciniasulphonic acid/ <i>Ircinia</i> sp.	P-gp	258
	Drug sensitive: KB-3-1; Drug resistant: KB-C2, KB-CV60.	agosterol A/ <i>Spongia</i> sp.	MRP-1	259, 261