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Diving for drugs: tunicate anticancer compounds

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The marine biosphere boasts tremendous biodiversity replete with structurally unique, active and selective secondary metabolites. Bioprospecting for antitumor compounds has been rewarding, and tunicates have been especially successful in yielding prospective cancer therapies. These compounds are now subjected to clinical trials in Europe and the USA. With the ongoing search for potent and specific anticancer drugs, in this article we discuss the unique perspectives, compounds and opportunities afforded by this rich source of potential pharmaceuticals. We discuss marine-derived antitumor drugs, their structures, and their various types and levels of antitumor activities in bench and bedside efforts.

Cancer research has made significant gains as improving technologies have revealed more detailed analyses of pathways, mechanisms and structures of antitumor compounds. The unique genomic profiles of tumor lines have been established, and many drugs used to target these particular lines have displayed remarkable successes [1]. While classic approaches to clinical oncology have focused on chemotherapy, irradiation, hemostasis and immunotherapy, other relatively unknown and unexplored approaches remain. These include analyzing purified natural products derived from plants and animals [2]. Many cytotoxic compounds have been isolated from tunicates, also known as urochordates, which belong to the subphylum protochordate [3]. Although both solitary and colonial tunicates have been identified, most are sessile as adults. Thus, they rely on an innate immune system for defense in their stationary state: an arsenal of toxins, each one possessing startling structural and functional diversity [4]. For example, compounds derived from the Didemnidae family are structurally unique, and include alkaloids and various peptides [5]. Remarkably, these compounds are found in animals within different phyla. For example, fascaplysin is an alkaloid isolated from two *Didemnum* tunicates, and four other distinct types of sponges [5]. Requiring clarification, this observation led many to hypothesize that these compounds are of microbial–bacterial origin. Supporting the claim, studies have confirmed that certain bacterial strains derived from tunicates seem to produce toxic metabolites [6,7]. This hypothesis is further supported by observations of compounds like Trabectedin (ET-743) with base structures that represent chemotypes similar to those originally described from microbes. Fortunately, symbionts have also aided the semi-synthetic production of some of these compounds [8]. These organic compounds serve as a natural defense against various predators, exerting anticancer, antiviral and immunosuppressive properties [9]. This assortment of structurally and functionally unique compounds has created a positive outlook regarding the potential of the marine ecosystem as the most lucrative and productive source of novel anticancer compounds of the future [10,11]. There has been excitement over developments in the aquaculture and biotechnology industries that have enabled rapid synthesis of certain, once trace compounds [12]. Consequently, there has been steady progress in determining the mechanisms of action of these compounds and their clinical efficacies [3]. Still, pertinent details for most of these compounds have yet to be fully elucidated. To this end, the inability to obtain necessary amounts of these compounds for research and clinical trials continues to be a major challenge, as technological advancements in biosynthesis have only eliminated some difficulties for a few compounds.

Despite these challenges, there has been a persistent effort in searching terrestrial and marine invertebrates for potential drugs [13]. This form of bioprospecting was initiated to stimulate research and development of newer natural products aimed at fighting disease, whether for complementary and alternative medicine

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GLOSSARY

Akt protein family A family of serine/threonine protein kinases that is involved in cell proliferation, apoptosis, transcription, metabolism and cell trafficking. In particular, Akt1 is an oncogene because of its ability to inhibit apoptosis.
Angiogenesis The growth and generation of new blood vessels from existing blood vessels. Although important for normal development, angiogenesis is also essential for tumors to transform from a dormant to a malignant state.
Apoptosis Programmed cell death. Unlike necrosis, apoptosis uses a different pathway that enables phagocytes to engulf the dying cells and prevents damage to other nearby cells.
Appendicularia Also known as Larvacea, this class of tunicates is transparent, solitary and free-swimming. Contrary to other tunicates, appendicularians primarily reside in the pelagic and photic zone of the ocean where more sunlight is present.
Aquafarming Also known as aquaculture, aquafarming involves the collection and/or cultivation of aquatic organisms.
Ascidiacea Also known as ascidians and sea squirts, the class of tunicates has a strong, rigid outer skin (or 'tunic') made of tunicin. Ascidians are sessile, that is they are attached to and grow on rocks and other objects in their environment. Of the 2300 plus ascidians, some are social while others are solitary. They are a commonly used model organism because of their relative simplicity, ease of manipulation and embryonic transparency.
Biodiversity Biodiversity is the extent of diversity in organisms within a particular system. It is often dependent upon the climate and region surrounding the system.
Bioprospecting Bioprospecting describes the process of discovering new compounds and biological details (e.g. mechanisms, interactions, among others) through analysis of natural sources.
Biosphere The biosphere is an ecological system that encompasses all ecosystems on earth. Interactions among organisms and their surroundings can also be considered a part of a biosphere, if a broader perspective is taken. Biospheres can also be any self-regulated ecosystem(s) that are enclosed, including artificial ones.
Cell cycle The cell cycle is a process that results in the division of a cell, in addition to its replication.
Gastrulation An early stage of embryonic development following cleavage and the formation of the blastula, during which the blastula forms into the three-layered gastrula (ectoderm, mesoderm and endoderm).
Inflammation Inflammation is a response to often-harmful stimuli such as dying or damaged cells, pathogens and irritants. This process facilitates removal of inflammatory agents and initiates recovery. When uncontrolled, chronic inflammation can lead to cancer by optimizing the microenvironment around cancer cells to support their growth and expansion.
Innate immune system The innate immune system acts as the host's immediate defense against foreign pathogens without conferring protective immunity. It is found throughout plant and animals of simple to complex organization, and is natural, non-specific, non-anticipatory, non-clonal and without memory of previous activity.
Metastasis Spread of cancer from one organ or site to another beyond the original territory.
MTT/MTS assay A colorimetric assay that measures the purple color that results from the reduction of MTT or MTS (and other similar dyes) to formozan dyes [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), respectively]. This allows for the determination of cell proliferation and viability, and conversely the cytotoxic potential of various compounds that can either increase or decrease those two properties.
Natural killer cell Natural killer (NK) cells are large granular lymphocytes that differentiate from normal lymphoid progenitor lymphocytes. They are widely used in the innate immune system. Importantly, NK cells are cytotoxic, causing target cells to undergo apoptosis secondary to perforin and granzyme release. As such, NK cells can reject tumors.
Necrosis Cell and tissue death secondary to external factors. Necrosis does not function through the same mechanism as apoptosis, and results in a build-up of cell debris and dead tissue that can harm other nearby cells.
Neutropenia Neutropenia is a disorder characterized by low numbers of neutrophils, an essential type of white blood cell. Neutropenia generally results in one of two ways: disruption of neutrophil production by bone marrow, or their destruction elsewhere in the body.
Nucleotide excision repair Nucleotide excision repair is a common DNA repair mechanism. This process can reverse UV-induced DNA damage, which can result in the formation of thymine dimers. Failure of this process can significantly increase the risk of cancerous mutations.
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells regulates DNA transcription. It mediates cellular responses to stress, immune responses to infection, and improper control of NF-κB has been associated with cancer and inflammation.
P53 family Tumor protein 53 (P53) is a tumor suppressor with key roles in cell cycle regulation.
Platelets Platelets are produced from the bone marrow during blood cell formation, and are involved in maintaining hemostasis by blood clot formation. Platelets are also important for hematogenous metastasis.
P-selectin P-selectin is a cell adhesion molecule present on endothelial cells that line the inner layer of blood vessels and activated platelets. P-selectin recruits leukocytes to sites of inflammation.
Sorberacea This class of tunicates resides in the ocean's deep Benthic zones.
Spindle The spindle apparatus is used during cell division to separate the chromosomes into their respective daughter cells. During mitosis, it is also known as the mitotic spindle, whereas during meiosis, it is known as the meiotic spindle. The proper formation of the spindle apparatus serves as a checkpoint before anaphase; defects at this checkpoint might contribute to cancer.
Thaliecea This class of tunicates is free-floating, and some members are colonial (pyrosomes), whereas others like the doliolids and salpids change from solitary to colonial. There is a complex cell cycle that depends on the anima's life cycle. Pharmacokinetics/pharmacopdynamics.
The danger model The danger model was presented in 1994 to compete with the self-non-self model. It hypothesized that cells with surface antigens respond to 'danger signals' from other cells undergoing injury and harmful processes (e.g. necrosis). These signals warn and arm the immune system – in particular T-cells – to respond to the threat. This model

argued that the immune system was dynamic, and was not strictly based upon a genetic, in-born definition of self.
Therapeutic index A ratio of the dosage of a drug that would be toxic for 50% of the population over the minimum effective dosage of the drug for 50% of the population, that is, it is the toxic dose divided by the therapeutic dose. Quantal dose curves are used to measure the toxic and minimum effective dosages, and how narrow or wide these curves are determines the drug's therapeutic index.
Thrombosis Formation of a blood clot.
Thy-1 Also known as cluster of differentiation 90 (CD90), Thy-1 is a 25–37 kDa cell surface antigen often used as a marker for many types of stem cells and the axonal projections of mature neurons. Thy-1 also regulates apoptosis, assists in T-cell activation, and importantly, is a tumor suppressor.
Tubulin Tubulin belongs to a family of globular proteins. Two forms, α- and β-tubulin, form microtubules, which help transport supplies across cells. These tubulin dimers regulate cell-mediated supply trafficking, and are often important targets for anticancer treatments.
Urochordata Tunicates belong to the subphylum Tunicata, also known as Urochordata. They are underwater filter feeders that often live at the ocean floor, but can also be found in higher zones. Tunicates are close relatives to vertebrates, and appeared around 540 million years ago.
VCAM-1 Vascular cell adhesion molecule-1 (VCAM-1), also known as cluster of differentiation 106 (CD106), is involved in the adhesion of various cells to the endothelium and regulates leukocyte-to-endothelial cell pathways.

(CAM), or traditional Western medical practices [14]. Tunicates can also be a useful model organism for studying cancer, because neoplastic disease has not been documented in these animals, despite the presence of oncogenes that are evolutionarily conserved in humans, such as *ras* and *src* [15]. The innate immune system and its immunosurveillance of tumors could provide insight into the decreased frequency of tumors in invertebrates. Hence, it is fitting to search tunicates for antitumor compounds, because these could be the factors that explain this observation; however, it is still unclear whether these compounds are produced in response to tumors, or what their roles in the native tunicate microenvironment are. Although there is no conclusive evidence to explain the rarity of tumors in many invertebrates, the innate immune system has been a key component in many hypothetical models, because evidence has shown that natural killer-like cells and hemocytes comprise an antitumor response in tunicates (Fig. 1) [16,17].

Bioprospecting for tunicate extracts has taken place for several decades on many levels across different continents [18,19]. These initiatives identified many new compounds, and major findings into marine natural products are presented by Faulkner, Blunt and Mayer (Midwestern University, Marine Pharmacology: http://marinepharmacology.midwestern.edu/preclinPipeline.htm) [20,21]. Many tunicates in the Eastern hemisphere have yielded potential antitumor compounds, justifying a sustained effort elsewhere, principally the West, to continue diving for extracts [22]. In particular, many antitumor drugs on the market are from natural products, their derivatives, or synthetic analogs, identifying new compounds with unique structures and activities can aid a continuing process of drug discovery [23]. Previous studies have identified many exciting new structure–activity relationships (SARs) that can be used to create potent semi-synthetic derivatives [24]. In this article, we discuss general mechanisms by which these antitumor compounds function, we present a list of miscellaneous antitumor compounds that have been discovered in tunicates, and review the most successful compounds in clinical trials.

SARs: antitumor effects and cancer-type specificity

An investigation of extracts derived from marine organisms reveals striking diversity in their structure, mechanisms of action and selectivity against certain cancer cell lines [4]. Although the specific functions and canonical pathways of these compounds have not all been identified, their structures have been well-characterized

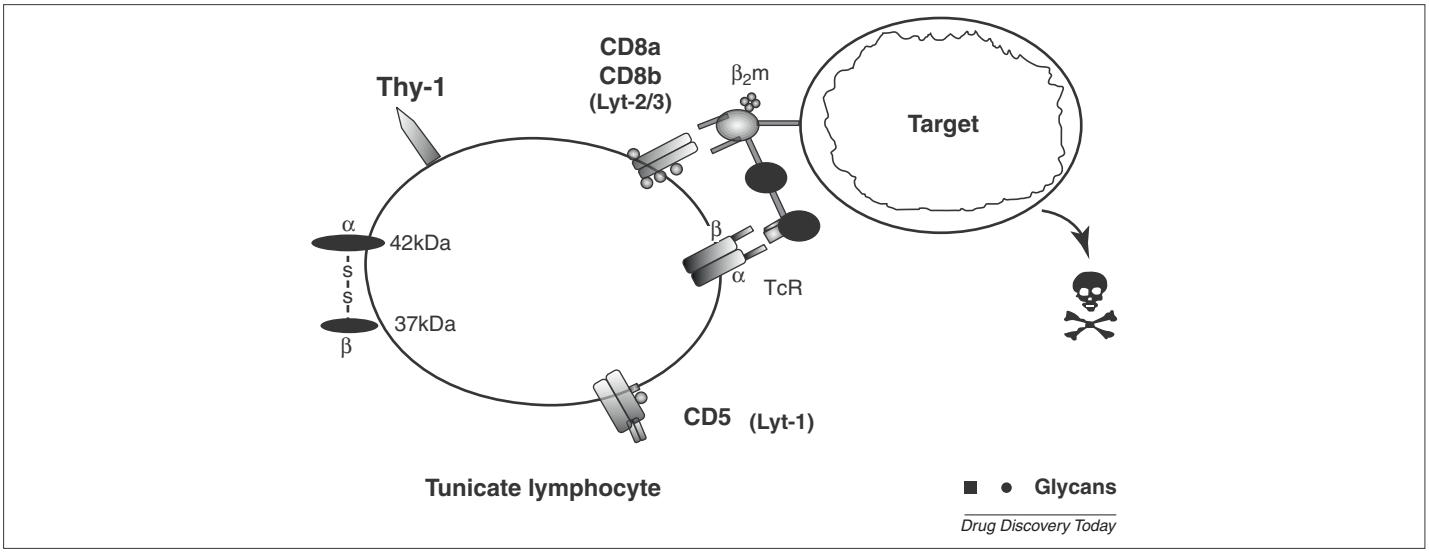

FIGURE 1

A cytotoxic signaling cascade involving antigen-recognition cell membrane molecules on lymphocytes and their respective target cells. This interaction results in a cytotoxic reaction leading to the destruction of the target. Thy-1 is found on tunicate lymphocytes which include natural killer cells, and also has a mammalian ortholog. These natural killer cells have been implicated in an antitumor response in invertebrates, thus providing a hypothetical model for an immune-mediated antitumor pathway that might support the observation of a decrease frequency of tumors among invertebrates such as chordata.

through extensive structural, spectral and chemical analysis. Valuable information can be derived from these features: a thorough understanding of the structure of each compound, selectivity against individual cancer lines and level of effect can help determine SAR [25]. Furthermore, many species of tunicates can each yield many compounds that are similar, except for a few differences in their stereochemistry and structure that might arise through sequential structural degradation of precursors or other evolutionary pathways [26,27]. The bacterial symbionts that produce many of the antitumor compounds found in tunicates can also be used to observe the reactions underlying the evolution of these metabolites. Asides from providing an attractive method of studying pathway evolution, examining these symbionts can address previous problems in comparative gene sequence analysis and rational pathway engineering, such as the confounding effect of point mutations that needs to be considered when studying more distantly related peptides. Because these symbionts and their hosts can be thoroughly traced by maternal transmission, independent point mutations that can otherwise significantly alter structural changes do not pose as a great problem in the analysis of complex enzymatic processes in metabolite pathways [28]. This enables a more direct comparison of common groups and biological activities among structurally similar compounds.

Didemnid ascidians hosting the symbionts *Prochloron* spp. have yielded distinctively related cyclic peptides with cytotoxic activity [27]. Other pertinent examples of the SAR of some antitumor compounds are observed in haterumaimides F–I, J–K and N–Q, isolated from *Ascidian lissoclinum*. Compounds F–I are monochlorinated diterpene alkaloids that each have different levels of cytotoxic potential against P388 leukemia cells, and haterumaimides N–Q are labdane alkaloids that are cytotoxic against similar lines [29]. By comparing the structural differences between these largely similar compounds to their levels of toxicity, Uddin and colleagues determined that an imido NH group in a ring and the positions of hydroxyl groups and chlorine atoms might be important for cytotoxicity against P388 cells. Knowledge of the antitumor effects of the compound along with SAR can help researchers synthesize drugs that target specific types of cancer.

For many of the compounds, only specific configurations exert a significant effect against specific cancer lines. Miyata *et al.* report this observation while evaluating the hyousterones from *Synoicum adareanum* which differ by a 14α-hydroxy or 14β-hydroxy group – the former has a cytotoxic response, and the latter, no noticeable cytotoxic properties [30]. The authors hypothesize that the β-inactive form enables the thermodynamically favorable conversion of hyousterone to abeohyousterone, a cytotoxic compound with even greater potency against several cancer lines. While conversion of hyousterone to abeohyousterone has not been forced experimentally, their respective structures can still be studied to determine potential SARs mediating abeohyousterone's cytotoxicity. The SAR of anticancer compounds is important; a major goal of cancer therapy is to design drugs that can kill cancer cells without disturbing healthy ones. Many tunicate-derived compounds are only selective against specific cancer lines. The two hydroperoxysterols from the tunicate *Eudistoma* sp. only differ by a single carboxylic acid, yet the beta form only exhibits cytotoxicity towards Hep3B hepatoma and A549 lung adenocarcinoma lines, whereas the alpha form is cytotoxic against Hep3B, HepG2 liver, MCF7 breast and MDA-MB-231 breast carcinomas [31]. By analyzing structural and conformational differences between compounds, and the resulting changes in their specificity or effects, the synthesis of even more efficient and effective antitumor pharmaceuticals will be possible.

Antitumor mechanisms of tunicate-derived compounds

Of the numerous marine-derived antitumor compounds, only a few have been extensively analyzed [11]. Those that have been rigorously scrutinized are prospective drug-candidates for clinical trials, or compounds that have ended up on the market [32]. Researchers observed that these compounds have differing mechanisms of action, possess unique targets, and have different pharmacokinetics and pharmacodynamics [22]. We have obtained a wealth of information by determining how these compounds act on different cancers; bioprospecting for new drugs and determining their mechanisms has helped identify new subtypes of cancers, and even provide potential targets for treating them [33].

It is important to expand our arsenal of anticancer drugs because tumors with similar histological or pathological marks might respond differently to the same drugs [34]. This first requires extensive analysis of the prospective antitumor compounds available. Hence, we review some of the mechanisms for compounds isolated from tunicates, with the goal of inspiring and encouraging further analysis. Most published works on these compounds used simple assays for antitumor activity, whereas others have been subjected to more detailed experiments to uncover the exact mechanisms of action. The following discussion on the compounds and their various mechanisms is summarized in Table 1.

Cytotoxicity

Cytotoxic compounds generally induce cell death by means of apoptosis, necrosis or decreased cell growth and division, and can thereby halt uncontrolled cancerous proliferation [35]. Of note, non-specific cytotoxicity that is apoptosis-independent can result in the harmful release of intracellular contents to the surrounding environment and promote inflammation [36]. Most antitumor compounds in Table 1 have been tested for cytotoxicity against cancer lines through the simple quantitative MTT/MTS (see Glossary) assay. However, this test provides little information regarding the mechanisms of action of these compounds [37]. Evidence of cytotoxicity from the MTT assay warrants further examination, because other tests are still necessary to provide details regarding possible mechanisms. For researchers identifying drugs that target specific pathways, clarifying these details can streamline searching for potential treatments.

Ogi *et al.* used the MTT assay to confirm the cytotoxicity of compounds isolated from *Diplosoma virens*, and then conducted additional experiments to determine how cell death occurred [38]. They examined the common apoptotic pathway involving caspase 3/7, and hypothesized that activated caspases in the mitochondrial cytochrome C pathway supported the apoptosis-induced cytotoxicity of the compound. Many other methods of determining cytoxicity can be employed, for example, Uddin *et al.* demonstrate that haterumaimides F–I are cytotoxic through its inhibition of the first cleavage of fertilized sea urchin eggs, an assay that was used extensively several decades ago, and is still just as applicable today [25]. However, more details still need to be uncovered after

TABLE 1

A structural and functional characterization of antitumor compounds from tunicates[a]

Compound name	Species	Comments on structure	Mechanisms (preliminary observations)	Refs
Haterumaimide J [$C_{20}H_{30}N_1O_4$] (14) Haterumaimide K [$C_{22}H_{32}N_1O_5$] (15)	***Lissoclinum* sp.**	Secondary hydroxyl groups at C-6, C-7, C-8 and C-12, an imido NH in the C ring, and the chlorine atom on C-2, all seem to be important for *in vitro* cytotoxicity.	Inhibited the first cleavage of fertilized sea urchin eggs and exhibited a gradient of its cytotoxicity against murine leukemia P388 cells.	[25]
Dichlorolissoclimide [$C_{20}H_{29}Cl_2N_1O_4$] Chlorolissoclimide [$C_{20}H_{30}ClN_1O_4$]	***Lissoclinum voeltzkowi Michaelson***	Nitrogenous labdane.	Antiproliferative effect due to blockage of G1 phase cells against the non-small-cell bronchopulmonary carcinoma line NSCLC-N6.	[77]
Cyclopentenones 1–7 [$C_{11}H_{14}O_4$] (1–2) [$C_{11}H_{12}O_4$] (3) [$C_{12}H_{14}O_4$] (4) [$C_{11}H_{10}O_4$] (5) [$C_{11}H_{14}O_5$] (6) [$C_{22}H_{24}O_7$] (7)	***Lissoclinum* sp.**	Didemnenone-related compounds 1–7 have a common carbon skeleton of 4-methyldecane.	Compounds 1, 2 and 8 were significantly cytotoxic against human colon carcinoma HCT116, epidermal cancer line A431 and the human alveolar basal epithelial adenocarcinoma line A549. Compounds 3, 4 and 7 were significantly cytotoxic against HCT116 and A431.	[78]
Lissoclinolide [$C_{11}H_{12}O_4$]	***Lissoclinum patella***	No specific comments.	Strong arrest at the G2/M phase of the cell cycle of human colon carcinoma HCT 116. The mechanism is thought to be independent of tubulin-interactions, ubiquitin-specific isopeptidases, p53 or p21. There was moderate selectivity towards colon tumor cell lines COLO 205, HCC-2998, HCT-116 and HCT-15 following NCI-60 panel tests. According to these tests, Lissoclinolide has a greater antiproliferative than a cytotoxic effect.	[79]
Lissoclibadin [$C_{39}H_{57}N_3O_6S_7$] (1) Lissoclibadin [$C_{26}H_{38}N_2O_4S_5$] (2) Lissoclinotoxin [$C_{26}H_{38}N_2O_4S_5$] (F)	***Lissoclinum* cf. *badium***	Compound 1 is trimeric. Compound 2 and 4 are dimeric structural isomers in either a *trans* or *cis* orientation around a disulfide or sulfide bond, respectively. Dimeric or trimeric compounds with a disulfide bond were shown to have greater activity than dimeric compounds with more than one disulfide bond, or monomeric compounds.	Lissoclibadin 2 has a wide range of inhibitory effects, effective against the human colon cancer lines DLD-1 and HCT116, breast cancer lines MDA-MB-231, renal cancer line ACHN and non-small-cell lung cancer line NCI-H460. Lissoclibadin 2 increases IL-8 production, indicating a link between cancer-killing and the immune system. The strongest cytotoxic responses were demonstrated by Lissoclibadins 1 and 2, and Lissoclinotoxin F.	[80]
Pibocin B [$C_{17}H_{21}N_2OBr$] (2)	***Eudistoma* sp.**	First representative of marine alkaloids with a unique structural feature, an *N-O*-methylindole group. (8 beta)-2-bromo-*N-O*-methyl-6,8-dimethylergoline.	Moderate cytotoxic activity against mouse Ehrlich carcinoma cells.	[81]
7 beta and alpha-hydroperoxycholesterol (1–2)	***Eudistoma* sp.**	No specific comments.	Sterol 1 shows weak inhibitory effects on human neutrophil elastase release. Sterol 1 has cytotoxic effects against human hepatoma cells Hep3B and human lung adenocarcinoma A549. Sterol 2 is cytotoxic against Hep3B, human hepatocellular carcinoma HepG2, human breast carcinoma MCF7, and human breast carcinoma MDA-MB-231.	[31]
Eilatin	***Eudistoma* sp.**	No specific comments.	Inhibited the proliferation of CFU-C from normal individuals and chronic myeloid leukemia patients in a concentration-dependent manner.	[47,48]

Dichloromethane extract (14–17)	***Eudistoma vannamei***	^{1}H NMR analysis suggests compounds 14–17 are structurally similar and might contain heterocyclic aromatic amines, as is observed with other compounds derived from the *Eudistoma* species.	Compound 14 demonstrated the greatest cytotoxicity. Treatment with compound 16 decreased BrdU uptake the most. Cells treated with compounds 15 and 17 demonstrated mixed apoptosis and necrosis, whereas compounds 14 and 16 only exhibited apoptosis. Compound 17 produced the greatest degree of apoptosis and necrosis. The compounds were tested against the promyeloblastic leukemia line HL-60.	[82]
2-Methyleudistomin D [$C_{12}H_{10}N_2O_3Br$] (1) 2-Methyleudistomin J [$C_{12}H_{10}N_2O_3Br$] (2) 14-Methyleudistomidin C [$C_{16}H_{18}N_3OBrS$] (3)	***Eudistoma gilboverde***	β-Carboline alkaloids.	14-Methyleudistomidin C had the strongest cytotoxic effect.	[83]
Shishijimicins A [$C_{46}H_{52}N_4O_{12}S_4$] (1) Shishijimicins B [$C_{45}H_{50}N_4O_{12}S_3$] (2) Shishijimicins C [$C_{45}H_{50}N_4O_{12}S_4$] (3)	***Didemnum proliferum***	Novel sugar component, which is a conjugation product of a hexose and a β-carboline, attached to the calicheamicinone aglycone.	Cytotoxic against HeLa, fibroblast line 3Y1 and murine leukemia P388 cells.	[84]
Mollamide B [$C_{36}H_{53}N_6O_6S$] (1) Mollamide C [$C_{30}H_{46}N_6O_6S$] (2)	***Didemnum molle***	Mollamide B: amide functionality, blocked N-terminus or a cyclic peptide.	Mollamide B had significant percentage growth inhibition in a non-small cell lung cancer line H460, breast cancer line MCF7 and the CNS cancer line SF-268; NCI-60 cell line panel was unremarkable beyond the mean for antitumor effects in any cancer cell line. Mollamide C was tested in an *in vitro* disk diffusion assay, and was not considered to be solid tumor selective.	[56]
Methanolic extract: Methyl myristate (1) Methyl palmitate (2) Methyl stearate (3)	***Didemnum psammatodes***	Compounds 1–3 are methyl esters (1 – methyl myristate; 2 – methyl palmitate; 3 – methyl stearate).	Compounds 1–3 demonstrate the greatest antiproliferative effect against acute promyeloblastic leukemia line HL-60, lymphoblastic leukemia line CEM, chronic myelogenic leukemia line K-562 and the T-cell leukemia line Molt 4. Compounds 1 and 3 were effective against HL-60 and Molt 4 lines; compound 2 was only effective against Molt 4. Compounds 1–3 also demonstrated an antiproliferative effect. The mechanisms involved include decreased DNA synthesis (reduced BrdU nucleotide uptake), necrosis (cells had significant eosin-stained proteins) and apoptosis (nuclear fragmentation and condensed chromatin).	[85]
Haouamine A [$C_{32}H_{27}NO_4$] (1) Haouamine B [$C_{42}H_{37}NO_{10}$] (2a; more stable, peracetylated derivative of 2)	***Aplidium haouarianum***	Each haouamine exists as an unseparable mixture of two interconverting isomers derived by the presence of a highly strained 3-aza-[7]-paracyclophane moiety in their structures.	Compound 1 exhibits a selective cytotoxic activity against the HT-29 human colon carcinoma cell line.	[86]

TABLE 1 (*Continued*)

Compound name	Species	Comments on structure	Mechanisms (preliminary observations)	Refs
2,3-Dimethoxy-5-(3′,7′-dimethyl-octa-2′(E),6′-dienyl)-[1,4]benzoquinone (1) and 12 synthetic analogs (3–14)	***Aplidium glabrum***	Quinones 1 and 3–6 have two isoprene groups in their side chains (10 carbons). Quinones 7–11 contain three isoprene groups (15 carbons). Quinones 12–14 have four isoprene groups (20 carbons). Activity is directly proportional to the length of the terpenoid side chain for quinones 1 and 3–11, but inversely proportional for quinones 12–14.	Quinones 1, 3–14 induce apoptosis in JB6 Cl41 cancer cells, and inhibit p53 while increasing transcription of AP-1 and NF-κB. Inhibition of cell transformation was found to be inversely proportional to the length of the terpenoid side chain. Quinones 1, 3–6 are more toxic towards transformed JB6 Cl41 cells than quinones 7–11, which conversely are more toxic towards normal JB6 cells. The relative positions of the terpenoid and methoxy groups also determine the extent of AP-1 transcription increase.	[87]
5-Hydroxy-7-prop-2-en-(E)-ylidene-7,7-adihydro-2*H*-cyclopenta[*b*]pyran-6-one (1) 5-Hydroxy-7-prop-2-en-(Z)-ylidene-7,7-adihydro-2*H*-cyclopenta[*b*]pyran-6-one (2)	***Diplosoma virens***	No specific comments.	Compounds 1 and 2 showed cytotoxity against HCT116 cells (human colorectal cancer cells) by triggering apoptotic cell death via the cytochrome C/mitochondrion stress pathway.	[38]
Hyousterone A [$C_{27}H_{42}O_5$] (2) Hyousterone B [$C_{27}H_{42}O_5$] (3) Hyousterone C–D [$C_{27}H_{42}O_6$] (4–5) Abeohyousterone [$C_{27}H_{42}O_5$] (6)	***Synoicum adareanum***	Hyousterones B (3) and D (5) are unusual ecdysteroids in bearing the 14β-hydroxyl group, and abeohyousterone incorporates the 13(14 → 8) abeo steroid skeleton, reflecting a rearrangement of the steroid C/D ring system. Hyousterones, abeohyousterone and diaulusterol B are the first ecdysteroids reported from tunicates.	Abeohyousterone has moderate cytotoxicity towards several cancer cell lines. Hyousterones bearing the 14α-hydroxy group (2 and 4) were weakly cytotoxic, while the 14β-hydroxy hyousterones (3 and 5) were devoid of cytotoxicity. The 14β-hydroxy function might be a thermodynamic pathway to the 13(14 → 8) abeo steroid skeleton.	[30]
Tuberatolide A [$C_{18}H_{26}O_3$] (1) Tuberatolide B [$C_{27}H_{34}O_4$] (2) 2′-Epi-tuberatolide B (3)	***Botryllus tuberatus***	Isoprenoid, tuberatolide A (1). Meroterpenoids tuberatolide B (2).	These terpenoids inhibited the chenodeoxycholic acid (CDCA)-activated human farnesoid X receptor (hFXR) without significant effect on steroid receptors. Furthermore, they released the activator protein from the CDCA-bound hFXR ligand binding domain in cell-free surface plasmon resonance experiments.	[88]
1-Heptadecanyl sulfate [$C_{17}H_{35}O_4S$] (1) 1-Octadecanyl sulfate [$C_{18}H_{37}O_4S$] (2) Sodium (2*S*)-2,6,10, 14-tetramethylpentadeca-1,18-diyl sulfate [$C_{19}H_{38}$ S_2O_8Na, $C_{19}H_{40}O_2$](3,3a) Sodium 3,7,11, 15-tetramethylhexadeca-1, 19-diyl sulfate [$C_{20}H_{40}S_2O_8Na_2$](4) 1-Hexyl sulfate [$C_6H_{13}SO_4Na$] (5)	***Sidnyum turbinatum***	Compounds 1–3 and 5 are alkyl sulfates. Compounds 1 and 2 are homologs. Compound 3 is a lower homolog of the diterpene 4.	Compounds 1–5 exhibited *in vitro* antiproliferative activity against the mice fibrosarcoma cell line (WEHI 164).	[89]
Stolonic acid A [$C_{26}H_{42}O_5$] (1) Stolonic acid B [$C_{26}H_{44}O_5$] (2)	***Stolonica* sp.**	Compounds 1 and 2 are 3,6-epidioxy-7,10-tetrahydrofurano C(26) unsaturated fatty acids.	The compounds exhibited antiproliferative activity against the LOX human melanoma and OVCAR-3 ovarian tumor cell lines.	[90]
Kottamide D [$C_{19}H_{20}Br_2N_4O_2$] (1)	***Pycnoclavella kottae***	Novel 2,2,5-trisubstituted imidazolone-containing alkaloids.	The kottamides exhibited anti-inflammatory and antimetabolic activity in addition to cytotoxicity towards tumor cell lines.	[58]

Hemocyte sub-populations from the buccal siphons	***Styela plicata***	No specific comments.	The hemocytes are cytotoxic towards the KS62 cancer line. There are two separate cytotoxic pathways contributing to this effect, both dependent on metabolic activity.	[91]
JBIR-66 [$C_{19}H_{32}N_2O_4$] (1)	***Saccharopolyspora* sp.**	(3*Z*,6*E*,8*E*)-*N*-(4-acetamido-3-hydroxybutyl)-2-hydroxy-4,8-dimethylundeca-3,6,8-trienamide. Structurally similar to BE-52211.	Weak cytotoxicity against human lymphoblastoid namalva cells.	[6]

[a] The mechanisms by which these compounds function, and special comments given by the original investigators on their structures is presented. Only compounds with demonstrated and significant effects are listed. Species have been bolded to emphasize compounds belonging to the same group. While a predictable trend in the mechanisms of the cytotoxic compounds is not noticeable, it might be of value to see what types of products can be isolated from within the same species. There are still hundreds more compounds that have not been added.

utilizing these assays, because different cytotoxic pathways can yield the same results. By discovering new antitumor compounds and exploring their mechanisms, we can expand our arsenal of potential therapeutics to target the specific pathways that differentiate various cancer lines. Many recent antitumor and cytotoxic compounds from the marine biosphere have been extensively reviewed by Gustafson and Mayer over the past decade [39].

Triggered anti-inflammatory pathways

Inflammatory agents trigger tumorigenesis by preventing apoptosis and initiating angiogenesis, the recruitment of blood vessels that aids tumor maintenance and promotes metastasis [40,41]. Agents formed from inflammatory–immune pathways and free radicals are released into the tumor microenvironment during inflammation, and contribute to cancer [42,43]. Inhibiting or controlling inflammation can thus prevent or delay the onset of cancer [44]. Many compounds derived from marine creatures display anti-inflammatory effects. One such compound, chondroitin sulfate, found in the tunic of *Styela clava*, has a fairly well-characterized mechanism of action [45]. When tested with JB6 cells, the investigators determined that chondroitin sulfate inhibits Akt, a serine/threonine kinase involved in transcription, proliferation and apoptosis, consequently blocking NF-κB dependent expression of vascular cell adhesion molecule-1 (VCAM-1) and nitric oxide synthase. Other compounds inhibit pathways that relate the processes of thrombosis and inflammation to metastasis; P-selectin mediates the interaction between platelets that promote clotting, the endothelium that lines blood vessels and tumor cells that precede metastasis [46]. Dermatan sulfates derived from the ascidians *Styela plicata* and *Phallusia nigra* inhibit inflammatory mediators and P-selectin, such that metastasis and thrombus formation were attenuated in MC-38 colon carcinoma and B16-BL6 melanoma cells. Although there are few marine-derived compounds whose mechanisms have been determined as clearly as the above examples, the potential for new antitumor compounds with anti-inflammatory mechanisms is astounding.

Inhibition of cell growth

Two definitive hallmarks of cancer are its uncontrollable growth and self-renewing capabilities [42]. Various antiproliferative compounds have been isolated from tunicates and ascidians that target growth-regulating pathways. Two from the Red Sea purple tunicate *Eudistoma* sp., Eilatin and Norsegoline, yielded a significant effect by halting self-renewal of leukemic progenitor cells through downregulation of CD34 and upregulation of CD11 and CD14 [47–49]. Analogs of compounds with potential therapeutic applications have also been synthesized (Fig. 2). Bouffier *et al.* created an analog of Eilatin, although it was not as effective because its low water solubility limits its use to low concentrations [50].

Many antiproliferative compounds target tubulin. These include Vitilevuamide, derived from *Polysyncraton lithostrotum* and *Didemnum cuculiferum*, and Diazonamide A from *Diazona angulata*. Microtubules are formed from dimers of α- and β-tubulin, and are necessary for cellular maintenance and trafficking. Hence, inhibiting tubulin polymerization can effectively impair cell growth. Vitilevuamide arrests the cell cycle at G2/M phase. The compound inhibits tubulin polymerization by targeting a tubulin binding site different from those used by dolastatin 10 (another

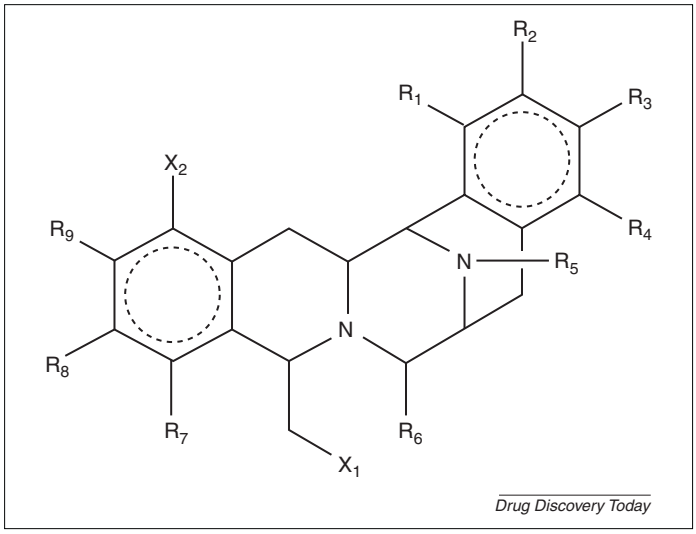

FIGURE 2

Synthetic analogs of ET-743 have been created, as shown below. Discovering new compounds can help elucidate novel structure–activity relationships which can then be used to construct and derive other synthetic compounds with potential clinical applications (patent information [92]).

marine-derived compound), vinca alkaloids and colchicine [51]. Preclinical studies with Vitilevuamide have demonstrated potent toxicities against P388 cells.

The difficulty of collecting *D. angulata* and extracting Diazonamide A from it initially prevented researchers from extensively analyzing its properties. Similar to many other marine natural products, the structure of Diazonamide A was also complex. Since its discovery in 1991, more than a dozen laboratories participated in a decade-long effort to crack its structure and synthesize it. While synthesizing Diazonamide A, researchers created the potent structural and functional analog AB-5 [52,53]. These efforts and a discussion of Diazonamide A are reviewed by Lachia and Moody [54].

Diazonamide A and its analog demonstrate their antitumor effects by inhibiting microtubule assembly at interphase [52]. For rapidly proliferating cancer cells, blocking tubulin prevents spindle formation and slows cancer cells in their tracks; of course, healthy dividing tissues that depend on microtubule transport will also be adversely affected. However, Diazonamide A and its analog AB-5 function by disrupting the mitochondrial enzyme ornithine δ-amino transferase (OAT) during mitosis, and inhibiting tubulin-dependent GTP hydrolysis [52,55]. Diazonamide A and its analog do not bind at the same regions on tubulin as dolastatin 10 and 15, two other potent anticancer compounds, even though there are similarities in the biochemical properties between these two groups. Of note, mice treated with AB-5 did not exhibit neutropenia similar to most tubulin-interacting antitumor drugs [55]. The relative lack of toxicity and potent anticancer properties make Diazonamide A and AB-5 promising candidates for further investigation in clinical trials.

However, while compounds such as mollamides B and C demonstrate some potential in inhibiting growth in various cancer lines, they can end up having no significant growth-inhibitory activities beyond the mean values when compared with other compounds [56]. These relative potencies can be efficiently measured when tested against the NCI-60, a panel of tumor cell lines obtained from adult human tumors including: melanoma, leukemia and non-small cell lung, colon, central nervous system (CNS), ovarian, renal and breast cancer [57]. Prospectively, the NCI-60 cell line panel can filter and find prospective drugs with exceptional determining the quantitative cytotoxic or antiproliferative effects of several candidate compounds relative to one another, the NCI-60 cell line panel can filter and find prospective drugs with exceptional antitumor activities. Of course, the NCI-60 cannot identify new mechanisms of action, because it will only report the cytotoxic or antiproliferative capability of the compound based upon the degree of growth inhibition among the tumor lines. With over 18,000 compounds isolated from marine animals, and over 150 of these demonstrating cytotoxic potential, measures to screen out the best compounds are of long-term interest, and can be a practical necessity to pick the promising candidate compounds for examination.

Mechanistic complexity

Many marine antitumor compounds exert individual effects, such as cytotoxic, anti-inflammatory, or antiproliferative, but others can use several different mechanisms to exhibit a complex array of antitumor activities. Kottamide D, isolated from the ascidian *Pycnoclavella kottae*, exhibited antimetabolic, antiproliferative and anti-inflammatory activities [58]. Microplate assays have proven especially useful in efficiently screening for both anti-inflammatory and antimetabolic activity by simply exchanging the type of salts used in the protocol [59]. With cancer drug-therapy focused on finding specific, targeted treatments, it is important to clearly understand how these natural products function. However, precise targets involved in the antitumor activities of even well-studied antitumor compounds are not conclusively defined. An incomplete picture of the mechanisms of action of a compound carries several risks. Practically, it is difficult to determine which compounds should be prioritized for further investigation. Applicably, translation to a clinical setting becomes more difficult and inefficient [60]. Even the compounds in Table 1 that are currently described as only having one mechanism might in fact have more.

From bench to bedside: marine natural immune defenses to practical pharmaceuticals

A few successful antitumor compounds derived from tunicates and ascidians have made significant progress through clinical trials in the USA and Europe. These include Didemnin B, Aplidine (Dehydrodidemnin B) and Trabectedin. Many other compounds from the marine biosphere have had ample success through clinical trials, and are thoroughly reviewed by Mayer *et al.* [61]. Didemnin B was first isolated from *Tridemnum solidum* in 1981, and evidence of its potent antiviral and cytotoxic properties made it the first marine anticancer drug to enter clinical trials [62,63]. The compound inhibits protein synthesis and cell growth by preventing EF-2-dependent ribosomal translocation and also induces protein tyrosine kinase-dependent apoptosis [64,65]. Didemnin B was introduced to Phase I and II clinical trials, following its early successes against various cancer lines. However, owing to side effects such as anaphylaxis and neuromuscular toxicity, these clinical trials were halted in the 1990s. Still, the precedent had been established: antitumor compounds could be derived from

TABLE 2

Successful marine natural products in clinical development

Compound name	Company	Clinical Phase I	Clinical Phase II	Clinical Phase III
Yondelis; Trabectedin; ET-743	PharmaMar Johnson & Johnson	Advanced adult solid tumors (Closed; NCT00002904) Refractory solid tumors (Completed; NCT00006463) Doxorubicin and Yondelis in soft tissue sarcoma (Completed; NCT00102609) Relapsed or refractory solid tumors (Closed; NCT00437047) Effects of Rifampin on the pharmacokinetics of Trabectedin in advanced malignanies (Active; NCT01273480) Effects of Ketoconazole on the pharmacokinetics of Trabectedin in advanced malignancies (Active; NCT01267084) Pharmacokinetic study of Trabectedin in advanced malignancies and hepatic dysfunction (Active; NCT01273493) Gemcitabine and Trabectedin in L-sarcomas (Active; NCT01426633)	Effects of Trabectedin on the heart in patients with advanced cancer (Completed; NCT00786838) Soft tissue sarcomas (Completed; NCT00003939) Previously treated metastatic osteosarcoma (Completed; NCT00005625) Previously treated unresectable advanced or metastatic soft tissue sarcoma (Completed; NCT00017030) Unresectable malignant mesothelioma (Completed; NCT00027508) Pediatric patients with recurrent or refractory soft tissue sarcomas or Ewing's sarcoma family of tumors (Completed; NCT00070109) Men with advanced prostate cancer (Completed; NCT00072670) Advanced ovarian cancer (Completed; NCT00050414) Advanced breast cancer (Completed; NCT00050427) Persistent or recurrent endometrial carcinoma (Completed; NCT00050440) Men with advanced prostate cancer (Completed; NCT00147212) Advanced, persistent, or recurrent uterine leiomyosarcoma (Temporarily Closed; NCT00379145) Docetaxel and Trabectedin with growth factor support in recurrent or persistent ovarian epithelial, fallopian tube or primary peritoneal cavity (Closed; NCT00569673) Localized myeloid/round liposarcoma (Closed; NCT00579501) Worsening metastatic adenocarcinoma of pancreas after Gemcitabine-containing first-line chemotherapy (Active; NCT01339754) Continuing versus intermittent Trabectedin in advanced soft tissue sarcoma after the 6th cycle (Active; NCT01303094) Doxorubicin and Trabectedin in first line treatment in advanced, non-operable and/or metastatic soft tissue sarcomas (Active; NCT01104298) Doxorubicin and Trabectedin in previously untreated advanced or metastatic malignant soft tissue sarcoma (Active; NCT01189253) Specific subtypes of metastatic breast cancer (Approved, not active; NCT00580112) Localized myeloid and round cell liposarcoma (Active; EudraCT Number: 2007-000035-25)	Relapsed ovarian cancer (Closed; NCT00113607) Trabectedin in patients with soft tissue sarcoma who have persistent or recurrent disease and are not expected to benefit from current standard of care (Active; NCT00210665) Advanced L-sarcoma (Active; NCT01343277) INOVATYON study of ovarian cancer (Active; NCT01379989) Trabectedin versus Doxorubicin-based chemotherapy in translocation-related sarcomas (Active; NCT00796120) Trabectedin with Pegylated Liposomal Doxorubicin (PLD) versus Carbopaltin with PLD in ovarian cancer (Active; EudraCT Number: 2010-022949-17) Trabectedin versus Doxorubicin in advanced or metastatic untreated soft tissue sarcoma (Active; EudraCT Number: 2009-014889-26)

TABLE 2 (*Continued*)

Compound name	Company	Clinical Phase I	Clinical Phase II	Clinical Phase III
PM01183	PharmaMar	Acute Leukemia (Active; NCT01314599) Non-colorectal cancer (Active; NCT01405391) Clinical and pharmacokinetic study of PM01183 in advanced solid tumors (Completed; NCT00877474)		
Aplidin; Dehydrodidemnin B; Plitidepsin	PharmaMar		Relapsing or refractory multiple myeloma (Completed; NCT00229203) Relapsing or refractory aggressive non-Hodgkin lymphoma (Completed; NCT00884286) Plitidepsin and Sorafenib or Gemcitabine in advanced solid tumors or lymphomas (Completed; NCT00788099) Primary myelofibrosis and post polycythemia vera/essential thrombocythemia myelofibrosis (Completed; NCT01149681) Plitidepsin with Cytarabine in relapsing or refractory leukemia (Closed; NCT00780143) Advanced prostate cancer (Closed; NCT00780975)	Plitidepsin and Dexamethasone in relapsing or refractory myeloma (Active; NCT01102426)

The most recent clinical successes and applications of the two most successful antitumor compounds derived from tunicates: ET-743 and Aplidin. For reference, we have also provided NCT and EudraCT numbers for the clinical trials. Many of the clinical trials are multi-center studies taking place in the USA, EU, Canada and many other regions.

marine tunicates, and many quickly followed, demonstrating even greater potential.

Aplidin, derived from the tunicate *Aplidium albicans*, is much more promising. Also known as Plitidepsin and Dehydrodidemnin B, it is structurally identical to Didemnin B except for a pyruvyl group at the *N*-lactyl side chain. However, its antitumor potency is significantly greater than Didemnin B [66]. The structural similarity to Didemnin B and its relatively small size advanced the production of effective Didemnin analogs. Structural modifications made during total synthesis enabled these analogs to better escape enzymatic turnover and remain active for longer durations. Aplidin has many mechanisms of actions, including mitochondria-initiated apoptosis, blockage of vascular endothelial growth factor (VEGF) release, p53-independent apoptosis, cell cycle arrest at G1–G2, disruption of angiogenesis, and inhibition of receptor proteins [22]. These mechanisms helped distinguish Aplidin's antiproliferative effects when it was first studied [67]. In the USA, there is still an active clinical trial assessing Aplidin and Dexamethasone in relapsing or refractory myeloma.

Marine creatures had another great success with the discovery of Trabectedin. Derived from the tunicate *Ecteinascidia turbinata*, it was the first antitumor drug derived from a marine source to be introduced to the market. However, as with Didemnin B and Aplidine, the initial problem with studying Trabectedin was that it could only be isolated in extremely trace amounts, quantities insufficient for proper clinical trials. Two developments helped break this impasse: increased aquafarming for Trabectedin, and new semi-synthetic approaches to create the compound [68,69]. In the process of determining the multi-step synthetic method for creating Trabectedin, researchers also created Phthalascidin, which has similar activities compared to Trabectedin [70]. More recently, the C subunit analog PM01183 of Trabectedin has been identified as both structurally and functionally similar, and has already entered clinical Phase I studies [71].

Trabectedin was found to be particularly effective against solid tumors. A synopsis of its DNA minor-groove binding mechanism to the guanine N^2 position is provided in a review by D'Incalci and Galmarini and Gajdos and Elias [60,72], and its effectiveness against various cancer lines is discussed by Amant *et al.* and Schoffski *et al.* [73,74]. Although Trabectedin was previously denied approval by the FDA owing to hepatotoxicity, it was approved in 2007 and 2009 in Europe for soft tissue sarcomas and ovarian cancer, demonstrating the difficulty and international differences in assessing new cancer regimens. However, it is still active in Phase III trials in the USA for use against ovarian cancer and soft tissue sarcomas. Importantly, Trabectedin is ineffective against cells without functional nucleotide excision repair, or that are sensitive to cisplatin, UV rays and alkylating compounds [75]. Fortunately, these disadvantages are offset by the finding that Trabectedin initiates apoptosis in cells that are actively transcribing genes; a deadly trap for overactive cancer cells [76].

Because antitumor compounds destroy cancer cells by disrupting essential cellular processes, this will also mean that normal cells are destroyed. To counter uncontrolled proliferation, indispensable cellular processes, such as transcription become targets for these chemotherapeutic compounds. Perfect, antitumor chemotherapeutic drugs that avoid such collateral damage are still the 'holy grail' of cancer therapy. For now, medical doctors and

biomedical researchers must determine which cancer lines certain antitumor compounds are most effective against, and whether or not they are safe to use. To assess the later, side effects are monitored in clinical trials, and the therapeutic index also becomes an important measure. Many clinical trials are terminated or suspended if a particular compound is too non-specific and destroys significant amounts of healthy tissue relative to cancerous tissue (i.e. it has a dangerously narrow therapeutic index as determined by quantal dose–response curves for the toxic and therapeutic doses). Different types of cancer have various properties, some of which make them more tolerant to drugs; unfortunately, patients might not be as tolerant to increased dosages, and this can cripple a drug's chances in clinical trials. The clinical progress of Aplidin, Trabectedin, and a new synthetic relative to Trabectedin, PM01183, are summarized in Table 2.

Concluding remarks

The potential hidden in the marine biosphere cannot be underestimated. Numerous compounds have been isolated from marine animals that display specific and non-specific cytotoxicity. Advancements in molecular biology and emerging tools have enabled for better screening and understanding of these compounds, and how they function. As cancer research progresses and new tumor-specific genes are identified, pharmaceutical and health industries are still extensively searching for drugs that can target these genes and proteins. With the potentially limitless amount of marine biodiversity, there are reasonable chances that compounds derived from tunicates, among other marine species like sponges, can provide long awaited therapeutics. Many compounds from tunicates have already demonstrated clinical relevance by targeting specific tumors. Although there are no guarantees of success, isolating and elucidating the nature of crude extracts can at least provide novel SAR for future pharmaceutical designs. Our hope is to inspire efforts to continue searching the sea for antitumor compounds. With this, and in-depth analysis at the bench, more antitumor compounds can finally be used as practical pharmaceuticals at the bedside.

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