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The expanding role of marine microbes in pharmaceutical development

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Abstract

Marine microbes have received growing attention as sources of bioactive metabolites and offer a unique opportunity to both increase the number of marine natural products in clinical trials as well as expedite their development. This review focuses specifically on those molecules currently in the clinical pipeline that are established or highly likely to be produced by bacteria based on expanding circumstantial evidence. We also include an example of how compounds from harmful algal blooms may yield lead both tools for measuring environmental change as well as leads for pharmaceutical development. An example of the karlotoxin class of compounds isolated from the dinoflagellate *Karlodinium veneficum* reveals a significant environmental impact in the form of massive fish kills but also provides opportunities to construct new molecules for the control cancer and serum cholesterol assisted by tools associated with rational drug design.

Introduction

Marine natural products are a continued focus for drug discovery and have provided many important therapeutic agents [1]. Lead compounds with biomedical potential have been isolated from marine invertebrates, bacteria and fungi. Each year numerous compounds with an array of biological activities are reported [2], but to-date only 13 molecules have entered into the clinical pipeline. Four molecules have been approved for clinical use, one of which is approved only in the EU. The approved molecules include two nucleosides based on sponge-derived nucleosides, a cone snail peptide, and a metabolite isolated from a tunicate [3].

Marine microbes have received growing attention as the sources for bioactive metabolites and have great potential to increase the number of marine natural products in clinical trials. The sustainable and economic supply of the active pharmaceutical ingredient (API) is often easier to achieve for compounds produced through microbial fermentation approaches vs the cultivation of slower growing macroorganism. Bacterial derived marine natural products

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have been the subject of two recent reviews, one dealing with symbiotic bacteria and one on marine microbes as drug leads in general [4,5]. In this volume, marine actinomycetes (Jensen), cyanobacteria (Gerwick), symbionts of ascidians (Schmidt and Donia) and bryozoans (Trindade-Silva et al.) are discussed separately.

A sometimes controversial and challenging field of marine natural products discovery is characterizing the relative importance of marine invertebrates and their bacterial symbionts in the actual production of the compounds found in the invertebrates. The fact that this issue has been debated for decades indicates the difficulty in unequivocally ascribing production of compounds to the hosts, the symbionts or a combination of both. The remarkable structural similarity that has been shown between some marine invertebrate-derived compounds from taxonomically distinct groups and those found in bacteria provides some circumstantial evidence that the compounds isolated from the invertebrates may be of bacterial origin, but it is hardly convincing since bacteria produce a great structural diversity of compounds. Localization of specific compounds within invertebrate or symbiont cells is also insufficient evidence since bioactive metabolites are routinely produced by one cell to elicit an effect elsewhere. The most convincing evidence is provided by the isolation of symbionts and demonstration of compound production by the microbes in pure culture on artificial media. This has been achieved in remarkably few cases and challenges in growing and controlling biosynthesis outside of the host persist. Strong circumstantial evidence has been provided by molecular approaches (e.g. see Haygood and Schmidt in this volume) [6,7]. This review focuses specifically on those molecules currently in the development pipeline that are clearly established or highly likely to be produced by bacteria. It is notable that approximately half of these compounds are of certain or likely bacterial origin. This provides good evidence for the importance of exploring microbial sources for marine natural products discovery. However, the number of cases where compounds originally discovered in invertebrates that were subsequently shown to be of bacterial origin does not indicate that all or most invertebrate-derived compounds are of bacterial origin. The likelihood that a particular lead will move forward into clinical trials is certain to be improved if bacterial production is established and the potential for sustainable production provided. We consider the relative importance of marine invertebrates and their microbial symbionts as producers of bioactive compounds still to be an area with many unanswered questions that need to be carefully resolved on a case-by-case basis.

In addition to marine bacterial products currently in clinical trials, we consider the toxins of harmful algae as a promising area for future investigations. The example of karlotoxins (KmTx2) from the dinoflagellate *Karlodinium veneficum* is discussed in detail to exemplify the potential of this area of research.

Microbes' involvement in lead entry into the clinical pipeline

Molecules in the clinical pipeline that are bacterial derived and others likely produced by bacteria are shown in Table 1. Two of the molecules, soblidotin (Figure 1-1) and tasidotin (Figure 1-2), are synthetic analogs of dolastatin 10 (Figure 1-3) [8,9,10]. Dolastatin and its analogs have been shown to be produced by cyanobacteria in the genera *Lyngbya* and *Symploca* [11,12]. Salinosporamide A (Figure 1-4) is produced by the marine actinomycete *Salinispora tropica* [13,14] (see Jensen in this volume). Although bryostatin (Figure 1-5) was initially identified from the bryozoan *Bugula neritina*, there is now strong evidence that it is produced by the uncultured symbiotic bacteria, "*Candidatus* Endobugula sertula" [15]. Plinabulin (Figure 1-6) is a synthetic product inspired by the *Aspergillus ustus*-produced phenylhistin (Figure 1-7) and halimide (Figure 1-8) [16,17,18]. These molecules are clearly produced by marine microbes.

There is circumstantial evidence that several additional molecules in the pharmaceutical product development pipeline may be produced by microbes. In some cases, this evidence is limited to conserved chemical characteristics between the marine derived compound and known bacterial compounds. Trabectedin (Figure 1-9), approved for cancer treatment in the EU, is highly likely produced by symbiotic bacteria [19]. Trabectedin and Zalypsis have strikingly similar ring systems to saframycin produced by bacteria and the semisynthesis of Trabectedin is based on the *Pseudomonas fluorescens* product cyanosafracin B (Figure 1-10) [20,21,22]. Eribulin mesylate (Figure 1-11) is a variant of the sponge isolated polyether halichondrin B (Figure 1-12) [23]. Bacterial produced polyethers such as tetronamycin (Figure 1-13), nigericin (Figure 1-14) and K41A (Figure 1-15) have similar structures to the halichondrin family of compounds suggesting the possibility of microbial production [24,25,26].

Despite the importance of these molecules only the bryostatins and salinisporamides have known biosynthetic pathways [27]. The gene cluster responsible for the production of bryostatin was identified and offers hope that heterologous production of the compound may lead to sufficient quantities for pharmaceutical therapy [28].

Of particular significance to the complex and unique chemical space covered by marine natural products is the fact that microbes can provide a sustainable supply of starting materials for the construction of unique classes of drugs. This is best exemplified by the development of trabectedin [19]. Although total synthesis of this molecule has been completed, none were practical for pharmaceutical development. Cyanosafracin B from *Pseudomonas fluorescens* provided a crucial starting material for a semisynthetic approach to the production of trabectedin. Similar results can be developed from identification and mutation of the microbe that produces the active compound, as is the aim for derivatives of manzamine as kinase inhibitors and for derivatives of the kahalalides class for cancer treatments [29]. Manzamine A (Figure 1-16) is active against malaria, Mtb and in neuroprotection assays which has been linked to its kinase inhibitory activity; however entry into the clinical pipeline has been hindered by supply of ircinal as a starting material and toxicity associated with the β -carboline group [30]. The entire molecule is required for activity as the β -carboline or aliphatic moiety, ircinal A (Figure 1-17), are essentially inactive as independent ligands [31]. Modeling experiments suggest that the kinase inhibition is due to hydrogen bonding and hydrophobic interaction in the allosteric site of GSK3- β ; while toxicity can be linked to DNA intercalation in the major groove [32]. These differing routes of action provide the opportunity for replacement of β -carboline ring to generate a better lead molecule. Ideally the aliphatic moiety (ircinal) could be assembled in just a few steps including an intramolecular Diels-Alder reaction. Biosynthetic studies for the manzamine class of compounds have been very difficult due to the lack of compound production by the organism in minimal media conditions and the difficulty of incorporating a labeled starting material in rich media conditions [33,34]. In the proposed biosynthesis of manzamine A, a terminal step is the addition of tryptamine by a Pictet-Spengler reaction to generate the β -carboline moiety [35]. The precursor ircinal provides a key starting material to semisynthetic approaches to replace the planar β -carboline ring with a ring system incapable of DNA intercalation that would retain the desirable selective kinase inhibiting affects.

Marine microbes will clearly play a greater role in the expansion of the drug development pipeline both in form of drug leads directly as well as the biosynthesis of starting material to be used in the construction of drugs and drug like molecules.

Turning marine Harmful Algal Bloom (HAB) toxins into new treatments for human health

HABs are becoming more frequent and problematic with global climate and other environmental changes [36]. These massive bloom events occur worldwide. A major concern is the presence of marine biotoxins in blooms. While normally only trace amounts of toxin are present in the environment, during an HAB toxin concentrations rise drastically. Filter-feeding organisms such as shellfish, zooplankton, and herbivorous fish may ingest the toxins, which are then concentrated through the aquatic food chain to humans. There are 50,000–500,000 incidents per year of marine biotoxins affecting humans with a mortality rate of 1.5% [37]. According to the National Oceanic and Atmospheric Administration, the economic impact of HABs on the United States is \$82 million per year. An interesting human health benefit for these molecules is that their stability in the environment, metabolic stability, unique chemical structures and biological targets all fit the desirable criteria for pharmaceutical prototypes that can be modified into functional drug leads. As an example the karlotoxins are a serious liability as a HAB toxin causing massive fish kills as well as intriguing molecules for the design and synthesis of novel cholesterol targeted drug leads.

The dinoflagellate *K. veneficum* produces a toxic suite of metabolites known as karlotoxins (KmTx). The structures associated with the known toxicity of the species were determined in the last decade. So far only three KmTx compounds have been published with complete mass and UV data, but up to six structures have been identified by LC-TOF MS [38,39]. The structure and complete absolute configuration of KmTx2 (Figure 1-18), has been successfully characterized by extensive 2D NMR experiments followed by degradation, Mosher's analyses and comparison with enantiomerically pure standards prepared by synthesis [40]. These compounds have shown hemolytic, cytotoxic, and ichthyotoxic activity which is reproducible from toxin produced in culture as well as from the environment [41]. Interestingly, *K. veneficum* strains can be found across the globe from Europe to Australia and especially in the United States along the Chesapeake Bay. The geographic location can affect whether or not this dinoflagellate produces a toxic KmTx or a relatively non-toxic analog. The mechanism of toxicity of KmTx is based on the membrane pore formation where the toxin forms a pore in the cell membrane destroying the osmotic balance of the cell, leading to cell death [42]. The pore formation is dependent on the sterol composition of the target cell. Cells containing 4 α -methyl sterols are immune, whereas cells containing 4-desmethyl sterols (like cholesterol) are susceptible to KmTx. *K. veneficum* is a mixotrophic organism and the preference for 4-desmethyl sterols may play an important role in prey immobilization since the main source of food possesses mainly these sterols as membranes while *K. veneficum* possesses mainly 4 α -methyl sterols [43]. The KmTx suite of molecules presents a unique opportunity to explore the design and synthesis of drug leads for the treatment of severe human health issues including heart disease and cancer where cholesterol is involved.

Cholesterol is a waxy, steroid lipid that is crucial for normal cellular and cell membrane formation. It is a precursor to many important vitamins and hormones including vitamin K, cortisol, cortisone, aldosterone, progesterone, estrogen, and testosterone [44]. A typical individual biosynthesizes approximately 1000 mg of cholesterol each day in their liver through the HMG-CoA reductase pathway which utilizes acetyl CoA starting units. This accounts for roughly 75% of cholesterol introduced into the body each day. The other 25% of cholesterol is consumed in foods such as egg yolks, meat, poultry, shellfish, and dairy products. The two main lipoproteins that transport cholesterol, Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL), are important markers for human health problems. The main health issue associated with cholesterol is coronary heart disease (CHD) which can lead to heart attacks, strokes, congestive heart failure and death. CHD is the

leading cause of death in the United States [45]. HDL cholesterol is considered to be the “good” cholesterol because it carries cholesterol away from the arteries and back to the liver. LDL cholesterol is the “bad” cholesterol carrying cholesterol from the liver to the arteries leading to blockages. The HDL major core lipids include endogenous cholesterol esters with an apoprotein A, and transfers its cholesterol esters to other lipoproteins including LDL. Its main function is to collect cholesterol from the blood stream and using an acyltransferase convert cholesterol to cholesterol esters and either return the molecule to the liver or transfer it to LDL or another waiting lipoprotein.

The karlotoxins provide an interesting opportunity to create through synthesis a non-toxic cholesterol pharmacophore that could potentially transport cholesterol from the arteries to the liver or kidneys for excretion. This would provide innovative HDL mimics. A variety of unique applications for a sterol binding molecule could be envisioned, adding to the six current classes of drugs for lowering total serum cholesterol levels and raising HDL cholesterol levels: niacin, fibrates, resins, statins, and selective cholesterol absorption inhibitors.

Although the primary focus of cholesterol research is on CHD, cholesterol is implicated in numerous other human health issues including cancer, viral infections (HIV-1 and Ebolavirus) [46,47], neurological disease (Creutzfeldt-Jakob, Alzheimer's and Parkinson's disease and obsessive compulsive disorder) [48,49,50], immunological responses and some bacterial infections (*Mycobacterium tuberculosis* and *Vibrio cholera*) [51]. The role cholesterol plays in each of these diverse human health issues is through its involvement with lipid rafts. Lipid rafts are part of the cell membrane between 10 and 50 nm in diameter, composed of sphingolipids, cholesterol and other proteins [52]. Cholesterol acts as both the adhesive and the spacer-unit between the sphingolipids and other proteins to hold the lipid raft together and ensure correct biological function. If cholesterol is depleted from a lipid raft formation in the cell membrane by a cholesterol sequestering agent, the raft components dissociate and the raft is rendered nonfunctioning. Lipid rafts have been shown to play important roles in membrane sorting, trafficking, cell polarization, cell growth, survival, death, and signal transduction processes of T cells, B cells, and allergic responses [53]. KmTx's mechanism of cytotoxicity appears to be linked specifically to perturbation of lipid rafts.

Cholesterol has been shown to play a key role in some cancers through its involvement in lipid rafts and lipid raft function. Cholesterol accumulation has been reported in some solid tumor cell lines. Breast and prostate cancer cell lines contain more lipid rafts than their comparable non-cancerous cell lines and are more sensitive to cholesterol depletion-induced cell death. Prostate cancer patients with hypercholesterolemia have accelerated growth of their carcinomas and conversely patients with reduced serum cholesterol levels show retarded prostate tumor growth [54]. Simvastatin, a common cholesterol lowering statin, increases apoptosis of certain types of cancer cells and Ezetimibe, a selective cholesterol absorption inhibitor, inhibits certain tumor angiogenesis [55]. These reports establish a link between cholesterol and cancer and highlight the potential for karlotoxin to lead to unique cholesterol targeting agents.

Conclusions

Marine natural products provide an excellent opportunity to study diverse and unique compounds not readily accessible from any other source leading to expansion of the pharmaceutical pipeline. Marine microbes can produce unique compounds covering new chemical space and the utility of marine natural products is expanding beyond its original role in identification of new prototype drug leads into fields of study involving sustainable

supplies of unique molecule using biosynthesis in conjunction with synthesis. Perhaps the greatest impact marine natural products has played is in revealing that unexplored and previously inaccessible chemical space can contribute to growth in the pharmaceutical pipeline. Improved methodologies in fermentation technologies, biosynthesis and synthesis provide opportunities to both create and supply drug leads which would not be available by any single method independently. As a result pharmaceutical biotechnology in the future is certain to provide increasingly sophisticated molecular architecture assembled using biosynthesis and synthesis in concert.

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Figure 1.

Structures of molecules in the preclinical/clinical pipeline and the prototype natural product.

(1) Solblidotin, (2) Tasidotin, (3) Dolastatin, (4) Salinosporamide A, (5) Bryostatin, (6) Plinabulin, (7) Phenylahistin, (8) Halimide, (9) Trabectedin, (10) Cyanosafrafracin B, (11) Eribulin mesylate, (12) Halichondrin B, (13) Tetronamycin, (14) Nigericin, (15) K41A, (16) Manzamine A, (17) Ircinal A, (18) Karlotoxin 2

Approved marine natural products and those in clinical trials (adapted from Mayer et al., 2010). Products known or likely to be produced by bacteria are highlighted in **bold**.

Table 1

Status	Name (Trademark)	Marine Organism	Chemical Class	Disease Area	Evidence for Bacterial Production of Parent Compound
Approved	Cytarabine (Cytosar-U®)	Sponge	Nucleoside	Cancer	None
	Vidarabine (Vira-A®)	Sponge	Nucleoside	Antiviral	None
	Ziconotide (Prialt®)	Cone snail	Peptide	Analgesic	None
Phase III	Trabectedin (Yondelis®)	Tunicate	Alkaloid	Cancer	Weak circumstantial evidence (structure)
	Eribulin Mesylate	Sponge	Macrolide	Cancer	Weak circumstantial evidence (structure)
	Soblidotin	Bacterium	Peptide	Cancer	Bacterial production
Phase II	DMXBA	Worm	Alkaloid	Cognition/Schizophrenia	None
	Plinabulin	Fungus	Diketopiperazine	Cancer	Fungal production
	Pittidepsin (Aplidin®)	Tunicate	Depsipeptide	Cancer	Weak circumstantial evidence (structure)
	Elisidepsin (Irvalec®)	Mollusk	Depsipeptide	Cancer	Bacterial production
	PM-00104 (Zalypsis®)	Nudibranch	Alkaloid	Cancer	Weak circumstantial evidence (structure)
	Tasidotin	Bacterium	Peptide	Cancer	Bacterial production
	Pseudoptererosins	Soft coral	Diterpene glycoside	Wound healing	Circumstantial evidence
	Bryostatins 1	Bryozoa	Polyketide	Cancer	Bacterial production (molecular evidence)
Phase I	Hemiasterlin	Sponge	Triptide	Cancer	None
	Marizomib	Bacterium	Beta-lactone-gamma-lactam	Cancer	Bacterial production