Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: informahealthcare.com/journals/iphb20

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To cite this article: William Fenical, Paul Jensen, Christopher Kauffman, Stephanie Mayhead, D. Faulkner, Catherine Sincich, M. Rao, Eric Kantorowski, Lyndon West, Wendy Strangman, Yuzuru Shimizu, Bo Li, Sudhakararao Thammana, Katherine Drainville, Michael Davies-Coleman, Robert Kramer, Craig Fairchild, William Rose, Robert Wild, Gregory Vite & Russell Peterson (2003) New Anticancer Drugs from Cultured and Collected Marine Organisms, Pharmaceutical Biology, 41:sup1, 6-14, DOI: 10.1080/1388020039051741

To link to this article: https://doi.org/10.1080/1388020039051741

Published online: 20 Oct 2008.

Pharmaceutical Biology
2003, Vol. 41, Supplement, pp. 6–14

Taylor & Francis
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New Anticancer Drugs from Cultured and Collected Marine Organisms*

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Abstract

This paper provides an outline of a collaborative research project between researchers at the University of California, San Diego, University of Rhode Island, and the Bristol-Myers Squibb Pharmaceutical Research Institute, with participating members from the Developmental Therapeutics Branch of the National Cancer Institute. The program, formally funded by the National Cancer Institute under the National Cooperative Drug Discovery Groups (NCDDG) program, seeks to discover new anticancer drugs from marine organisms, in particular invertebrates such as sponges and ascidians, and marine microalgae, marine bacteria and fungi. In this report, the program and results obtained since its beginning in 2000 will be summarized.

Keywords: Sponge metabolites, marine protoctista, marine fungi and bacteria, cytotoxic metabolites, marine anticancer agents.

Introduction

This National Cooperative Drug Discovery Group (NCDDG) is organized to discover and develop new natural product anticancer therapeutic agents from chemically-prolific groups of marine invertebrates and unexplored marine microorganisms. Novel chemical sources have been interfaced with a drug development program employing state of the art, cell and mechanism-based screening coupled with effective *in vivo* evaluation of potential drug candidates.

This program is composed of three university-based laboratory programs, coupled with a central oncology discovery and development core. The biotesting component, the Bristol-Myers Squibb Oncology Drug Discovery Group, provides comprehensive cancer pharmacology and drug developmental and marketing capabilities.

The following Project Leaders are associated with this Program:

Principal Investigator: **William Fenical**, Scripps Institution of Oceanography (SIO)

Program Leaders: **a) D. John Faulkner**, SIO; **b) William Fenical**, SIO; **c) Yuzuru Shimizu**, University of Rhode Island; *BMS Oncology Program:* **Robert A. Kramer**, Oncology Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute; *South Africa Field Program Leader:* **Michael T. Davies-Coleman**, Rhodes University.

* We dedicate this paper to the late D. John Faulkner.

Accepted: December 30, 2003

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10.1080/1388020039051741

Goals of the cooperative drug discovery group

The overall goal of this program was to bring together a diverse group of scientists with backgrounds in natural products chemistry and cancer drug discovery to form a unique collaborative program to discover new anticancer drugs. The benefit of this approach is the fertility of joining academic researchers with access to diverse natural resources, with industrial pharmacologists who possess in-depth capabilities in anticancer drug development. To achieve this goal, the group established an aggressive laboratory and field program to discover new anticancer agents from selected marine invertebrates and cultured marine microorganisms. Our sources for novel compounds are uncommon sponges, ascidians, bryozoans and uninvestigated soft corals, as well as three very diverse sources of unusual marine microorganisms: marine microalgae, marine bacteria, and marine fungi. These sources are among the most unique in the marine environment, and are known to produce novel metabolites of utility in cancer drug discovery.

Another goal of this Group is to employ effective and innovative mechanism-based bioassays to discover structurally novel, non-toxic anticancer agents from unusual marine organisms.

This project, which began in the year 2000, was the first renewal of a previous 5-year program. During that period several notable discoveries were made. These included the taxol mimic eleutherobin (Lindel et al., 1997; Long et al., 1998), the tubulin depolymerizers diazonamide A (Lindquist et al., 1991) and scleritodermin (Schmidt, 1999), the unique cytotoxin halimide, and the hormone-dependent prostate specific agents avrainvillamide and derivatives (Qian-Cutrone et al., 2002).

Group structure and interactions

This NCDDG program is structured to facilitate joint collecting expeditions and regular communication among the PI's, Bristol-Myers Squibb and the NCI. The role of the NCI, through the Staff Coordinator, Dr. Yali Hallock, is to facilitate the effective development of new anti-cancer leads and to avoid overlap with other NCDDG programs.

Sample collection, screening and processing

Each of the project leaders in this program has developed their specific capabilities for the collection of marine inver-

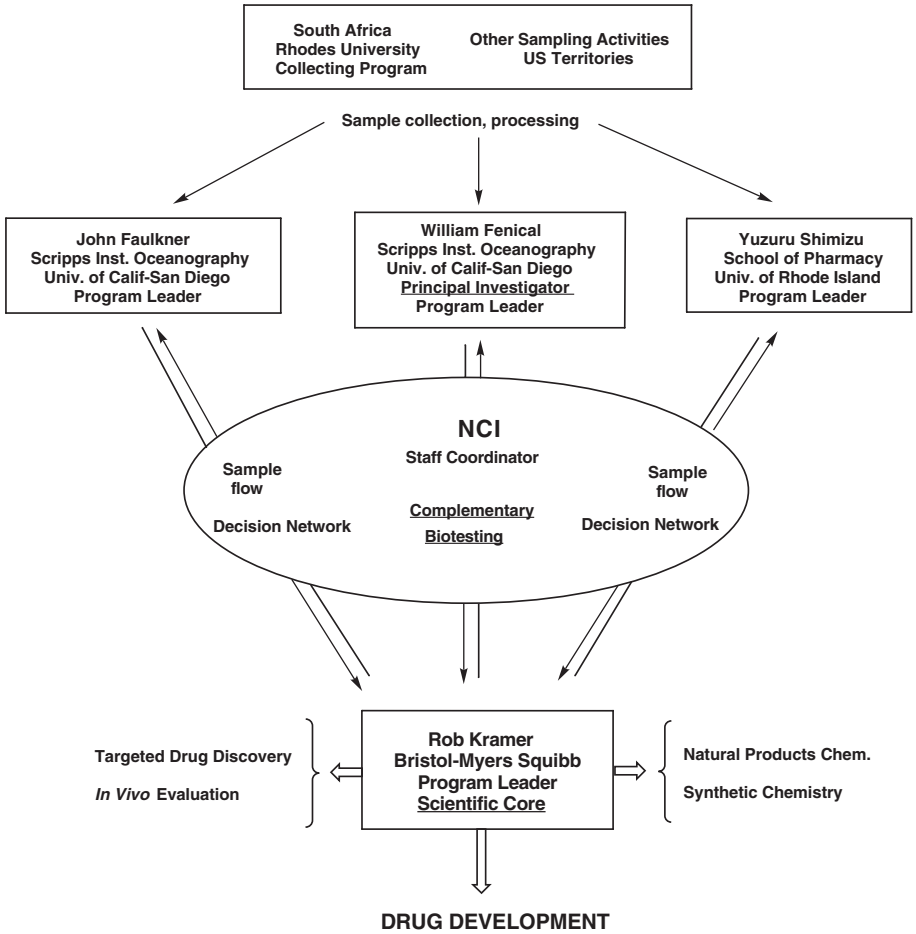


Figure 1. Diagram of the NCDDG Program "New Anticancer Drugs from Cultured and Collected Marine Organisms".

tebrates and microorganisms. However, to insure a robust supply of new samples for study, this program incorporated a subcontract with Professor Mike Davies-Coleman at Rhodes University in South Africa. Under this contract, yearly collecting trips to various regions of the South African Coast have been undertaken. Approximately 150 samples have been collected each year, they have been extracted at Rhodes University and then distributed to the relevant Program Leaders. Crude extracts were then sent to BMS in a 96-well format such that daughter plates can be prepared for multiple whole cell and mechanism-based screens. Over the past 3 years, over 5100 marine extracts were prepared and evaluated for biological activity.

Bristol-Myers Squibb screening targets

Over the past 3 years, BMS has provided access to numerous screening opportunities generally involving the following areas of cancer cell biology: **Targets aimed at Angiogenesis; Targets involving Hormonal Pathways; Targets involving Cell Cycle Control; Targets involving Signal Transduction; Novel Targets for Cytotoxicity** (Oncology Diverse Cell Panel); and ***In vivo* Screening** (Leukemia models and conventional xenograft systems).

Because of the proprietary nature of many of the above assays, it is not possible to provide further details of the BMS program. The general approach used is to simultaneously examine both *in vitro* cytotoxicity in the BMS Oncology Diverse Cell Panel (ODCA) and activity in mechanism-based assays. The ODCA is a small panel of cancer cell lines selected to represent a diversity of cancer tissue types some with specific sensitivities and thus selectivity patterns to a range of standard anticancer drugs. Using the ODCA panel, BMS personnel rank samples as to their priority for comprehensive examination. Priority 1 samples are potent cell division inhibitors and show high cell line selectivity. Priority 2 samples demonstrated good potency but lack significant cell line selectivity. Low priority samples (priority 3) are those that show poor selectivity and are less potent. These criteria will be mentioned in the subsequent sections which define new molecules being isolated as part of this collaborative effort.

Studies of marine invertebrates

This component of the NCDDG effort was under the direction of D. John Faulkner until his untimely death in November, 2002. The Faulkner program mainly involved examination of invertebrates collected in South Africa – 305 specimens (best hits overall), California – 89 specimens (modest results), the Red Sea – 54 specimens (modest results), US Virgin Islands – 61 specimens (a good source), and deep water in the Monterey Canyon (2000–3400 m) – 10 specimens (poor results) as part of a collaboration with MBARI (Monterey Bay Aquarium Research Institute).

Notable results from this effort are the discovery of at least 4 "priority 1 and 2" hits from the BMS ODCA panel. Fractionation of the highly cytotoxic extract of the sponge *Leiosella* cf. *arenifibrosa* yielded three novel molecules, **1–3** (Fig. 2) with excellent cytotoxic potency but little cell line selectivity. The icadamide derivative **1** showed an average IC_{50} of 0.11 nM against the ODCA cell lines, while the halipeptin derivative **2** showed an average IC_{50} value of 0.42 µM. Unfortunately, these potent molecules have yet to be evaluated in the BMS *in vivo* assays due to limited material. Additional supplies will likely be difficult to obtain. The third compound from *Leiosella* is the highly potent cytotoxin **3**, a rare analog of dihydroxytedanolide (Fig. 2). This interesting and potent macrolide demonstrated an average IC_{50} value of 4.1 nM when evaluated for *in vitro* cytotoxicity, and marginal activity *in vivo* (35% increase in lifespan versus vehicle treated control) against i.p. implanted P388 murine lymphocytic leukemia when compound was delivered by the i.p. route (i.p./i.p. P388). A 25% lifspan increase is required for a compound to be considered active. A maximum tolerated dose was reached, so increased compound could not be used. However, when evaluated in mouse (P388 and M109 lung) and human ovarian (A2780) xenograft distal site models, compound **3** failed to show activity when dosed to the MTD in these *in vivo* assays. In these more stringent antitumor models, the tumor is implanted into the mouse i.v. (P388) or s.c. (M109 and A2780) while the drug is administered by the i.v. route. Failing in these models, the compound was subsequently dropped from further consideration.

Marine invertebrates have also yielded several new molecules active in BMS mechanism of action screens. One of the targets currently under investigation is the intracellular proteasome, the enzyme complex responsible for degrading spent peptides and proteins. Examination of several invertebrates led to the discovery of two interesting inhibitors of proteasome function. From a sponge of the genus *Dysidea*, the Faulkner group isolated and described the polybrominated biphenyl ether **4**. This compound showed an IC_{50} value of 3.5 µg/ml in an *in vitro* proteasome assay. Similarly, from an unidentified dictyoceratid sponge, the sulfated merosesterterpenoid **5** was isolated. Compound 5 showed an IC_{50} value of 2.9 µg/ml. Although these compounds were active in the proteasome assay, they lacked the selectivity and potency in the ODCA panel (>10 µg/ml) and a chemotype with sufficient interest to be considered for further *in vivo* evaluation.

Chromatographic separation of the extract of the sponge *Aplysinella* sp. generated fractions which were highly active in the BMS HDAC (Histone Deacetylase) assay. Final purification of the active fractions yielded psammaplin A (**6**, Fig. 3) which showed amazing potency with an IC_{50} of 26 pM! We became aware, however, that this compound had previously been isolated by another research group that developed it further, and thus we dropped it from further evaluation.

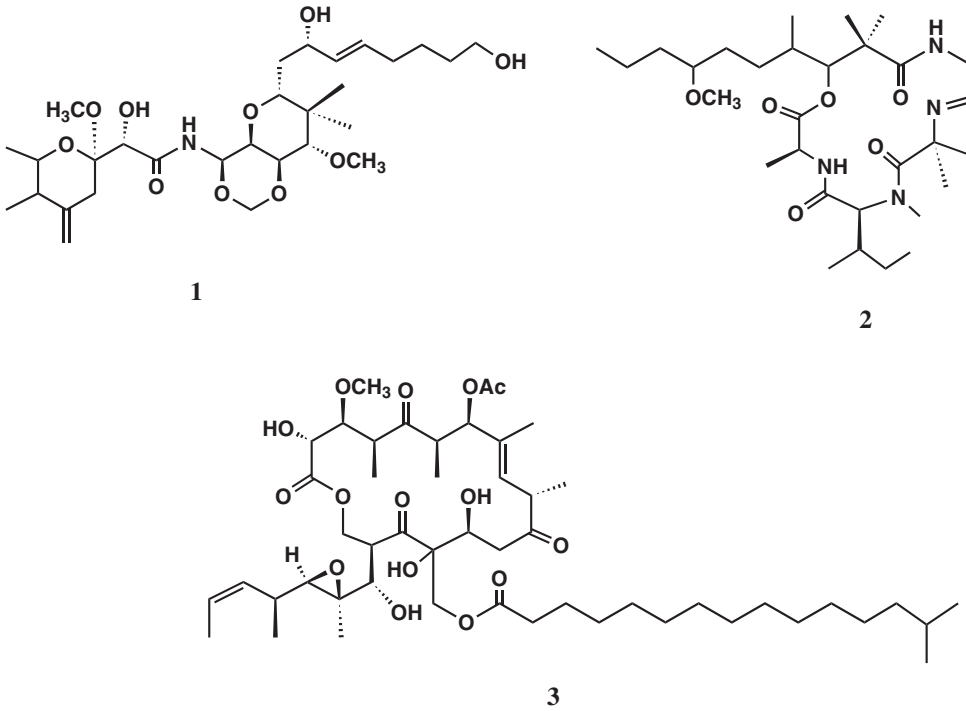


Figure 2. Cancer cell growth inhibitors from marine invertebrates.

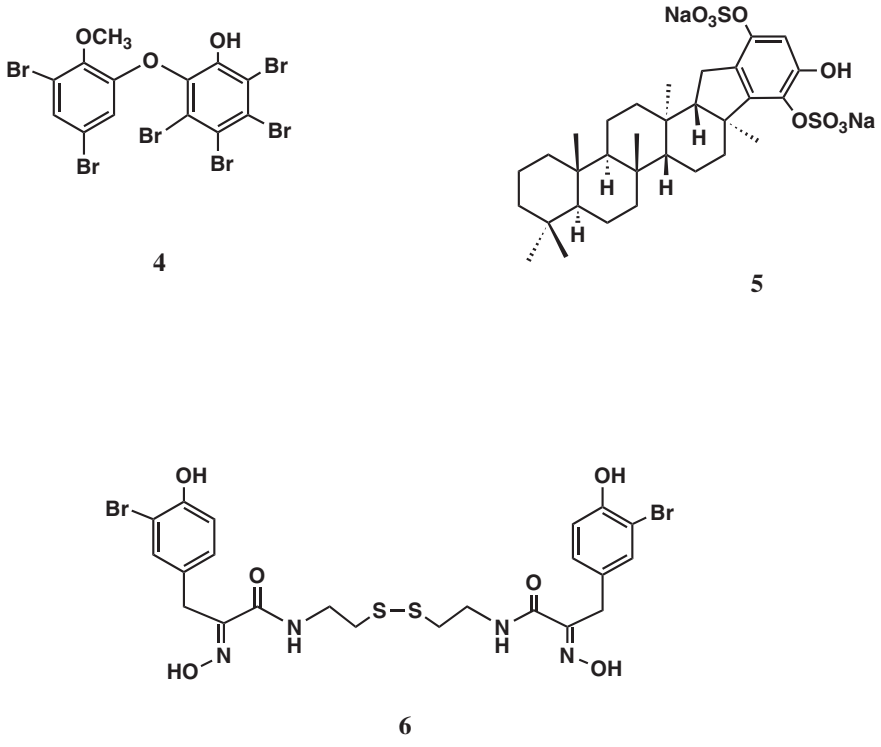


Figure 3. Cell growth inhibitors from marine invertebrates with activities in Histone Deacetylase (HDAC) and proteasome assays.

Investigations of selected marine microalgae

Marine microalgae. which taxonomically span more than 10 full plant divisions, represent an exciting frontier for natural products chemistry (Round, 1980). For more than 15 years, the Shimizu group has been examining numerous groups of marine algae, first as a source of marine toxins such as brevetoxin, saxitoxin, etc., and second as a new resource for the discovery of novel, bioactive natural products. The taxonomy of the microalgae is complex, but the group known as the Protoctista is rapidly emerging as a chemically prolific group. This group of eukaryotes includes the dinoflagellates, the chrysophytes and the haptophytes, complex microalgae that are thought to have evolved by the assimiliation of other microorganisms (Fig. 4). The dinoflagellates (Pyrrophyta), for example, possess extremely large genomes, at times up to 3–5 times the size of the human genome. These are also the same organisms that produce some of the most complex polyketide-derived compounds ever isolated. Examples of the former are the large polyether ciguatoxin and the impressive C_{145} polyketide maitotoxin.

There are two major challenges that must be addressed when working with microalgae: 1. The isolation of diverse strains, and 2. The mass scale cultivation for the preparative isolation of facilitating amounts of compounds of potential use in the treatment of cancer. When this project began, it was envisioned that progress to solve these two obstacles would be readily achieved. We were only partially correct.

Isolation of diverse microalgal strains

Over the past 3 years, the Shimizu group has developed new methods to selectively isolate microalgal strains that had not been observed before. This new discovery will greatly expand the numbers of species that will be available for future chemical studies. In brief, this new method is a modified enrichment technique that has never before been applied to microalgae. Using this method, the Shimizu group took natural seawater samples, low in microalgal counts, and treated them with a diversity of commercial antibiotics. For example, when Naragansett Bay water was treated with ampicillin and incubated, a massive bloom of an *Olistodiscus* sp. was observed. Over time, this species would dissipate and other species would replace it. Remarkably, despite having studied samples from this environment for more than 2 decades, the blooms consisted of unprecedented species. Since these new algae had bloomed in the incubation water, they were readily isolated and easily cultivated in the laboratory. Likewise, when another sample of Bay water was also treated with ampicillin, a different bloom of an unidentified cryptophyte was obtained. Extending this work, other antibiotics were employed and equally interesting blooms were obtained. For example, treatment with oleandomycin generated a significant bloom of a specific coccolithophorid alga, while treatment with streptomycin resulted in a significant bloom of a new dinoflagellate, probably an *Amphidinium* species. While this method has not eliminated the difficulties in isolating microalgae from complex environmental

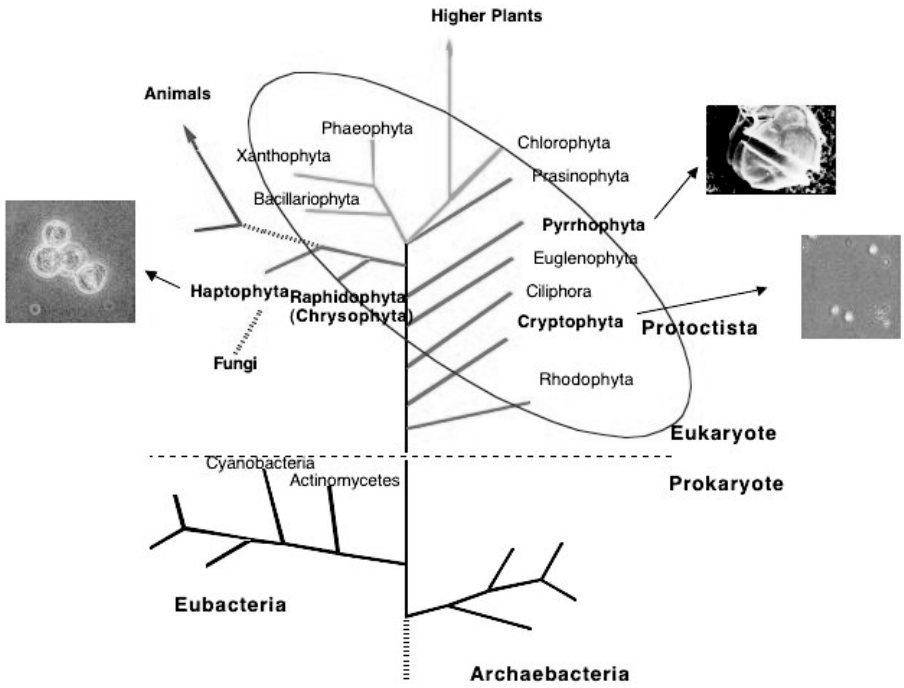


Figure 4. Taxonomic relationships of the eukaryotic microalgae with special reference to the Protoctista.

samples, it is the first example of a new selective isolation procedure that can be successfully applied to water samples from many marine habitats.

Using the above antibiotic method, and classic isolation techniques, the Shimizu group has isolated, cultivated, extracted and screened the extracts of over 255 microalgal species (dinoflagellates and hyptophyte algae) over the past 3 years. Of these, 62 samples (24.3%) scored as priority or 2 in the BMS ODCA panel. This is a higher percentage than any other biological resource yet studied, and it points to the major potential of this group of marine algae. By contrast, in earlier studies, the Shimizu group examined over 200 strains of another algal group, the diatoms, and found zero hits, showing that not all marine microalgae are exciting resources for bioactive metabolites.

Mass scale cultivation and isolation of metabolites

Examination, by the Shimizu group, of several strains of dinoflagellates of the genus *Amphidinium* collected in St. Thomas, US Virgin Islands, led to the discovery of numerous cytotoxic polyketides (Fig. 5). One example is the macrolide, amphidinolide B (**7**) (Bauer et al., 1994), which showed a mean IC_{50} of 0.2 μM in the BMS ODCA panel, minimal cell line selectivity and no activity against i.p./i.p. P388. Of even greater potency was the macrolide caribenolide (**8**) (Bauer et al., 1996; Shimizu & Fairchild, 1996), which showed a mean IC_{50} of 1.6 nM and, likewise, had limited cell line selectivity. Using limited supplies, activity against ip/ip P388 was established (50% lifespan increase), but no distal site antitumor activity could be shown against i.v. implanted P388 or s.c. implanted M109 (mouse lung tumor). Similarly, the Shimizu group cultured and investigated the dinoflagellate, a *Symbiodinium* sp., isolated as a symbiont of the Sargasso Sea jellyfish. This unusual dinoflagellate was found to produce the major polyol polyketide, symbiopolyolide 1 (**9**), which showed a mean IC_{50} of 0.2 μM in the ODCA panel and flat cell line selectivity. Symbiopolyolide 1 is one of the largest polyketides known possessing the molecular formula $C_{134}H_{225}NO_{54}S$ with a molecular weight of 2746.3 amu. The Shimizu group also isolated and comprehensively defined the protocerotins I–III (**10–12**), sulfated "ladder" polyether glycosides which possess impressive mean cytotoxicities of 0.5 nM in the ODCA panel, although with little cell line selectivity.

In viewing these impressive molecules, one naturally asks about their *in vivo* activities and consequently their stages of development. Given that these dinoflagellate-produced molecules generally can be quite potent, although with little cell line selectivity, some would be priority 2 projects. Yet, few of these molecules have reached advanced stages of development. Why? The answer lies in the great difficulty in mass culture and in providing the hundreds of milligrams needed for comprehensive *in vivo* analyses. We view examination of the bioactive secondary metabolites of marine dinoflagellates to be one of the most underdeveloped areas in natural products chemistry. Great potential exists here, but advances in culture methodologies and in photoreactor design will be required before these potent cytotoxic molecules become available in mass quantities. The Shimizu group has repeatedly approached this problem designing new reactors and experimenting with new culture conditions only to fail to achieve the cultivation scale required to solve this serious supply problem. We hope that molecules will be discovered of such importance that the monumental efforts to design new methods and create new facilities will be undertaken. Unfortunately designing and implementing such methods is beyond the capabilities and circumstances of University research.

Investigations of marine fungi and bacteria

Research efforts in the Fenical Program are focused on the discovery of high priority lead molecules from the cultivation broths of marine fungi and bacteria. Over the three year period since the inception of the grant, microbial collection activities have focussed on Southern California, Guam, Hawaii and South Africa. With the exception of South Africa, this program emphasizes collections from American waters. The results of these collections were the isolation and purification of 1600 new strains. Cultivation of these strains under 4 different conditions generated 6400 extracts which were tested at SIO in the HCT-116 assay and at BMS in the ODCA panel. Of the priority 1 and 2 results obtained, chemical studies have generated 40 new, bioactive molecules. Of these, one has been shown by BMS researchers to be active *in vivo* and is currently under further evaluation. Working with the NCI program, 27 new compounds have been requested and submitted to the 60 cell line panel cytotoxicity screen. Of these, 8 cytotoxic metabolites have advanced to the Hollow Fiber assay and one compound is currently scheduled for evaluation in a murine distal site xenograph model.

One of the success stories from this program involves the cytotoxic dioxopiperazine derivative, halimide (**13**) (Fig. 6). Halimide, produced by a fungal strain recovered from the surface of the green alga *Halimeda* sp., was discovered as part of the BMS screening activities. The *S* isomer shows a mean GI_{50} = 5 ng/mL in the NCI 60 cell line panel and was active *in vivo* (T/C = 153, P388 i.p./i.p in BMS screening. Further work at BMS demonstrated that halimide inhibited tubulin polymerization, similar to the Vinca alkaloids although at 100-fold greater concentrations. Of additional interest was, that unlike Vinca alkaloids vincristine and vinblastine which are cross resistant to multidrug resistant cancer cells which overexpress the P-glycoprotein drug efflux pump, halimide showed no such cross resistance. Similar to the Vinca alkaloids, halimide was collaterally sensitive to a paclitaxel resistant cell line with altered β-tubulin. Further development of this compound at BMS was discontinued due to research priorities for other tubulin active natural products, such as the epothilones. Consequently,

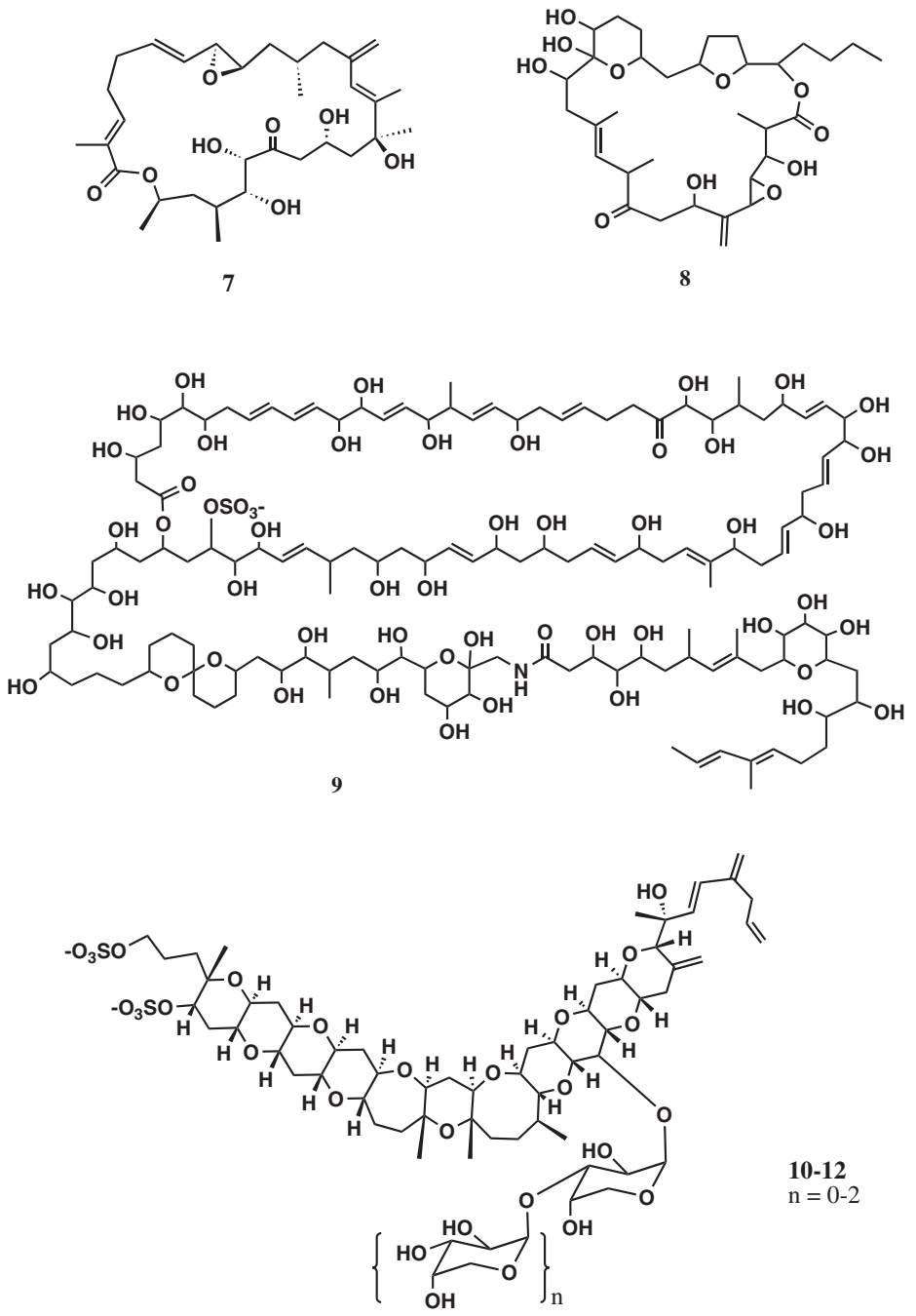
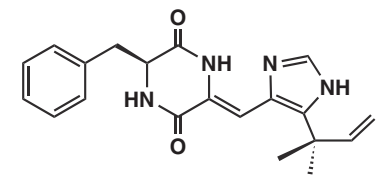


Figure 5. Various cancer cell inhibitory polyketides produced by marine dinoflagellates.

Nereus Pharmaceuticals in San Diego licensed halimide, demonstrated significant distal site *in vivo* activity, and invested heavily in an SAR program. The result was the development of a very potent derivative of halimide with sub-nanomolar IC_{50} values and significant *in vivo* activity. This new compound is an inhibitor of tubulin polymerization, but seems to possess a new mechanism of action.

Several other fungi-produced metabolites are under current study. This includes the destruxin analog **14** (Fig. 6) isolated from the culture broth of a *Gliocladium* sp. This chlorinated cyclic peptide showed a mean BMS ODCA value of 0.34 uM, weak cell line selectivity and was inactive *in vivo* in the P388 assay (T/C of 111% although no MTD was reached at 30 mg/kg/inj X 5). This compound is currently being examined by NCI in the Hollow Fiber assay. From an unidentifiable fungal strain, we also isolated the napthoquinone derivative **15**. This quinone showed an IC_{50} against HCT-116 = 0.34 µg/ml, a mean value in the BMS ODCA panel of 3.1 µM, and a mean NCI cell panel value GI_{50} of 0.5 µM. Extensive *in vivo* testing was subsequently under-



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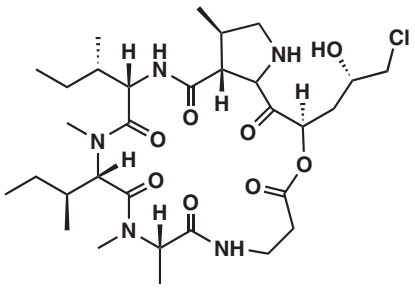

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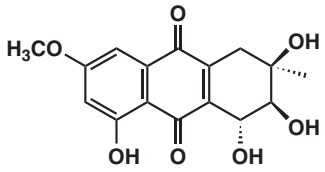

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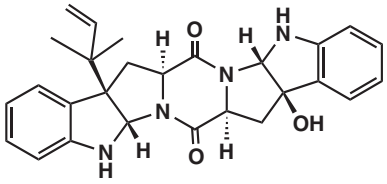

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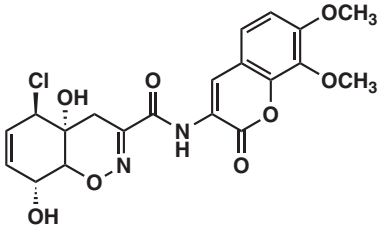

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Figure 6. New cancer cell growth inhibitors from marine fungi.

taken at BMS. Compound **15** was marginally active in the *in vivo* i.p./i.p. P388 model (33% lifespan increase) but failed in murine distal site models for P388 and M109 (lung). Although the mechanism remains unknown, it was determined by direct measurement that this compound does not intercalate DNA as its structure might suggest. This compound was subsequently dropped from further consideration.

Continued studies of marine fungi led to other molecules with significant bioactivities. From an unidentified fungal strain, we isolated the dioxopiperazine derivative **16**. Although chemically interesting, this molecule showed a mean IC_{50} value in the BMS ODCA panel of only 48 μM. Low cytotoxic potency and low fermentation yields resulted in termination of this project compound. From a marine isolate of the biomedically important fungus *Trichoderma*, we isolated the cyclic oxime **17**. This compound showed an IC_{50} = 0.32 μg/mL against HCT-116 and a mean value of 0.79 μM in the BMS ODCA panel. Unfortunately, due to difficulties in obtaining sufficient quantities of this compound for *in vivo* evaluation, further work on this compound was discontinued.

While most of our activities over the past 3 years have involved marine fungi, recent events will, no doubt, alter our strategy for the next two years. Working with Paul Jensen and Tracy Mincer on another project, we have discovered that deep ocean sediments are a treasure chest for the isolation of new, truly marine actinomycete bacteria. While these explorations began over 10 years ago, only recently were we able to confirm our suspicions that these bacteria were taxonomically unique. Using a combined culture and phylogenetic approach, we have recently described the genus *Salinospora*, the first truly marine actinomycete genus (Mincer et al., 2002). Members of the genus *Salinospora* are ubiquitous in marine sediments and appear to be concentrated in tropical ocean bottoms. They can also be found in the sediments in more shallow waters and on the surfaces of numerous marine plants and animals. These organisms often reach concentrations up to 10^4 per cc of sediment and are readily culturable using the appropriate selective isolation techniques. Aggressive efforts to obtain members of this new group in culture have led to the recovery of more than 3000 strains that are currently housed in our culture collection.

Given our recent discovery of the *Salinospora* group, and the realization that terrestrial actinomycetes were the single most prolific natural source for bioactive compounds ever discovered, we began to extend our studies to search for other unique groups of marine actinomycetes. Using a combined culture and phylogenetic approach, we began to observe other genera of actinomycetes not described from terrestrial environments. In total, 7 new taxonomic groups have been identified, and these are being described currently. These results suggest that the oceans harbor a unique reservoir of previously unknown actinomycetes that could represent a new source of bioactive compounds.

To begin to better understand the chemical potential of these marine actinomycetes, we cultivated 120 *Salinospora* isolates and subjected them to cytotoxicity and antibiosis bioassays. Amazingly, more than 80% of these strains inhibited HCT-116 colon carcinoma *in vitro* with IC_{50} values less that 20 µg/mL. Likewise, in a series of antimicrobial screens, these extracts were active against drug resistant bacterial and fungal pathogens with MIC values under 30 µg/mL. Subsequently, we cultured several strains and identified a very potent cytotoxin, salinosporamide A, which is perhaps the most potent proteasome inhibitor ever isolated (IC_{50} = 1.3 nM) (Feling et al., 2003). Because these preliminary results have been highly encouraging, we intend to focus our NCDDG research on several of the new genera currently under biological evaluation.

Summary

In this paper, we have highlighted some of the discoveries that have been made during the first three years of this NCDDG program. Although many more discoveries were made than could be presented, the goal was to emphasize the structural diversity and biological activities of the compounds that have been obtained from select marine invertebrates and cultured microorganisms. From these studies, we can conclude that it is becoming increasing difficult to discover new molecules from well studied invertebrates such as sponges. However, if unique sampling sites are targeted along with unusual taxa, new structures can still be found. We also recognize that certain groups of cultured micoalgae, such as dinoflagellates, are an exceptional source of structurally unique metabolites that possess potent biological activities. It is clear, however, that new methods for microalgal cultivation will continue to yield new species and exciting new chemistry; however the barriers to producing these compounds in sufficient yields for pre-clinical development remain significant. Great promise for the discovery of new biologically active natural products can also be found in heterotrophic marine bacteria. Now that it is clear that uniquely marine taxa belonging to chemically prolific groups such as the actinomycetes can be readily isolated from marine sources, these microorganisms warrant continued study of not only the secondary metabolites that they produce but also their culture requirements and diversity and distributions in the marine environment. Finally, our participation in this program has provided a unique opportunity for industrial and academic scientists to collaborate in a drug discovery effort. This has made it clear to the academics in the group that, without industrial resources and know-how, there would not be an effective mechanism by which the pharmaceutical potential of unique marine metabolites can be effectively explored.

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