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Targeted Natural Products Discovery from Marine Cyanobacteria Using Combined Phylogenetic and Mass Spectrometric Evaluation

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Abstract

Combined phylogenetic and HPLC-MS-based natural products dereplication methods aimed at identifying cyanobacterial collections containing the potent cytotoxins largazole, dolastatin 10 and symplostatin 1 were developed. The profiling of the phylogeny, chemical space, and antiproliferative activity of cyanobacterial collections served to streamline the prioritization of samples for the discovery of new secondary metabolites. The dereplication methods highlighted the biosynthetic potential and combinatorial pharmacology employed by marine cyanobacteria. We found that largazole was always coproduced with dolastatin 10 or with symplostatin 1 and consequently tested combinations of these agents against colon cancer cells. Combinatorial regimens of largazole and dolastatin 10 aimed at curbing the growth of HCT116 cancer cells showed cooperative activity.

Benthic marine cyanobacteria are a validated source of antiproliferative agents, having yielded several of the best-in-class inhibitors of malignancies.^{1,2} Cytotoxins from marine cyanobacteria also display not just a variety in structure but mechanisms of action as well.³ Marine cyanobacteria corresponding morphologically with the genus *Symploca* have yielded several modified linear peptides that target tubulin polymerization.^{4–9} The most potent among these are the related dolastatin 10 and symplostatin 1 (Figure 1),¹⁰ with the former serving as the template for the design of the clinically approved anti-Hodgkin's and

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Dedicated to Dr. William Fenical of Scripps Institution of Oceanography, University of California-San Diego, for his pioneering work on bioactive natural products.

anaplastic large cell lymphoma drug brentuximab vedotin.¹¹ Another novel agent from a cyanobacterium identified as *Symploca* sp. is the histone deacetylase inhibitor largazole (Figure 1), which displayed potent activity in preclinical evaluations.^{12,13} However, recent phylogenetic inferences of marine collections previously identified as *Symploca* and responsible for the natural products (NPs) dolastatin 10, symplostatin 1, and largazole have revealed that these cyanobacteria are evolutionarily dissimilar to the genus *Symploca*.¹⁴ These specimens have, as well as many other groups of tropical and subtropical marine cyanobacteria, simply been forced into established classification systems and were identified as *Symploca* based on morphological similarities.¹⁴ As a consequence, this taxon will soon be described as a new genus. The taxonomic revision demonstrates the current incomplete understanding of the taxonomy of marine cyanobacteria and stresses the need to implement phylogenetic inferences for proper identification of these microbes. In fact, molecular phylogenetic methods have been shown to be essential not only for taxonomic identification, but also for the prediction of secondary metabolite production and, thus, are useful for enhancing or directing NP-discovery efforts.^{15,16}

The isolation of a large number of antiproliferative agents from marine cyanobacteria, however, also increases the possibility of re-isolating known compounds as bioactive components. Thus, it is advantageous to employ a screen of the chemical space and phylogenetic relatedness as well. Several dereplication methods – identification of known metabolites from sample collections with the least effort and resources – have been developed for both terrestrial and marine cyanobacteria, employing UV spectroscopy and mass spectrometry. To distinguish known bioactive compounds in a screen for phorbol dibutyrate receptor binding activity, a HPLC-UV dereplication was utilized.¹⁷ Members of the aplysiatoxin class of compounds are known to be phorbol dibutyrate receptor binders, and comparison of the retention time and UV profile of authentic debromoaplysiatoxin allowed the identification of this compound as the active principle for several *Lyngbya majuscula* collections.¹⁷ This method also accounted for debromoaplysiatoxin as the bioactive constituent of seagrasses and macroalgae, possibly due to cyanobacterial contamination.¹⁷ More compound-specific techniques emerged with the development of new technologies in mass spectrometry such as MALDI-TOF-MS and ESI-MS. The initial utilization of MALDI-TOF-MS for dereplication was a serendipitous discovery, but nonetheless, demonstrated the presence of microcystins, micropeptin and anabaenopeptolin from collections of *Microcystis*, *Anabaena* and *Oscillatoria*.¹⁸ The application of MALDI-TOF for dereplication has been extended to determination of the spatial distribution of secondary metabolites in cyanobacteria themselves and other marine organisms, in addition to identification.¹⁹ Structure determination of nonribosomal peptides have also greatly benefited from mass spectrometry, with tandem mass spectrometry yielding the identity of these compounds via characteristic fragmentation patterns. Recently introduced is comparative dereplication using tandem mass spectrometry and spectral alignment algorithms to identify identical compounds and related analogs.²⁰ The requirement for minimal material to perform mass spectrometric analysis and its amenability to a high-throughput format makes this method an attractive choice for dereplication.

Here, a phylogenetic approach was used to classify marine cyanobacteria and use their inferred evolutionary relationships to known NP-producing strains as a way of predicting their production of secondary metabolites. Marine cyanobacteria putatively identified as *Symploca* spp. were used as model taxa to evaluate this phylogenetic approach, due to the richness in secondary metabolites and the taxonomic ambiguity associated with this group. Additionally, an HPLC-MS dereplication method utilizing multiple reaction monitoring (MRM) was developed to improve the resolution of known cytotoxins in collections of marine *Symploca*. This information enhanced our understanding of the phylogenetic distribution of known cytotoxins within these cyanobacteria. The novelty of the cyanobacteria based on phylogenetic inference and mass spectrometric analysis, together with antiproliferative screening against HT29 colorectal adenocarcinoma cells, was utilized to prioritize cyanobacterial collections for further studies. Based on the results of the chemical profiling, the combinatorial effect of largazole and dolastatin 10 against human colorectal adenocarcinoma cells was also interrogated.

RESULTS AND DISCUSSION

A total of 67 marine cyanobacterial samples were collected in Florida, Guam, Bonaire, and the US Virgin Islands from 2007–2012 (Supporting Information Table S1). The specimens were selected based on similar phenotypic features that corresponded with the traditional taxonomic definition of *Symploca* spp. All specimens were composed of fine filaments with barrel-shaped cells organized into entangled bundles. The colony morphologies were upright and formed various puffball-shaped erect colonies. It should be noted that, although many of the specimens were shown microscopically to be composed of mixed assemblages of morphologically-different cyanobacteria, the bulk in all specimens were species of *Symploca*.

Phylogenetic profiling

The small ribosomal subunit (16S rRNA) genes were sequenced from 21 of the specimens. The gene sequences were then aligned with all 16S rRNA gene sequences available for known NP-producing marine cyanobacteria in public databases (see ref 14 for a comprehensive list of NP-producing strains). Phylogenetic inference revealed a degree of polyphyly among the *Symploca* specimens and diversification into several distinct and distantly related lineages (Figure 2). As a result of this phylogenetic analysis, nine of the specimens were shown to nest within a known NP-rich group (highlighted with a blue box in Figure 2). All specimens within this clade were genetically similar with a p-distance of less than 1%. It should be noted that specimens within this clade have previously been shown to produce the NPs dolastatin 10, symplostatin 1, and largazole (this group is designated as Clade III in ref 14). We will refer to this NP-rich lineage as Clade III.

Despite phenotypic similarities, the remaining 12 specimens were phylogenetically distantly related with uncorrected 16S rRNA gene sequence divergences of over 7% to the Clade III specimens. Instead, these specimens form four separate and distinct lineages (highlighted with green boxes in Figure 2). All four lineages were only distantly related to any known taxa, suggesting a large amount of novel biodiversity among these specimens. Furthermore,

these specimens were also distantly related to any NP-producing cyanobacterial strains with available gene sequences.

Metabolite and bioactivity profiling

Collected organisms were lyophilized and extracted with either CH₂Cl₂–MeOH (1:1) or EtOAc–MeOH (1:1) to yield the nonpolar extracts. These extracts were further subjected to a C18 solid phase extraction (SPE) cleanup using a MeOH–H₂O elution. Initial elution using 25% MeOH removed the majority of the salts and ensured minimal non-specific bioactivity and interference in HPLC-MS arising from these polar compounds. The fraction collected from 100% MeOH elution was tested for antiproliferative activity against HT29 colorectal adenocarcinoma cells and concurrently profiled by HPLC-MS.

Antiproliferative activity was assessed based on the fractional survival of HT29 cancer cells, detected using the MTT reagent. Extracts which caused <60% survival of HT29 cells were considered bioactive at the specified concentration. From the 67 samples screened for antiproliferative activity, only seven sample collections were inactive at all concentrations tested (Figure 3). There were 22 sample collections that exhibited moderate antiproliferative activity against HT29 cells at concentrations of 1,000 and 10,000 ng/mL (Figure 3). The remaining 57% of the screened cyanobacterial collections exhibited antiproliferative activity at concentrations of 10 and 100 ng/mL (Figure 3). With the large number of cyanobacterial collections showing antiproliferative activity, additional information for prioritization of sample collections was needed. Also, with potent cytotoxins such as dolastatin 10, symplostatin 1 and largazole being produced by several collections, the contribution of these known compounds to the bioactivity should be assessed at an early stage of the discovery process.

The dereplication method for the known compounds largazole, dolastatin 10 and symplostatin 1, consisted of a gradient HPLC run using CH₃CN–H₂O (+ 0.1% HCOOH) and MRM as MS detection mode. This provided a sensitive, specific and high-throughput format for the dereplication of previously isolated metabolites from sample collections identified as *Symploca* spp. The MRM mode relies on the detection of both the parent ion mass (Q1) and a specific daughter ion resulting from fragmentation (Q3), giving a significant reduction in background, improvement in signal-to-noise ratio and limits of detection. This dereplication format permitted automation, short run times per sample (<20 min) and simultaneous monitoring of largazole, symplostatin 1 and dolastatin 10. This method does not have specific structural requirements and can be done using commonly available mass spectrometers. However, authentic standards are required for optimization of the HPLC-MS parameters. Because MRM is also a compound-specific detection, no information on the presence of related congeners may be derived using this method.

The HPLC-MS dereplication revealed a correlation between the phylogenetic grouping of cyanobacterial specimens and their production of NPs. Specifically, the specimens that were genetically related to known producers of dolastatin 10, symplostatin 1, and largazole were all shown to produce these compounds (Figure 2).

Based on the HPLC-MS dereplication, the majority of the sample collections with antiproliferative activity at 10 and 100 ng/mL contained combinations of largazole, dolastatin 10 and symplostatin 1 (Figure 3). All cyanobacterial collections bioactive at a concentration of 10 ng/mL contained these metabolites (Figure 3). Extracts containing symplostatin 1 or dolastatin 10 alone or lower concentrations of these metabolites in combination showed activity at a concentration higher than 100 ng/mL. Interestingly, largazole was consistently detected in combination with dolastatin 10 and symplostatin 1, whenever present (Figure 3), suggesting a correlative relationship and that the coproduction may serve a function to the cyanobacteria, which we exploited for combinatorial pharmacology studies (see below).

Samples without detectable levels of largazole, symplostatin 1 or dolastatin 10 showed varied antiproliferative activity and thus presented as priority candidates for both bioactivity- and ^1H NMR-guided purification. The fact that these groups have not yet been exploited for chemical characterization support that these specimens could be attractive targets for future NP discovery efforts.

The phylogenetic information together with the bioactivity data, the dereplication results and available material of the cyanobacterial collection were considered in the prioritization of crude samples for further purification. Bioactive collections at concentrations $< 10\text{ }\mu\text{g/mL}$ with sufficient amounts of lyophilized cyanobacteria and/or nonpolar extract were given highest priority. Non-cytotoxic or weakly cytotoxic samples were further subjected to a silica SPE and ^1H NMR profiling to check for relevant functionalities such as $N\text{-CH}_3$, $O\text{-CH}_3$, -NHs and $\alpha\text{-hydrogens}$. Through this dereplication method, sample collections that ultimately yielded the new compounds veraguamides A–G²¹, symplostatins 5–10²² and caylobolide B²³ were prioritized and analyzed.

To validate our current HPLC-MS dereplication method, largazole and dolastatin 10 were isolated from a cyanobacterial collection from Pickles Reef in Florida using a HPLC-MS-guided purification. Monitoring by HPLC-MS required minimal amounts of sample, while still permitting sensitive detection. Using this approach, sub-milligram quantities of largazole and dolastatin 10 were isolated. The identities of the purified compounds were verified using ^1H NMR and LRESIMS measurements, and comparison with literature values. We were interested to determine if the co-production of these compounds may provide a competitive advantage to the cyanobacterium or cyanobacterial community that may also translate into cooperative anticancer activity.

Combinatorial pharmacology

Combinatorial regimens are actively pursued in the treatment of malignancies to improve current treatments and also curb drug resistance.²⁴ Histone deacetylase (HDAC) inhibitors are widely evaluated for combination regimens, with several in clinical trials. HDAC inhibitors lower the apoptotic threshold of cancer cells treated with classical anticancer drugs such as microtubule disrupting agents and topoisomerase inhibitors, then rendering the cells susceptible to apoptosis, leading to additive or synergistic effects of therapeutic agents.^{24–26} Taking the cue from the observed coproduction of largazole with dolastatin 10 and/or analogues (Figure 3B) and attempting to translate this potentially functional

correlation into biomedical utility, we assessed the combinatorial pharmacology of largazole and dolastatin 10 on the growth of HCT116 human colorectal adenocarcinoma cells. A concentration of largazole (4.0 nM) was selected that did not produce drastic effects on cell growth (~80%) when used as single agent, while multiple concentrations of dolastatin 10 (0.32, 0.50, 0.70 nM) were utilized (Figure 4). Largazole and dolastatin 10 when used in combination decreased the proliferation of HCT116 cells (Figure 4). The effects of the combination regimens were significantly different from those of both largazole and dolastatin 10 alone (Figure 4). For example, HCT116 maintained 80% growth when treated only with either dolastatin 10 (0.32 nM) or largazole (4.0 nM). When dolastatin 10 (0.32 nM) and largazole (4.0 nM) were used in combination, a significant decrease in HCT116 cell growth to $55 \pm 4\%$ was observed (Figure 4). To assess whether largazole and dolastatin 10 display an additive or synergistic relationship, the predicted additive effect was calculated based on the Loewe additivity.^{27,28} The predicted concentrations of largazole and dolastatin 10 required to yield the same effect as the combination regimen was extrapolated from the nonlinear regression analysis. Based on the relationship of $(CI_1/I_1) + (CI_2/I_2)$, largazole and dolastatin 10 co-treatment had an additive effect. To further validate this, the predicted additive effect of largazole and dolastatin 10 was calculated based on the % cell growth of the individual agents. The predicted additive effect ($60 \pm 7\%$) and the experimental combinatorial effect ($55 \pm 4\%$) did not show any significant difference based on unpaired t-test ($p < 0.05$), corroborating that dolastatin 10 and largazole have an additive effect in preventing the growth of HCT116 cells (Figure 4). These results further validate that the combinatorial biosynthesis employed by marine cyanobacteria can also be exploited for combinatorial pharmacology, as evidenced by previous work on the synergistic relationship between the metabolites largazole and symplostatin 4,⁷ and also various laxaphycins from *Anabaena* sp.^{29,30}

CONCLUSION

A phylogeny-based classification approach was used to catalog marine cyanobacteria of the genus *Symploca*. This cladistics approach found a direct correlation between the phylogenetic position and the production of secondary metabolites in the various collections. In principle, this approach was shown to allow the prediction of secondary metabolites, and it can be utilized to identify specific NP-producing groups as well as target groups that have not yet been exploited for their NP production. The bioactivity and chemical space of crude extracts of 67 cyanobacterial collections were screened using the MTT cell viability assay and HPLC-MS-based dereplication method, respectively. The majority of the screened cyanobacteria collections with potent bioactivity contained combinations of the cytotoxins largazole, dolastatin 10 and symplostatin 1. These compounds were rapidly identified as the major cytotoxic constituents through comparison of the HPLC-MS profiles with authentic standards, using multiple reaction monitoring. The dereplication method was further validated using large-scale isolation from a dolastatin 10- and largazole-containing cyanobacterial collection. By combining phylogeny, dereplication information, antiproliferative activity profiles against HT29 cancer cells, availability of material, and/or initial ¹H NMR profile, sample collections were prioritized for the discovery of novel bioactive secondary metabolites. The co-production of potent antiproliferative agents

employed by marine cyanobacteria also permitted the design of combinatorial regimens of largazole and dolastatin 10, which displayed cooperative activity in preventing the growth of a human colorectal adenocarcinoma cancer cell line.

Experimental Section

General Experimental Procedures

^1H NMR spectra were recorded in CDCl_3 or CD_2Cl_2 on a Bruker Avance II 600 MHz spectrometer equipped with a 5-mm TXI cryogenic probe using residual solvent signals [$(\text{CDCl}_3: \delta_{\text{H}} 7.26)$, $(\text{CD}_2\text{Cl}_2: \delta_{\text{H}} 5.32)$] as internal standards. LRESIMS measurements, MRM analysis and MS/MS fragmentation were done on an ABI 3200Q TRAP mass spectrometer.

Sampling and Morphological Identification

Cyanobacterial collections corresponding with the genus *Symploca* were collected by hand at various sites in Florida, Guam, Bonaire, Curacao, and the US Virgin Islands. The selected specimens were putatively identified based on traditional classification systems^{31–33} and with references to available field guides.³⁴ Microscopic identification was performed using an Olympus IX51 Leica epifluorescent microscope (Olympus) equipped with a Nikon Coolpix camera (Olympus). Voucher specimens were preserved in 100% EtOH or in seawater with 5% formalin, for morphological analysis and deposited in the University of Guam Herbarium and at the Smithsonian Marine Station, Fort Pierce, FL. Samples were kept in RNAlater (Ambion) for genetic analysis and frozen at -20°C after collection. Frozen cyanobacterial samples were lyophilized prior to chemical extraction. The freeze-dried cyanobacteria were extracted with EtOAc–MeOH (1:1) or CH_2Cl_2 –MeOH (1:1) to yield the nonpolar extracts.

Gene Sequencing

The cyanobacterial specimens were partly cleaned under a dissecting microscope (Wild Heerbrugg) prior to DNA extraction. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega), following the manufacturer's specifications. DNA concentration and purity was measured on a ND-1000 spectrophotometer (Thermo Scientific). The 16S rRNA genes and the 16S–23S ITS regions were PCR-amplified using the 106F primer³⁵ on the 16S rRNA gene and the 340 primer³⁶ on the 23S rRNA gene. The PCR reaction volumes were 25 μL containing: 1 μL (~100 ng) of DNA, 5 μL of 5X Green GoTaq Flexi buffer, 2.5 μL MgCl_2 (10 mM), 0.5 μL (10 mM) of dNTP mix, 1.0 μL of each primer (10 μM), 0.25 μL of GoTaq DNA polymerase (5 $\text{u}\cdot\mu\text{L}^{-1}$), and 14.75 μL dH_2O . The PCR reactions were performed in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad) as follows: initial denaturation for 2 min at 95°C , 25–28 cycles of 45 sec at 95°C , 45 sec at 50°C and 2 min at 72°C , and final elongation for 3 min at 72°C . All PCR products were purified using a MinElute PCR Purification Kit (Qiagen) before subcloning using the pGEM-T Easy Vector system (Promega), following the manufacturer's specifications. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced bidirectionally with M13 vector primers as well as the internal primers 359F, 785R, and 1509R.³⁵ Gene sequence anomalies, including chimeric sequences, were predicted using the

Pintail software with the cut-off size set at >600 bp,³⁵ and were manually confirmed by comparison of (NJ) phylogenetic trees for different regions (>300 bp) of the sequences. Genetic sequencing was performed at the Laboratories of Analytical Biology, NMNH. All gene sequences are available in the DDBJ/EMBL/GenBank databases under the accession numbers: KP164815-KP164826.

Phylogenetic Inference

The 16S rRNA genes (1,306 bp) and the 16S–23S ITS regions (480 bp) from a total of 21 *Symploca* specimens were used for phylogenetic inferences. Gene sequences from other cyanobacterial taxa were obtained from the National Center for Biotechnology Information (NCBI) web pages. Representative reference-strains were selected from Bergey's Manual³² and the type species were selected from CyanoDB³⁷ and algaeBASE.³⁸ The unicellular *Gloeobacter violaceus* PCC 7421^R (GenBank acc. nr. NC005125) was included as an evolutionarily distant out-group. All gene sequences were aligned using the MUSCLE algorithm.³⁹ The 16S rRNA gene sequence alignment was visually compared and refined using the SSU secondary structures model of *Escherichia coli* J01695.⁴⁰ Pair-wise sequence divergences were calculated in MEGA 5.1 without model selection.⁴¹ Appropriate nucleotide substitution models were compared and selected using uncorrected/corrected Akaike Information Criterion, Bayesian Information Criterion, and the Decision-theoretic in jModeltest 0.1.1.⁴² The Maximum likelihood (ML) inference was performed using PhyML⁴³ with the GTR+I+G model assuming heterogeneous substitution rates and gamma substitution of variable sites (proportion of invariable sites (pINV) = 0.510, shape parameter (α) = 0.452, number of rate categories = 4). Bootstrap resampling was performed on 1,000 replicates. Bayesian analysis was conducted using MrBayes 3.1 for the GTR+I+G model.⁴⁴ Four Metropolis-coupled MCMC chains (one cold and three heated) were run for 1,000,000 generations and the first 100,000 generations (10%) were discarded as burn-in and the following data sets were sampled with a frequency of every 100 generations.

HPLC-MS Profiling

Nonpolar cyanobacterial extracts (10–20 mg) were fractionated by C18 SPE column using MeOH–H₂O. In each case, the fraction eluting with 100% MeOH was dried under N₂, weighed, and methanolic stock solution (1 mg/mL) was prepared. A dilution (10,000 ng/mL) of the stock solution was prepared in MeCN and spiked with the internal standard harmine and was used as test solution. A 10 μ L portion of the test solution was injected for HPLC-MS analysis, using the following conditions: column, Kinetex (100 $\times$ 2.1 mm), Phenomenex; linear gradient of 0.1% HCOOH in MeCN–0.1% HCOOH in H₂O [50%–100% MeCN in 10 min and then 100% MeCN for 5 min, flow rate, 0.5 mL/min; detection by ESIMS in positive ion mode (MRM scan)]. The retention times (t_R , min; MRM ion pair) of the analytes were as follows: harmine (1.5; 214 $\rightarrow$ 170.9), dolastatin 10 (2.2; 785.6 $\rightarrow$ 753.7), symplostatin 1 (2.4; 799.6 $\rightarrow$ 767.6), largazole (2.5; 623 $\rightarrow$ 497).

Validation of Dereplication Method

A *Symploca* sp. collection from Pickles Reef, Florida was lyophilized and the dried material (13.3 g) was extracted with EtOAc–MeOH (1:1) to yield the nonpolar extract (1.7 g). The

nonpolar extract was adsorbed on a Diaion HP-20 resin and eluted with 100% H₂O, 25%, 50%, 75% and 100% MeOH and 50% CH₂Cl₂ in MeOH. Each fraction was monitored for the presence of largazole, symplostatin 1 and dolastatin 10 using the HPLC-MS method. The fraction eluting from 50% CH₂Cl₂ (33 mg) showed peaks corresponding to largazole and dolastatin 10 and was applied onto a silica SPE column, eluting with increasing gradients of *i*-PrOH in CH₂Cl₂, until 100% *i*-PrOH. The fractions eluting from 10% *i*-PrOH and 20% *i*-PrOH contained largazole and dolastatin 10, respectively, based on HPLC-MS profiling. These fractions were further purified by semipreparative HPLC (Phenomenex Synergi-HydroRP, 4 μ m; flow rate, 2.0 mL/min) using a linear gradient of MeOH–H₂O (70%–100% MeOH in 60 min and then 100% MeOH for 15 min). The 10% *i*-PrOH fraction yielded largazole (t_R 41.7 min, 0.3 mg). Using the same chromatographic condition, the 20% *i*-PrOH fraction afforded dolastatin 10 (t_R 40.0 min, 0.2 mg). The ¹H NMR and LRESIMS of the isolated compounds were identical to those of the literature values.

Largazole: colorless, amorphous solid; ¹H NMR spectrum is identical to that of an authentic sample,¹² LRESIMS m/z 623.0 [M + H]⁺.

Dolastatin 10: colorless, amorphous solid; ¹H NMR spectrum is identical to that of an authentic sample,^{4,6} LRESIMS m/z 785.6 [M + H]⁺.

Cell Viability Assay

HT29 colorectal adenocarcinoma cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) under a humidified environment with 5% CO₂ at 37 °C. HT29 (12,500) cells were seeded in 96-well plates. These were treated with varying concentrations (10, 100, 1,000, 10,000 ng/mL) of the 100% MeOH fraction of the nonpolar extract, dissolved in EtOH, 24 h post-seeding. Cells were incubated for an additional 48 h before the addition of the MTT reagent. Cell viability was measured according to the manufacturer's instructions (Promega, Madison, WI). The antiproliferative activity of single agents and combinations of largazole and dolastatin 10 was determined using the same procedure, employing the cancer cell line HCT116 human colorectal carcinoma (10,000 cells/well). Treatments were done in duplicate. Nonlinear regression analysis was carried out using GraphPad Prism software.

Combinatorial Screening

HCT116 cells were treated with largazole (4.0 nM), dolastatin 10 (0.32 nM), combination of largazole (4.0 nM) and dolastatin 10 (0.32 nM) or ethanol (solvent control). Treatments were done in quadruplicate and the effect on cell growth was assessed using the MTT reagent. Analysis of the additive effect was performed using Loewe additivity [(CI₁/I₁) + (CI₂/I₂) = 1], where CI₁ and CI₂ are the concentrations used in treatments, I₁ and I₂ are the isoeffective concentration calculated from the dose-response analysis.^{27,28} Statistical analysis was carried out using GraphPad Prism software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Mechanism of Action: Microtubule-disrupting agent

Dolastatin 10 R = H
Symplostatin 1 R = Me

Mechanism of Action: Histone deacetylase inhibitor

Largazole

Figure 1.

Potent cytotoxins from marine cyanobacteria identified as *Symploca* and their mechanisms of action.

Figure 2.

Evolutionary tree used to catalog the specimens (indicated in bold font) and place them in phylogenetic perspective with known natural products (NP) producing marine cyanobacteria. All gene sequences for NP-producing marine cyanobacteria available in public databases were included in the phylogenetic inferences. Groups with taxonomic reference strains or type strains were condensed and identified by the genus name. The phylogenetic inferences were based on the SSU (16S) rRNA nucleotide sequences using Bayesian Inference (MrBayes) and Maximum Likelihood (PhyML). The support values are

indicated as posterior probability (MrBayes) and bootstrap support (PhyML). The scale bar is indicated at 0.05 expected nucleotide substitutions per site using the GTR+I+G substitution model. The presence of dolastatin 10, symplostatin 1 and largazole is indicated by colored dots.

Figure 3.

Summary of bioactivity and natural products screening of cyanobacterial collections. A. The majority of the cyanobacterial collections displayed antiproliferative activity against HT29 human colorectal adenocarcinoma cells as assessed using the MTT reagent. The majority of potent bioactive extracts showed combinations of dolastatin 10, largazole and symplostatin 1 based on mass spectrometry-based dereplication using multiple reaction monitoring. B. Detailed distribution of the three known antiproliferative agents in profiled cyanobacterial collections.

Figure 4.

Combinatorial pharmacology of dolastatin 10 and largazole on HCT116 cell growth. HCT116 cells were treated with either single agents or combinations of largazole and dolastatin 10. The predicted additive effects were extrapolated from the dose-response analysis for the single agents and calculated based on Loewe's additivity. Statistical analysis using two sample t-test indicated significant difference between treatments using single agents versus combination ($p < 0.05$). However, there was no statistically significant difference between the predicted additive effect and experimental values obtained,

indicating an additive effect of largazole and dolastatin 10. Data are presented as mean $\pm$ SD ($n = 4$).

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