Published in final edited form as:
Nat Prod Commun. 2009 December ; 4(12): 1717–1728.

Cancer Chemopreventive and Anticancer Evaluation of Extracts and Fractions from Marine Macro- and Micro-organisms Collected from Twilight Zone Waters Around Guam[]1

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Abstract

The cancer chemopreventive and cytotoxic properties of 50 extracts derived from Twilight Zone (50–150 m) sponges, gorgonians and associated bacteria, together with 15 extracts from shallow water hard corals, as well as 16 fractions derived from the methanol solubles of the Twilight Zone sponge *Suberea* sp, were assessed in a series of bioassays. These assays included: Induction of quinone reductase (QR), inhibition of TNF-α activated nuclear factor kappa B (NFκB), inhibition of aromatase, interaction with retinoid X receptor (RXR), inhibition of nitric oxide (NO) synthase, inhibition 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH), and inhibition of HL-60 and MCF-7 cell proliferation. The results of these assays showed that at least 10 extracts and five fractions inhibited NFκB by greater than 60%, two extracts and two fractions inhibited DPPH by more than 50%, nine extracts and two fractions affected the survival of HL-60 cells, no extracts or fractions affected RXR, three extracts and six fractions affected quinone reductase (QR), three extracts and 12 fractions significantly inhibited aromatase, four extracts and five fractions inhibited nitric oxide synthase, and one extract and no fractions inhibited the growth of MCF-7 cells by more than 95%. These data revealed the tested samples to have many and varied activities, making them, as shown with the extract of the *Suberea* species, useful starting points for further fractionation and purification. Moreover, the large number of samples demonstrating activity in only one or sometimes two assays accentuates the potential of the Twilight Zone, as a largely unexplored habitat, for the discovery of selectively bioactive compounds. The overall high hit rate in many of the employed assays is considered to be a significant finding in terms of “normal” hit rates associated with similar samples from shallower depths.

Keywords

Chemoprevention; anticancer; NFκB; aromatase; quinone reductase; nitric oxide synthase; DPPH; RXR; MCF-7 and HL-60 proliferation; extracts; fractions; Twilight Zone; Guam; micro-organisms; marine organisms

The marine environment is a proven rich source of natural products that have a wide variety of biological activities. During the last three decades, more than 15,000 natural products have

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been isolated from marine organisms [2]. Sessile organisms, such as sponges, ascidians and gorgonians, often lack any physical protection, and are of special interest in this respect. Sponges, with more than 600 million years of evolution, have evolved a vast number of bioactive secondary metabolites to protect themselves against predation, bacterial and fungal infection, and overgrowth by other organisms [3,4]. The ecological bioactivity of sponge, ascidian and gorgonian secondary metabolites often correlates remarkably well with bioactivity in pharmaceutical assays [5,6]. Many of these more than 5000 metabolites [2] revealed strong activity in pharmaceutical bioassays and thus became promising targets for further preclinical studies as agents against a series of human diseases (for example, cancer, AIDS, malaria, and inflammation) [7–9].

One major reason for the slow progress of a newly discovered biologically active natural compound is the issue of ‘supply’ [10]. Often, the bioactive compounds are found in only minute quantities in the source organism, making it necessary to collect and extract large amounts of material to supply appropriate amounts for preclinical assays. This cannot be justified from an ecological point of view nor is it congruent with the laws of countries in which the invertebrates occur [11]. Thus, alternatives to harvesting specimens from the wild are often required to take a compound beyond phase I clinical trials. Unfortunately, the supply problem has largely been ignored by marine natural products researchers [10].

For the commercial production of an active compound discovered from nature the pharmaceutical industry would almost certainly select synthesis when practicable over all other possible methods of compound generation. However, many bioactive natural products are structurally extremely complex and require multi-step syntheses that typically are not economically viable for drug development [10,12], even with the advent of approaches used by companies such as Eisai. Mariculture or aquaculture, the farming of marine organisms, is sometimes considered as an alternative to collecting specimens from the wild [13]. A promising example in this respect was *Bugula neritina*, a bryozoan that is the source of bryostatins, a group of anticancer compounds already in clinical use [14]. Although *B. neritina* is now grown in mariculture [10], Lopanik and colleagues [15] discovered that bryostatins are actually produced by a microbial symbiont (*Endobugula sertula*) that protects *Bugula* larvae from predators by its production of bryostatins. This example clearly shows that culturing marine invertebrates can only be an economically relevant alternative if the organisms lend themselves to a viable cost-effective cultivation, and if they produce the metabolites of interest in large and constant quantities.

Another interesting and promising approach to the “supply problem” is the possibility that in many cases, as mentioned above, it is not the marine invertebrates themselves, but their associated microbes that are the true producers of the pharmaceutically interesting compounds [10,16–19]. In this respect, the micro-organisms, assuming they can be cultured, would represent a more attractive source of marine natural products since fermentation is more feasible than synthesis or massive collections [10]. Again, sponges are of special interest in this respect, as they often harbor significant amounts of bacteria in their tissues. In some cases bacteria make up more than 40% of sponge biomass [20,21]. So far, only few studies have identified the actual producers of secondary metabolites of interest, indicating either the sponge itself [22] or the associated bacteria [23–25]. In many cases it is only an assumption that ”sponge” metabolites are actually produced by bacterial symbionts, based on the structural characteristics of the metabolites that are typical of prokaryotic rather than eukaryotic biosynthetic processes [17,26].

Determining the true origin of secondary metabolites in marine invertebrates is a difficult task. Bacterial communities in sponges and gorgonians are often complex, making interactions between the macro-organism and bacterial symbionts highly intricate [27–29]. This

relationship complicates the process of defining culture conditions for many of the invertebrate (e.g., sponge) associated bacteria and it is currently accepted that only a small percentage of the total associated bacterial community in a given sponge can be cultured. Hence, the goal of the current study was to establish the feasibility of collecting Twilight Zone macro- and micro-organisms in waters around Guam, and to assess biological activity relevant to cancer prevention and treatment. Based on these data, more advanced studies can be designed for the isolation and testing of active chemical constituents. By targeting bacteria from unusual sources and relatively untouched locations (i.e., sponges, ascidians and gorgonians from Twilight Zone habitats around Guam) and by tapping into Guam's enormous, unexploited resources, we are confident we have been able to identify numerous extracts with interesting biological activity from the macro-organisms as well as from bacterial strains isolated from these sources. The Key to our approach was the use of experienced technical divers who are comfortable working at depths typically not approached by the average scientific diver (50–150 m).

The project involved the screening of 65 extracts from unusual sources; 25 represented sponges and gorgonians from the Twilight Zone (50–150 m depth), 25 were bacterial isolates obtained from the Twilight zone sponges, and 15 were extracts from hard (Scleractinian) corals, obtained through access to a NAVY-dredging project. By tapping this diverse, yet largely untapped biodiversity, we were able to obtain a normally unthinkable hit rate of 42% active samples in the various screens employed. A closer look at the results (Table 1), reveals that although the coral samples and bacterial isolates from the sponges generated 27% and 20% hits, respectively, extracts from the Twilight Zone sponges and gorgonians resulted in an astonishing 72% hit rate. Based on these screening results we are now in the process of fractionating the most promising lead extracts to ensure that these activities are "real" and not caused by so called nuisance compounds, these being usually too toxic or generalist for further development.

When these results are compared with reports in the literature, the true value of this unexplored biodiversity becomes even more apparent. Laird and Kate [30] estimated that screening 5 million compounds/extracts would roughly translate to 1000 hits; 1/5000 or a 0.02% hit rate. Following fractionation, purification, dereplication and structure elucidation only an estimated 10 out of these 1000 hits would become a lead.

Cragg and Newman [31] point to a similar bleak reality referring to the commonly used quote that, "only 1 in 10,000 biologically active leads will result in a commercial drug". They also reiterate that for natural products the ratio might be even worse, as it does not take into account the initial phase of fractionation and purification of compounds from the respective extracts.

In a more detailed analysis, Cragg and colleagues [32] evaluated the number of marine specimens with antileukemic activity as a percent of the total tested specimens for different phyla. When comparing their results with our outcomes, it becomes apparent that extracts of invertebrates from the Twilight Zone have a much higher hit rate than their shallow water counterparts in comparable assays with comparable cut-offs. For example, Cnidaria samples produced a hit rate of 4.4% active samples of the total tested, while our samples demonstrated a hit rate of 8%. The higher hit rates were most pronounced for Porifera, with a hit rate of 8.7% in their analysis; our Twilight Zone specimens exhibited an unprecedented 47% hit rate. The bacterial strains obtained from the Twilight Zone sponges also showed considerable promise with a hit rate of 20%. However, it should be noted here that isolated bacteria were first sequenced to obtain information regarding their taxonomy, and only those bacteria from known producing genera (genera reported in the literature to produce active metabolites) were selected for grow-out and subsequent extract screening. This approach was chosen because limited resources necessitated prioritizing samples, and focusing on bacterial genera already known

to be producers of novel, pharmacologically active secondary metabolites was deemed to be the most likely approach to yield positive outcomes.

In order to investigate the potential of extracts from Twilight Zone organisms to mediate chemopreventive and anticancer effects, a series of bioassays were employed. The assays were selected to monitor a broad spectrum of activity. First, the potential to inhibit tumor necrosis factor-α induced NFκB was determined. NFκB is a transcription factor that plays roles associated with apoptosis, cell differentiation, and cell migration. Upon activation, it may promote cell proliferation and further prevent cell death through apoptosis. Inhibition of NFκB signaling has potential application for the treatment or prevention of cancer. The greatest activity was observed in the NF-*κ*B assay, with 15 extracts having activity above 65% at the 20 μg/mL level, with at least 50% cell survival (Table 1).

Next, antioxidant activity was assessed. Free radicals, due to their high chemical reactivity, can react with other macromolecules, including DNA, proteins, carbohydrates and lipids, leading to destructive oxidative damage. Oxidative damage has been implicated in the cause of various diseases including cancer, and also has an impact on the body's aging process. In this assay, stable purple chromogen 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) is reduced by antioxidants or free radical scavengers to the corresponding pale yellow hydrazine and the scavenging capacity is evaluated by monitoring this process. Only three extracts were active in the DPPH assay with inhibition levels of 50% or higher.

Retinoids are derivatives of vitamin A that influence cellular proliferation, differentiation, and apoptosis in a retinoid-specific and cell-type specific manner. Retinoids have shown efficacy as anti-cancer drugs that intervene in the carcinogenic process by regulating proliferation and differentiation at several stages [33]. Retinoid receptors belong to the family of nuclear hormone receptor proteins. There are two major classes of retinoid nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each class contains three subtypes (α, β, γ). We utilized a cell line-based retinoid X receptor responsive element (RXRE)-luciferase reporter gene assay; no extracts were deemed to be active.

Quinone reductase 1 (QR1) is considered a detoxifying enzyme. Induction correlates with induction of related phase 2 detoxification enzymes, such as glutathione *S*-transfrease [34]. Of the extracts tested, three mediated an induction ratio of ≥2.0.

Aromatase is an enzyme that catalyzes the conversion of androgen to estrogen. Therefore, aromatase inhibition blocks the production of estrogen, which in turn can reduce the growth of breast cancer cells. Aromatase inhibitors have been used in anticancer therapy to treat breast cancer in postmenopausal women, and animal studies have shown their potential as chemopreventive agents [35,36]. As shown in Table 1, three of the extracts derived from Twilight Zone organisms were effective inhibitors of aromatase.

Nitric oxide (NO) is an ubiquitous signaling molecule that impacts many physiological and pathological processes. It has been shown to be associated with the development of cancers in the early stages with *in vivo* studies [37]. The blocked production of NO is a potential mechanism for chemoprevention; four extracts inhibited production by 50% or more.

Finally, the cytotoxic potential of the extracts was determined employing HL-60 and MCF-7 cells in culture. Inhibition of proliferation or induction of apoptosis in cancer cells is one characteristic of many anti-cancer drugs. Seven of the extracts were active in the HL-60 cell assay, one of which was active against MCF-7 cells as well.

The assays utilized for evaluation of these samples have been devised to monitor potential inhibition of multiple stages of carcinogenesis [38]. Based on past experience, as described in

the literature references for each assay, threshold levels of test material concentrations have been established to yield an active hit rate in the range of 3%. Known active compounds are used in each case to assure the accuracy of the test. Although activity in a single test system may be deemed desirable, it is becoming increasingly clear that pleiotropic mechanisms are facilitated by many promising chemopreventive agents [39]. Accordingly, it is appropriate to analyze the overall response mediated by the unique collection of extracts reported herein.

In sum, of the 65 extracts, 24 showed a positive result in at least one of the applied tests. Of these, five were active in two of the assay systems, three in three tests, and only one in four assays. Of the extracts showing positive results in two assays, two of them (PS 430, PS 432) were positive in both the NFκB (67.5 and 78.1% inhibition, respectively, with cell survival >80% at 20 μg/mL) and the HL-60 (100% cell mortality at 20 μg/mL, and IC_{50} values of 13.3 and 10.5 μg/mL, respectively) test systems, the remaining three being active in QR (IR 2.1 at 20 μg/mL) and NO (52.9% inhibition with 100% cell survival at 20 μg/mL) (PS 454), NFκB (79.7% inhibition with 100% cell survival at 20 μg/mL) and QR (IR 2.0 at 20 μg/mL) (GUDS 477), and HL-60 (50.8% cell mortality at 20 μg/mL, and an IC_{50} value of 19.5 μg/mL) and NO (55.4% inhibition at 20 μg/mL, with 92.9% cell survival). Extract PS 341 gave a high cytotoxic response towards HL-60 cells (100% cell mortality at 20 μg/mL, and an IC_{50} of 7.7 μg/mL), aromatase (94.9% inhibition at 20 μg/mL and an IC_{50} of 0.6 μg/mL) and NO assay (54.9% inhibition with 100% cell survival), while extract PS 431 was active in NFκB (72.3% inhibition at 20 μg/mL with 69.1% cell survival), HL-60 (100% cell mortality at 20 μg/mL, and an IC_{50} of 11.9 μg/mL) and NO (54.9% inhibition with 81.3% cell survival) assays. Extract PS 383 showed activity in four assays; activation of QR I (IR 2.4, with a 74.6% survival at 20 μg/mL), inhibition of aromatase (89.1% inhibition at 20 μg/mL, and an IC_{50} of 3.14 μg/mL) and cytotoxicity towards HL-60 and MCF-7 cells (80.3% cell mortality at 20 μg/mL, IC_{50} of 1.0 μg/mL, and 96.6% mortality at 20 μg/mL, respectively).

Aside from these, all other extracts were active in either only one assay system or apparently devoid of any activity in the applied assays. Eight extracts were active in the HL-60 assay with survival less than 50%. In both the aromatase (greater than 70% inhibition) and NO (greater than 50% inhibition and 80% survival) assays, three extracts were found to have activity. Finally, only one extract (PS 383) was found to have significant cytotoxicity towards MCF-7 cells (greater than 50% cell mortality).

On the basis of these findings, fractionation was initiated with one of the active extracts, a sample of the sponge *Suberea* sp (Table 1, Extract No. 5) that demonstrated significant activity in three assays; aromatase, NO and HL-60. As outlined in the experimental section, this extract was fractionated several times employing various chromatographic methods to yield 16 further sub-fractions for testing. The sub-fractions fall into two groups: 1–11 and 12–16, based on the original fractions from which they were derived. As shown in Table 2, the weak NFκB activity observed with the original extract was enhanced in sub-fractions 1, 2, 4 and 7, as was the original weak DPPH activity of the extract, which was enhanced in sub-fractions 5 and 6. The original significant cytotoxicity towards HL-60 cells of the extract was borne out by similar levels of activity being seen for sub-fractions 6 (IC_{50} 7.1 μg/mL) and 13 (IC_{50} 19.3 μg/mL), which is probably attributable to at least two different chemical entities based on the fractionation scheme. The significant QRI activity of sub-fractions 8–11 and 15 was somewhat unexpected based on the activity found for the original extract, and may also be attributable to two chemical entities, different from the one(s) responsible for the observed HL-60 activity. The aromatase activity observed for sub-fractions 3, 7–11, and 13–15 is consistent with the activity of the original extract and certainly translates into at least two chemical entities that may have similar structures to those responsible for the observed QR activity. Finally, the NO activity demonstrated by sub-fractions 9–11 and 13–14 is entirely consistent with the similar level of activity seen for the extract, if not better. Interestingly, it appears that the QR, aromatase

and NO activity is localized in these groups of sub-fractions, indicating that the same or similar compounds are responsible for these activities.

Although preliminary in nature, the current body of work illustrates the vast biological potential of Twilight Zone macro- and micro-organisms. Based on this prototype study, with only 50 Twilight Zone organisms and 15 shallow water hard corals out of a myriad of organisms sampled, it is clear that larger collections will yield additional leads of significant interest. Further, as judged by sub-fractionation of the sponge *Suberea* species, the rich bioactivity profile shown by this sponge's extract, and fractions, is indicative of the presence of a series of active natural products each having a specific type of activity, not of one or two compounds with a broad spectrum of activity. As this work continues, we are confident that unique and unpredictable chemical entities will emerge from the investigation of Twilight Zone and hard coral samples that will have inspiring spectra of biological activities, some of which will be relevant to human health.

Conclusion

This study is one of the first to document that tapping into largely unexplored marine diversity has great potential to yield active extracts and at hit rates much larger then previously reported. The Twilight Zone and hard corals represent a relatively unexplored habitat and organism group, due to the fact that the Twilight Zone is typically too deep for regular SCUBA diving and only a small group of professionals have undertaken the effort to become trained to dive to these depths, and the fact that hard corals are often difficult to collect due to permit issues. Additionally, for the Twilight Zone samples, submarines are rarely deployed at these depths, because of their high operating costs. It is more usual that such equipment is employed for deep-sea exploration; depths beyond 500 m. Access to these unique samples in terms of location and organism class, combined with a large array of chemopreventive and anticancer assays, enabled us to identify a higher than usual number of leads to follow up and clearly validated our approach to finding new sources of biologically active extracts.

Experimental

Chemicals

Methanol (HPLC grade), ethyl acetate (HPLC grade), marine broth, actinomycetes agar, cyclohexamide, agar, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), sulfanilamide, 0.1% *N*-(1-naphthyl) ethylenediamine, sulforhodamine B (SRB), tetradecanoylphorbol 13-acetate (TPA), sodium-diethyldithiocarbamate trihydrate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), non-essential amino acids, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM), Antibiotic-Antimycotic, Hygromycin B, MEM, sodium pyruvate, Roswell Park Memorial Institute (RPMI) 1640 medium, and Lipofctamine™ 2000 transfection reagent were purchased from Invitrogen Co. (Carlsbad, CA). Reporter Lysis Buffer, Luciferase Assay System, *Renilla reniformis* luciferase vector (pRL-SV40), and dual luciferase assay system were purchased from Promega (Madison, WI). Tumor necrosis factor-α (TNF-α) was purchased from Calbiochem (Gibbstown, NJ). Retinoid X receptor responsive element encoding firefly luciferase reporter vector (pRXRE) was purchased from Panomics Inc. (Fremont, CA). Human RXRα protein expressing vector (phRXRα) was bought from Addgene (Cambridge, MA). All other chemicals were obtained from commercial sources and were of the highest grade and purity.

Sample Collection

Sponges and gorgonians were collected by a commercial diver from various sites around Guam. Locations were marked with GPS coordinates to allow possible recollection of active samples. Samples were collected in the Twilight Zone from depths of 50–150 m, and bagged underwater in Ziploc bags, and stored in a cooler at ambient seawater temperature until return to the laboratory for processing. Upon return to the laboratory, samples were photographed, DNA samples were taken and a voucher for taxonomic identification made. Vouchers were taken of all collected specimens, preserved in 70% ethanol. Subsamples taken for DNA analysis were preserved in 95% ethanol. Voucher specimens have been deposited at the National Museum of Natural History, Leiden, the Netherlands. After processing, samples were immediately frozen at −20°C and freeze-dried prior to extraction.

Macro-organisms-Extract Preparation

Freeze-dried samples were exhaustively extracted with a 1:1 mixture of methanol and ethyl acetate. Solvent was removed under reduced pressure and the resulting extract evaporated to dryness in a speed vac (Labconco). Extracts were weighed to calculate yields and a portion of each prepared for pharmacological screening.

Bacterial Isolation

Bacterial isolation has sofar focused on sponges, since they are known to harbor large and diverse microbial consortia (up to 40% biomass). Small sub-samples (3 cm^2) of collected sponges were cut in half and one freeze-dried and the other ground wet and kept cool. The dried and finely ground samples were “stamped” with a sterile cotton swab over different agar plates. Dried specimens were used for actinomycetes isolation, since these groups produce spores. Wet processing and plating of the ground extract targeted unicellular bacteria. Approximately 1 g of sponge tissue was homogenized in 1 mL of sterile seawater and serially diluted from 1:10 to 1:10,000. One hundred μL of each dilution was used to inoculate five media with varying nutrient levels: MA (Difco marine agar), M1 (1.8% agar, 1% starch, 0.4% yeast extract, 0.2% peptone, cyclohexamide (100 μg/mL), filtered (80 μm) seawater, M5 1.8% noble agar, 0.05% mannitol, 0.01% casamino acids, cyclohexamide (100 μg/mL), filtered (80 μm) seawater), M8 (1.8% agar), cyclohexamide (100 μg/ml), filtered (80 μm) seawater), AC (Actinomycete Isolation Agar). Bacteria were purified by repeatedly transferring single colonies onto Petri plates. Cryo-stocks of isolates were created by growing the purified strains in 10 mL liquid cultures of the respective isolation media and once the culture reached high densities, 1 mL of the culture was diluted with sterile glycerol, vortexed and frozen.

Bacterial Characterisation

Purified bacteria, either “fresh” or from cryo-stocks, were cultivated and single colonies used as templates for Hot Start PCR. 16S rDNA fragments were amplified with the primer set 27f and 1492r and were sequenced. The NIH BLAST database was used to search for nearest sequence matches.

Bacteria Extract Preparation

Bacterial isolates were grown in 250 mL of Difco Marine Broth until turbid. Liquid cultures were exhaustively extracted by liquid: liquid partition with ethyl acetate. Solvent was removed under reduced pressure and extracts transferred to vials for final evaporation in a speed vac (Labconco). A portion of each extract was used for pharmacological screening. Extracts exhibiting pharmacological activity were grown up in 10 × 1 L cultures to obtain sufficient material for subsequent fractionation and purification of the active principles. This process is ongoing.

Fractionation of Suberea sp. Extract

Initial tests revealed that the extract (Table 1, Extract No. 5) of the Guamenian sponge *Suberea* sp., was extremely active in several of the employed bioassays (inhibition of NFκB, Aromatase inhibition, IC_{50} of 7.7 μg/mL in MTT assays with HL-60 leukemia cells). Based on these results it was selected for further fractionation. The freeze dried sponge was extracted exhaustively with a 1:1 mixture of methanol and ethyl acetate. This extract was screened on a normal phase silica gel TLC plate for its chemical composition, such as number of possible compounds and their polarity range. Based on the results of this TLC analysis, the extract was partitioned with *n*-hexane to remove the more lipophilic components. The remaining methanol layer was concentrated under reduced pressure and fractionated using flash column chromatography employing reversed phase silica gel (RP-18) with solvent mixtures of methanol: water (40:60; 80:20; 100:0), to yield 10 fractions. Resultant fractions were pooled, according to results of an HPLC analysis, into 2 major fractions. The more polar fraction was further fractionated using flash chromatography over a RP18 sep-pack column (methanol: water; 8 solvent mixtures from 5:95 to 100:0), while the fractions containing compounds of medium polarity were fractionated using column chromatography with Sephadex LH20 and methanol. The flash chromatography with sep-pack columns yielded 8 fractions, which were again screened for activity in the various assays (7 fractions, Fractions 5–11, Table 2). One of the active fractions was further purified by column chromatography over RP18 (methanol: water; 90:10) to yield a further 4 fractions. These (Fractions 1–4, Table 2) were again screened. The Sephadex LH20 column gave 5 fractions, all of which were further screened for their pharmacological activity (Fractions 12–16, Table 2).

Biological Testing

Biological assays were performed to evaluate the potential chemopreventative and anticancer activity of extracts and fractions generated from 65 organisms. A brief description of each assay method follows:

NFκB Luciferase Assay

Studies were performed with NFκB reporter stably-transfected human embryonic kidney cells 293 from Panomics (Fremont, CA). This cell line contains chromosomal integration of a luciferase reporter construct regulated by NFκB response element. The gene product, luciferase enzyme, reacts with luciferase substrate, emitting light, which is detected with a luminometer. Data were expressed as % inhibition or IC_{50} values (i.e., concentration of test sample required to inhibit TNF-α activated NFκB activity by 50%). After incubating treated cells, they were lysed in Reporter Lysis buffer. The luciferase assay was performed using the Luc assay system from Promega, following the manufacturer's instructions [40]. In this assay Nα-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was used as a positive control; IC_{50} = 5.09 μM.

DPPH Radical Scavenging Assay

In this assay, stable purple chromogen 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) was reduced by antioxidants or free radical scavengers to the corresponding pale yellow hydrazine, and the scavenging capacity was evaluated by monitoring the absorbance decrease at 515 nm [41]. DPPH radical scavenging capacity of samples was performed according to Lee *et al*. [42]. DPPH radical solution (316 μM) was added to 96-well plates containing samples dissolved in DMSO. The absorbance of each well was measured at 515 nm using a microplate reader. The DPPH radical scavenging activity of each sample was evaluated by calculating % of inhibition: $\%\ \text{of Inhibition} = (1 - \frac{A_{\text{sample}}}{A_{\text{control}}}) \times 100$

In this assay, ascorbic acid was used as a positive control; IC_{50} = 36.30 μM.

Retinoid X Receptor Responsive Element (RXRE)-Luciferase Reporter Gene Assay

COS-1 cells were plated and incubated for 24 h and then transiently transfected with RXR responsive element encoding vector (pRXRE), human RXRα protein expressing vector (phRXRα), and *Renilla reniformis* luciferase vector (pRL-SV40) by using Lipofctamine™ 2000 transfection reagent. After 24 h of transfection, cells were treated with samples and further incubated for 24 h. Then cells were lysed and a dual luciferase assay was performed. Results were presented as a relative value calculated by fold increase over control after normalizing ratios of firefly luciferase/Renilla luciferase [43]. In this assay, the cut-off was set at 4-fold induction and 9-*cis*-retinoic acid was used as a positive control; 23.7-fold induction at 100 nM.

Quinone Reductase (QRI) Assay

Hepa 1c1c7 (mouse hepatoma) cells were used in the assay. Cells were incubated in a 96-well plate with test compounds at a maximum concentration of 20 μg/mL, digitonin was used to permeabaize cell membranes, and enzyme activity was measured by the reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan. Production was measured by absorption at 595 nm. A total protein assay using crystal violet staining was run in parallel [44]. 4'-Bromoflavone (CD = 0.01 μM) was used as a positive control.

Aromatase Assay

Aromatase inhibition was determined by measuring the fluorescent intensity of fluorescein, the hydrolysis product of dibenzylfluorescein by aromatase. The test substance was preincubated with a NADPH regenerating system before the enzyme and substrate mixture were added. The reaction mixture was then incubated for 30 min to allow for generation of product, and then quenched with 2 N NaOH. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission) [45]. Naringenin (IC_{50} = 0.23 μM) was used as a positive control.

Nitric Oxide (NO) Synthase Assay

RAW 264.7 cells were incubated in a 96-well culture plate for 24 h. The cells were treated with various concentrations of compounds dissolved in phenol red-free DMEM for 30 min followed by 1 μg/mL of LPS treatment for 24 h. NO was oxidized to stable end product, nitrite, by the addition of Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 2.5% H_3PO_4], and absorbance was measured at 540 nm. A standard curve was created by using known concentrations of sodium nitrite [46]. The positive control in this assay was Na-L monomethyl arginine (L-NMMA); IC_{50} = 19.7 μM.

Evaluation of Proliferation Inhibitory Potential MTT Assay

HL-60 cells (2×10^5 cells/well in 96-well plates) were treated with various concentrations of samples for 72 h. After treatment, MTT solution was added to the incubated plate at a concentration of 500 μg/mL and incubated for an additional 4 h. The purple formazan crystal was dissolved in DMSO and the absorbance was measured at 540 nm. The effect of samples on cell proliferation was calculated as a percentage, relative to vehicle-treated control [47].

SRB Assay

The cytotoxic potential of test substances towards MCF-7 cancer cells was determined as described previously [48]. Briefly, various concentrations of test compounds in DMSO were transferred to 96-well plates and incubated for 72 h at 37°C in a CO_2 incubator. The incubation was stopped with trichloroacetic acid. The cells were washed, air-dried, stained with SRB solution, and optical densities were determined at 515 nm using a microplate reader. In each case, a zero-day control was performed by adding an equivalent number of cells to several wells, incubating at 37°C for 30 min, and processed as described above. Percent of cell survival

was calculated using the formula: $(OD_{cells+tested\ compound} - OD_{day\ 0})/OD_{cells+10\%DMSO} - OD_{day\ 0}) \times 100$.

Acknowledgments

This research was supported in part by P01 CA48112 and 5U56CA096278 grants awarded by the National Cancer Institute and NIH MBRS SCORE grant S06-GM-44796. We are indebted to J. Pinson for his assistance with sample collection. This is contribution 641 of the University of Guam Marine Laboratory.

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Table 1

Results of cancer chemopreventative and anticancer assays performed with extracts from 65 samples of marine macro- and micro-organisms.

Ext. No.	Taxonomy[a,b]	NFκB[c] % Inhib	NFκB[d] % Survival	DPPH[e] % Inhib	DPPH[f] IC_{50} (ug/mL)	MTT (HL-60)[g] % Survival	MTT[h] IC_{50} (ug/mL)	RXR[i] Fold induction	QR[j] IR	QR[k] % Survival	Aromatase[l] % Inhib	Aromatase[m] IC_{50} (ug/mL)	[NO][n] % Inhib	[NO][o] % Survival	SRB (MCF-7)[p] % Survival
1	Thorectidae	41.4	65.2	12.8		53.8		1.8	1.6	89.9	0.00		38.2	131.9	91.2
2	Callyspongiidae	67.5	80.8	5.6		−6.4	13.3	2.4	1.1	75.9	0.00		26.0	115.4	96.8
3	Halichondriidae	63.8	100.9	76.9	97	83.4		2.1	1.3	93.8	0.00		34.0	113.7	96.7
4	Callyspongiidae	73.8	93.1	4.5		51.5		1.8	1.3	89.3	7.38		21.6	103.6	96.9
5	*Suberea* sp.	54.4	111.6	41.5		−4.9	7.7	0.9	1.8	75.7	94.93	0.6	54.9	102.1	53.8
6	Halichondriidae	64.4	84.0	2.6		19.7	1.0	0.9	2.4	74.6	89.14	3.14	31.7	94.5	3.4
7	Thorectidae	72.3	69.1	11.5		−13.2	11.9	0.6	1.5	67.7	11.47		54.9	81.3	67.6
8	Callyspongiidae	74.0	132.5	5.6		66.9		0.9	1.1	108.2	5.57		28.7	111.1	95.2
9	Mycalidae	78.1	111.4	5.6		−2.2	10.5	1.2	1.3	53.4	9.47		23.2	105.0	91.7
10	Porifera	93.0	99.0	0.2		100.6		1.5	0.9	55.8	17.43		20.9	119.8	97.9
11	Nephtheidae	77.2	111.8	0.2		102.7		1.2	1.2	95.7	0.00		27.4	130.7	92.7
12	Callyspongiidae	51.8	109.6	6.1		−2.6	11.0	1.3	1.1	48.1	3.54		34.0	96.0	88.6
13	Porifera	47.6	113.6	24.1		74.6		1.3	1.4	113.3	93.20	0.29	40.9	137.1	94.0
14	Thorectidae	48.2	101.3	2.1		−0.3	8.4	0.9	1.9	60.3	17.14		25.3	102.6	92.5
15	Octocorallia	66.3	112.5	16.3		81.6		1.2	1.2	110.7	12.91		29.4	115.6	100.5
16	Octocorallia	62.8	110.7	55.7	164.1	66.4		1.4	1.4	87.4	15.90		31.3	121.8	95.8
17	Octocorallia	48.5	122.4	1.3		79.0		1.1	1.1	102.8	3.84		29.7	115.1	98.9
18	Octocorallia	38.1	112.1	8.5		78.0		1.0	1.9	74.4	10.75		38.2	101.9	94.2
19	Octocorallia	69.5	92.5	2.9		86.4		1.0	1.3	104.6	4.43		29.5	111.1	99.1
20	Octocorallia	51.0	102.6	11.7		65.5		0.7	2.1	66.0	16.84		52.9	117.7	84.6
21	Octocorallia	59.8	120.0	28.9		76.1		1.3	1.6	78.6	9.07		29.9	116.6	89.7
22	Octocorallia	56.7	99.2	2.6		94.7		1.0	1.2	102.2	5.56		22.3	132.6	96.6
23	Petrosiidae	55.3	65.6	0.2		104.0		1.1	1.0	126.4	49.2		48.8	83.4	78.0
24	Agelasidae	28.4	95.4	76.7	97.4	118.4		1.4	0.6	132.8	15.1		31.5	108.7	106.6
25	Octocorallia	31.5	119.5	0.2		82.3		1.2	0.8	127.8	16.8		29.0	102.6	99.5
26	*Kytococcus* sp.	59.5	116.4	−10.3		98.1		0.9	1.0	110.4	10.36		23.9	139.9	90.1

Ext. No.	Taxonomy[a,b]	NFκB[c] % Inhib	NFκB[d] % Survival	DPPH[e] % Inhib	DPPH[f] IC_{50} (ug/mL)	MTT (HL-60)[g] % Survival	MTT[h] IC_{50} (ug/mL)	RXR[i] Fold induction	QR[j] IR	QR[k] % Survival	Aromatase[l] % Inhib	Aromatase[m] IC_{50} (ug/mL)	[NO][n] % Inhib	[NO][o] % Survival	SRB (MCF-7)[p] % Survival
27	*Gordonia* sp.1	61.7	98.1	−6.0		84.3		1.3	0.8	130.5	18.43		32.0	91.2	90.1
28	*Gordonia terrae*	54.9	137.4	−5.7		100.8		1.3	1.0	115.3	13.43		33.6	138.5	95.1
29	*Streptomyces* sp.1	56.6	102.8	−10.0		104.2		1.2	1.1	106.0	13.38		22.5	114.2	98.7
30	*Ruegia* sp.	64.0	119.2	−13.2		94.6		1.5	1.0	116.4	12.56		29.5	112.0	94.6
31	*Gordonia* sp.2	64.5	124.4	−8.9		81.6		1.6	1.6	78.3	11.87		27.1	106.1	97.7
32	*Streptomyces* sp.2	38.8	101.9	−11.1		97.2		1.0	1.2	103.4	8.29		22.5	118.0	95.6
33	Not identified	64.5	114.2	1.5		97.7		1.3	1.8	75.6	16.70		25.1	100.3	100.2
34	*Pseudoalteromonas* sp.7	77.4	109.2	4.2		85.0		0.8	1.4	103.0	0.00		20.5	115.6	89.7
34	*Gordonia* sp.3	79.7	112.1	−5.2		74.8		1.7	2.0	74.8	14.06		22.1	119.6	95.8
35	*Staphylococcus* sp.	59.0	112.6	−7.6		107.7		1.3	1.4	86.7	21.29		20.9	119.2	94.3
36	*Micrococcus* sp.	59.9	85.9	2.6		110.6		1.4	1.1	111.0	14.39		20.2	134.7	93.5
37	*Gordonia* sp.4	54.1	105.9	−8.7		118.5		1.0	1.0	109.9	19.82		26.2	143.2	91.0
38	*Streptomyces* sp.3	59.7	105.7	−11.6		104.7		1.9	1.0	115.3	21.21		39.3	78.9	92.8
39	Rhodobacteraceae	32.4	132.4	6.6		103.5		0.9	1.0	109.5	4.23		33.4	128.8	96.0
40	*Gordonia* sp.5	39.3	126.9	−11.9		91.4		0.9	1.4	86.6	7.54		22.5	109.5	97.7
42	*Pseudoalteromonas* sp.	42.2	125.9	2.6		88.3		1.1	1.3	90.5	16.25		21.6	104.5	93.8
43	*Pseudoalteromonas citrea*	41.3	125.4	−6.0		111.0		1.6	1.2	93.5	8.76		22.5	101.7	93.7
44	*Streptomyces bellus*	7.2	125.3	−8.4		93.9		0.8	1.2	95.1	0.00		23.7	106.9	96.8
45	*Pseudoalteromonas* sp.1	24.7	112.5	−0.1		88.6		1.2	1.1	102.7	0.00		20.2	112.0	99.7
46	*Pseudoalteromonas* sp.2	49.5	116.7	−22.3		90.3		1.1	1.2	103.3	13.42		25.1	118.0	97.8
47	*Pseudoalteromonas* sp.3	48.2	93.3	−0.9		107.3		1.2	1.6	83.1	15.78		25.6	125.1	94.6
48	*Pseudoalteromonas* sp.4	76.1	112.2	1.3		108.3		1.1	1.2	101.0	15.58		21.2	112.8	93.6
49	*Pseudoalteromonas* sp.5	74.3	116.7	1.8		100.8		1.2	1.3	95.8	16.88		18.9	125.1	95.8
50	*Pseudoalteromonas* sp.6	72.9	127.4	5.3		111.8		1.0	1.2	107.1	2.85		25.1	134.7	92.5
51	Poritidae	73.1	52.7	14.7		101.0		1.2	0.9	119.5	56.2		41.2	130.8	91.8
52	Siderastreidae	49.8	95.7	2.9		110.0		0.9	1.0	128.1	55.4		29.4	121.5	99.0
53	Poritidae	34.1	80.4	7.4		98.7		1.2	1.2	129.6	45.7		26.9	101.2	97.1
54	Pocilloporidae	38.9	96.7	−5.4		104.8		1.1	1.0	111.0	44.9		33.1	113.7	101.2
55	Poritidae	46.3	121.3	3.7		86.9		1.1	0.9	122.2	42.5		31.1	100.0	97.9

Ext. No.	Taxonomy[a,b]	NFκB[c] % Inhib	NFκB[d] % Survival	DPPH[e] % Inhib	DPPH[f] IC_{50} (ug/mL)	MTT (HL-60)[g] % Survival	MTT[h] IC_{50} (ug/mL)	RXR[i] Fold induction	QR[j] IR	QR[k] % Survival	Aromatase[l] % Inhib	Aromatase[m] IC_{50} (ug/mL)	[NO][n] % Inhib	[NO][o] % Survival	SRB (MCF-7)[p] % Survival
56	Pocilloporidae	45.0	92.8	−0.9		99.9		1.0	1.3	123.9	30.8		28.8	109.9	97.0
57	Faviidae	46.8	101.6	−3.3		99.1		1.1	1.1	128.1	32.3		29.9	121.1	101.7
58	Faviidae	23.6	96.4	1.8		99.4		0.8	1.1	116.8	35.2		28.3	125.5	96.8
59	Acroporidae	46.6	93.5	−4.6		74.6		1.8	1.0	128.1	33.3		32.4	126.9	94.5
60	Acroporidae	57.7	104.4	−7.3		99.7		0.9	1.1	122.9	28.1		29.4	133.1	96.7
61	Acroporidae	55.5	87.8	−2.8		49.2	19.5	0.8	1.0	124.3	27.9		47.1	150.8	88.1
62	Merulinidae	58.9	74.5	1.8		94.1		1.3	1.2	134.9	23.9		39.8	98.8	93.0
63	Faviidae	61.4	69.2	8.8		84.2		0.9	0.8	121.8	3.5		39.8	116.5	94.1
64	Faviidae	49.7	82.1	0.2		86.5		1.1	1.1	121.8	25.1		32.4	105.7	99.0
65	Acroporidae	33.8	87.1	1.8		33.6	3.4	1.8	1.4	90.6	29.6		55.4	92.9	94.3

Test Concentrations in each of the assays: NF_B: 20 ug/mL; DPPH: 200 ug/mL; MTT with HL-60 leukemia cells: 20 ug/mL; RXR: 20 ug/mL; QR: 20 ug/mL; Aromatase: 20 ug/mL; NO: 20 ug/mL; Cytotoxicity SRB assay: 20 ug/mL

[a] Samples 1–25 are either sponges or gorgonians, samples 26–50 are bacteria, samples 51–65 are hard corals

[b] Taxonomic identifications of samples 26–50, the bacteria, were based on comparisons of sequence data with closest taxonomic matches in the NIH BLAST data base

[c] 100 % activity is with TNF as activator

[d] To avoid false positives and to determine cytotoxic effect on NFkB 293/Luc cells, 100% is with TNF and DMSO

[e] 100% activity is with scavenging DPPH free radical

[f] Sample concentration which caused 50% inhibition of DPPH

[g] HL-60 leukemia cells. 100% survival is with DMSO

[h] Sample concentration which caused 50% inhibition in HL-60 cell survival

[i] One (1.0) fold is with control induction level of RXR transcriptional activity

[j] QR the induction ratio (IR) of QR represents the specific enzyme activity of agent-treated cells compared with DMSO-treated control

[k] 100% survival is with DMSO

[l] 100% activity is with DMSO

[m] Sample concentration which caused 50% inhibition of aromatase

[n] 100% activity is with LPS as activator

[o] To avoid false positives and to determine cytotoxic effect on RAW 264.7 cells, 100% is with LPS and DMSO

[p] 100% survival is with DMSO

Table 2

Results of cancer chemopreventative and anticancer assays performed with fractions from the extract of the sponge sponge *Suberea* sp.

Fract. No.	NFκB % Inhib *a*	NFκB % Survival *b*	NFκB IC_{50} *c*	DPPH % Inhib *d*	DPPH IC_{50} (ug/mL) *e*	MTT (HL-60 leukemia cells) % Survival[f]	MTT IC_{50} (ug/mL) *g*	RXR Fold induction *h*	QR IR *i*	QR % Survival *j*	Aromatase % Inhib *k*	Aromatase IC_{50} (ug/mL)[l]	[NO] % Inhib *m*	[NO] % Survival *n*	SRB (MCF-7) % Survival[o]	SRB (KB) % Survival *p*
1	81.7	55.0	13.8	21.9		102.9		1.1	0.9	100.0	19.90		12.25	155.25	89.2	75.0
2	78.9	77.6	16.0	26.1		82.3		1.1	1.0	100.0	46.73		15.80	143.05	93.5	72.1
3	31.7	67.3	8.9	17.2		136.3		1.3	1.5	100.0	68.75		64.45	155.95	72.7	70.0
4	68.7	62.1	2.6	19.4		103.9		1.2	0.8	100.0	23.85		15.90	134.00	91.3	75.5
5	43.1	76.9	4.2	74.7	155.2	98.4		1.3	2.2	36.4	71.77	13.32	37.10	149.15	62.8	71.9
6	31.3	47.6		61.0	181.3	-31.5	7.1	1.2	1.8	42.0	67.52		48.00	137.85	70.6	72.0
7	60.4	90.2		37.7		53.7		1.2	1.8	100.0	70.54	13.8	60.60	148.65	72.3	79.6
8	-7.8	12.0		36.3		76.3		1.0	2.0	100.0	75.71	6.16	46.10	143.85	62.0	71.2
9	-17.5	28.2		18.4		64.4		1.2	2.0	100.0	83.87	1.89	78.20	145.95	85.6	73.7
10	9.0	3.5		10.4		54.9		1.2	2.3	100.0	89.89	0.56	89.70	134.60	70.2	65.5
11	55.3	13.7		4.8		124.4		1.0	3.5	69.7	87.23	2.89	82.65	131.05	75.4	56.9
12	66.4	16.8		5.8		91.9		1.1	1.0	100.0	25.06		15.95	125.10	84.5	73.8
13	5.8	39.0		3.7		-20.4	19.3	1.2	1.1	100.0	82.76	7.45	40.10	130.15	87.8	64.0
14	14.6	56.8		5.0		93.8		1.3	1.1	100.0	89.03	0.74	89.25	129.75	68.1	71.1
15	5.3	33.6		11.2		78.0		1.3	2.2	100.0	77.13	1.73	81.95	129.60	50.6	60.8
16	-18.7	43.6		7.6		91.9		1.2	1.0	100.0	66.41		27.60	121.80	72.4	74.4

Test concentrations in each of the assays: NFkB: 20 ug/mL; DPPH: 200 ug/mL; MTT with HL-60 leukemia cells: 20 ug/mL; RXR: 20 ug/mL; QR: 20 ug/mL; Aromatase: 20 ug/mL; NO: 20 ug/mL; Cytotoxicity SRB assays: 20 ug/mL

[a] 100 % activity is with TNF as activator

[b] To avoid false positives and to determine cytotoxic effect on NFkB 293/Luc cells, 100% is with TNF and DMSO

[c] Sample concentration which caused 50% inhibition of NFkB

[d] 100% activity is with scavenging DPPH free radical

[e] Sample concentration which caused 50% inhibition of DPPH

[f] 100% survival is with DMSO

[g] Sample concentration which caused 50% inhibition in HL-60 cell survival

[h] One (1.0) fold is with control induction level of RXR transcriptional activity

[i] QR the induction ratio (IR) of QR represents the specific enzyme activity of agent-treated cells compared with DMSO-treated control

[j] 100% survival is with DMSO

[k] 100% activity is with DMSO

[l] Sample concentration which caused 50% inhibition of aromatase

[m] 100% activity is with LPS as activator

[n] To avoid false positives and to determine cytotoxic effect on RAW 264.7 cells, 100% is with LPS and DMSO

[o] 100% survival is with DMSO

[p] 100% survival is with DMSO