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EVALUATION OF MARINE SPONGE METABOLITES FOR CYTOTOXICITY AND SIGNAL TRANSDUCTION ACTIVITY

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ABSTRACT.—Twenty-four metabolites derived from marine sponges were evaluated for their cytotoxicities against two human tumor cell lines, non-small cell lung carcinoma A549 and colon adenocarcinoma HT-29, and against one murine leukemia cell line, P-388, and evaluated for their ability to effect signal transduction in a newly developed cell adhesion assay using an EL-4 cell line. The compounds included latrunculin A [**1**], batzelline A [**2**], chondrillin [**3**], aureol [**4**], epihippuristanol, theonellamine B, discorhabdins A and C, kabiramide C, dercitin, meridine, manzamines A, B, and C, 8,15-diisocyano-11(20)-amphilectene and the corresponding C-15 formamide, a 20-carbon acetylenic alcohol, 4,5-dihydro-6″-deoxybromotopsentin, epispongiadiol, isospongiadiol, puupehenone, reiswigin A, and demethyl- and demethyloxyaaptamine. Latrunculin A [**1**], batzelline A [**2**], chondrillin [**3**], and aureol [**4**] expressed the desired profile of a greater than five-fold level of cytotoxicity against A549 relative to P-388, and an effect in the cell adhesion assay. In this group of compounds, cytotoxicity toward A549 was equal to or more pronounced than against HT-29. Latrunculin A was evaluated in an sc-implanted human A549 lung tumor xenograft mouse model and yielded a T/C of 146%. Batzelline A was evaluated in the cancer cell line panel at the National Cancer Institute and found to express selective cytotoxicity against several melanoma cancer cell lines.

Marine natural products, in particular those derived from sponges, have proved to be a rich source of novel compounds with varying degrees of antitumor activity (1–13). However, until very recently, the classical approach to the discovery of such agents has been routinely restricted to the screening of natural product extracts using rapidly growing murine leukemia and lymphoma cell lines (10–13). While this approach has yielded a number of new and interesting compounds with demonstrable antileukemic activities in vitro (14–18), the majority of these compounds also demonstrate a broad spectrum of cytotoxic activity towards cell lines derived from many other tissue types and organs as well as cytotoxic activity directed against normal cells, thus rendering them less suitable as potentially new and clinically useful chemotherapeutic agents. In addition, cytotoxicity as the sole endpoint of the test of a potentially new chemotherapeutic reveals little, if anything, about its mechanism of action, an important criterion for determining potential therapeutic utility (19–21). Thus a combined approach, utilizing a mechanism based screen along with a defined chemotherapeutic target (tissue or organ specific) should aid in the identification of more efficacious antitumor agents.

Recently, we have implemented such a mechanism-based approach to our screening of marine natural products for potential antitumor activities. This approach includes the parallel evaluation of selective cytotoxicity towards two human cell lines, HT-29 colon adenocarcinoma and A549 non-small cell lung carcinoma, and using the measured activity observed towards the murine leukemia line P-388 as a negative screening parameter. The mechanistic screening focus encompasses the ability of the marine extract/compound to perturb signal transduction pathways as measured by a newly developed cell adhesion assay, using the EL-4.IL-2 cell line (22).

As an initial test of the validity of this approach to discover compounds with unique mechanism(s) and selectivity associated with their antitumor activities, twenty-four metabolites derived from marine sponges were evaluated for selective cytotoxicity against human cell lines and for their ability to perturb signal transduction pathways associated with EL-4.IL-2 cell adherence. The results are described in this report.

RESULTS AND DISCUSSION

The twenty-four compounds that were evaluated in this study are listed in Table 1, arranged according to their selective cytotoxicity toward the human lung cancer cell line (A549) relative to the murine leukemia P-388. With one exception, the selective cytotoxicity against A549 roughly paralleled the effects against the human colon cancer cell line (HT-29). This first group of compounds expressed a fivefold or greater difference in cytotoxicity. The compounds which expressed selective cytotoxicity towards A549, latrunculin A [**1**] (23,24), batzelline A [**2**] (25), chondrillin [**3**] (26,27), and aureol [**4**] (28), were also active in the EL-4 assay. The two exceptions were epihippuristanol (29) and theonellamine B (30).

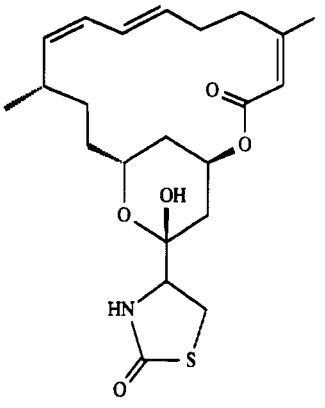
Both batzelline A [**2**] and chondrillin [**3**] were evaluated in the cancer cell line panel at the National Cancer Institute. Selective cytotoxicity was observed for batzelline A in several of the melanoma lines tested, i.e., MALME-3M ($\log_{10} LC_{50} = -4.81$) M19-MEL ($\log_{10} LC_{50} = -5.18$), and SK-MEL-5 ($\log_{10} LC_{50} = -5.18$). However, no selectivity was observed for the lung or colon cancer cell lines, relative to the leukemia cell lines. Chondrillin was not found to be selectively cytotoxic in this panel.

Latrunculin A [**1**], batzelline A [**2**], and chondrillin [**3**] were selected for in vivo evaluation against sc-implanted human A549 lung tumor xenografts in mice. At a dosage of 0.3 mg/kg, latrunculin A [**1**] yielded a T/C of 146%. Although none of the survivors were completely tumor-free, there were no deaths associated with drug toxicity. The remaining two compounds tested were not shown to be efficacious.

The remaining list of compounds tested were shown not to be selectively cytotoxictoward A549; however, some of these compounds affected EL-4.IL-2 cell adhesion, which correlates with protein kinase C (PKC) agonism and antagonism (22). This latter group included discorhabdins A and C (31,32), kabiramide C (33), demethyloxyaaptamine (34,35), dercitin (14,36), manzamine C (37), 8-isocyano-11(20)-amplilectene-15-formamide (38), and meridine (39). Meridine was the only compound that demonstrated substantial selective cytotoxicity toward the human colon cancer cell line, HT-29, relative to the other cell lines. The former group included a 20-carbon compound containing a terminal acetylene-alcohol group (40), dihydrodeoxy-bromotopsentin (41), espongiadiol (42,43), manzamine A and B (44), puupehenone (45,46), and reiswigin A (47). Interestingly, demethyloxyaptamine was the most potent inducer of EL-4 adhesion of all the compounds tested. This curious finding is currently under investigation in our laboratory.

Finally, two compounds expressed selective cytotoxicity against P-388 relative to A549, i.e., demethylaaptamine (34,35) and 8,15-diisocyano-11(20)-amplilectene (38).

Of all pure compounds Table 1, latrunculin A proved to be the most interesting. Latrunculin A has been the subject of considerable research since its structure was first reported by Kashman *et al.* in 1980 (23). Its biological profile includes its ability to alter cell shape, disrupt microfilament-organization, and inhibit the microfilament mediated processes of fertilization and early development in sea urchin eggs and mouse oocytes (48–50). These effects have been partially explained by in vitro experiments in which polymerization of pure actin was inhibited by the direct binding of latrunculin A to G-actin in a 1:1 molar proportion (50). Our findings of selective cytotoxicity of latrunculin A for A549 human lung cells and the apparent antagonism for EL-4.IL-2 cell adherence might relate to the previous findings of others in relationship to cytoskeletal changes which could result in an observed inhibition of adhesion of EL-4.IL-2 cells for the plastic substratum. However, in separate experiments (data not shown) we tested latrunculin A for its ability to antagonize the activity of protein kinase C itself and found it to be active. This would indicate another potential new mechanism of action of this compound



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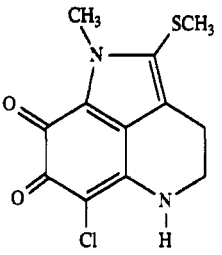

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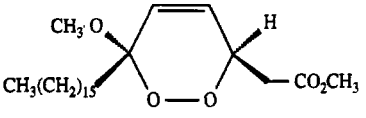

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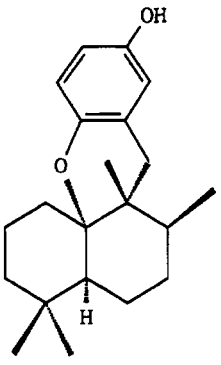

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which has not been previously reported. Further studies regarding these findings are currently underway.

In summary, twenty-four marine-derived compounds were analyzed using a cellular adherence mechanism-based assay and cytotoxicity assays. Several compounds were found to exhibit selective cytotoxicity against human A549 lung and colon (HT-29) cell lines and also affected EL-4.IL-2 cell line adherence. Several of these compounds were further tested in A549 lung tumor xenografts in mice, and one (latrunculin A) showed efficacy.

EXPERIMENTAL

ISOLATION OF COMPOUNDS FOUND IN TABLE 1.—All of the compounds in Table 1 were isolated by methods reported previously (23–47).

CELL LINES.—P-388 cells were obtained from Dr. J. Mayo, National Cancer Institute, Bethesda, MD; HL-60, HT-29 and A549 cells were obtained from American Type Culture Collection, Rockville, MD. All cell lines were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% horse serum. All cell lines were cultured in plastic tissue culture flasks and kept in an incubator at 37° in humidified air containing 5% CO_2. Prior to testing, antibiotic-free stock cultures of each of the cell lines were subcultured to 10^6 cells/ml by dilution in fresh growth medium at 2- to 3-day intervals.

CYTOTOXICITY ASSAYS.—To assess the antiproliferative effects of agents against the P-388 cell line, 200-μl cultures (96-well tissue culture plates, Nunc, Denmark) were established at 1×10^5 cells/ml in drug-free medium or medium containing the pure compounds at 10.0, 1.0, 0.10, and 0.010 μg/ml. Solvent for all dilutions was MeOH, which was removed from plates under vacuum. All experimental cultures were initiated in medium containing gentamicin sulfate (50 μg/ml; Schering Corporation, Kenilworth, NJ). After 48-h exposures, P-388 cells were enumerated using 3-[4,5-dimethylthiazol-2-yl]-2,5-

TABLE 1. Cytotoxicity and Signal Transduction Activity for Marine Sponge Metabolites.[a]

Compound	Cell line			
	A549	HT-29	P388	EL-4[b]
Selective (A549<P388)				
Latrunculina A [1]	0.06	0.06	4.1	0.1 (H)
Batzelline A [2]	0.7	2.8	5.3	22.9 (D)
Chondrillin [3]	0.3	1.1	2.4	0.4 (D)
Aureol [4]	4.3	4.7	>20.0	9.9 (H)
Epihippuristanol	0.04	0.05	0.5	ND
Theonellamine B	5.3	5.1	>20.0	ND
Non-Selective: (A549 vs. P388)				
Discorhabdin A	0.04	0.01	0.1	4.8 (D)
Discorhabdin C	0.3	0.1	0.5	0.5 (D)
Kabiramide C	0.8	NT	0.3	0.3 (D)
Demethyloxyaaptamine	5.2	1.5	1.6	<0.05 (D)
Dercitin	0.07	NT[c]	0.2	<6.0 (H)
Meridine	4.5	0.2	2.0	8.8 (D)
Manzamine C	3.5	1.5	2.6	12.0 (D)
Amphilectene formamide	5.3	3.5	4.2	2.9 (D)
Acetylenic Alcohol	3.0	1.2	3.6	ND
Dihydrodeoxybromotopsentin	6.2	5.0	18.1	ND
Epispongiadiol	2.2	1.3	6.2	ND
Isospongiadiol	5.3	6.5	10.2	ND
Manzamine A	1.3	0.8	2.4	ND
Manzamine B	4.5	1.6	6.6	ND
Puupepehenone	0.4	0.2	1.3	ND
Reiswigin A	7.2	6.0	8.5	ND
Selective: P388<A549				
Demethylaaptamine	6.7	2.2	0.3	1.2 (D)
Diisocyanoamphilectene	13.0	1.2	0.7	ND

[a]Table entries are IC_{50} in μg/ml. NT=not tested; ND=not detected.
[b]H=Inhibitor of EL-4.IL-2 cell adherence; D=Inducer of EL-4.IL-2 cell adherence.
[c]Lit. (15) HT-29 IC_{50} value is 63 nM (0.02 μg/ml).

diphenyltetrazolium bromide (MTT) as described below (51). A549 and HT-29 cells required an additional 48h exposure prior to MTT addition. Results were expressed as percent inhibition compared to the negative (no drug) control. Positive drug controls were included to monitor drug sensitivity of each of the cell lines. These included varying dilutions of 5-fluorouracil and adriamycin.

To quantitate the effects on cell proliferation and resulting IC_{50} values, 75 μl of warm growth medium containing 5 mg/ml MTT was added to each well, cultures returned to the incubator, and left undisturbed for 90 min. To quantitate formation of reduced formazan spectrophotometrically, plates were centrifuged (900×*g*, 5 min), culture fluids were removed by aspiration, and 200 μl of acidified iPrOH (2 ml concentrated HCl/liter iPrOH) added per well. The absorbance of the resulting solutions was measured at 570 nm with a plate reader (MR700 Microplate Reader, Dynatech, Laboratories, Chantilly, VA). The absorbance of test wells was divided by the absorbance of drug-free wells, and the concentration of agent that results in 50% of the absorbance of untreated cultures (IC_{50}) was determined by linear regression of logit-transformed data. A linear relationship between tumor cell number and formazan production has been routinely observed over the range of cell densities observed in these experiments. The four standard drug controls (indicated above) were included in each assay as a check to monitor the drug sensitivity of each of the cell lines, and IC_{50} values were determined for each drug-cell combination.

ADHERENCE-BASED ASSAY FOR PKC.—The El-4.IL-2 adherence-based assay (22) was used to assess the ability of the sponge metabolites to perturb signal transduction pathways associated with the activation of protein kinase C (PKC). EL-4.IL-2 cells (TIB 181), were obtained from American Type Culture Collection (Rockville, MD) and maintained in culture by in vitro passage with RPMI 1640 medium supplemented with 10% v/v heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 60 μg/ml L-glutamine, and 5×10^{-3} M 2-mercaptoethanol (TCM) (Grand Island Biologicals Co., Grand Island, NY).

Cells were passaged every 3 to 4 days. Viability was determined by Trypan blue exclusion and always exceeded 80%. For the adherence assay, cells were utilized within 15 passages of frozen stock cultures. Phorbol-12-myristate-13-acetate (PMA), and 4α-phorbol-12-myristate-13-acetate (4α-PMA) were obtained from LC Services Corporation (Woburn, MA). Stock solutions of PMA and 4α-PMA were prepared in DMSO and were stored at −70° prior to use.

EL-4.IL-2 cells (2.5×10^5/well) were cultured in 96-well, flat-bottomed microtiter test plates at 37° with the sponge metabolites in triplicate concentrations in the presence (Plate #1) or absence (Plate #2) of PMA (16 nM final concentration). The test compounds were diluted in TCM in such a manner that the final concentration of EtOH did not exceed 1.25%. Controls consisted of cells incubated alone (negative control) and cells incubated with PMA (positive control). An additional control plate (Plate #3), consisting of EL-4.IL-2 cells and identical dilutions of compounds in the absence of PMA served as a cytotoxicity plate and was not processed for adherence as described below, but used to monitor the potential cytotoxic effect of compounds for EL-4.IL-2 cells. Incubation time was 1 h at 37°. Following the incubation period, Plates #1 and #2 were processed as follows. The contents of each well were removed by flicking the plates into a catch tray and placing the plates inverted onto a paper towel in order to drain and blot the remaining draining wells. To each well, a volume of 100 μl of TCM was added at room temperature and the plates placed on a rotating platform shaker (Mini-Orbital Shaker, BELLCO Biotechnology, Vineland NJ) at setting 5 for 4 min. The plates were then rotated 180° and subjected to an additional 4 min of shaking. The plates were removed and their contents again removed as described, blotted, TCM added to each well, and the plates placed on the rotating shaker. This process was repeated for two additional wash cycles.

Following the removal of well contents, a volume of 200 μl of TCM and 75 μl of MTT solution (2 mg/ml in TCM) was added to each well of all plates. Plates were incubated at 37° for an additional 4 h. Following incubation, the contents of Plates #1 and #2 were again removed by flicking the plate into the sink and the plates blotted onto paper towels. Plate #3 was centrifuged and likewise blotted. A volume of 200 μl of iPrOH was then added to each well of all plates in order to dissolve the resulting formazan crystals. The plates were read at 570 nm using a plate reader (Microplate Autoreader, Model EL-311, BIO-TEK Instruments, Inc., Winooski, VT). The absorbances for each set of triplicate wells were averaged and the standard error determined. Results were expressed graphically by plotting the mean absorbance and standard error of each test well. The results were interpreted as follows.

Agonist of PKC (inducer of adherence).—Compound induces adherence of EL-4.IL-2 cells in the absence of PMA (Plate #2), as evidenced by absorbance of MTT metabolized by adherent cells which is greater than that of cells incubated alone (negative control).

Antagonist of PKC (inhibitor of adherence).—Compound does not induce adherence of EL-4.IL-2 cells in the absence of PMA (Plate #2), as evidenced by no absorbance of MTT. Compound inhibits adherence of EL-4.IL-2 cells in the presence of PMA (Plate #1) as evidenced by no absorbance of MTT. Compound is not cytotoxic as measured by Plate #3.

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