Brief Articles

Aldisine Alkaloids from the Philippine Sponge *Stylissa massa* Are Potent Inhibitors of Mitogen-Activated Protein Kinase Kinase-1 (MEK-1)

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Raf/MEK-1/MAPK cascade inhibitor activity-directed fractionation of the sponge *Stylissa massa* afforded eight known alkaloids: aldisine (**1**), 2-bromoaldisine (**2**), 10*Z*-debromohymenialdisine (**3**), 10*E*-hymenialdisine (**4**), 10*Z*-hymenialdisine (**5**), hymenin (**6**), oroidin (**7**), and 4,5-dibromopyrrole-2-carbonamide (**8**). Both **4** and **5** showed significant enzyme inhibitory activity (IC_{50} 3 and 6 nM, respectively). Secondary assays identified these compounds as potent MEK-1 inhibitors. Compounds **4** and **5** also inhibited the growth of human tumor LoVo cells.

Introduction

Raf/MEK/MAPK proteins play crucial roles in cellular signaling processes. The Ras-MAPK signaling cascade (MAPK module) is found in all eukaryotic organisms and is involved in transmitting signals from the extracellular compartment into the cytosol and nucleus.[1] This cascade is activated by guanosine 5′-triphosphate-loaded Ras, which recruits Raf-1 (c-Raf) to the inner cell membrane where it is activated by phosphorylation. Activated Raf-1 phosphorylates and activates the dual specificity kinase, MEK (MAP kinase kinase), on two different serine residues.[2,3] Activated MEK-1 phosphorylates and activates MAPKs (mitogen-activated protein kinases), which can translocate to the nucleus, and through the phosphorylation of a variety of substrates modulate cytoplasmic events such as cell proliferation and differentiation.[3,4]

Because the oncogenic form of Ras is associated with 30% of all cancers, Ras and the downstream kinase effectors of Ras represent attractive targets for pharmacological intervention.[5] Both in vitro and in vivo studies have shown that selective Raf and/or MEK-1 inhibitors are important pharmacological targets.[6−8] Raf/MEK-1/MAPK cascade assays, as well as individual Raf, MEK-1, or MAPK activity assays, have been described.[9−11] A sensitive, nonradioactive, and high-throughput Raf/MEK-1/MAPK cascade enzyme-linked immunosorbent assay (ELISA) was recently developed.[12] Using this assay, we have screened several thousand marine sponge extracts to identify inhibitors of this signaling cascade. The extract of the sponge *Stylissa massa* collected from the Philippines showed significant activity in this assay. Bioassay-guided fractionation yielded eight known pyrrole alkaloids, **1**−**8** (see Chart 1).[13] In secondary assays, 10*E*-hymenialdisine (**4**) and 10*Z*-hymenialdisine (**5**) were shown to be potent inhibitors of MEK-1 with IC_{50} values of 3 and 6 nM, respectively. Hymenialdisine has recently been reported to display significant inhibitory potential against a number of cytoplasmic kinases;[14,15] however, this represents the first report of activity for this class of compounds specifically against MEK-1. The structure−inhibitory activity relationships among **1**−**8** are also discussed.

Results and Discussion

The sponge *S. massa*, previously classified as *Stylotella aurantium*,[16] was extracted with MeOH and MeOH:$CHCl_3$ (8:2) mixtures followed by a standard solvent partitioning protocol (see Experimental Section). The Raf/MEK-1/MAPK cascade inhibitory effect was tracked to the $CHCl_3$ and MeOH solubles. Further chromatography of the these extracts furnished aldisine (**1**), 2-bromoaldisine (**2**), 10*Z*-debromohymenialdisine (**3**), a 1:1 mixture of **4** and **5**, hymenin (**6**), oroidin (**7**), and 4,5-dibromopyrrole-2-carbonamide (**8**). Separation of the mixture of **4** and **5** required repeated use of C-18 analytical high-performance liquid chromatography (HPLC) to provide geometrically pure **4** and **5**.

The Raf/MEK-1/MAPK cascade ELISA, which uses activated Raf to activate (phosphorylate) MEK-1, which in turn activates (phosphorylates) MAPK, was used to examine compounds **1**−**8**. The ELISA end point was the measurement of MAPK phosphorylation on Threonine (T) 202 and Tyrosine (Y) 204 by a phospho-specific monoclonal antibody. The specific target of inhibition in the Raf/MEK-1/MAPK ELISA was subsequently identified by secondary assays that directly measure Raf

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Chart 1

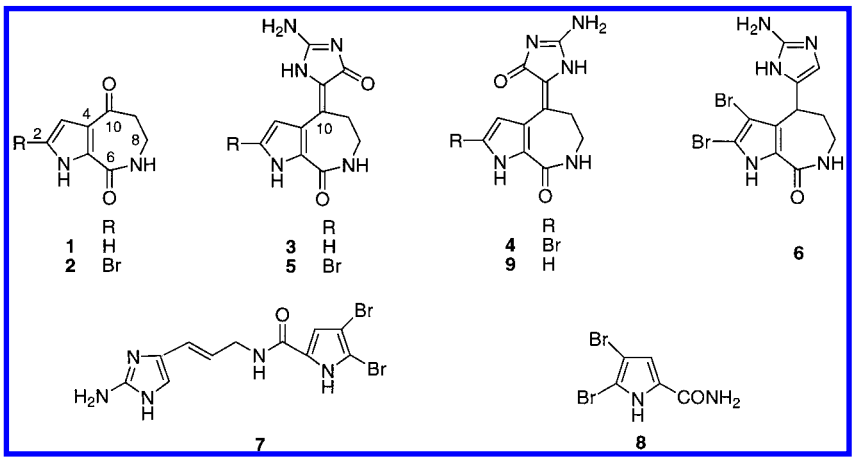

Table 1. Kinase Enzyme Inhibitory Activity and In Vitro Antitumor Activity of Compounds **1**−**8** (IC_{50} nM)[a]

compd	Raf/MEK1/ MAPK ELISA	Raf to MEK-1 Assay	MEK-1 to MAPK Assay	Caco-2	LoVo
1	>2500	2500	>2500	>10 000	>10 000
2	539	2500	539	>10 000	>10 000
3	881	2500	824	>10 000	>10 000
4	3	2500	6	>3867	586
5	6	2500	9	>7799	710
6	1288	2500	1288	>10 000	>10 000
7	>2500	2500	>2500	>10 000	>10 000
8	>2500	2500	>2500	>10 000	>10 000
staurosporine	2.5	2.5	2.5	NT	NT
PD98059	2800	>10 000	2800	NT	NT
Hamilton FTI	NT	NT	NT	>10 000	50

[a] NT = not tested.

phosphorylation of MEK-1, or MEK-1 phosphorylation of MAPK, by using either MEK-1 or MAPK phospho-specific antibodies. The sensitivity of the Raf/MEK-1/MAPK ELISA was confirmed by testing specific and generic kinase inhibitors, such as staurosporine, tyrphostin, olomoucine, genistein, lavendustin A, and apigenin. The Raf/MEK-1/MAPK ELISA sensitivity to staurosporine (IC_{50} = 2.5 nM) and the lack of response to tyrphostin, olomoucine, genistein, lavendustin A, and apigenin confirmed assay performance.

Several compounds were active in the initial Raf/MEK-1/MAPK signaling cascade assay (Table 1), with **4** and **5** being the most potent. It is noteworthy that **4** and **5** showed virtually indistinguishable inhibitory activity, with IC_{50} values of 3 and 6 nM, respectively. Although the initial samples were geometrically pure, we believe that the activity observed for **4** is not significant, because **4** is quite unstable and smoothly converts to **5** upon standing in dimethyl sulfoxide (DMSO), the solvent used as a vehicle in the current study. A facile and irreversible conversion of 10*E*-debromohymenialdisine (**9**) and **4** into their respective *Z* isomers (**3** and **5**) has been proposed.[17]

Compound **2** was found to be a reasonably potent inhibitor of the Raf/MEK-1/MAPK cascade but was still 90-fold less active (IC_{50} 539 nM) than **5**, suggesting that the aminoimidazolidinone ring is important for activity. Its debromo analogue, **1**, was inactive at concentrations up to 2500 nM. Similarly, **3** exhibited only moderate inhibition (IC_{50} 881 nM) in comparison to its 2-bromo derivative, **5**, indicating the importance of the bromine for enzyme inhibition. Compound **6**, which lacks the ketone function and instead bears an amino function on the imidazole appendage, is at least 200-fold less potent than **5**. None of the compounds displayed activity in the Raf to MEK-1 assay, whereas all compounds showed essentially identical IC_{50} values in the MEK-1 to MAPK assays (Table 1) as in the Raf/MEK-1/MAPK cascade assay. For comparison, the IC_{50} values of staurosporine and PD98059,[18] in both Raf/MEK-1/MAPK ELISA and secondary assays, are shown in Table 1.

This activity profile prompted us to test compounds **1**−**8** for their ability to inhibit growth of two human colon tumor cell lines, LoVo and Caco-2. LoVo cells have been shown to be sensitive to growth inhibition by farnesyl protein transferase inhibitors (FTIs) at low nanomolar levels.[19] Sensitivity of LoVo cells to FTIs is attributed to the presence of mutant activated K-Ras. Mutant K-Ras causes the activation of the Raf/MEK-1/MAPK signaling cascade that emanates from Ras; thus, inhibitors of Raf or MEK-1 should inhibit LoVo growth. Table 1 shows that the most potent MEK-1 inhibitors, **4** and **5**, also inhibited the growth of LoVo cells (IC_{50} values 586 and 710 nM, respectively). Caco-2 contains wild-type K-Ras, which correlates with resistance to the growth inhibitory effects of FTIs.[19] Compounds **4** and **5** were significantly less active against Caco-2 cells (Table 1). Hamilton FTI-276, a known Ras inhibitor, is also included in Table 1 for reference.

Conclusions

This study showed that **4** and **5** are potent inhibitors of the Raf/MEK-1/MAPK signaling cascade. They block the cascade by selectively inhibiting the phosphorylation of MAPK by MEK-1.

Hymenialdisine has recently been reported to inhibit the glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase 1 (CDK1), and CK1 at nanomolar levels.[14] Interestingly, activity against MEK was also reported but only at much higher concentrations (>1200 nM).[14] During the preparation of this paper, Curman and co-workers published the inhibition of the G_2 DNA checkpoint and Chk1 and Chk2 kinases by hymenialdisine and debromohymenialdisine at micromolar levels.[15] Here, we report selective MEK-1 inhibitory activity of hymenialdisine in the Raf/MEK-1/MAPK pathway. Our enzyme inhibition activity was observed at low nanomolar levels and presents the most potent in vitro activity to date for hymenialdisine. We believe that the discrepancy with published data stems from the assay format used for Raf and MEK-1 kinase inhibition. The ELISA format for Raf-1 and MEK-1 activity allows for inhibitor detection within the linear (most sensitive) response range of enzyme activity.[12]

Experimental Section

Nuclear magnetic resonance (NMR) spectra were recorded in CD_3OD or DMSO-d_6 at 500 MHz for ^1H and 125 MHz for ^{13}C. Mass spectra were taken on Finnigan MAT 95 (fast atom bombardment mass spectroscopy, FABMS) and Finnigan LCQ DECA ion trap (electrospray ionization mass spectroscopy, ESIMS) spectrometers. Reversed phase (C-18) HPLC separations were carried out on a Rainin Dynamax 60 Å semi-preparative column (10 × 250 cm, 8 mm, 6 mL/min) and an Alltech analytical column (4.6 × 150 mm, 5 mm, 1 mL/min) using a Beckman 168 diode array system. Flash column chromatography (CC) was performed on J. T. Baker C-18 material (40 mm, 275 Å).

Animal Material. The specimen of *S. massa* (sample # PS97-1-21) was collected by SCUBA in Surigao, Philippines, in 1997 and kept frozen until extraction.

Extraction and Isolation. Thawed sponge material was extracted with MeOH and MeOH:$CHCl_3$ (8:2) mixtures. The crude extract (3.42 g) was dissolved in 10% H_2O in MeOH and partitioned against hexanes. The water content of the MeOH phase was then adjusted to 30% before partitioning against $CHCl_3$. The $CHCl_3$ extract (220 mg) was fractionated by C-18 flash CC using a multistep gradient from 0 to 100% MeOH in H_2O. The fractions eluting with 30 (fraction 3), 40 (fraction 4), and 60% (fraction 6) aqueous MeOH were subjected to further chromatographic separations. Fraction 3 (15 mg) was purified by semipreparative C-18 HPLC using an isocratic mobile phase composition of 10% aqueous MeOH (0.05% trifluoroacetic acid, TFA) to furnish **1** (1.5 mg) and **3** (3.7 mg). Fraction 4 (25 mg) was also subjected to a semipreparative C-18 HPLC. Elution with 15% MeOH/85% aqueous TFA (0.05%) afforded **2** (1 mg) and **5** (14.7 mg). The separation of fraction 6 (13 mg) on a C-18 column (HPLC) using 30% aqueous MeOH (0.05% TFA) yielded **7** (3.6 mg) and **8** (2.5 mg).

The aqueous MeOH extract was triturated with MeOH three times to remove salts. The initial fractionation of the MeOH-soluble material (1.0 g) was also accomplished by C-18 flash CC. A step gradient was used with the mobile phase composition varied from 0 to 100% aqueous MeOH. Bioactivity-guided isolation was carried out on fractions 3 and 4, eluted with 30 and 40% MeOH in water, respectively. Fraction 3 (78 mg) was subjected to a semipreparative C-18 HPLC (10% aqueous MeOH with 0.05% TFA solution) and afforded additional **3** (8 mg) and a mixture (1:1) of **4** and **5**. This mixture was purified by repeated C-18 analytical HPLC employing a 15% MeOH/85% aqueous TFA (0.05%) solution to yield compounds **4** (2 mg) and **5** (3 mg) in a pure state. The separation of fraction 4 (68 mg) by C-18 HPLC using 15% MeOH in water (0.05% TFA) yielded **6** (14.5 mg).

Raf/MEK-1/MAPK ELISA. Recombinant human c-Raf was produced in Sf9 (*Spodoptera frugiperda*) insect cells, and GST tagged MEK-1 and MAPK was made in *Escherichia coli*.[12] Raf/MEK-1/MAPK cascade kinase activity was measured in ELISA format by a phospho-specific MAPK antibody (Sigma) that recognized the phosphorylation of amino acids T202 and Y204 on MAPK. Assay reactions (in 96 well plate format) were run in 0.1 mL AB (assay buffer: 20 mM MOPS, pH 7.2, 25 mM glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol) that contained activated Raf (50 μg protein/mL), nonactivated GST-MEK-1 (30 nM), nonactivated GST-MAPK (60 nM), 100 mM adenosine 5′-triphosphate (ATP), 5 mM $MgCl_2$, and various inhibitors at 1−10 000 nM concentrations. Inhibitor compounds in 100% DMSO were diluted so that the solvent concentration never exceeded 1% of the total reaction volume. Reactions were incubated for 0−300 min at 30 °C and stopped by the addition of ethylenediaminetetraacetic acid (EDTA, final concentration 70 mM). Reactions were then transferred to α-GST antibody coated 96 well plates.[12] After transfer, reactions were incubated for 60 min at 30 °C and then washed three times with TBST. α-Phospho MAPK (1:3000) in 0.1 mL TBST was added and incubated for 60 min at 30 °C. Plates were washed three times with TBST and then 0.1 mL of α-mouse-antibody/Europium conjugate (1:500, Wallac) was added. Incubation was for 60 min at 30 °C. Plates were washed three times with TBST, and the assay signal was read in a Wallac Victor model plate reader. Data were analyzed in Excel for single point percent inhibition and IC_{50} determinations. The ELISA was DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay) in format.

Raf Enzyme Selectivity Assay. Activated Raf (50 μg protein/mL) phosphorylation of MEK-1 (30 nM) was carried out in 0.1 mL of AB with $MgCl_2$ (5 mM) and ATP (100 μM). Inhibitor compounds, when present, were at 1−10 000 nM, with a compound solvent (DMSO) concentration of 1% in all reactions. Raf kinase reactions were run for 0−300 min at 25 °C, and reactions were stopped by the addition of EDTA (final concentration of 70 mM.) Completed reactions were resolved by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Proteins were blotted onto nitrocellulose, and MEK-1 phosphorylation was determined by Western analysis using α-phospho-MEK-1 antibody (New England Biolabs). MEK-1 phosphorylation was quantified by densitometry of the X-ray film from MEK-1 Western blots.

MEK-1 Enzyme Selectivity Assay. MEK-1 phosphorylation of MAPK was directly measured by the use of activated MEK-1 (Upstate Biotechnology) in an ELISA protocol that omitted the addition of activated Raf. All other aspects of the MEK-1/MAPK ELISA were identical to the Raf/MEK-1/MAPK ELISA. When kinase inhibitor compounds were tested in the MEK-1/MAPK ELISA, they were at 1−10 000 nM.

Cellular Assays. The human adenocarcinoma cell line LoVo, which is growth-inhibited by low nanomolar concentrations of a reference standard inhibitor of Ras (Hamilton, FTI-276), and the human adenocarcinoma cell line Caco-2, which is resistant to the same reference compound, were exposed to varying concentrations of the isolates, **1**−**8**. Cells were grown in RPMI 1640 with 10% fetal bovine serum supplemented with L-glutamine and Penicillin/Streptomycin. Compounds in 100% DMSO were diluted with complete growth media to the desired concentrations and applied to cells. Growth inhibition was measured by staining the cells with Sulforhodamine B.[20]

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