Small Molecule Therapeutics

Marine Sponge Cribrochalina vasculum Compounds Activate Intrinsic Apoptotic Signaling and Inhibit Growth Factor Signaling Cascades in Non–Small Cell Lung Carcinoma

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Abstract

Marine-derived compounds have been explored and considered as possible antitumor agents. In this study, we analyzed extracts of the sponge Cribrochalina vasculum for their ability to inhibit tumor cell proliferation. Screening identified two acetylenic compounds of similar structure that showed strong tumor-specific toxicity in non–small cell lung carcinoma (NSCLC) cells and small-cell lung carcinoma cells, and less prominent toxicity in ovarian carcinoma, while having no effect on normal cells. These acetylenic compounds were found to cause a time-dependent increase in activation of apoptotic signaling involving cleavage of caspase-9, caspase-3, and PARP, as well as apoptotic cell morphology in NSCLC cells, but not in normal fibroblasts. Further analysis demonstrated that these compounds caused conformational change in Bak and Bax, and resulted in loss of mitochondrial potential and cytochrome c release in NSCLC cells. Moreover, a decreased phosphorylation of the growth factor signaling kinases Akt, mTOR, and ERK was evident and an increased phosphorylation of JNK was observed. Thus, these acetylenic compounds hold potential as novel therapeutic agents that should be further explored for NSCLC and other tumor malignancies. Mol Cancer Ther; 13(12); 2941–54. ©2014 AACR.

Introduction

Marine organisms have been shown to be a great source of pharmaceutical compounds that can be used for anticancer, antiviral or anti-inflammatory purposes. Since the 1970s, there is a steady increase in the number of new compounds discovered and the number of registered patents is also growing (1–3). Among the different marine organisms, sponges (Porifera) have been identified as the most promising source of new compounds with anticancer potential (2–5). The leading role of Porifera as a source of new anticancer compounds is likely attributed to their long evolutionary history, extreme plasticity, and their remarkably rich associated microbiota (4). Indeed many marine-derived compounds have been explored and considered as possible antitumor agents. In this study, we analyzed extracts of the sponge Cribrochalina vasculum for their ability to inhibit tumor cell proliferation.

Screening identified two acetylenic compounds of similar structure that showed strong tumor-specific toxicity in non–small cell lung carcinoma (NSCLC) cells and small-cell lung carcinoma cells, and less prominent toxicity in ovarian carcinoma, while having no effect on normal cells. These acetylenic compounds were found to cause a time-dependent increase in activation of apoptotic signaling involving cleavage of caspase-9, caspase-3, and PARP, as well as apoptotic cell morphology in NSCLC cells, but not in normal fibroblasts. Further analysis demonstrated that these compounds caused conformational change in Bak and Bax, and resulted in loss of mitochondrial potential and cytochrome c release in NSCLC cells. Moreover, a decreased phosphorylation of the growth factor signaling kinases Akt, mTOR, and ERK was evident and an increased phosphorylation of JNK was observed. Thus, these acetylenic compounds hold potential as novel therapeutic agents that should be further explored for NSCLC and other tumor malignancies. Mol Cancer Ther; 13(12); 2941–54. ©2014 AACR.
Caribbean sponge *Cribrochalina vasculum* (family *Niphatidae*, order *Haplosclerida*). Structure elucidation and literature search of these compounds revealed that they both were previously described (15–17) and reported to have antitumor activity, yet their mechanism of action for induction of tumor cell death has not been described. Beside these compounds, several other related acetylenic alcohols were previously reported from the sponge *C. vasculum*. Such related acetylenic alcohols were shown to exhibit immunosuppressive activity, toxicity toward brine shrimp, and also to possess antitumor activity (8, 15, 18, 19). Here, we show that compound 1 causes strong cytotoxic activity in tumor cells from NSCLC, SCLC, and ovarian carcinoma but not in normal and primary cells tested. Yet we see differences also among the tumor cells with NSCLC being more sensitive than SCLC and ovarian carcinoma. For compound 2, a cytotoxic effect was evident in NSCLC and SCLC whereas in ovarian carcinoma the difference compared with normal cells was less prominent. Nevertheless, in NSCLC, we can demonstrate that both these compounds, isolated from sponge *C. vasculum*, activate intrinsic apoptotic signaling cascades, that is, activation of Bak/Bax, decreased phosphorylation of Bad, depolarization of mitochondria, cytochrome c release from mitochondria, and activation of caspases 9 and 3. Moreover, a decreased phosphorylation of Akt, mTOR, and ERK growth factor signaling kinases was evident and an increased phosphorylation of JNK was observed. Thus, compound 1, and to some extent compound 2, hold potential as therapeutic agents that should be further explored for different tumor malignancies with specific emphasis on NSCLC and SCLC.

**Materials and Methods**

**Collection and purification of extracts**

Samples of the sponge *C. vasculum* were collected in Key Largo, Florida in June 2011. Further details about the collection of the material can be found in Supplementary Materials and Methods, section collection of sponges. The freeze-dried sample (206 g) was extracted with 45:45:10 mixture of ethyl acetate:methanol:water to yield crude extract (43 g). Fractionations of crude extract were guided by MTT cell viability assay (see cell viability assay below) and each fraction was tested for cell cytotoxicity in NSCLC U-1810 cells or in normal diploid fibroblasts WI-38 after 72 hours of continuous exposure. The fraction that gave the best therapeutic window was chosen for further purification. In the last step of purification, the semi-pure fractions from the second Sephadex LH-20 column (fractions 8–11) were combined and separated on a reversed phase semipreparative YMC C-8 HPLC (high-performance liquid chromatography) column with a mixture 40:51:9 of acetonitrile:methanol:water as the isocratic mobile phase. Several products were obtained from this last purification and pure compounds (Supplementary Fig. S1B) were tested for tumor-specific toxicity. Two pure substances (3S)-icos-4E-en-1-yn-3-ol (1) (tq 41.8 minutes; 70.1 mg, 95% purity) and (3S)-14-methyldocos-4E-en-1-yn-3-ol (2) (tq 67.0 minutes, 109.0 mg, 99% purity) were found to have the best therapeutic window, and were therefore chosen for further analysis.

The following characteristics were noted for compounds 1 and 2: (3S)-icos-4E-en-1-yn-3-ol (1): colorless oil, [α]D 1.5 (c 0.35, CHCl3); UV (MeOH) (c) 204 (1400) nm; IR (ATR) 3,292; 2,917; 2,850 cm/L; for NMR data see Supplementary Table S1; GCMS SMB El m/z 292.3 M- C20H38O (19).

(3S)-14-methyldocos-4E-en-1-yn-3-ol (2): Colorless oil; [α]D 6.4 (c 5.8, CHCl3); UV (MeOH) (c) 204 (1300) nm; IR (ATR) 3,303; 2,922; 2,852; 2,098 cm/L; For NMR data see Supplementary Table S2; GCMS SMB El m/z 334.3 M+, C23H42O (19).

**Cell culture and treatments**

*C. vasculum* parental extract (fraction 10) and compounds 1 and 2 were diluted in DMSO to make 10 mg/mL stock solutions that were kept at −20°C and diluted in cell culture media before use. Data on stability of compound 2 after long-term storage in DMSO and in cell culture media after 72 hours incubation time at concentrations used in experiments can be found in Supplementary Materials and Methods, sections stability of compound 2 after long-term storage and stability of compound 2 in cell culture media.

The human SCLC U-1285 (20) and NSCLC U-1810 (21) were kind gifts from Uppsala University where they were established and characterized (22). SCLC H69 and H82, ovarian cancer cell lines A2780 and SKOV-3, foreskin fibroblasts immortalized with hTERT BJ-5ta, bronchial epithelial cells BEAS-2B and hTERT immortalized retina epithelial cells RPE-1 were all purchased from the ATCC. The lung fibroblast cell line WI-38 (23) was obtained from Coriell Cell Line Repository. Human cardiomyocyte primary cell culture was acquired from Celprogen Inc. BJ-5ta was obtained in 2014, BEAS-2B and cardiomyocytes in 2012, RPE-1 and SKOV-3 in 2011, A2780 in 2008, and U-1810, U-1285, H69, and H82 in 1996. Cell lines were authenticated by cell banks using the short tandem repeat profiling. No authentication for these cell lines was done by the authors. Upon receipt cultures were passaged for not more than 2 months and aliquots were frozen. For experiments each cell line was thawed according to the protocol and grown out for no more than 6 weeks for tumor and 3 for normal cells before use in an experiment. Human peripheral blood mononuclear cells (PBMC) were...
isolated from buffy coats of healthy donors (provided by the Department of Clinical Immunology and Transfusion Medicine, Karolinska Hospital, Stockholm, Sweden) by using a standard Ficoll-Hypaque gradient.

The U-1810, H69, H82, U-1285, A2780 cells, and PBMCs were cultured at 37°C and 5% CO2 in RPMI-1640 medium (Sigma-Aldrich) containing 2 mmol/L l-glutamine (Invitrogen) and 10% heat-inactivated FBS (HyClone). The ovarian cancer cell line SKOV-3 was grown in McCoy’s 5A medium (Sigma-Aldrich) containing 2 mmol/L l-glutamine and 10% FBS. Lung fibroblasts WI-38 were maintained in Eagle’s Minimum Essential Medium (Sigma-Aldrich) supplemented with 15% FBS and 2 mmol/L l-glutamine. hTERT immortalized retina epithelial cells (RPE-1) were cultured in DMEM/F12 (Lonza) with 10% FBS, glutamine, and antibiotics using Celprogen’s human cardiomyocyte culture extracellular matrix precoated flasks (Celprogen Inc.). BJ-5ta was cultured in DMEM (HyClone) supplemented with 2 mmol/L l-glutamine and 10% FBS. BEAS-2B was grown in BEGM k media (Lonza) and flasks were precoated with a mixture of 0.01 mg/mL fibronectin (Sigma-Aldrich), 0.03 mg/mL bovine collagen type I glutamine (Invitrogen), and 0.01 mg/mL BSA (Sigma-Aldrich) dissolved in BEBM (Lonza) to allow proper growth.

Cell viability assay

The cytotoxicity induced by extracts or pure compounds was determined with the previously described 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (24) with minor modifications. Cell viability experiments on tumor cells were carried out 24 hours after seeding, at a confluence of about 80%, whereas normal cells were completely confluent to mimic a non-dividing state, which is their natural state in the body. For all cells, the amount of cells required were titrated out in preparatory experiment and the following amounts were seeded in each well of a 96-well plate in 90 μL of complete growth media: 5,000 cells (U-1810, H82, SKOV-3 cell lines, and cardiomyocytes), 7,500 cells (H69, A2780, and BJ-Sta), 10,000 cells (U-1285), 18,000 cells (WI-38), 20,000 cells (BEAS-2B and RPE-1), and 400,000 cells (PBMCs). All technical replicates were done in triplicates and for each experiment the three biologic replicates were carried out. Different concentrations of parental extract and negative control (1, 2 or equal volumes of DMSO or 3 µmol/L of 1 or 2 for 24 hours) were assessed by staining the cells with tetramethylrhodamine ethyl ester perchlorate (MitoPT TMRE; Immunochemistry Technologies Bloomington) as previously been described (25). Briefly, cells were treated with DMSO or 3 µmol/L of 1 or 2 for 16 hours. Blocking was carried out with buffer (3% BSA, 0.2% TritonX100) for 60 minutes at room temperature and the slides were subsequently incubated with primary antibody against cytochrome c (1:200; Cell Signaling Technology) for 16 hours at 4°C, followed by three steps of washing in PBS and incubation with goat-anti-rabbit Alexa 488 secondary antibody (Invitrogen). To visualize nuclei, slides were counterstained with DAPI (Vector Laboratories). Staining was examined using a Zeiss Axioplan 2 imaging microscope with Zeiss 40x objective. Mitochondrial permeability

Mitochondrial permeability

The loss of mitochondrial membrane potential upon treatment with DMSO or 3 µmol/L of 1 or 2 for 24 hours was assessed by staining the cells with tetramethylrhodamine ethyl ester perchlorate (MitoPT TMRE; Immunochemistry Technologies Bloomington) as previously been described (25). Briefly, cells were treated with DMSO or 3 µmol/L of 1 or 2 for 24 hours. As a positive control for depolarization of mitochondria, the proton gradient uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 50 µmol/L, 45 minutes) was used. After harvesting, cells were stained with TMRE (150 nmol/L) for 20 minutes in the dark. Cells were centrifuged and resuspended in 1X Assay buffer provided by the MitoPT TMRE Staining Kit. Cells (10,000/sample) were analyzed on the FL-2 channel of Calibur flow cytometer (BD Biosciences). The percentage of cells that lost their TMRE staining was gated and quantified and values shown are the mean of three independent experiments ± SD.
Analysis of proapoptotic conformational changes of Bak and Bax

During apoptosis, the proapoptotic Bcl-2 family proteins Bak and Bax undergo N-terminal conformational changes leading to a proapoptotic state required for formation of pores in the mitochondria and subsequent cytochrome c release (26, 27). To analyze N-terminal changes in Bak or Bax in response to treatment, NSCLC U-1810 cells were treated with DMSO or 3 μmol/L of 1 or 2 for 16 hours. Cells were harvested and fixed in 4% paraformaldehyde for 10 minutes and washed in PBS. Fixed cells were stained with antibodies recognizing N-terminal activation-related conformational changes in either Bak (AM03, clone TC100; Oncogene Research Products) or Bax (clone 6A7; BD Biosciences Pharmingen). For staining cells were resuspended in 100 μL of primary antibody diluted in PBS (Bak 1:50 and Bax 1:250) containing digitonin (100 μg/mL) as permeabilizing agent. After 1 hour incubation at 4°C, samples were washed in PBS and incubated with secondary antibody Alexa 488-labeled anti-mouse antibody for 30 minutes at 4°C in the dark to enable monitoring by flow cytometry. Before FACS analysis, samples were washed in PBS and resuspended in 200 μL of PBS. The Bak- or Bax-associated IFL signal was measured in the FL-1 channel of the FACS Calibur flow cytometer (BD Biosciences). Data were processed using Cell Quest software (BD Biosciences) and change in fluorescence intensity was determined by comparing the percentage of gated cells after treatment with that of untreated cells, which was set to 100%. Data shown are the mean values of three independent experiments ± SD.

Western blot analysis

NSCLC U-1810 cells and WI-38 fibroblasts were seeded in 10-cm cell culture dishes at density of 1.2 million cells for U-1810 and 2 million for WI-38. After 24 hours, cells were treated for 4, 16, 24, 48, and 72 hours with 3 μmol/L of 1 or 2 with equal volume of DMSO or with equal volume of DMSO for 24 hours and fixed in 70% ethanol. Cells were stained with propidium iodide as described previously (28). Signals were recorded using FACSCalibur (BD Biosciences) and analyzed with ModFit LT (Verity Software House).

Results

Isolation of antitumor extracts from C. vasculum

Several compounds with antitumor activity have been isolated from marine-derived organisms and some of them are already in clinical use (8–12). Here, we set out to isolate and characterize antitumor compounds from the sponge C. vasculum with aim to reveal their antitumor mechanism of action. For that purpose material was collected and extracted as described in Materials and Methods. Resulting fractions were tested for antitumor activity against NSCLC U-1810 cells and for normal cell cytotoxicity using diploid fibroblasts WI-38 during 72 hours continuous exposure. Fraction 10 (named parental extract) from the C. vasculum extract induced significant cytotoxicity of NSCLC cells and almost completely inhibited cell growth at a concentration of 0.5 μg/mL, whereas only minor cytotoxicity was evident in a normal diploid fibroblasts WI-38 up to a concentration of 25 μg/mL (Fig. 1A and B).

Chemical structure elucidation of C. vasculum antitumor active fractions identifies (35)-icos-4E-en-1-yn-3-ol (1) and (35)-14-methyleneicos-4E-en-1-yn-3-ol (2)

HPLC fractionation of the parental extract from C. vasculum (Supplementary Fig. S1B) yielded two known alkyl-4-en-1-yn-3-ol derivatives; 70.1 mg (0.035% of methanol. After blocking nonspecific binding with the Odyssey blocking buffer (Li-Cor Biosciences; Bad Homburg Germany) diluted in PBS (1:1), membranes were probed with primary antibodies overnight at 4°C and with secondary antibody for 1 hour at room temperature.

Following primary antibodies diluted in blocking buffer were used: phospho-Akt (Thr308), phospho-Akt (Ser473), Akt, phospho-Bad (Ser112), Bad, Bak, Bax, Bcl-xL, caspase-3, caspase-9, phospho-mTOR (Ser2448), phospho-p44/42MAPK (Erk1/2), Thr202/Tyr204, p44/42MAPK (Erk1/2; Cell Signaling Technology); JNK (FL), PARP (H250; Santa Cruz Biotechnology), and phospho-JNK1/JNK2 (Thr183/Tyr185; Abcam). Where indicated, antibodies against β-tubulin, β-actin (Abcam), or GAPDH (Trevigen) were used as control of equal loading. IR-Dye–linked secondary antibodies (LI-COR Biosciences) was performed at 30 V for 90 minutes in transfer buffer (NuPAGE; Invitrogen). Transfer to polyvinylidene fluoride membranes (Hybond-C Extra; Amersham Biosciences) was performed at 30 V for 90 minutes in transfer buffer (NuPAGE; Invitrogen) supplemented with 10% methanol. After blocking nonspecific binding with the Odyssey blocking buffer (Li-Cor Biosciences; Bad Homburg Germany) diluted in PBS (1:1), membranes were probed with primary antibodies overnight at 4°C and with secondary antibody for 1 hour at room temperature.

Following primary antibodies diluted in blocking buffer were used: phospho-Akt (Thr308), phospho-Akt (Ser473), Akt, phospho-Bad (Ser112), Bad, Bak, Bax, Bcl-xL, caspase-3, caspase-9, phospho-mTOR (Ser2448), phospho-p44/42MAPK (Erk1/2; Thr202/Tyr204), p44/42MAPK (Erk1/2; Cell Signaling Technology); JNK (FL), PARP (H250; Santa Cruz Biotechnology), and phospho-JNK1/JNK2 (Thr183/Tyr185; Abcam). Where indicated, antibodies against β-tubulin, β-actin (Abcam), or GAPDH (Trevigen) were used as control of equal loading. IR-Dye–linked secondary antibodies (LI-COR Biosciences) were used to image bands on the Odyssey platform.

Cell-cycle distribution

To assess cell-cycle distribution, U-1810 and WI-38 cells were treated with 1.3 μmol/L (IC50 in U-1810 at 24 hours), 3.4, 6.8, and 16.2 μmol/L (IC50 in WI-38 at 24 hours) of compound 1 or an equal volume of DMSO for 24 hours and fixed in 70% ethanol. Cells were stained with propidium iodide as described previously (28). Signals were recorded using FACSCalibur (BD Biosciences) and analyzed with ModFit LT (Verity Software House).

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sponge dry weight) of 95% pure (3S)-icos-4E-en-1-yn-3-ol (1) and 109 mg (0.055% of sponge dry weight) of 99% pure (3S)-14-methyldocos-4E-en-1-yn-3-ol (2) (ref. 19). The structures of 1 and 2 were confirmed by 1D and 2D NMR techniques. EIMS and MS-MS techniques were used to elucidate the position of the branched methyl substituent in compound 2 (Supplementary Fig. S1C; ref. 19). The absolute stereochemistry of 1 and 2 was determined by the Mosher method (29) because their optical rotations were small. Analysis showed that compounds 1 and 2 are structurally related acetylenic alcohols both containing a C4-C5 trans double bond and the same absolute configuration of the hydroxy group. The structure determination also showed that the compounds differed in the length of their carbon chains and that 2 has a branched methyl substituent at position 14 (Fig. 1C).

**C. vasculum compounds 1 and 2 have tumor-specific cytotoxicity**

We next continued with the acetylene containing compounds 1 and 2 to reveal their antitumor effect and delineate their mechanism of action. Similar to the parental extract, compounds 1 and 2 induced toxicity in NSCLC U-1810 cells but not in normal diploid fibroblasts WI-38 until 3 μmol/L was used (Fig. 2A). A clear cytotoxicity was also evident when treating the NSCLC U-1810 cells with different concentrations of 1 and 2 for 72 hours and examining cell viability with MTT assay (Fig. 2B). Thus, at concentration of 3 μmol/L 1 and 2 reduced U-1810 cell viability for about 90% and 60%, respectively. Importantly, applying the same concentration of these compounds to WI-38 fibroblasts caused little or no cytotoxicity, suggesting a tumor-selective window for these compounds. Thus, the concentration of compounds 1 and 2 needed to inhibit 50% of cell viability (IC50) was more than 10 times higher for WI-38 than for the U-1810 cells (Supplementary Table S4).

Figure 1. *C. vasculum* semi-pure fraction shows specific tumor cell cytotoxicity and generates two pure compounds. A, fraction 10 [crude mixture of (3S)-alkyl-4E-en-1-yn-3-ol, named parental] after three fractionation steps of sponge *C. vasculum* extract was tested for capacity to induce tumor-specific cytotoxicity in NSCLC U-1810 cells or in diploid fibroblasts WI-38 after 72 hours of continuous exposure to indicated concentrations (phase-contrast images, ×10 magnification; scale bar, 0.5 mm). B, cells were treated as in A and cell viability was examined using MTT cell viability assay. Cell viability is given as the percentage of cell survival as compared with DMSO solvent–treated cells. Data, mean of three independent experiments ±SEM. C, structure of (3S)-icos-4E-en-1-yn-3-ol (1) and (3S)-14-methyldocos-4E-en-1-yn-3-ol (2).
C. *vasculum* compounds 1 and 2 induce prominent cytotoxicity in SCLC but not in normal cells

Next, we extended our analyses onto three SCLC cell lines (U-1285, H69, and H82) and two ovarian cell lines (SKOV-3 and A2780; Fig. 2C; Supplementary Table S4). To assess normal cell toxicity human cardiomyocytes, human PBMCs, foreskin fibroblasts immortalized with hTERT (BJ-5ta), bronchial epithelial cells (BEAS-2B), and hTERT immortalized retina epithelial cells (RPE-1) were used in the analyses (Fig. 2D; Supplementary Table S4).

Of the SCLC cell lines, H82 showed the highest sensitivity toward compound 1 with an IC50 value of 1.1 μmol/L and with an almost complete inhibition of cell survival at 1.5 μmol/L (Fig. 2C, left). The toxicity of compound 1 was slightly lower for U-1285 and H69, yet at 3 μmol/L both these cell lines showed an 80% decrease in cell viability and the corresponding IC50 values were 1.6 and 2.2 μmol/L, respectively (Supplementary Table S4). For compound 2 U-1285, H69, and H82 responded with about 80% decrease in cell viability upon treatment with 3 μmol/L concentration (Fig. 2C, right). IC50 values were 1.8 μmol/L for U-1285, 1.3 μmol/L for H69, and 1.1 μmol/L for H82 (Supplementary Table S4). In ovarian carcinoma cells, compound 1 caused a similar 80% reduction in cell viability at 3 μmol/L as seen in SCLC whereas for compound 2 both A2780 and SKOV-3 cells were less responsive (Fig. 2C). Compound 1 reached IC50 in A2780 at 1.8 μmol/L and in SKOV-3 at 2.1 μmol/L. Toxicity of compound...
toward ovarian carcinoma was not so prominent, IC50 was reached only at 3.4 μmol/L in A2780 and 4.9 μmol/L in SKOV-3 cell lines (Supplementary Table S4). Taken together, compounds 1 and 2 both demonstrate toxicity toward SCLC, whereas for ovarian carcinoma compound 1 has a greater potency than compound 2.

Both compounds were tested against several normal cells. Treatment with 3 μmol/L concentration of compound 1, which caused high cytotoxicity in all tumor cell lines tested, induced only 20% reduction in cell viability in cardiomyocytes and BEAS-2B whereas in PBCM and BJ-5ta about 40% cytotoxicity was evident (Fig. 2D, left). With the applied concentrations, the IC50 value could not be reached for cardiomyocytes and BJ-5ta whereas for RPE-1 it was 6.6 μmol/L, PBMC 7.8 μmol/L, and for BEAS-2B 12.9 μmol/L (Supplementary Table S4). Compound 2 at 3 μmol/L, a concentration that caused at least a 50% cell cytotoxicity in LC cells induced less than 15% cytotoxicity in cardiomyocytes, BJ-5ta, BEAS-2B, and RPE-1 whereas in PBMC about a 35% cytotoxic effect was evident (Fig. 2D, right). With compound 2, the IC50 value was reached only for PBCM and RPE-1 (8.5 μmol/L for PBMC and 10.4 for RPE-1; Supplementary Table S4).

In summary, the cytotoxic effect of compound 1 was significantly lower in all normal cells than in tumor cells, whereas for compound 2 the tumor-selective effect was less prominent but evident in NSCLC and SCLC.

C. vasculum Compounds Possess Antitumor Potential

To further understand mechanism of action of these C. vasculum compounds, their effect on apoptosis induction was examined in NSCLC cells (Supplementary Fig. S3A and Fig. 3A). Treatment of NSCLC U-1810 cells with either 1 or 2 (3 μmol/L, 48 hours) or cisplatin (20 μmol/L, 72 hours) caused a prominent induction of apoptotic morphology of the cell nuclei, whereas no major alteration was observed in diploid fibroblasts WI-38 upon treatment (Supplementary Fig. S3A). Induction of apoptotic morphology in U-1810 cells was also quantified at 24 to 72 hours after treatment with 1 or 2 (3 μmol/L; Fig. 3A). Already after 24 hours, 1 induced apoptotic morphology in about 30% of the cells with a further increase after 48
hours and with 80% of the cells showing apoptotic nuclear morphology at 72 hours after treatment (Fig. 3A). Compound 1-induced apoptosis was delayed as compared with 1 but nevertheless 48 hours after treatment, about 40% of the U-1810 cells had fragmented nuclei and at 72 hours almost 60% showed a prominent apoptotic response (Fig. 3A). Both compounds induced less than 2% apoptosis in fibroblasts treated for 72 hours (data not shown). Cisplatin, which was used as a positive control, induced apoptotic morphology in 26% of U-1810 cells showing that these cells are capable to undergo apoptosis (data not shown).

C. vasculum compound 1 induces G2 arrest in NSCLC but not in normal diploid fibroblasts

Loss of proliferation capacity and induction of cytotoxicity may also be attributed to inhibition of cell-cycle progression. The effect of 1 on cell-cycle distribution was, therefore, also evaluated (Fig. 3B). NSCLC U-1810 or WI-38 fibroblasts were treated with different concentrations of 1 for 24 hours. In NSCLC U-1810 cells, treatment with 1 was found to induce a significant dose-dependent increase in the number of cells arrested in the G2–M phase (Fig. 3B, left graph). In contrast, cell-cycle distribution of normal fibroblasts showed no alteration after treatment with compound 1 even at IC50 concentration (Fig. 3B, right graph). Thus, the results showed that compound 1 clearly induces cell-cycle arrest in G2–M in NSCLC tumor cells and this is a tumor-specific effect.

C. vasculum compounds 1 and 2 induce mitochondrial depolarization, activate caspases 9 and 3, and cause PARP cleavage in NSCLC cells but not normal diploid fibroblasts

One important gateway to apoptosis is mitochondrial depolarization allowing cytochrome c to be released to the cytosol where it subsequently causes the pro–caspase-9 to be activated within the apoptosome complex. Caspase-9 in turn triggers cleavage of pro–caspase 3/7 into active proteases, thereby giving rise to apoptotic morphology (30). The effect of 1 and 2 on depolarization of mitochondria and release of cytochrome c were, therefore, examined. NSCLC U-1810 cells were treated with compound 1 or 2 (3 μmol/L) for 24 hours and mitochondrial depolarization was examined with TMRE (Fig. 4A). Upon membrane depolarization, the potential of mitochondria is lost and the TMRE dye leaks into cytosol. Thus, cells that have a decrease in TMRE staining represent cells with depolarized mitochondria and these cells were quantified. The mitochondrial uncoupler CCCP (50 μmol/L, 45 minutes) was used as positive control showing that TMRE is capable of detecting alterations in mitochondrial potential. Importantly, treatment with either compounds resulted in prominent mitochondrial depolarization. Hence, both compounds were capable of disrupting mitochondrial membrane potential.

Next, the release of cytochrome c after treatment of NSCLC U-1810 cells with 1 and 2 (3 μmol/L) for 16 hours was analyzed by immunofluorescence staining (Fig. 4B). A punctate staining pattern of cytochrome c indicative of mitochondrial localization was found in untreated cells, whereas upon treatment with 1 or 2 diffuse staining pattern indicating release of cytochrome from mitochondria to cytosol was evident (Fig. 4B).

To study whether depolarization of mitochondria and release of cytochrome c in response to 1 or 2 also resulted in the downstream activation of caspase 9/3 and subsequent apoptotic-associated cleavage of the caspase-3 substrate PARP-1 NSCLC U-1810 cells were treated with 1 or 2 for 24 hours at a concentration that caused 50% and 70% of cell death, respectively (Fig. 4C). As can be seen in U-1810 cells both caspases 9 and 3 were cleaved into active forms (35–37 and 15–17 kDa, respectively) upon treatment with IC50 or IC70 of either compound. Treatment also resulted in clear cleavage of PARP-1 into the proapoptotic-associated 89 kDa fragment confirming that these compounds indeed triggered an apoptotic response (Fig. 4C). Normal fibroblasts WI-38 were used for comparison and treated with same concentrations as U-1810 cells. Importantly, under these conditions no caspase-3 or PARP-1 cleavage was evident in normal WI-38 fibroblasts (Fig. 4C). However, the lower proapoptotic activity of compounds observed in fibroblasts could be due to cell permeability rather than to a difference in actual mode of cell kill. To see whether these compounds at all were able to induce apoptosis in these normal lung fibroblasts, concentrations that induced 50% and 70% kill of fibroblasts at 24 hours were applied. Even at these concentrations, no PARP-1 or caspases cleavage was observed in the fibroblast further demonstrating a NSCLC cell–specific proapoptotic activity of these compounds (Fig. 4D).

We also examined the kinetic of caspases and PARP-1 cleavage after treatment with 3 μmol/L of 2 for 24, 48, and 72 hours (Supplementary Fig. S3B). In line with MTT and nuclear apoptotic morphology data, cleaved versus full-length caspase-3, caspase-9, and PARP-1 all clearly showed an increase over time in response to 2. Thus, there is a clear NSCLC-specific activation of apoptosis by both compounds.

C. vasculum compounds 1 and 2 trigger activation of Bax and Bax and JNK signaling and inhibit PI3K/Akt and MAPK ERK survival signaling in NSCLC cells

Activation of the Bcl-2 proteins Bak and Bax is one important gateway for the intrinsic mitochondrial pathway of apoptosis (26, 27). The effect of compounds 1 and 2 on Bax and Bak conformational changes associated with activation was, therefore, analyzed (Fig. 5A and B). Indeed, treatment of NSCLC U-1810 cells with 3 μmol/L of 1 or 2 resulted in prominent activation of Bak indicated by a shift of histogram peak to the right (Fig. 5A and B, right). An activation of Bax was also observed after treatment of NSCLC U-1810 cells with either 3 μmol/L of 1 or 2, yet less pronounced than for Bak (Fig. 5A and B, left).
The total expression of Bax and Bak was also examined using conformation-independent antibodies in Western blot analysis (Fig. 5C). However, no changes in their expression levels were evident upon treatment, indicating that the observed Bak/Bax activation upon 1 or 2 relates to N-terminal conformational changes of these proteins.

The antiapoptotic Bcl-xL protein is reported to inhibit Bak/Bax complex formation and subsequent cytochrome c release (31). The expression level of Bcl-xL was, therefore, examined after treatment with compound 1 or 2 and a clear decrease in Bcl-xL expression was evident after treatment with 1, whereas no change was observed after treatment with 2 (Fig. 5C). All in all, these results show that both 1 and 2 can activate the Bak/Bax rheostat in NSCLC cells, which is in line with the observed depolarization of mitochondria, release of cytochrome c, and

Figure 4. Compounds 1 and 2 induce depolarization of mitochondria, cytochrome c release, and activation of caspases in NSCLC cells but not in diploid fibroblasts. A, NSCLC U-1810 cells were treated with 3 μmol/L 1 or 2 for 24 hours. CCCP (50 μmol/L) was added to cells 45 minutes before staining as positive control. Depolarization of mitochondria was examined by staining with 150 nmol/L TMRE for 20 minutes in media. Left, the signal was recorded in the FL-2 channel on FACS Calibur. Histogram showing TMRE-associated fluorescence. Filled gray, DMSO; solid line, CCCP, 1 or 2 treated cells. Right, quantification of cells that lost their TMRE signals (i.e., depolarized mitochondria). Data, mean with bars representing SD; *, P < 0.05. B, cytochrome c release was examined by immunofluorescence staining of U-1810 cells after treatment with either DMSO or 3 μmol/L of 1 or 2 for 16 hours (magnification ×100; scale bar, 15 μm). Blue, DAPI staining of nucleus; green, cytochrome c. C, NSCLC U-1810 cells or diploid fibroblasts WI-38 were treated with compound 1 or 2 at concentrations that induced IC50 and IC70 in U-1810 cells at 24 hours. Cleavage of caspase-3, caspase-9, or PARP-1 was examined after 24 hours. β-Tubulin was used to visualize equal loading. D, diploid fibroblasts WI-38 were treated with 1 or 2 at concentrations that at 24 hours inhibited growth to 50% or 70% (i.e., IC50 and IC70), respectively. Cleavage of caspase-3, caspase-9, or PARP-1 was examined after 24 hours. β-Tubulin was used to visualize equal loading.
activation of caspases via the intrinsic route after treatment with these compounds.

Bak and Bax complex formation is in part regulated by the BH3-only protein Bad, which upon phosphorylation is sequestered by binding to 14-3-3 protein preventing its proapoptotic action (32). The effect of 1 or 2 in causing dephosphorylation of Bad was, therefore, examined. Indeed a decreased phosphorylation of Bad at Ser112 was clearly evident in response to treatment with 1 already at 16 hours after treatment and still evident at 24 hours in NSCLC U-1810 cells (Fig. 5C). In response to treatment with 2, a moderate decrease in Bad phosphorylation was observed only at 24 hours (Fig. 5C). The observed alteration on Bad Ser112 in response to 1 was confirmed to be a result of altered signaling as no difference in total Bad protein expression was evident upon treatment (Fig. 5C). Thus, both compounds partly inhibit Bad phosphorylation, indicating a putative proapoptotic action of Bad at mitochondria-mediated apoptotic signaling in NSCLC cells.

Several growth factor signaling cascades are reported to control Bad phosphorylation, thereby inhibiting its proapoptotic signaling capacity. The effect of 1 and 2 on the

Figure 5. Compounds 1 and 2 activate proapoptotic Bcl-2 family members and JNK signaling, decrease Bad and Akt phosphorylation, and inhibit MAPK-ERK survival signaling in NSCLC cells. A, conformational changes in Bax and Bak in response to 1 or 2 were examined in NSCLC U-1810. Histograms showing Bax and Bak activation. Filled gray, DMSO; solid line, 1 or 2 treated cells. B, quantification of Bax- (left) and Bak- (right) associated IFL. Data, mean of three independent experiments ± SD. C, NSCLC U-1810 cells were treated with 3 μmol/L of either 1 or 2 for 4, 16, and 24 hours or with equal volume of DMSO for 24 hours and Bcl-xL, Bax, Bak, and Bad expression and Bad phosphorylation at Ser112 were examined by Western blot analysis. GAPDH or β-tubulin served as loading controls. D, deactivation of AKT on phosphoresidues Ser473, Thr308, mTOR Ser2448, p42/44MAPK(Erk1/2) on Thr202/Tyr204, and JNK1/JNK2 on Thr183/Tyr185 and total forms of AKT, p42/44MAPK(Erk1/2) ERK, and JNK1/JNK2 were examined at 4, 16, and 24 hours after addition of 1 or 2 or 24 hours treatment with equal volume of DMSO. GAPDH or β-actin were used as loading controls.
phosphorylation status of the Bad-regulating kinases Akt, Erk1/2 as well as on JNK, a kinase shown to promote apoptotic signaling (13, 33) was, therefore, evaluated. A decreased phosphorylation of Akt at both Ser473 and Thr308 was evident in NSCLC U-1810 cells after treatment with compound 1 or 2 with an earlier response seen with 1 (Fig. 5D). Deactivation of mTOR was also evident after 1 as the phosphorylation on Ser2448 declined (Fig. 5D). These data suggest that 1 and 2 impair PI3K/AKT pathway signaling and may in this way inhibit proliferation and survival of NSCLC cells. A decreased phosphorylation on Thr202/Tyr304 of ERK1/2 was also evident after treatment with both 1 and 2 again with a more prominent deactivation caused by compound 1 (Fig. 5D). Hence, a decreased prosurvival signaling via the MAPK–ERK may also contribute to the observed cytotoxic activity of these two compounds in NSCLC cells. A sustained increase in JNK phosphorylation is reported to be critical for efficient induction of apoptotic signaling (33). In line with this 1 caused increased phosphorylation of JNK1/JNK2 at 16 and 24 hours, whereas in response to 2 a similar increase in phosphorylation was seen only at 24 hours in these NSCLC cells. Thus, this increased JNK phosphorylation may in part contribute to the observed apoptotic signaling in response to these compounds in this tumor type.

Discussion

Here, we analyzed the ability of C. vasculum–derived metabolites to inhibit proliferation, induce cytotoxicity, and trigger apoptotic signaling in NSCLC, SCLC, and ovarian carcinoma. It has previously been shown that compounds isolated from this sponge can kill tumor cells of different origin albeit through unknown mechanism of action (19). We show here that the two acetylenic compounds 1 and 2 as well as the parental mixture of these compounds from which they were separated, indeed cause tumor-specific cytotoxicity in NSCLC and SCLC. Importantly, we demonstrate that for both compounds this cytotoxicity is tumor specific, as all normal cells tested representing heart tissue, bronchial and retina epithelium, normal PBMCs, and foreskin or lung fibroblasts remained unaffected at concentrations that were toxic for NSCLC and SCLC. In ovarian carcinoma cells compound 1 but not compound 2 caused tumor-specific cell death. For compound 2, a cytotoxic effect was evident in NSCLC and SCLC whereas in ovarian carcinoma the difference compared with normal cells was less prominent.

With respect to NSCLC cytotoxicity, we for the first time show that compounds from C. vasculum can induce a prominent apoptotic response whereas in normal fibroblasts no induction of apoptosis was seen after exposure to the same concentration of 1 or 2. Moreover, no apoptosis was seen in normal fibroblasts even when concentrations that caused 50% or 70% cytotoxic response in cell viability assay were used. This indicates that the induction of apoptosis is a tumor-selective path that might be a consequence of inhibition of cell survival signaling pathways, which tumor but not normal cells rely upon for protection against apoptotic cell death.

At an early stage of the intrinsic apoptotic pathway, the proapoptotic Bcl-2 family proteins Bak and Bax are activated leading to permeabilization of the mitochondrial membrane, release of cytochrome c, and activation of caspases within the apoptosome (34, 35). We demonstrate that both 1 and 2 cause all these events in NSCLC cells. However, a more prominent activation of Bak over Bax was evident in these NSCLC cells. This is likely attributed to the basic signaling propensity of these NSCLC cells rather than specific action of these compounds to activation of Bak preferentially over Bax. This conclusion is based on the fact that we showed similar less pronounced activation of Bax also after treatment with other agents, for example, cisplatin and radiation in these NSCLC cells (25).

When comparing 1 and 2 with respect to Bak activation and mitochondria-mediated apoptotic signaling, compound 2 was found to be less efficient. Compound 1 is, at the applied concentration 3 μmol/L, more toxic toward U-1810 cells than is equimolar concentration of compound 2, likely explaining the more pronounced Bak and Bax activation and cytochrome c release. There was also a less efficient decrease of Bcl-xL protein expression upon exposure to 3 μmol/L of compound 2. The presence of Bcl-xL could sequester Bak in an inactive state, thereby preventing cytochrome c release and proper intrinsic route to caspase activation (34). We demonstrate that both 1 and 2 subsequently trigger the cleavage and activation of caspase-9 and thereafter also caspase-3 showing that a full apoptotic signaling cascade is active in these NSCLC tumor cells but not in normal diploid fibroblasts. At their IC50 and IC70 values, both compounds are equally efficient in causing of PARP and caspases cleavage, suggesting that they have similar potency of apoptosis induction. Altogether, our results demonstrate that both C. vasculum 1 and 2 induce cell death by activation of the intrinsic apoptotic pathway and this effect is tumor cell specific. One of the regulators of Bax and Bak activation is the BH3 only protein Bad that antagonize the antiapoptotic proteins Bcl-2 and Bcl-xL enabling Bak/Bax complex formation and cytochrome c release (36). Bad function as an integrator of multiple growth factor signaling cascades, including the PI3K/Akt and the Raf/MEK/ERK pathway, which antagonize Bad function by phosphorylation causing its binding and sequestration to 14-3-3 protein (37, 38). Akt inactivates Bad by phosphorylation at Ser136 (39) whereas MAPK signaling cascade inactivates Bad protein at Ser112 and Ser155 (40–43). With respect to 1 and 2, Bad phosphorylation at Ser112 was markedly inhibited, indicating a role for the MAPK signaling pathway whereas the basal level of phosphorylation of the other sites was weak in untreated cells making effect of treatment unsecure (data not shown). Given this altered phosphorylation of Bad Ser112 by compounds 1 and 2, we next examined how multiple growth and apoptosis-promoting kinases in NSCLC cells were affected. A decrease in ERK phosphorylation was observed, which is in line with the decrease in antiapoptotic activity. It is therefore likely that this decrease in ERK phosphorylation contributed to the observed apoptosis activity.
Bad protein phosphorylated at Ser112. Although results for inactivation of Bad protein at site Ser136 were inconclusive, a major impairment of phosphorylation of Akt at Ser473 and Thr308 upon treatment with these compounds was evident. Deactivation of Akt is reported to cause activation of proapoptotic proteins, cause alterations in mitochondrial membrane potential, release of cytochrome c, and caspase activation leading to activation of apoptosis (37, 43, 44). Hence, the impaired Akt phosphorylation upon treatment with these compounds fits well with observed apoptotic changes in these NSCLC cells.

We and others have linked NSCLC apoptotic response to a sustained JNK activation (25, 33, 45–48). In line with this and the observed apoptotic response, phosphorylation of JNK was increased by these C. vasculum–derived compounds. All in all, our data clearly demonstrate that 1 and 2 inhibit proliferation and induce apoptosis through regulation of the Ras/Raf/MAP kinase pathway as well as deactivation of Akt signaling in NSCLC cells. As both Akt and MAPK kinase signaling is controlled by growth factor receptors, these results may indicate that a common growth factor receptor instrumental in controlling their activity could indeed be a target of C. vasculum compounds. Further studies are, however, needed to reveal whether that is the case. We observed that Bak and Bax activation, cytochrome c release, downregulation of Bcl-xL, Bad, Akt, mTor, p43/p44MAPK (ERK1/2), JNK1/JNK2 phosphorylation alteration were delayed or less affected by 2 than by 1 in these NSCLC cells when used at equimolar concentrations. Yet given that 1 induces a higher toxicity than 2, one cannot rule out that this is the underlying effect. Therefore, at this point, we can only say that both compounds are capable of inducing apoptosis in tumor cells.

We show that 1 is also capable of enhancing cytotoxicity through induction of G2–M cell-cycle arrest in tumor cells. It has been shown that Akt regulates cell cycle by controlling of cyclins and CDK inhibitors (49–52). In addition, JNK is involved in cell-cycle progression and mediates G2–M phase cell-cycle arrest in different tumor cells (53–58). As both, decreased phosphorylation of Akt and increased phosphorylation of JNK are evident after treatment with 1, these signaling cascades may be responsible for the observed G2–M arrest upon treatment. Further studies are, however, required to uncover whether this is the case.

Albeit compounds 1 and 2 only show structural variance in the length of their carbon chains and the methyl substituent at position 14, a great difference in their potency is evident when it comes to induction of cytotoxicity in NSCLC, SCLC, and more importantly in ovarian carcinoma where 1 but not 2 shows tumor-specific cytotoxicity. The cytotoxic response by 1 was much more prominent in NSCLC and also appeared at an earlier time point. One possible reason for the less prominent effects observed in tumor cells, especially in ovarian carcinoma, by compound 2 could be lower affinity of drug to target a growth factor receptor upstream of these pathways. Yet another possibility is that their target upstream of these kinases are different and with the compound 2 targeting a signaling component, which play a less prominent role in ovarian carcinoma. Permeability of different cell types for the different compounds could also clearly have a

Figure 6. Schematic illustration of the proposed molecular mechanism of C. vasculum compounds in NSCLC cells. Experimental data from NSCLC cells support that compounds 1 and 2 indirectly or directly target growth factor receptors, thereby interfering with downstream proliferative signaling pathways Akt and MAPK, that is, decrease phosphorylation of ERK and Akt. Both compounds were found to deactivate Akt signaling leading to impaired Bad phosphorylation and activation of proapoptotic proteins Bak and Bax. Compounds were also found to trigger depolarization of mitochondria, release of cytochrome c, and activation of caspases. Compound 1 was also found to inhibit progression of cell cycle by arresting NSCLC cells in G2–M phase.
role. Hence, further research is required to put an answer to these issues.

In conclusion, our data reveal that 1 isolated from marine sponge C. vasculum causes tumor-specific cell death in NSCLC, SCLC, and ovarian carcinoma. For compound 2, we found such an activity to be evident in NSCLC and SCLC but not in ovarian carcinoma. Our findings are summarized in Fig. 6. We show that in NSCLC these compounds trigger a prominent response of the intrinsic apoptotic pathway (activation of Bak/Bax, decreased phosphorylation of Bad, depolarization of mitochondria, release of cytochrome c, and activation of caspases 9 and 3) and cell-cycle arrest in G2–M phase with a concomitant inhibition of both Akt and ERK/MAPK growth–promoting pathways. Moreover, we show that in NSCLC these compounds trigger a sustained JNK activation, which may contribute to the observed proapoptotic capacity. Taken together, our results, therefore, implicate that one or several growth factor receptors could be targets of 1 and 2 either directly or indirectly and in this way trigger cell death through the intrinsic apoptotic pathway and cell-cycle arrest. Further studies of the effects of compounds on growth factor receptors are, therefore, warranted to reveal mechanism of action, develop these compounds into suitable pharmacologic leads as well as to understand their role in treatment of NSCLC and SCLC but also other tumor malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
22. Srizer F, Zhivotovsky B, Nilsson A, Bergh J. Lewensohn R. Spon- taneous and radiation-induced apoptosis in lung carcinoma cells...
**Marine Sponge *Cribrachalina vasculum* Compounds Activate Intrinsic Apoptotic Signaling and Inhibit Growth Factor Signaling Cascades in Non–Small Cell Lung Carcinoma**

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