Eupalmerin acetate, a novel anticancer agent from Caribbean gorgonian octocorals, induces apoptosis in malignant glioma cells via the c-Jun NH₂-terminal kinase pathway

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Abstract

The marine ecosystem is a vast but largely untapped resource for potential naturally based medicines. We tested 15 compounds derived from organisms found in the Caribbean Sea (14 gorgonian octocoral–derived compounds and one sponge-derived compound) for their anticancer effects on human malignant glioma U87-MG and U373-MG cells. Eupalmerin acetate (EPA) was chosen as the lead compound based on its longer-term stability and greater cytotoxicity than those of other compounds we tested in these cell types. EPA induced G₂-M cell cycle arrest and apoptosis via the mitochondrial pathway; it translocated Bax from the cytoplasm to the mitochondria and dissipated the mitochondrial transmembrane potential in both cell types. EPA was found to increase phosphorylated c-Jun NH₂-terminal kinase (JNK) by > 50% in both U87-MG and U373-MG cells. A specific JNK inhibitor, SP600125, inhibited EPA-induced apoptosis, confirming the involvement of the JNK pathway in EPA-induced apoptotic cell death. Furthermore, 7 days of daily intratumoral injections of EPA significantly suppressed the growth of s.c. malignant glioma xenografts (P < 0.01, on day 19). These results indicate that EPA is therapeutically effective against malignant glioma cells in vitro and in vivo and that it, or a similar marine-based compound, may hold promise as a clinical anticancer agent. [Mol Cancer Ther 2007;6(1):184–92]

Introduction

More than 60% of anticancer drugs have originated from natural materials, such as plants, microbes, and marine organisms (1). Compared with totally synthetic drugs, compounds derived from natural sources tend to have well-defined three-dimensional structures, fitting biological target structures that are conserved across species; thus, natural compounds may act more specifically and have fewer or less severe adverse side effects than synthetics (2). Marine organism–derived medicines have several features that make them particularly suitable for consideration as sources of antineoplastic agents. For example, the vast majority of marine invertebrates have only primitive immune systems, and thus, they produce toxic substances as a form of defense; these substances would be expected to have high potency and low solubility, given that they are immediately and tremendously diluted by water (3). Therefore, an increasing number of compounds derived from sponges, algae, mollusks, and other marine organisms are being tested for their therapeutic effects against cancer and other diseases in clinical and preclinical trials (2–4).

Apoptosis is a cell death mechanism by which many chemotherapeutic drugs kill cancer cells (5). This process is characterized by chromatin condensation and fragmentation, cell shrinkage, and membrane blebbing. The Bcl-2 family, which consists of antiapoptotic and proapoptotic proteins, is the central regulator of apoptosis (6). When apoptosis is triggered, proapoptotic proteins, such as Bax and Bak, translocate from the cytoplasm to the mitochondrial membrane, where they interact with antiapoptotic proteins, such as Bcl-2 and Bcl-XL, causing a loss of mitochondrial transmembrane potential and the release of cytochrome c. The release of cytochrome c, in turn, activates caspases, which then cause the apoptosis to occur.

Serine/threonine protein kinases, including the mitogen-activated protein kinases (MAPK) and Akt, are upstream molecules that play an important role in regulating the apoptotic response of a cell to various extracellular stimuli (7). Three MAPK groups have been identified: c-Jun NH₂-terminal kinase (JNK), extracellular signal-regulated kinase, and p38 (8). In most instances, JNK and p38 induce apoptosis, whereas extracellular signal-regulated kinase and Akt inhibit it.
Malignant glioma is the most common primary malignancy in the brain. Despite of aggressive combination of surgery, radiotherapy, and chemotherapy, the average survival time is <1 year from the diagnosis (9). This dismal outcome prompted the development of new drugs for the treatment of patients with malignant glioma. In the present study, we screened 15 compounds derived from organisms collected from the Caribbean Sea (14 from Caribbean gorgonian octocorals and 1 from a marine sponge) for cytotoxicity in human malignant glioma U87-MG and U373-MG cells. The lead compound, eupalmerin acetate (EPA), was then analyzed for its mechanism of action and found to induce apoptosis in the malignant glioma cells through activation of the JNK pathway. Furthermore, treatment of s.c. xenografts derived from U87-MG cells with EPA injections significantly inhibited tumor growth compared with the control treatment (P < 0.01).

Materials and Methods

Cell Lines

The human malignant glioma cell lines U87-MG and U373-MG were purchased from the American Type Culture Collection (Manassas, VA). These cell lines have different p53 status: U87-MG has wild-type p53 and U373-MG has mutant p53 (10). Cells were cultured and maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 4 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂.

Marine Compounds

Specimens of the Caribbean gorgonian octocoral *Eunicea succinea* were collected at 25 m depth by SCUBA from Mona Island, Puerto Rico. The gorgonian was stored at 0°C immediately after collection and then frozen at −10°C on arrival, freeze dried, and kept frozen until extraction. The dried *E. succinea* (2.5 kg) was blended with MeOH-CHCl₃ (1:1), and after filtration, the crude extract was evaporated under vacuum to yield a residue (323 g) that was partitioned between hexane and H₂O. The hexane extract was concentrated to yield 171 g of a dark green oily residue, which was treated to yield 171 g of a dark green oily residue, which was

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Cell Proliferation Assay

Cells (1 × 10⁴ per well, 100 µL) were seeded in 96-well flat-bottomed plates and incubated overnight in 5% CO₂-95% air at 37°C. The cells were then exposed to various concentrations of the marine compounds (0–100 µmol/L) for 72 h, and relative rate of surviving cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay as described previously (14). Briefly, an aliquot of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (3 mg/mL in PBS) was added to each well (25 µL per 200 mL of medium), and the plate was incubated at 37°C for 2 h. Cells were then spun in a centrifuge at 300 × g for 5 min, and the medium was removed carefully. A 50-µL aliquot of DMSO was added, and the absorbance of each well at 570 nm was measured on a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The IC₅₀ was defined as the concentration that inhibited the viability of cells by 50% compared with that of control cells. In the assays using a specific JNK inhibitor, SP600125, cells were pretreated with 5 µmol/L SP600125 for 1 h and then treated with EPA for 3 days. SP600125 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Calbiochem (La Jolla, CA) and Sigma Chemical, respectively.

Flow Cytometry

For cell cycle analysis, cells were treated with EPA at 0, 5, 10, and 20 µmol/L for 72 h; fixed with ice-cold 70% ethanol; and then stained with propidium iodide using the Cellular DNA Flow Cytometric Analysis Reagent Set (Boehringer Mannheim, Indianapolis, IN). The stained cells were analyzed for DNA content using a FACScan (Becton Dickinson, San Jose, CA) as described previously (15). The percentages of cells in the sub-G₁, G₁, S, and G₂-M populations were determined with the CellQuest software program (Becton Dickinson).

For analysis of mitochondrial membrane potential, we used rhodamine 123 (Invitrogen), a cationic voltage-sensitive probe, as described previously (16). Cells were treated with 20 µmol/L EPA for 72 h, trypsinized, washed with PBS, and stained with 1 µmol/L rhodamine 123 in PBS for 1 h by exposure in the dark at 37°C. Cells were then washed, resuspended in PBS, and analyzed with the FACScan using CellQuest software.

Apoptosis Detection Assay

Tumor cells were seeded on Lab-Tek chamber slides (Nunc, Rochester, NY), incubated overnight, and then treated with 0 to 10 µmol/L EPA for 72 h and stained with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technique using an ApopTag apoptosis detection kit (Invitrogen) as described previously (17). Two hundred cells were counted and scored for the incidence of positive staining under a microscope.

Immunocytochemical and Immunohistochemical Staining

To detect Bax translocation to the mitochondria, treated cells were fixed with 4% paraformaldehyde for 15 min and then incubated with 50 nmol/L MitoTracker Green FM probe (Invitrogen) for 20 min at room temperature while
being shielded from the light. PBS was used to remove excess probe. The samples were then blocked with 3% normal goat serum and incubated with anti-Bax (N-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution in PBS containing 0.5% Triton X-100 (Sigma Chemical) overnight at 4°C. After a subsequent washing with PBS, the samples were visualized using Alexa Fluor 594–conjugated goat anti-rabbit IgG (Invitrogen).

For immunocytochemical and immunohistochemical staining, samples were fixed with 4% paraformaldehyde

Figure 1. Structures of 15 potential anticancer compounds derived from organisms found in the Caribbean Sea. Plakortide O was derived from a marine sponge, *P. halichondroides*, whereas the remainders were derived from gorgonian soft corals (subclass Octocorallia).
for 15 min, washed with PBS, blocked with 3% normal goat serum, and incubated with anti–phosphorylated stress-activated protein kinase/JNK antibody (for phosphorylation at Thr183/Tyr185; Cell Signaling, Danvers, MA) or anti-Ki-67 antibody (Abcam, Cambridge, MA) in PBS overnight at 4°C. A secondary biotinylated antibody was then applied followed by incubation with avidin-biotin complex method solution (Vector Laboratories, Burlingame, CA). Samples were visualized by means of a standard diaminobenzidine reaction. The number of phosphorylated JNK-positive and Ki-67-positive cells per 100 cells was determined in each of three or more areas, which were chosen at random for each sample.

Immunoblotting

Cells were lysed in extraction buffer as described previously (18). Thereafter, for each immunoblot preparation, 40 μg protein was separated by SDS-PAGE (Bio-Rad, Richmond, CA) and transferred to a Hybond-P membrane (Amersham Co., Piscataway, NJ). The membranes were treated with antibodies against poly(ADP-ribose) polymerase (Cell Signaling), Bax, and Bcl-2 (Santa Cruz Biotechnology) and then subjected to immunoblotting. To detect upstream signaling for apoptosis, we also did immunoblotting using PathScan Multiplex Western Cocktail kits I, II, and III (Cell Signaling). Anti-Pin1 and anti-eIF4E antibodies were used as loading controls.

Animal Studies

All animal studies were done in the veterinary facilities of The University of Texas M. D. Anderson Cancer Center in accordance with institutional, state, and federal regulations and institutional and international ethical guidelines for the care and use of experimental animals. U87-MG cells (1.0 × 10^6 in 20 μL of serum-free DMEM) were inoculated s.c. into the right flank of 5- to 10-week-old female nude mice (six mice for each treatment group). Tumor growth was measured daily with calipers. Tumor volume was calculated as (L × W^2) / 2, where L is the length in millimeters and W is the width in millimeters, as described previously (19). When the tumors reached a mean volume of 50 mm^3, intratumoral injections of EPA (50 mg/kg in 40 μL of DMSO and PBS) or injections of 40 μL of DMSO and PBS alone (vehicle) were given daily for 7 days. Tumor volume was measured every other day with calipers. Mice were euthanized by exposure to CO2 when the tumor volume reached ≥1,500 mm^3 (for mice in the control group) or on day 19 (for mice in the EPA group). Tumors were removed, snap frozen, and kept at −80°C until use. Tumor specimens were sliced to a thickness of 10 to 20 μm with a cryostat, mounted on glass slides, fixed with 4% paraformaldehyde in PBS, and processed for immunohistochemical and TUNEL staining.

Statistical Analysis

The data were calculated as mean ± SD. Statistical analysis was done using the Student’s t test (two tailed). Statistical significance was defined as P < 0.05.

Results

EPA Was Chosen for Its Potent Anticancer Effect and Stability

The 15 marine compounds isolated from organisms found in the Caribbean Sea and purified in our laboratory are shown in Fig. 1. These compounds were preselected based on their anticancer activity or on the anticancer activity of their derivative that had been assessed previously (20–22).

Table 1. IC_{50} values of the top eight marine compounds studied

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plakortide O</td>
<td>4.0</td>
</tr>
<tr>
<td>EPA</td>
<td>5.1</td>
</tr>
<tr>
<td>Isoeupalmerin acetate</td>
<td>6.7</td>
</tr>
<tr>
<td>3'-O-acetyl-pseudopterosin U</td>
<td>14.0</td>
</tr>
<tr>
<td>3-Epi-14-deoxyxcrassin</td>
<td>25.8</td>
</tr>
<tr>
<td>Asperdicin</td>
<td>26.3</td>
</tr>
<tr>
<td>Pseudoplexauric acid methyl ester</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2-Deoxyasperdiol acetate</td>
<td>57.5</td>
</tr>
</tbody>
</table>

Figure 2. EPA induces G_{2/M} arrest and apoptosis in U87-MG and U373-MG cells. A, cell cycle analysis. Cells were treated with different concentrations of EPA for 72 h, incubated with propidium iodide, and subjected to flow cytometric cell cycle analysis. Percentage of the sub-G_1 population at each EPA concentration. Data are representative of three independent experiments. B, quantification of TUNEL-positive cells. Cells were treated as described above and subjected to the TUNEL assay. Columns, mean of triplicate experiments; bars, SD.
All the compounds but one were derived from gorgonian octocorals; plakortide O was derived from a marine sponge, *Plakortis halichondroides* (21, 22). All these compounds were coded and examined blindly for their relative cytotoxicity against human malignant glioma U87-MG and U373-MG cells. Cells were treated with each compound at 0 to 100 μmol/L for 72 h, and viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Table 1 shows the IC₅₀ values of the eight most potent compounds in descending order of potency. The other seven compounds had IC₅₀ values of >100 μmol/L in both cell types and were therefore considered not to be potent enough to serve as anticancer drugs for these cell types. Although plakortide O was the most potent of the tested compounds, its cytotoxicity significantly decreased after it was stored in solution for several weeks (IC₅₀ value decreased from 4.0 to 31.0 μmol/L in U87-MG cells and from 4.0 to 15.4 μmol/L in U373-MG cells). The second most potent compound, EPA, was stable at least over several months, and it had IC₅₀ values of 5.1 μmol/L for U87-MG cells and 6.9 μmol/L for U373-MG cells. This range of IC₅₀ is compatible with that of cisplatin, which is in the range of 5 to 10 μmol/L for these cell types (data not shown). These results prompted us to focus on EPA and determine the mechanisms by which it asserted its anticancer effect.

**EPA Translocates Bax to the Mitochondria and Induces Apoptosis in U87-MG and U373-MG Cells**

To examine the effect of EPA on cell cycle and apoptosis, we treated cells with different concentrations of EPA for 72 h and did cell cycle analysis using flow cytometry (Fig. 2A). The percentage of cells in the sub-G₁ population, which indicates the level of cell death, increased from 0.7% at baseline to 35.6% and 91.4% in U87-MG cells treated with 10 and 20 μmol/L EPA, respectively. Similarly, the sub-G₁ population increased from 2.1% to 57.2% in U373-MG cells treated with 20 μmol/L EPA. For both cell lines, the percentages of cells in the G₁ population decreased and that of cells in the G₂-M phase increased in a dose-dependent manner after treatment with up to 10 μmol/L EPA (Fig. 2A), indicating that EPA also induced G₂-M arrest. Because apoptosis is a cell death mechanism by which many chemotherapeutic drugs kill cancer cells (5), we did TUNEL staining to examine whether EPA induces apoptosis in these cells (Fig. 2B). At 72 h after treatment with 10 μmol/L EPA, the percentage of TUNEL-positive cells increased from 1.3 ± 0.6% to 10.4 ± 1.9% in U87-MG cells and from 0.8 ± 0.3% to 6.7 ± 1.5% in U373-MG cells (P < 0.05 in both cell types). These results indicate that EPA induces apoptosis and G₂-M arrest in both cell types.

Bcl-2 family proteins regulate apoptosis by controlling the integrity of the mitochondrial membrane (6). To determine whether EPA induces apoptosis by loss of the integrity of the mitochondrial membrane through Bcl-2...
family proteins, we measured the mitochondrial membrane potential using rhodamine 123 staining and did immunoblotting for poly(ADP-ribose) polymerase, Bcl-2, and Bax. EPA increased the proportion of cells with mitochondrial transmembrane potential dissipation to 86.9 ± 0.4% in U87-MG cells and to 70.1 ± 2.4% in U373-MG cells (Fig. 3A). Poly(ADP-ribose) polymerase was cleaved effectively at 24 h after EPA treatment in both cell types (Fig. 3B). The expression of Bcl-2 decreased after 48 h of EPA treatment in U373-MG cells, although it did not change in U87-MG cells (Fig. 3B). The expression level of Bax did not change during 48 h of EPA treatment in either cell type (Fig. 3B).

Although Bax remains inactivated, it localizes diffusely in the cytoplasm. Once apoptosis is induced, Bax translocates to the mitochondria, neutralizes antiapoptotic protein Bcl-2, changes its own conformation, and opens the mitochondrial pore, resulting in apoptosis (23). To determine whether Bax plays a role in EPA-induced apoptosis, we did double staining using an anti-Bax antibody and a mitochondrial marker (MitoTracker) in U87-MG and U373-MG cells. Twenty-four hours after EPA treatment, immunoreactivity to Bax showed punctate pattern and was colocalized with MitoTracker dye in both cell types (Fig. 4A). Quantification of double-stained cells showed that the percentage of the cells with Bax translocation to the mitochondria increased significantly from 4.3% to 11.3% in U87-MG cells and from 2.5% to 10.2% in U373-MG cells (P < 0.01 for both; Fig. 4B). These results indicate that EPA induces apoptosis in both cell types at least in part by activating the mitochondrial pathway and inducing Bax translocation to the mitochondria.

EPA Activates the JNK Pathway to Induce Apoptosis in U87-MG and U373-MG Cells

To determine which upstream signal pathways were activated or inhibited in EPA-induced apoptosis, we used PathScan Multiplex Western Cocktail kits. The following pathways were examined: the Ras/Raf/MAPK/extracellular signal-regulated kinase/extracellular signal-regulated kinase/p90RSK pathway, the phosphatidylinositol 3-kinase/
Eupalmerin Acetate Induces Apoptosis in Glioma

The level of phosphorylated JNK increased by 50% after 1 h of EPA treatment in U87-MG cells and by 130% after 2 h of EPA treatment in U373-MG cells (Fig. 5). Levels of phosphorylated Akt and phosphorylated S6RP, the latter of which is a downstream target of Akt, increased in U87-MG cells by 20% after 1 to 2 h of EPA treatment in U87-MG cells; in U373-MG cells, the level of phosphorylated Akt did not increase but that of phosphorylated S6RP did (by no more than 15%) after 1 to 2 h of EPA treatment (Fig. 5). The level of phosphorylated p90 S6RP decreased by 35% after 5 h of EPA treatment in only U373-MG cells (Fig. 5). Collectively, these results indicate that EPA activates the JNK pathway in both U87-MG and U373-MG cells, suggesting that EPA induces apoptosis via the JNK pathway.

To confirm the involvement of the JNK signaling pathway in EPA-induced apoptosis, we first used immunocytochemical staining to determine whether phosphorylated JNK was present in the cell nuclei. Two hours after treatment with EPA, some malignant glioma cells showed enhanced immunoreactivity to anti–phosphorylated JNK antibody in the nucleus; the cells also had begun to take on a rounded shape, which is characteristic of apoptosis (Fig. 6A). The percentage of phosphorylated JNK-positive cells increased significantly from 1.9% to 20.6% in U87-MG cells and from 2.2% to 13.6% in U373-MG cells after EPA treatment (P < 0.01 for both cell types; Fig. 6B). Further, the effect of a JNK-specific inhibitor, SP600125, on cell cycle and apoptosis was examined. Pretreatment of U87-MG and U373-MG cells with SP600125 did not inhibit EPA-induced G2/M arrest (Fig. 6C). On the other hand, pretreatment with SP600125 significantly inhibited EPA-induced apoptosis from 10.3% to 4.3% in U87-MG cells and from 6.7% to 3.3% in U373-MG cells (P < 0.05 for both cell types; Fig. 6D). These results indicate that EPA induces apoptosis, at least in part, by activating the JNK pathway.

**EPA Inhibits Tumor Growth in the S.c. Tumor Model**

To determine whether the in vitro antitumor effect of EPA is recapitulated in vivo, we treated nude mice bearing s.c. tumors with EPA. When s.c. xenografts derived from U87-MG cells reached a mean volume of 50 mm³ (defined as day 1), we injected EPA (50 mg/kg, dissolved in DMSO and PBS) or control vehicle (DMSO and PBS alone) directly into the tumors twice a day for 7 days and measured tumor size every other day until day 19. Mice treated with EPA had tumors ~80% smaller than those in mice treated with vehicle control on day 19 (mean volume: 2,090 ± 842 mm³ in control mice and 492 ± 424 mm³ in EPA-treated mice; P < 0.01; Fig. 7A). Tumors were removed on day 19 and assessed by TUNEL staining and immunohistochemical staining for phosphorylated JNK and the proliferation marker Ki-67. Some TUNEL-positive cells were detected in the tumors treated with EPA, but few were found in those treated with vehicle (Fig. 7B); phosphorylated JNK was not detected in tumors of either treatment group (data not shown). These results suggest that the majority of apoptotic cells were removed by phagocytes by day 19 and that EPA-induced JNK activation was transient, which was consistent with the results of immunoblotting analysis using PathScan (Fig. 5A). The expression of Ki-67 was strikingly inhibited in tumors treated with EPA relative to those treated with vehicle (Fig. 7B); only 1.5% of treated cells stained positive for Ki-67, whereas 19.9% of control cells did so (P < 0.01; Fig. 7C). These results clearly indicate that EPA can inhibit the growth of malignant glioma cells in vivo.

**Discussion**

We have collected a large number of samples from organisms collected throughout the Caribbean Sea over the last 15 years and have isolated, purified, and tested >200 compounds for their anticancer and anti-inflammatory effects (21, 22, 24, 25). In the present study, EPA showed the best combination of cytotoxicity and stability among 15 such compounds we screened. In vivo, EPA induced...
apoptosis effectively by activating a mitochondrial pathway (specifically the JNK pathway) in both U87-MG and U373-MG cells regardless of their p53 status. In vivo, the growth of s.c. xenograft tumors was remarkably inhibited by intratumoral injections of EPA, indicating that the agent also holds potential for clinical anticancer activity. Further work must, of course, involve development of a suitable formulation for either systemic administration or convection-enhanced delivery of the compound to animals and/or humans as well as appropriate toxicology tests. Convection-enhanced delivery is a recently developed technique of direct injection to increase drug uptake and distribution to large regions of the brain by applying a pressure gradient (26–28). With convection-enhanced delivery, chemotherapeutic and toxic materials can be delivered to brain tumors efficiently and homogeneously while limiting toxicity to surrounding areas and the whole body system. Our initial results as a pilot study, however, suggest that natural compounds derived from marine organisms, including EPA, are promising candidates for consideration as anticancer drugs.

JNK is a serine-threonine kinase and a member of the MAPK family. It modulates apoptotic pathways in response to a variety of extracellular stimuli (8). JNK induces apoptosis by means of several mechanisms, including the activation of transcription factors and through cytoplasmic substrates. For example, JNK activates the downstream transcription factor c-Jun, which increases transcription of Fas ligand, resulting in the induction of apoptosis (29). c-Jun is also implicated in the up-regulation of a proapoptotic member of the Bcl-2 family, Bim, which leads to apoptosis (30). JNK also directly phosphorylates Bim-related proteins, such as Bim and Bmf, which activate Bax and Bak, resulting in apoptosis (31). Further, JNK also phosphorylates 14-3-3 proteins and promotes Bax translocation to the mitochondria (32). Our results show that EPA mediates induction of apoptosis through activation of JNK mainly in the nucleus (Figs. 5 and 6A), suggesting the involvement of c-Jun activation.

Our immunoblot analysis revealed that the EPA-induced phosphorylation of JNK was transient in both U87-MG and U373-MG cells (Fig. 5). In U87-MG cells, the level of phosphorylated JNK after EPA treatment returned to the level in untreated cells by 5 h after the treatment. Indeed, no tumor cells expressed detectable phosphorylated JNK in EPA-treated s.c. tumors. Although some apoptotic cells were detected in the tumors treated with EPA, the incidence was very low. Instead, we found that the expression of Ki-67 was dramatically suppressed in treated tumors on day 19 (Fig. 7B). These results suggest the possibility that proliferating tumor cells had undergone apoptosis and had been removed during a relatively early period of EPA treatment and that only tumor cells that escaped apoptosis and had a low proliferation rate remained, indicating the potential for EPA to have a long-lasting antitumor effect in vivo.

In summary, we tested 15 compounds derived from Caribbean marine organisms, mainly from gorgonian soft corals, for their anticancer effects on U87-MG and U373-MG malignant glioma cells. Our lead compound, EPA, showed high potency in killing these cells: EPA induced apoptosis by translocating Bax to the mitochondria and depolarizing the mitochondrial membrane. Our analysis of upstream signaling revealed that EPA activates the JNK pathway in its induction of apoptosis; treatment with a JNK inhibitor was able to inhibit the cytotoxicity of EPA. Furthermore, EPA significantly inhibited the growth of s.c. xenograft tumors. Our results suggest that EPA may be a promising candidate for use as an anticancer drug.

Acknowledgments
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15. Kanzawa T, Iwado E, Aoki H, et al. Ionizing radiation induces apoptosis and inhibits neuronal differentiation in rat neural stem cells via the cell death factor BNIP3 in ceramide-induced autophagic response to a variety of extracellular stimuli (8). JNK mediates induction of apoptosis through activation of JNK mainly in the nucleus (Figs. 5 and 6A), suggesting the involvement of c-Jun activation.

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