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Rottlerin stimulates apoptosis in pancreatic cancer cells through interactions with proteins of the Bcl-2 family

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Rottlerin and the Bcl-2/Bcl-xL inhibitor, BH3I-2, activate a common signaling pathway in PaCa cells by preventing sequestration of BH3-only proteins by Bcl-xL. However, the effects of rottlerin and BH3I-2’ on the complex formation between Bcl-xL and BH3-only proteins are different. BH3I-2’ disrupts complexes of Bcl-xL with Bad but not with Bim or Puma, whereas rottlerin had no effect on the Bcl-xL interaction with Bad. Also BH3I-2’, but not rottlerin, required Bad to stimulate apoptosis. In conclusion, our results demonstrate that rottlerin has a potent proapoptotic and antitumor activity in pancreatic cancer, which is mediated by disrupting the interaction between prosurvival Bcl-2 proteins and proapoptotic BH3-only proteins. Thus rottlerin represents a promising novel agent for pancreatic cancer treatment.

pancreatic adenocarcinoma is an aggressive malignancy and is resistant to chemo- and radiotherapy (7, 38). One mechanism mediating pancreatic cancer aggressiveness and unresponsiveness to treatment is its resistance to apoptosis. Finding approaches to stimulate the death-signaling mechanisms is critical for the development of effective therapeutic strategies in pancreatic cancer.

Rottlerin is a polyphenolic compound derived from Mallotus philipinensis (Euphorbiaceae) (15). Rottlerin is widely used as an inhibitor of the δ-isosform of protein kinase C (15). Another cellular target of rottlerin is mitochondria. Rottlerin was shown to cause uncoupling of mitochondrial respiration from oxidative phosphorylation and a collapse of mitochondrial membrane potential (ΔΨm) in several cell types (8). Recently, rottlerin was shown to stimulate apoptosis in various cancer cells, including colon (40) and lung (6) cancer cells, chronic leukemia, and multiple myeloma cells (31, 34). The mechanisms whereby rottlerin stimulates apoptosis have not yet been elucidated.

The key signaling event in apoptosis pathway is the release of mitochondrial cytochrome c into the cytosol, which is usually accompanied by a decrease in ΔΨm. Once in the cytosol, cytochrome c activates caspases, leading to apoptosis. Proteins of the Bcl-2 family are major regulators of cytochrome c release. On the basis of their function and structure, Bcl-2 proteins can be divided into three groups, namely proapoptotic proteins containing three homologous BH domains (BH1–BH3), proapoptotic proteins containing BH3 domain only, and prosurvival proteins containing four BH domains (BH1–BH4). Proapoptotic multidomain Bax and Bak form channels in the outer mitochondrial membrane through which mitochondrial cytochrome c is released into the cytosol (25, 29). BH3-only proteins, such as Bad, Bim, and Puma, activate Bax/Bak channels (1, 24, 46). Oppositely, prosurvival Bcl-2 proteins, such as Bcl-xL and Bcl-2, bind to and sequester Bad, Bim, and Puma, preventing them from activating Bax/Bak channels (1, 24, 28, 46).

During the past several years, small-molecule pharmacological inhibitors of prosurvival Bcl-2/Bcl-xL have been developed and shown to stimulate apoptosis in cancer cells, demonstrating prosurvival Bcl-2 proteins as promising therapeutic targets in cancer treatment. At the same time, there is a growing interest in testing the therapeutic potential and studying the mechanisms of action of natural phytochemical compounds as anticancer drugs (3, 21, 27).

The present study shows that rottlerin inhibits tumor growth in orthotopic models of pancreatic cancer and stimulates apoptosis both in vivo in the pancreatic tumor and in vitro in pancreatic cancer (PaCa) cells. BH3-only proteins Bim and Puma are required for rottlerin to stimulate apoptosis. Rottlerin prevents sequestration of Bim and Puma by Bcl-xL, resulting in cytochrome c release, loss of ΔΨm, and PaCa cell death. Rottlerin and the Bcl-2/Bcl-xL inhibitor, BH3I-2’, activate a common signaling pathway in PaCa cells by preventing se-
questration of BH3-only proteins by Bcl-xL, thus stimulating apoptosis.

MATERIALS AND METHODS

Materials. Antibodies against caspase-3 caspase-9, PKC-ε, and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Bcl-xL, Puma, Bim, and Bad, total Akt, and phosphoTyr70S-Akt antibodies were from Cell Signaling Technology (Denver, MA); anti-cytochrome c antibody was from BD Pharmingen (San Diego, CA); anti-cytochrome c oxidase subunit IV antibody was from Molecular Probes (Carlsbad, CA); rottlerin was from Sigma (St. Louis, MO); BH3I-2 and PKC-ε peptide substrate were from CalBiochem (San Diego, CA). PKC assay kit was purchased from Upstate Biotechnology (Lake Placid, NY). All other reagents were purchased from Sigma. PKC inhibitors GF-109203X and Ro-32-0432 were from CalBiochem. A specific PKC-ε translocation inhibitor (δV1-1: S-F-N-S-Y-E-L-G-S-L) was synthesized as we described previously (35).

Pancreatic cancer animal models. Animal studies were approved by the Chancellor’s Animal Research Committee of the University of California, Los Angeles in accordance with the NIH Guide for the Care and Use of Laboratory Animals. One model used was the subcutaneous model. Pancreatic cancer cells (MIA PaCa-2, 2 x 10⁵) were subcutaneously injected into the flanks of nude mice. After the tumor reached a size of ∼2 x 2 mm, animals were randomly allocated to receive either control vehicle (n = 6) or rottlerin (0.5 mg/kg, n = 6). Drugs were prepared fresh each day and injected intraperitoneally in a total volume of 50 μl for 2 wk. The other model used was the orthotopic model. Pancreatic tumors grown subcutaneously were harvested, and tumor pieces (1 mm³) were transplanted into the tail of the pancreas of recipient nude mice (20). Animals were randomized and treated with either daily intraperitoneal injections of rottlerin (0.5 mg/kg, n = 8) or control vehicle (n = 4) for 4 wk. At euthanasia, pancreatic tumors, liver, spleen, lungs, and kidney were harvested. Tumor volume was assessed as described earlier (12). For further analyses, the tumor was either fixed in formalin and embedded in paraffin or frozen in 2-methyl-butane/dry ice and embedded in optimal cutting temperature compound.

Cell culture. Human pancreatic adenocarcinoma cell lines, the poorly differentiated MIA PaCa-2, and moderately differentiated PANC-1 were obtained from the American Type Culture Collection (Manassas, VA). MIA PaCa-2 and PANC-1 cells were grown in 1:1 DMEM-F-12 medium from Gibco Invitrogen (Grand Island, NY) supplemented with 15% FBS, 4 mM l-glutamine, and antibiotic/antimycotic solution from Omega Scientific (Tarzana, CA). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and were used between passages 4 and J2. For analyses, cells were plated at a density of 5 x 10⁵/ml in either 100-mm culture dishes or 150 cm², and cells were cultured for up to 48 h.

Transfection. Puma, Bim, Bad, and negative control siRNAs were obtained from Dharmacon (Lafayette, CO). PaCa cells were transiently transfected using Nucleofector system from Amaxa Biosystems (Cologne, Germany). The 2 x 10⁵ cells were resuspended in corresponding Nucleofector solution. siRNA and cells were mixed in the electroporation Amaza cuvette, and transfection was performed with the electrical setting T20. After nucleofection the cells were immediately transferred into prewarmed medium and cultured at 37°C, 5% CO₂.

Measurement of apoptosis in cells. Internucleosomal DNA fragmentation was measured by using Cell Death Detection ELISA™ kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Phosphatidylinerine externalization was analyzed with the annexin-V (AnV)-FLUOS Staining Kit from Roche Biochemicals (Indianapolis, IN) as we described before (9, 41, 42). Caspase-3, -9, and -8 activities were measured as we described previously (9, 41, 42) using a fluorogenic assay with the substrates specific for caspase-3 [Ac-DEVD-aminoo-4-methylcoumarin (AMC)], caspase-9 (Ac-LEHD-AMC), and caspase-8 (Ac-IETD-AMC). The data were expressed as moles of AMC/mg of protein/min and normalized to the control.

Cell fractionation for the measurement of cytochrome c release. Cells were resuspended in a lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1 mM Na-EGTA, 1 mM Na-EDTA, 2 mM MgCl₂, pH 7.0); disrupted by 80 strokes in a Dounce homogenizer, and centrifuged at 1,000 g to pellet nuclei and cell debris. Supernatants were further centrifuged at 13,000 g for 30 min, and the cytosolic fractions (supernatants) and the pellets (membrane fractions enriched with mitochondria) were collected. To validate the quality of cytosolic and mitochondrial separation, both fractions were assessed by immunoblotting for the mitochondrial marker cytochrome c oxidase subunit IV.

Total cell lysates. Cells were resuspended in RIPA phosphorylation buffer (50 mM NaCl, 50 mM Tris•HCl pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 10 mM Na₂HPO₄ + NaH₂PO₄, pH 7.0, 100 mM NaF, 2 mM Na₃VO₄, 20% glycerol, 80 μM glycophosphate, 1 mM PMSF, 5 μg/ml of pepstatin, leupeptin, chymostatin, antipain, and aprotinin), sonicated, and centrifuged for 15 min at 15,000 g at 4°C.

Immunoprecipitation. Cells were collected, washed twice in a buffer containing 20 mM Tris (pH 7.5) and 10 mM DTT, then resuspended in a lysis buffer (50 mM Tris•HCl, 150 mM NaCl, 2 mM EGTA, 10 μg/ml each leupeptin and aprotinin, 1 mM PMSF, 1% NP-40), and sonicated for 30 s. Lysates were clarified by centrifugation, and 500 μg of protein was subjected to overnight immunoprecipitation with either Bcl-xL or Bcl-2 antibody at 4°C using Catch and Release Reversible Immunoprecipitation System from Millipore (Billerica, MA).

Western blot analysis. Proteins from total cell lysate or immunoprecipitates were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked with 5% bovine serum albumin or nonfat dry milk in Tris-buffered saline (4 mM Tris base, 100 mM NaCl, pH 7.5) containing 0.05% Tween 20 for 1 h. Membranes were incubated with primary antibody for overnight at 4°C and then for 1 h with peroxidase-conjugated secondary antibody. The blots were developed with Supersignal Chemiluminescent Substrate from Pierce (Rockford, IL).

Measurements of ΔΨm in intact PaCa cells. Changes in ΔΨm were detected with the potential-sensitive probe MitoTracker Red (CMX-Ros) from Invitrogen (Carlsbad, CA) as we described previously (41). During the last 30 min of the incubation period, cells were loaded with 10 nM CMX-Ros for 30 min at 37°C in the dark, washed twice with phosphate-buffered saline, and analyzed by flow cytometry using the FACSscan with FL-3 detector. To completely dissipate ΔΨm, cells were treated with the uncoupling agent CCCP (50 μM) for 1 h before CMX-Ros staining.

Measurements of ΔΨm and cytochrome c release in PaCa cells permeobilized with digitonin. For permeobilization, cells were resuspended in the medium that mimics ionic composition of cytosol. It contained 250 mM sucrose, 22 mM KCl, 11 mM triethanolamine, 3 mM MgCl₂, 5 mM KH₂PO₄ (pH 7.0), 0.5 mM EGTA, and 10 mM succinate incubated for 3 min with 1.25 x 10⁻³ M digitonin as described (24, 26). Efficiency of permeabilization was confirmed by positive Trypan blue staining in greater than 90% of the cells. Changes in ΔΨm were measured with a ΔΨm-sensitive tetraphenyl phosphonium (TPP⁺)-selective electrode (23). An increase in ΔΨm causes TPP⁺ uptake by mitochondria and, correspondingly, a decrease in external TPP⁺ measured by the electrode. Cytochrome c levels in soluble and membrane fractions were measured with West-
ern blot. Cytochrome c release and ΔΨm were measured in the same preparations of permeabilized cells.

Fluorescence polarization assay. Complex formation between a fluorescein-labeled peptide [NLWAAQRYGERLRRMSDK(flourescein)FVD] of BH3 domain of Bad (synthesized by CURE Research Center Peptide CORE) and the recombinant Bcl-XL fragment lacking the flexible loop and the carboxyterminal hydrophobic region (Calbiochem) was performed as described in Ref. 48. Briefly, Bcl-XL was mixed with 15 nM fluorescently labeled Bad in a 96-well plate with and without rottlerin, BH3I-2’, or vehicle in a final volume of 50 μl, and fluorescence polarization was measured using Analyst 96-well plate reader (LJL; Molecular Devices, Sunnyvale, CA).

Measurements of PKC-δ activity. Measurements of PKC-δ activity were performed as we described in Ref. 35. Briefly, cells were homogenized in ice-cold homogenization buffer containing (in mM) 130 NaCl, 50 Tris-HCl (pH 7.5), 5 EGTA, 5 EDTA, 1.5 MgCl₂, 10 NaF, 1 Na₃VO₄, 10 Na₂P₂O₇, 1 PMSF, and 10% (vol/vol) glycerol plus 5 μg/ml each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin, sonicated five times for 10 s on ice, and incubated for 45 min at 4°C. PKC-δ was immunoprecipitated using specific antibody against PKC-δ isoform (1:100 dilution). The beads were washed and resuspended in the kinase buffer (in mM) 20 MOPS (pH 7.2), 25 β-glycerophosphate, 5 EGTA, 1 Na₃VO₄, and 1 DTT. The kinase assay was performed by using the PKC assay kit (Upstate Biotechnology).

Statistical analysis. For statistical analysis, the results were from at least three independent experiments. Differences between two groups were analyzed using Student’s t-test. P < 0.05 was considered statistically significant.

RESULTS

Rottlerin inhibited tumor growth and stimulated apoptosis in pancreatic cancer animal models. To determine the effects of rottlerin on the growth of xenografted pancreatic cancers we used a subcutaneous and orthotopic pancreatic cancer model in nude mice as described before (11, 12, 20). Mice received either 0.5 mg/kg of rottlerin or vehicle (DMSO) daily for 2 (subcutaneous model) or 4 wk (orthotopic model). There was no difference in body weight throughout the study period between both experimental groups (not shown). In both models, rottlerin significantly inhibited tumor growth by 60–70% (Fig. 1, A and B). The decrease in tumor growth was associated with an increase in apoptotic cell death in PaCa cells. In the orthotopic model, treatment of mice with rottlerin increased apoptosis in PaCa cells fourfold compared with control animals (Fig. 1C), suggesting that rottlerin inhibits pancreatic cancer growth by induction of apoptosis. Importantly, normal extra- tumoral tissues, e.g., liver and kidney, were histologically normal; there was no increase in apoptosis or necrosis in these organs (not shown).

Rottlerin stimulated PaCa cell death. Rottlerin at micromolar concentrations dose dependently stimulated apoptosis in PaCa cells (Fig. 2). Increase in DNA fragmentation in both MIA PaCa-2 and PANC-1 cells was already significant at 1.3 μM and reached maximum at 2.5 μM rottlerin (Fig. 2A). Of note, application of 2.5 μM rottlerin in vitro is approximately equivalent to the in vivo application of 0.5 mg/kg. Stimulation of apoptosis by rottlerin increased with the time and reached plateau at 24-h culturing with rottlerin.

To assess the differential effects of rottlerin on apoptosis and necrosis, we stained MIA PaCa-2 cells with AnV and propidium iodide (PI). As we showed before (41), AnV+/PI− group represents cells with early apoptosis; cells stained with PI only (AnV−/PI+) are early necrotic ones, whereas AnV+/PI+ group includes cells with primary necrosis and also cells with apoptosis associated with secondary necrosis (28a, 41). Rottlerin increased the number of cells in each group up to

Fig. 1. Rottlerin inhibited pancreatic cancer growth in vivo. The effects of rottlerin on pancreatic cancer growth were measured in a subcutaneous (A) and orthotopic (B) xenograft model in nude mice. Animals received either rottlerin (0.5 mg/kg) or vehicle by daily intraperitoneal injections. The tumor volumes of rottlerin- and vehicle- treated animals were determined after 2 wk in the subcutaneous (A) and after 4 wk in the orthotopic model (B). C: percentage of apoptotic cells in tissues sections was determined in cryostat sections fixed in 4% paraformaldehyde using terminal deoxynucleotidyl transferase-mediated nick end labeling assay. The number of apoptotic cells was determined in relation to the total number of cells. The values in A–C represent means ± SE (at least 5 animals in each group). *P < 0.05, **P < 0.02, ***P < 0.0005 vs. the same parameter in tumors from mice treated without rottlerin.
twofold and as a result decreased the number of live (AnV⁻/PI⁻) cells (Fig. 2B). These data indicate that rottlerin stimulated not only apoptotic but also necrotic death of PaCa cells.

We compared the proapoptotic effects of rottlerin with those induced by BH3I-2', an inhibitor of Bcl-2 and Bcl-xL. BH3I-2' is a mimetic of BH3 domain; it binds to the hydrophobic pocket of Bcl-2 and Bcl-xl, preventing their interaction with BH3-only proteins (13, 18). BH3I-2' also dose dependently stimulated cytochrome c release in both MIA PaCa-2 and PANC-1 cells (Fig. 2C). The effects of 25 μM of BH3I-2' and 2.5 μM of rottlerin on apoptosis were nonadditive, suggesting a common underlying mechanism (Fig. 2D).

**Rottlerin stimulated mitochondrial pathway of apoptosis in PaCa cells.** Rottlerin dose and time dependently stimulated cytochrome c release from mitochondria into cytosol in both MIA PaCa-2 and PANC-1 cells (Fig. 3, A and B). Cytochrome c release increased with the concentration of rottlerin in the range of 2.5 to 10 μM. In MIA PaCa-2 cells, the induction of cytochrome c release was evident as early as at 15 min and continued to increase for up to 48 h (Fig. 3, A and B). BH3I-2' also dose dependently stimulated cytochrome c release in MIA PaCa cells (Fig. 3C).

We next examined the direct effect of rottlerin and BH3I-2' on mitochondria using MIA PaCa-2 cells permeabilized with digitonin (Fig. 3D). When added to permeabilized cells both rottlerin and BH3I-2' dose-dependently stimulated cytochrome c release (Fig. 3D), suggesting that both these agents stimulate cytochrome c release by directly affecting mitochondria.

Cytochrome c release in apoptosis is usually associated with mitochondrial depolarization (14). Rottlerin dose dependently decreased ΔΨm in MIA PaCa-2 and PANC-1 cells (Fig. 4A). Depolarization was first observed at 15 min of rottlerin treatment. Importantly, low concentrations of rottlerin (2.5 μM) transiently depolarized mitochondria, whereas higher concent-
trations (5 and 10 μM) caused permanent loss of ΔΨm (Fig. 4A). The reason for transient depolarization remains to be determined. However, the differences in the time dependencies of rottlerin-induced cytochrome c release and depolarization suggest that cytochrome c release and depolarization are mediated through different mitochondrial permeability systems. BH3I-2 also dose dependently stimulated mitochondrial depolarization of MIA PaCa cells (Fig. 4B). In contrast to rottlerin, the effect of BH3I-2 on ΔΨm was not transient at concentrations tested.

Both rottlerin and BH3I-2 stimulated loss of ΔΨm in digitonin-permeabilized MIA PaCa-2 cells (Fig. 4C and D), indicating direct interaction of rottlerin and BH3I-2 with mitochondria.

Once in cytosol, cytochrome c activates the initiator caspase-9, followed by activation of effector caspase-3, resulting in apoptosis (33). We found that rottlerin increased activity of caspase-9 (LEHDase) as well as caspase-9 processing manifest by a decrease in pro-caspase-9 (47 kDa) and increase in 35-kDa cleaved form (Fig. S1A; supplemental material for this article is available online at the American Journal of Physiology Gastrointestinal and Liver Physiology website). Rottlerin dose and time dependently stimulated caspase-3 (DEVDase) activity and its processing, which also manifests by a decrease in 32-kDa band of procaspase-3 and by a concomitant increase in 17-kDa band corresponding to active (i.e., cleaved) caspase-3 (Fig. S1, A and C). Of note, apoptosis induced by rottlerin in MIA PaCa-2 cells was prevented by pan-caspase inhibitor z-VAD-fmk, indicating a necessary role of caspases (not shown). Importantly, rottlerin did not activate caspase-8 (Fig. S1B), which acts upstream of mitochondria (33). These confirm that it stimulates mitochondrial pathway of apoptosis.

The results of the Figs. 3, 4, and S1 suggest that rottlerin and BH3I-2 stimulate apoptosis through a common mechanism;
they both directly interact with mitochondria, stimulating cytochrome c release and loss of $\Delta\psi_m$. This leads to caspase-9 and -3 activation and apoptosis.

**BH3I-2** but not rottlerin stimulated apoptosis through disrupting complexes of Bcl-xL with Bad. The pharmacological inhibitors of prosurvival of Bcl-xL and Bcl-2 induce apoptosis by preventing sequestration by prosurvival proteins of proapoptotic BH3-only proteins (18). Our immunoprecipitation experiments showed that, in MIA PaCa-2 cells, the proapoptotic BH3-only protein Bad is in complex with Bcl-xL and Bcl-2 (Fig. 5A). BH3I-2 markedly decreased the amount of Bad coimmunoprecipitated with Bcl-xL and Bcl-2 (Fig. 5, A and B). Differently, rottlerin only slightly decreased the amount of Bad in complex with Bcl-xL (Fig. 5, A and B) and had no effect on the interaction of Bad with Bcl-2 (Fig. 5A).

We further showed that Bad knockdown with siRNA greatly decreased apoptosis induced by BH3I-2' but had no effect on apoptosis induced by rottlerin (Fig. 5, C and D), indicating that proapoptotic effect of rottlerin does not involve Bad.

To compare the effects of rottlerin and BH3I-2' on the interaction of Bcl-xL with Bad in cell-free system we examined the effect of rottlerin on the binding of fluorescein-labeled peptide of the BH3 domain of Bad protein to Bcl-xL using fluorescence polarization assay (48). Figure S2 shows that recombinant Bcl-xL forms complex with the BH3 domain of Bad manifested by an increase in the fluorescence polarization.

BH3I-2' dose dependently disrupted complex formation between Bcl-xL and BH3 domain of Bad, whereas rottlerin at concentrations up to 200 $\mu$M was without effect. Thus the results in MIA PaCa-2 cells, as well as the results in cell-free system, indicate that BH3I-2' but not rottlerin disrupts the complex of Bad with Bcl-xL and Bcl-2.

**Rottlerin stimulated apoptosis through disrupting complexes of Bcl-xL with Bim and Puma.** To determine whether the proapoptotic effect of rottlerin in PaCa cells is mediated through Puma and Bim we measured the effect of rottlerin on the sequestration of these BH3-only proteins by Bcl-xL (Fig. 6). Rottlerin greatly decreased the amount of Puma and Bim coimmunoprecipitated with Bcl-xL (Fig. 6, A and B). Differently, BH3I-2 had no effect on the interaction of Bcl-xL with Puma (Fig. 6A, B). Of note, neither rottlerin nor BH3I-2' affected the interaction of Bcl-2 with Puma or Bim (data not shown).

We further showed that Puma and Bim knockdown with siRNA prevented stimulation of apoptosis by rottlerin in MIA PaCa-2 cells, indicating a critical role for Puma and Bim in the proapoptotic effects of rottlerin (Fig. 6, C and D). Of note, Puma and Bim knockdown with siRNA also prevented stimulation of apoptosis by rottlerin in PANC-1 cells (not illustrated), suggesting that the mechanisms of proapoptotic effects of rottlerin are the same in both MIA PaCa-2 and PANC-1 cells.
Rottlerin-induced apoptosis was not mediated by the inhibition of PKC-δ or Akt. Apoptotic effects of rottlerin were often attributed to the inhibition of PKC-δ (32, 36). Therefore, we next examined the involvement of PKC-δ in the effects of rottlerin on apoptosis. We showed (Fig. 7A) that neither broad spectrum PKC inhibitor nor specific peptide inhibitor of PKC-δ (35) stimulated apoptosis in MIA PaCa-2 cells. We also showed that the inhibitors blocked PKC-δ activity in MIA PaCa-2 cells (Fig. 7B and Ref. 46a). Pretreatment with PKC inhibitors also did not prevent apoptosis induced by rottlerin (Fig. 7C). Furthermore, rottlerin similarly stimulated apoptosis in MIA PaCa-2 cells, which express PKC-δ, and in PANC-1 cells, which do not express this isoform of PKC.

As shown in several publications (33, 36), PKC-δ could inhibit apoptosis through activating prosurvival Akt kinase. However, rottlerin did not have any significant effect on Akt phosphorylation in PaCa cells (Fig. 7D). These data indicate that PKC-δ and Akt are not involved in proapoptotic effects of rottlerin.

DISCUSSION

The results of this study show that rottlerin inhibited tumor development and increased apoptosis in the orthotopic model of pancreatic cancer. Rottlerin also stimulated apoptosis in PaCa cell lines.
Several lines of evidence indicate that rottlerin induced apoptosis in PaCa cells through interacting with Bcl-2 proteins. 1) The proapoptotic effects of rottlerin were not mediated by PKC-δ inhibition, as other inhibitors of PKC-δ do not have such effect. 2) Rottlerin directly interacts with mitochondria, resulting in cytochrome c release and mitochondrial depolarization. 3) Rottlerin displaces Bim and Puma from complexes with Bcl-xL. 4) Bim and Puma are necessary for rottlerin to cause apoptosis because their knockdown prevented induction of apoptosis by rottlerin. Taken together, these findings provide strong support for our hypothesis that rottlerin acts to cause apoptosis through disrupting the ability of prosurvival Bcl-2 proteins to sequester proapoptotic BH3-only proteins, Bim, and Puma.

The conclusion that proapoptotic effects of rottlerin are mediated through Bcl-2 proteins is further supported by the findings that rottlerin has actions similar to a known inhibitor of Bcl-2/Bcl-xL, BH3I-2’ (13, 18). BH3I-2’ is a mimetic of BH3 domain; it binds to the hydrophobic pocket of Bcl-2 and Bcl-xL, preventing their interaction with BH3-only proteins (13, 18). The effects of BH3I-2’ and rottlerin on apoptosis were not additive. Rottlerin and BH3I-2’ both directly affected mitochondria, resulting in cytochrome c release and loss of ΔΨm. Rottlerin and BH3I-2’ both disrupted complexes of Bcl-xL and BH3-only proteins. Proapoptotic effects of rottlerin and BH3I-2’ are mediated by BH3-only proteins.

Importantly, we found that effects of rottlerin and BH3I-2’ on the interaction of Bcl-xL with BH3-only proteins are not the same. Rottlerin prevented coimmunoprecipitation of Bcl-xL with Bim and Puma, but it had little effect on the complex of Bcl-xL with Bad. Differently, BH3I-2’ decreased the level of Bad, but not Bim and Puma, in the complex with Bcl-xL. The mechanisms underlying such differences in the interaction of rottlerin and BH3I-2’ with Bcl-xL remain to be determined.

Rottlerin stimulated not only cytochrome c release but also loss of ΔΨm in PaCa cells. Data from other cell types show that cytochrome c release and mitochondrial depolarization are mediated through different mitochondrial permeability systems (5). Cytochrome c is released through the Bax/Bak channels in the outer mitochondrial membrane, whereas mitochondrial depolarization is mediated by permeability transition pore
(mPTP), which involves proteins of both inner and outer membranes. Bcl-2 proteins were shown to be involved in the regulation of both mPTP and Bax/Bak channels (2, 4), which explains the effect of rottlerin on both cytochrome c release and necrosis in PaCa cells.

In nontransformed cells, mitochondria are the major source of ATP production; therefore, mitochondrial depolarization leads to ATP depletion and necrosis. ATP depletion also prevents caspase activation, leading to the inhibition of apoptosis (10, 37, 47). Differently, in cancer cells, ATP production is mostly mediated through glycolysis (43–45) and is relatively independent on ΔΨm (19, 43, 44). In agreement with these considerations, we found that, in PaCa cells (differently from normal pancreatic acinar cells) (36, 39), rottlerin stimulated both apoptosis and necrosis.

Importantly, the results of in vivo experiments suggest that normal and cancer cells display different sensitivity to rottlerin. The doses of rottlerin we applied in vivo stimulated neither apoptosis nor necrosis in normal pancreas and liver. These same doses of rottlerin greatly potentiated apoptosis in pancreatic tumor. Although the mechanisms underlying these differences remain to be determined, the findings that low doses of rottlerin to preferentially stimulate apoptosis in cancer cells are important for possible rottlerin application in cancer treatment.

In summary, our data showed that rottlerin has a potent proapoptotic and antitumor activity in pancreatic cancer.

**Fig. 7.** Rottlerin-induced apoptosis is not mediated through PKC or Akt. MIA PaCa-2 cells were cultured for 48 h with indicated concentrations of specific PKC-δ translocation inhibitor, broad-spectrum PKC inhibitors GF-109203X and Ro32-0432, rottlerin, or vehicle. A and C: apoptosis was determined by measuring DNA fragmentation using Cell Death ELISA kit. The values were normalized to those in cells cultured without inhibitors. The values are means ± SE, n = 3. B: changes in PKC-δ kinase activities were measured in PKC-δ immunoprecipitates by kinase assay using PKC-δ-specific peptide substrate. Activities were normalized to the basal activity in the absence of the inhibitors. Values are means ± SE (n = 2). C: Akt phosphorylation, an indicator of Akt activation, was measured by immunoblots of Akt phosphorylated at S473 and total Akt. D: intensities of pAkt and total Akt were densitometrically quantified and expressed as the ratios of the intensities of phosphoAkt to that of total Akt. The values are means ± SE, n = 3.
proapoptotic effect of rotterlin is mediated by the disruption of the complexes between prosurvival Bcl-2 proteins and BH3-only proteins Puma and Bim. Thus rotterlin represents a promising novel agent for pancreatic cancer treatment.

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DISCLOSURES
No conflicts of interest are declared by the author(s).


