A New Fungal Diterpene Induces VDAC1-dependent Apoptosis in Bax/Bak-deficient Cells*

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§§These abbreviations used are: OMM, outer mitochondrial membrane; Bcl-2, B-cell lymphoma/leukemia-2; Bax, Bcl-2-associated X protein; DIDS, 4,4′-disothiocyanato-stilbene-2,2′-disulfonic acid disodium salt; DTIDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; DpC, N-phenylanthranilic acid; EGS, ethylene glycolbis(succinimidyl succinate); ΔΨm, mitochondrial membrane potential; MOMP, mitochondrial outer membrane permeabilization; VDAC, mitochondrial outer membrane permeability transition pore; mPTP, mitochondrial permeability transition pore; MEF, mouse embryonic fibroblast; NAC, N-acetylcysteine; PI, propidium iodide; PAO, phenylarsine oxide; ROS, reactive oxygen species; SITS, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid; VDAC, voltage-dependent anion channel; rVDAC1, recombinant voltage-dependent anion channel 1; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; TMRM, tetramethylrhodamine, methyl ester; Z, benzylxoycyanobyl; fmk, fluoromethyl ketone; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid.

Data Centre (www.ccdc.cam.ac.uk/data_request/cif).

Significance:

Background: VDAC1 functions in both cellular metabolism and mitochondria-mediated apoptosis. New compounds were identified that induce apoptosis by promoting VDAC1 oligomerization and apoptosis in a Bak- and Bak-independent manner. Conclusion: Bak and Bak are dispensable for VDAC1-mediated apoptosis, revealing a novel mechanism of apoptosis involving VDAC1 oligomerization. Significance: In cancers with Bax/Bak down-regulated, VDAC1-induced apoptosis offers a novel approach for tumor therapies.

The pro-apoptotic Bax and Bak proteins are considered central to apoptosis, yet apoptosis occurs in their absence. Here, we asked whether the mitochondrial protein VDAC1 mediates apoptosis independently of Bax/Bak. Upon screening a fungal secondary metabolite library for compounds inducing apoptosis in Bax/Bak-deficient mouse embryonic fibroblasts, we identified cyathin-R, a new cyathane diterpenoid compound able to activate apoptosis in the absence of Bax/Bak via promotion of the VDAC1 oligomerization that mediates cytochrome c release. Diphenylamine-2-carboxilic acid, an inhibitor of VDAC1 conduction and oligomerization, inhibited cyathin-R-induced VDAC1 oligomerization and apoptosis. Similarly, Bcl-2 overexpression conferred resistance to cyathin-R-induced apoptosis and VDAC1 oligomerization. Silencing of VDAC1 expression prevents cyathin-R-induced apoptosis. Finally, cyathin-R effectively attenuated tumor growth and induced apoptosis in Bax/Bak-deficient cells implanted into a xenograft mouse model. Hence, this study identified a new compound promoting VDAC1-dependent apoptosis as a potential therapeutic option for cancerous cells lacking or presenting inactivated Bax/Bak.

One of the enigmas in mitochondria-mediated apoptosis concerns the release pathway(s) used by apoptogenic factors residing in the mitochondrial intermembranal space to cross the outer mitochondrial membrane (OMM) and reach the cytosol. Indeed, mitochondrial outer membrane permeabilization (MOMP) is a critical event resulting in the release of cytochrome c into the cytosol, where it binds to Apaf1 to promote formation of the apoptosome (1). The apoptosome recruits and cleaves procaspase-9 to initiate a caspase cascade, ultimately leading to apoptosis (2). To date, several models of MOMP leading to cytochrome c release have been proposed (3), such as the Bax/Bak pore formation (4) and mitochondrial permeability transition pore (mPTP) models (5).

Bcl-2 family proteins Bax and Bak are believed to serve as central regulators of MOMP and thus of mitochondria-mediated apoptosis (6). In response to apoptotic stimuli, BH3-only proteins like Bim or Bid are activated via transcriptional up-regulation or post-translational modification. These subsequently bind to either Bcl-2 or Bax and Bak, leading to the translocation of Bax to the OMM, where the protein changes conformation. This structural alteration encourages the formation of Bax homo-oligomers or hetero-oligomers with OMM-anchored Bak, yielding porelike structures that mediate MOMP and apoptosis (7–9). Although the importance of Bax and Bak in MOMP and apoptosis is widely recognized, Bax/Bak-independent apoptotic pathways also exist (10–12).

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CCDC 881686 contains supplementary crystallographic data for cyathin-R. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre (www.ccdc.cam.ac.uk/data_request/cif).

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Indeed, Bax/Bak down-regulation or inactivation has been shown to be the mechanism for the development of resistance to apoptosis in some cancers (13–15). Therefore, identifying molecules that mediate apoptosis in tumor cells independently of Bax and Bak offers an opportunity for the development of novel tumor therapies.

The voltage-dependent anion channel 1 (VDAC1) is an OMM protein that serves as a mitochondrial gatekeeper, controlling metabolic and energy cross-talk between mitochondria and the rest of the cell (3, 16, 17). The involvement of VDAC1 in mitochondria-mediated apoptosis has been proposed based on several lines of experimental evidence. VDAC1 is involved in cytochrome c release and is associated with pro- and anti-apoptotic members of the Bcl-2 protein family (3, 17–21).

Experimental Procedures

Cell Culture and Reagents—E1A- and K-Ras-transformed Bax−/−/Bak−/− MEF, Bax−/− HCT116, and HCT116 colon cancer cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Antibodies to β-actin and Bad, Puma, and FLAG were from Sigma; antibodies to Bcl-2, Tim23, cytochrome c, and Bcl-xL were from BD Biosciences; antibody to VDAC1 was from Abcam (ab14734) (Cambridge, UK); and antibody to cyclophilin D was from Calbiochem. FITC-conjugated goat anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotechnology, Inc., and HRP-labeled goat anti-mouse and anti-rabbit IgG antibodies were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). ECL reagents and ethylene glycolbis(succinimidyl succinate) (EGS) cross-linker were from Thermo Scientific (Waltham, MA). MitoTracker Red CMXRos, MitoSOX Red mitochondrial superoxide indicator, and carboxy-H2DCFDA were from Invitrogen, whereas Z-VAD-fmk was from Enzo Life Sciences (Lausen, Switzerland). BAPTA-AM was obtained from Tocris Bioscience (Bristol, UK). Other chemicals were purchased from Sigma, unless otherwise specified.

Extraction and Isolation of Cyathin-R and X-ray Crystallographic Analysis—As reported in our earlier publication (47), *Cyathus africanus* was incubated on rice at 25 °C for 38 days to afford a solid culture. The culture was extracted repeatedly with ethyl acetate by exhaustive maceration (3 × 4 liters). The organic solvent was evaporated to dryness under vacuum to afford the crude extract (56.5 g). The residue was dissolved in H2O (400 ml) and partitioned with CHCl3 (400 ml). The CHCl3-soluble fraction (36 g) was subjected to silica gel column chromatography and eluted with a gradient of n-hexane-ethyl acetate (100:0, 100:1, 100:2, 100:5, 100:10, 100:15, 100:25, and 100:35 v/v) and dichloromethane-acetone (100:0, 100:1, 100:2, 100:5, 100:10, 100:15, 100:20, and 100:30 v/v) to afford the crude column chromatography (ODS column chromatography using a gradient of increasing methanol (30–100%) in water to afford 10 subfractions (CA-6-1 to CA-6-10). Cyathin-R (1.1 g) was obtained from CA-6-6 by recrystallization in methanol.

Cyathin-R was isolated as white crystalline, δ13C NMR, see
The absolute configuration of cyathin-R was confirmed as 5. The [13C]NMR spectrum of cyathin-R (Table 1) revealed 21 carbons and an oxymethine at H-17 (δ 0.97 (3H, s, H-16); 1.00 (3H, d, J = 5.5 Hz, H-13), and an oxymethylene at δ 4.63 (1H, d, J = 5.5 Hz, H-14). The [13C]NMR spectrum of cyathin-R (Table 1) revealed 21 carbons ascribable to four methyl, six methylene, and four methane groups, five quaternary carbons, one methoxyl carbon, and one carbonyl group.

Upon crystallization from MeOH using the vapor diffusion method, colorless needles of cyathin-R were obtained. Data collection was performed on an Agilent Gemini A Ultra diffractometer using graphite-monochromated copper Kα radiation, λ = 1.54184 Å at 100.2 K. Crystal data: C_{21}H_{32}O_{3}Na, M = 332.47, space group orthorhombic, P212121; unit cell dimensions were determined to be a = 8.2443(2) Å, b = 11.0732(4) Å, c = 20.5762(6) Å, α = 90.00°, β = 90.00°, γ = 90.00°, V = 1878.42(10) Å³, Z = 4, Dx = 1.176 mg/m³, F (000) = 728, μ (copper Kα) = 0.601 mm⁻¹, 18,044 unique reflections were collected to θmax = 71.90°, in which 3,586 reflections were observed (F2 > 4σ(F2)). The structure was solved by direct methods using the SHELXS-97 program and refined using SHELXL-97 and full-matrix least-squares calculations. In the structure refinements, non-hydrogen atoms were placed on the isogonal atom positions. The final refinement gave R1 = 0.0640, wR2 = 0.1865, S = 1.049, Flack parameter (0.05(16). The Flack absolute structure parameter 0.05(16) obtained by graphite-monochromated copper Kα radiation.

VITRO Two batches of VDAC1-expressing HCT116 cells (HCT116-pSUPER-VDAC1) were generated using the SuperScript VILO cDNA synthesis kit (Invitrogen). Real-time PCR was carried out using a Quant One Step quantitative RT-PCR (probe) kit (Tiangen) and a CFX96 real-time PCR detection system (Applied Biosystems).

The resulting cDNAs for Bcl-2 were PCR-amplified using 5′-GTTGGCCTTTTGTGAGTTG-3′ and 5′-CCTACCCAGCC-TCGTTAT-3′ (Bcl-2) as the sense and antisense primers, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA that served as a control was amplified using 5′-ACACACGTGGCATGCCATAC-3′ and 5′-TCCACCAC-CCTGTGGCTGTA-3′ as the sense and antisense primers, respectively.

Reconstitution of Purified Intact VDAC into a Planar Lipid Bilayer, Single Channel Current Recording, and Data Analysis—VDAC1 purified from rat liver mitochondria was solubilized with LDAO and purified using hydroxyapatite resin, as described previously (48). Purified VDAC was used for channel reconstitution into a planar lipid bilayer prepared from soybean asolectin dissolved in n-decane (50 mg/ml) as described previously (48). Purified VDAC was added to the cis chamber containing 1 M NaCl and 10 mM Hepes, pH 7.4. After one or more channels were inserted into the planar lipid bilayer, currents were recorded by voltage clamping using a Bilayer ClampBC-525B amplifier (Warner Instruments, Hamden, CT). Current was measured with respect to the trans side of the bilayer.
FIGURE 1. Screening for VDAC1-associated apoptosis inducers and structural analysis of cyathin-R. A, diagram of compound-screening strategy. B, DpC reduces VDAC1 channel conductance. VDAC1 was reconstituted into a planar lipid bilayer as described under "Experimental Procedures." Currents through VDAC1 in response to a voltage step from 0 to 10 mV were recorded before and 20 min after the addition of DpC (100 μM). C, VDAC1 conductance as a function of voltage, from 60 to −60 mV, was recorded before (□) and after (○) the addition of DpC. The average steady-state conductance at a given voltage (G) was normalized to the maximal conductance at 10 mV (G0). D, DpC inhibits VDAC1 oligomerization induced with selenite (30 μM, 3 h) in HeLa cells preincubated with DpC (1 h). VDAC1 oligomerization was revealed using EGS-based cross-linking and immunoblotting using anti-VDAC1 antibodies. E, Bax−/−/Bak−/− MEFs were pretreated with 0.5 mM DpC for 1 h and incubated for 24 h with the indicated compounds (10 μM X15-1, 53, 5 μM H8-5, and 1 μM PAO). Apoptotic cell death was analyzed by annexin-V/PI staining and flow cytometry. Means ± S.E. (error bars) (n = 3) are shown. H8-5, cyathin-R. F and G, Bax−/−/Bak−/− MEFs were treated with cyathin-R for the indicated times, stained with annexin-V/PI, and analyzed by flow cytometry. Data from one of the three independent experiments are shown on the left. Means ± S.E. (n = 3) are shown. H–J, chemical structure of cyathin-R (H), key heteronuclear multiple-bond correlations (HMBC) of cyathin-R (I), and the conformation of cyathin-R with minimized energy (J) are shown. The arrows show the NOE correlations. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. *, p < 0.05; ***, p < 0.001.
membrane (ground). The current was digitized on-line using a Digi data 1200 interface board and pCLAMP6 software (Axon Instruments, Union City, CA).

**Cellular [Ca^{2+}] Analysis**—Fluo-4-AM was used to monitor changes in cytosolic Ca^{2+} levels. Bax^{-/-}/Bak^{-/-} MEF cells (1 × 10^6 cells/ml) were harvested after the appropriate treatment, collected (1,500 × g for 10 min), washed with HBSS buffer (5.33 mM KCl, 0.44 mM KH2PO4, 138 mM NaCl, 4 mM NaHCO3, 0.3 mM Na2HPO4, 5.6 mM glucose, 0.03 mM phenol red), and incubated with 2 mM Fluo-4 in 200 ml of HBSS (+) buffer (HBSS buffer supplemented with 1.8 mM CaCl2) in the dark for 30 min at 37 °C. After washing the excess dye, the cells were incubated with 200 ml of HBSS (+) buffer, and changes in cellular free Ca^{2+} concentration were measured immediately via FACS analysis. At least 10,000 events were recorded on the FL1 detector, represented as a histogram, and analyzed by FACSCalibur flow cytometer software (BD Biosciences). Positive cells showed a shift to an enhanced level of green fluorescence (FL1).

**Gel Electrophoresis and Immunoblotting**—Cells were lysed in lysis buffer (150 mM NaCl, 25 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM DTT, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 μg/ml trypsin inhibitor, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin), diluted with sample buffer, subjected to SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and then incubated with specific antibodies overnight at 4 °C. Bands were detected with HRP-conjugated secondary antibodies and an enhanced chemiluminescence system. Quantitative analysis of bands was performed using Image Gauge software (Fujifilm).

**Cross-linking of VDAC1**—After treatment with compounds, cells were harvested and treated with DMSO as a vehicle control (2%, as used in compound-containing samples) or cross-linked with 0.5 mM EGS for 10 min at 30 °C. The reaction was quenched by the addition of 20 mM Tris-HCl (pH 7.4). Samples were analyzed by SDS-PAGE followed by immunoblotting.

**BRET Assay**—In BRET2 experiments, Bax^{-/-}/Bak^{-/-} MEF cells were transfected to express the proteins below using JetPRIME transfection agent (Iillkirch, France) according to the manufacturer’s instructions. Transfections were carried out with siRNA specific to human VDAC1 (50 nm), 0.2 μg of a plasmid coding for rat VDAC1 (rVDAC1)-Rluc (Renilla luciferase fused to the VDAC1 C terminus) and with 0.8 μg of a plasmid coding for rVDAC1-GFP2 (GFP2 fused to the VDAC1 C terminus) (34). Cells were subjected to the BRET2 assay 48–72 h post-transfection. As background, cells were transfected with plasmids encoding rVDAC1-Rluc (0.2 μg) and with plasmid pcDNA4/TO (0.8 μg). Cells transiently expressing rVDAC1-Rluc and rVDAC1-GFP2 were incubated with the apoptosis inducer, harvested, washed twice with PBS, resuspended in 200 μl of PBS, and divided between two wells of a white 96-well clear bottom plate (Grenier). Luciferase activity was assayed using the membrane-permeant substrate DeepBlue C in PBS supplemented with MgCl2 (1 g/liter) and glucose (1 g/liter), with DeepBlue C added to a final concentration of 5 μM just before luminescence measurements. The BRET2 signal represents the ratio of the GFP2 fluorescence, measured at its emission wavelength (510 nm), to the light intensity (luminescence) emitted at 395 nm. All measurements were performed using the Infinite 200 ELISA reader (Tecan). BRET2 signals were defined as the GFP2/Renilla luciferase intensity ratio and calculated as described previously (34).

**Xenograft Experiments Using Nude Mice**—Bax^{-/-}/Bak^{-/-} MEF and Bax^{-/-}/HCT116 and HCT116 colon cancer cells (5 × 10^6) were subcutaneously injected into the back of the upper limbs of 4–5-week-old nude BALB/c mice. The mice were separated into three groups (6 mice/group). One week postinjection, two groups were treated with 5 or 10 mg/kg cytharin-R, whereas the vehicle control third group was treated with propylene glycol (50% (v/v) in 1.8% NaCl every other day for 2 weeks. The three groups of mice were sacrificed, and tumors were excised. Tumor length (a) and width (b) were measured using a slide gauge to determine tumor volumes (V) according to the formula \( V = a \times b^2/2 \). Apoptotic cells in the tumors were visualized by fluorescence microscopy after TUNEL using an in situ cell death detection kit (Roche Applied Science). Mice were maintained in specific pathogen-free conditions, and all research work was approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

**Statistical Analysis**—Data are expressed as means ± S.E. Statistical evaluation was carried out using Student’s t test (two-tailed) to test for differences between control and experimental results. The level of significance of differences between control and treated samples was determined using the nonparametric Mann-Whitney U test. A difference was considered statistically significant when the p value was deemed <0.05 (*), <0.01 (**), or <0.001 (***) Tumor volume data were analyzed using one-way repeated measures analysis of variance.
VDAC1-mediated Apoptosis in Bax/Bak-deficient Cells

FIGURE 2. Cyathin-R induces apoptosis in Bax−/−/Bak−/− MEFs. A, cytochrome c release from mitochondria; Bax−/−/Bak−/− MEFs treated with 3 μM cyathin-R for the indicated times, as analyzed by immunohistochemistry using anti-cytochrome c antibodies, with nuclei stained with DAPI and mitochondria with MitoTracker. Cells were visualized by confocal microscopy. The enlarged images on the right are from the boxed areas. Scale bar, 20 μm. B, cytochrome c (Cyto c) release as assayed by immunoblotting of cytosolic and mitochondrial fractions. C, poly(ADP-ribose) polymerase (PARP) cleavage; Bax−/−/Bak−/− cells were treated with 3 μM cyathin-R for the indicated times. Poly(ADP-ribose) polymerase cleavage was analyzed by immunoblotting. D, caspase activation; Bax−/−/Bak−/− MEFs were treated as described in the legend to Fig. 1G and then stained with a CaspACE-FITC-conjugated caspase marker. Means ± S.E. (error bars) (n = 3) are shown. E, apoptosis inhibition with caspase inhibitor; Bax−/−/Bak−/− MEFs were pretreated with Z-VAD-fmk caspase inhibitor (1 h) and then incubated with cyathin-R (5 μM, 24 h). Cell death was analyzed by annexin-V/PI staining and flow cytometry. Means ± S.E. (n = 3) are presented. F, cell nuclear fragmentation; Bax−/−/Bak−/− cells were treated with 3 μM cyathin-R, stained with Hoechst 33342, and imaged by confocal microscopy. Scale bar, 20 μm. **, p < 0.01; ***, p < 0.001.

Results

Cyathin-R Is Able to Induce Bax/Bak-independent Apoptosis—To identify new compounds able to induce apoptosis in a Bax- and Bak-independent manner, Bax−/−/Bak−/− MEF cells were employed in a two-round screening strategy (Fig. 1A). We first screened 149 fungus-derived natural compounds for cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell survival assay. In this manner, we obtained seven compounds that strongly reduced cell viability. As the next step to select for VDAC1-dependent molecules, DpC, identified as an inhibitor of both VDAC1 channel conductance and selenite-induced oligomerization (Fig. 1E), was recruited (Fig. 1E). Purified mitochondrial VDAC1 were reconstituted into a planar lipid bilayer, and channel conductance was monitored under voltage clamp conditions. DpC reduced channel conductance of reconstituted VDAC1 assayed at −10 or 10 mV (Fig. 1B). VDAC1 channel conductance is voltage-dependent, showing decreased conductance at both high negative or positive potentials. DpC decreased the conductance regardless of the voltage gradient applied (Fig. 1C).

DpC inhibited VDAC1 oligomerization as analyzed following chemical cross-linking with cell-permeable EGS and Western blot analysis using anti-VDAC1 antibodies. Protein bands corresponding to VDAC1 dimers (72 kDa) and multimers were obtained upon exposure to selenite (Fig. 1D).

To identify molecules that induce VDAC1-dependent apoptosis, cells were pretreated with DpC before exposing them to the seven fungus-derived hit compounds, and then apoptosis was analyzed by annexin-V/propidium iodide staining and flow cytometry (Fig. 1E). This approach led to the finding that of the seven molecules inducing apoptosis in Bax−/−/Bak−/− MEFs, only H8-5, termed cyathin-R, was able to induce apoptosis that was inhibited by DpC (Fig. 1E) and in a time-dependent manner (Fig. 1, F and G). DpC alone had no effect on cell viability.

Cyathin-R, a cyathane-type diterpenoid from the medicinal fungus C. africanus, was isolated from solid culture by column chromatography on silica gel and C-18 reverse-phase silica, followed by recrystallization in methanol. The molecular formula of cyathin-R was established to be C21H32O3 based on high resolution electrospray ionization mass spectrometry, 1H NMR, and 13C NMR (Table 1). The structure of cyathin-R (Fig. 1H) was unambiguously defined by heteronuclear multiple-bond correlation and NOESY spectral analysis and x-ray crystallographic analysis (Fig. 1, H–J).

Whereas annexin-V/PI staining revealed cyathin-R-induced apoptosis in Bax−/−/Bak−/− MEF cells, other markers of apoptosis were also considered. Cyathin-R induced hallmarks of apoptosis, such as cytochrome c release from mitochondria (Fig. 2, A and B), poly(ADP-ribose) polymerase cleavage (Fig. 2C), and caspase activation (Fig. 2D), as well as nuclear fragmentation and condensation (Fig. 2F), all in the absence of Bax and Bak. Furthermore, the pan-caspase inhibitor Z-VAD prevented cyathin-R-induced apoptosis (Fig. 2E). Similar results were also obtained with Bax−/− HCT116 colon cancer cells (Fig. 3). We thus conclude that cyathin-R induced MOMP and apoptosis in a Bax- and Bak-independent fashion.
Cyathin-R Induces Increased VDAC1 Protein Levels and Oligomerization That Mediate Apoptosis in Bax- and Bak-deficient Cells—Cyathin-R induced apoptosis in Bax/H11002/Bak/H11002 MEFs and Bax/H11002 HCT116 colon cancer cells that was inhibited by DpC and other anion transport inhibitors, DIDS, DNDS, or SITS (Fig. 4, A and B). DIDS was shown to inhibit VDAC1 channel conductance (49) and oligomerization (34). Interestingly, the cyclophilin D-interacting molecule cyclosporin A, a known inhibitor of mPTP opening, failed to inhibit cyathin-R-triggered apoptosis but rather promoted it (Fig. 4, C and D). The direct interaction of cyathin-R with VDAC1 is reflected in the decreased channel conductance of bilayer-reconstituted VDAC1 (Fig. 4, E and F).

Moreover, cyathin-R did not activate PTP opening in the absence of Ca2+ and had no effect on Ca2+-activated PTP opening (Fig. 4, G and H). These findings imply that VDAC1 and not mPTP opening mediates cyathin-R-induced apoptosis in the absence of Bax and Bak.

We next asked whether oligomerization of VDAC1 is required for cyathin-R-induced cytochrome c release and apoptosis. In both Bax/H11002/Bak/H11002 MEF and HCT116 colon cancer cells, following chemical cross-linking with EGS and immunoblotting, VDAC1 dimers and multimers were obtained upon exposure to cyathin-R in an incubation time- and concentration-dependent manner, concomitant with the disappearance of monomeric VDAC1 (Fig. 5, A–D). The inhibitors DpC, SITS, DIDS, and DNDS completely prevented cyathin-R-induced VDAC1 oligomerization (Fig. 5, C and D).

Cyathin-R-induced VDAC1 oligomerization was also monitored in living cells by BRET. Energy transfer between VDAC1-luciferase (light-producing enzyme) as the donor and VDAC1-GFP2 (fluorophore) as the acceptor was obtained when the two VDAC1 molecules interacted physically (Fig. 5E). The BRET signal was increased about 7-fold upon apoptosis induced by cyathin-R.
These data are consistent with the ability of DpC, DIDS, DNDS, and SITS to inhibit cyathin-R-induced apoptosis (Figs. 1E and 4 (A and B)) and suggest that VDAC1 oligomerization induced by cyathin-R can mediate MOMP and apoptosis.

Cyathin-R also induced an increase in VDAC1 levels (Fig. 5, A (arrow), F, and G), as recently demonstrated for various other apoptosis stimuli (36). VDAC1 levels were increased several-fold upon cell treatment with cyathin-R, as compared with other mitochondrial proteins, such as cyclophilin D, which maintained a constant level (Fig. 5, F and G).

Stable and specific knockdown of human VDAC1 expression afforded these cells almost complete resistance to cyathin-R-induced apoptosis (Fig. 5H). More importantly, rescue experi-
ments showed that wild type rat VDAC1 could restore cyathin-R-induced apoptosis in such cells, whereas the VDAC1 L277R mutant, which shows weakened oligomerizing ability (33) (Fig. 5I), was significantly less able to restore cyathin-R pro-apoptotic potential (Fig. 5J).

Because different apoptosis inducers elevate cytosolic Ca\(^{2+}\) and induce VDAC1 overexpression and oligomerization (36), we examined the effect of cyathin-R on cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\(_i\)]) levels using the Ca\(^{2+}\) indicator Fluo-4 (Fig. 6A). Cyathin-R elevated [Ca\(^{2+}\)\(_i\)], concomitant with increasing apoptosis (Fig. 6B), whereas decreasing [Ca\(^{2+}\)\(_i\)] using the cell-permeable Ca\(^{2+}\)-chelating reagent BAPTA-AM inhibited cyathin-R-induced increases in VDAC1 expression level (Fig. 6C), oligomerization (Fig. 6D), and apoptosis (Fig. 6E).
Cyathin-R-mediated Changes in the Cellular Redox State and Mitochondrial Membrane Potential—Previous studies revealed that oxidative stress, especially due to the mitochondrial superoxide, could trigger VDAC1-dependent apoptosis (3, 17, 20, 21). Here, we found that cyathin-R induced reactive oxidative species (ROS) production, as measured by MitoSOX Red, a mitochondrial superoxide indicator (Fig. 7A). Cyathin-R elevated overall cellular ROS levels (H$_2$DCFDA fluorescence) in Bax$^{-/-}$/Bak$^{-/-}$ MEF cells (Fig. 7B), with cyathin-R-induced apoptosis being inhibited by the ROS scavenger NAC (Fig. 7C) or by DTT (Fig. 8A). Moreover, NAC completely inhibited cyathin-R-induced VDAC1 oligomerization (Fig. 7D). Because depletion of GSH, a vital cellular antioxidant, could lead to oxidative stress, we examined the cellular GSH content and found it to be significantly decreased upon cyathin-R treatment (Fig. 7E). Pretreatment with cell-permeable glutathione ethyl ester prevented cyathin-R-induced apoptosis (Fig. 7F).

Because cyathin-R induces ROS production, with ROS known to activate NF-κB, we analyzed the location of the NF-κB component p65 and found it to have translocated to the nucleus, as analyzed by immunocytochemistry and immunoblotting of the nuclear fraction. Such translocation was prevented by NAC (Fig. 8, B and C).

Finally, because loss of mitochondrial membrane potential (ΔΨ$_m$) is often considered to be indicative of MOMP, the effect of cyathin-R on ΔΨ$_m$ was monitored using TMRRM. Cyathin-R induced a complete loss of ΔΨ$_m$ in a dose-dependent manner (Fig. 7G).

Cyathin-R Attenuates the Growth of Bax$^{-/-}$/Bak$^{-/-}$ MEFs, Bax$^{-/-}$ HCT116, and HCT116 Colon Cancer Cells in Vivo—Because Bax or Bak mutation or deficiency was correlated with the occurrence of cancer and drug resistance in cancer cells (13–15), we examined the effect of cyathin-R on MEFs and cancer cells growth in a xenograft mouse model. Immortalized Bax$^{-/-}$/Bak$^{-/-}$ MEFs or wild type or Bax$^{-/-}$ HCT116 colon cancer cells were implanted into nude mice, and tumor growth was monitored. One week later, mice implanted with a specific cell type were divided into three groups (6 mice/group) and underwent intraperitoneal treatment every 2 days with the vehicle control (10% DMSO) or cyathin-R (5 or 10 mg/kg) for 2 weeks. Cyathin-R effectively inhibited the growth of Bax$^{-/-}$/Bak$^{-/-}$ MEFs (Fig. 9, A and B) and of wild type and Bax$^{-/-}$ HCT116 colon cancer cells in the mice in a concentration-dependent manner (Fig. 9, E, F, H, and I). Comparing tumor sizes at the end point revealed that the average tumor volume in the 10 mg/kg cyathin-R-treated tumors was decreased by about 75% of that of control, DMSO-injected tumors (Fig. 9A). At the dosage used, cyathin-R was tolerable, because no obvious weight loss was observed (Fig. 9, D, G, and J). In situ TUNEL staining showed that cyathin-R also effectively induced apoptosis of the neoplasm in vivo (Fig. 9C). These data indicate that as with cells in culture, cyathin-R induced cell death in the xenograft mouse model.

Bcl-2 Protects against Cyathin-R-induced Apoptosis—Bcl-2 and Bcl-xL are important antagonists of MOMP and apoptosis and were shown to directly interact with VDAC1 (25, 48, 50, 51). We observed that the overall levels and mitochondrial localization of Bcl-2 (and, to a lesser extent, of Bcl-xL) were dramatically decreased upon cyathin-R treatment of Bax$^{-/-}$/Bak$^{-/-}$ MEFs (Fig. 10, A and B). Quantitative real-time PCR analysis showed that cyathin-R treatment decreased Bcl-2 transcript levels (Fig. 10C). As expected, cells stably expressing Bcl-2 showed resistance to cyathin-R-induced apoptosis and VDAC1 oligomerization (Fig. 10D). Interestingly, the Bcl-2/
Bcl-xL inhibitor ABT737 failed to induce apoptosis in Bax-/Bak-deficient cells and had no effect on cyathin-R-induced apoptosis. Moreover, ABT737 did not abrogate Bcl-2-mediated protection against cyathin-R-induced apoptosis (data not shown).

Discussion

Investigations into the mechanisms of MOMP and cytochrome c release from mitochondria have centered mainly on Bax and Bak as major players in these critical processes that ultimately set off the apoptotic cascade. However, mitochondria-mediated apoptosis can be activated in the absence of Bax/Bak (10–12). This is significant because in several tumors, resistance to chemotherapy is due to down-regulation of Bax and Bak (13–15, 52), thus reflecting a substantial clinical challenge.

As such, it is important to identify novel apoptosis inducers that bypass Bax/Bak. The results presented here showing that a new compound, cyathin-R, that enhanced VDAC1 protein levels and promoted VDAC1 oligomerization and apoptosis in a manner that can be inhibited by the VDAC1 interaction and conductance inhibitors DpC, DIDS, or Bcl-2. Accordingly, we have identified a cyathane diterpenoid compound, cyathin-R, that enhanced VDAC1 protein levels and promoted VDAC1 oligomerization and apoptosis in a manner that can be inhibited by DpC, DIDS, SITS, or DNDS; by knockdown of VDAC1; or by ectopic expression of Bcl-2.

The results presented here, together with previous reports, clearly demonstrate that oligomerization of VDAC1 is critical for cytochrome c release (34–37). It is thus important to understand how VDAC1 oligomerization is regulated in the cell. We observed that VDAC1 levels are increased by cyathin-R treatment, providing the basis for subsequent oligomerization. Indeed, several other apoptosis inducers were found to increase VDAC1 levels (40–42, 55, 56). By extending these observations, we have proposed that apoptosis-inducing agents act by

FIGURE 7. Cyathin-R induces apoptosis via oxidative stress. A–E, Bax−/−/Bak−/− MEFs were pretreated (1 h) with NAC followed by incubation with cyathin-R (5 μM, 16 h) (A). The cells were stained with MitoSOX Red ROS indicator and analyzed by flow cytometry. Shown are means ± S.E. (error bars) (n = 3). B, cells were incubated with cyathin-R (6 h), stained with H2DCFDA (DCF), and analyzed by flow cytometry. Shown are means ± S.E. (n = 3). C, cells were incubated with cyathin-R (5 μM, 36 h) and analyzed for apoptosis by annexin-V/PI staining. Means ± S.E. (n = 3) are presented. D, cells were incubated with cyathin-R (1 h) and cross-linked by EGS, and VDAC1 oligomerization was analyzed by immunoblotting. E, cells were incubated with cyathin-R (16 h), and GSH content was assayed with a GSH and GSSG assay kit. Means ± S.E. (n = 3) are presented. F, Bax−/−/Bak−/− MEFs were pretreated with glutathione ethyl ester (GSHee) for 1 h and then with cyathin-R (16 h), and apoptosis was analyzed. Means ± S.E. (n = 3) are presented. G, analysis of mitochondrial membrane potential (Δψm). Bax−/−/Bak−/− MEFs were treated with cyathin-R (5 μM, 6 h) or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (10 μM, 6 h) as a positive control and incubated with TMRE (10 min, 37 °C), and fluorescence was analyzed by flow cytometry. The results shown are those obtained after subtracting the value measured with the carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) control. Means ± S.E. (n = 3) are presented. **, p < 0.01; ***, p < 0.001.

VDAC1-mediated Apoptosis in Bax/Bak-deficient Cells
increasing \([\text{Ca}^2+]\), and that this in turn leads to up-regulation of VDAC1 expression (43). The increased level of monomeric VDAC1 shifts the equilibrium toward VDAC1 oligomerization, resulting in changes in OMM permeability allowing for cytochrome c release, leading to cell death (34–37). Moreover, the cellular level of VDAC1 was shown to be a crucial factor in the process of mitochondria-mediated apoptosis, with exogenous overexpression of VDAC1 shown to be a crucial factor in the process of mitochondria-mediated apoptosis, with exogenous overexpression of VDAC1 resulting in cell death, regardless of the cell type or the origin of VDAC used (36, 57–60). Importantly, the causal relationship between VDAC1 levels and drug sensitivity was emphasized in several studies. A prostate cancer cell line resistant to G3139-induced apoptosis expresses low levels of VDAC1 relative to the G3139-sensitive cell line (61), whereas overexpression of VDAC1 sensitized carcinoma cells to apoptosis as induced by cisplatin and its derivatives (43). These observations suggest that the activity of numerous anticancer drugs and treatments is mediated via elevating VDAC1 levels.

Here, tight correlation between increased VDAC1 levels, VDAC1 oligomerization, and apoptosis was clearly demonstrated in cells lacking Bax/Bak, because all three processes were inhibited by the VDAC1-interacting molecules DpC and DIDS, the ROS scavenger NAC, or by ectopic expression of Bcl-2. Additionally, cyathin-R effectively attenuated tumor growth in Bax\(-/-\)/Bak\(-/-\) MEF and Bax\(-/-\)/HCT116 cells and in a colon cancer xenograft model.

The finding that cyathin-R-triggered oxidative stress is associated with increased VDAC1 levels and oligomerization is in agreement with a previous report showing that ROS induced VDAC1 up-regulation that could be prevented by a ROS chelator (56). \(\text{H}_2\text{O}_2\) was found to elevate intracellular \(\text{Ca}^{2+}\) levels, up-regulate VDAC1 expression, and induce VDAC1 oligomerization and apoptosis (36). These \(\text{H}_2\text{O}_2\)-mediated effects were prevented by BAPTA, suggesting their \(\text{Ca}^{2+}\) dependence (36), as also shown here for cyathin-R inducing increased intracellular \(\text{Ca}^{2+}\) and inhibition of its pro-apoptotic activity by BAPTA (Fig. 6). Several potential \(\text{Ca}^{2+}\)-dependent steps could contribute to the enhanced expression of VDAC1, including mRNA transcription, elongation, splicing, stability, and/or translation.
Another possible mechanism may be linked to the proposed role of VDAC1 in mediating ROS release from mitochondria to the cytosol (62). VDAC1 could be subjected to oxidative modifications that would reduce its degradation, thereby leading to its accumulation and subsequent oligomerization.

Finally, we found that treatment with cyathin-R led to downregulation of Bcl-2 levels. Thus, cyathin-R not only induces apoptosis via promoting VDAC1 oligomerization but also antagonizes the anti-apoptotic effects of Bcl-2 and Bcl-xL by decreasing their levels. Reduced Bcl-2 levels alter either mitochondrial oxidative stress, because Bcl-2 was previously shown to regulate ROS and cellular GSH (63), or its interaction with VDAC1 (25, 48, 50, 51), thereby releasing VDAC1 for subsequent oligomerization.

The findings that the Bcl-2/Bcl-xL inhibitor ABT737 (64) failed to induce apoptosis in Bax−/−/Bak−/− cells and Bak−/− HCT116 or HCT116 colon cancer cells in a xenograft mouse model. Immunodeficient nude mice were implanted with Bax−/−/Bak−/− MEFs (A–D), Bax−/− HCT116 (E–G), or HCT116 (J–L) colon cancer cells. One week later, the mice were divided into three groups (6 mice/group) and underwent intraperitoneal treatment with the vehicle control (DMSO, 10%) or cyathin-R (5 or 10 mg/kg/2 days) for 2 weeks, during which time tumor size was measured. Two weeks post-cyathin-R treatment, the mice were euthanized, and the xenografts were excised for further studies. Tumor volume curves (A, E, and H) and representative images of isolated tumors (B, F, and I) and average body weights of nude mice after 2 weeks of treatment (D, G, and J) are shown. C, fluorescence images show staining of apoptotic Bax−/−/Bak−/− cells by the in situ TUNEL assay. Scale bar, 100 μm. Error bars, S.E.

some reagents, such as As$_2$O$_3$ and Koenig’s polyanion, were shown to reduce channel conductance but induce apoptosis, whereas others inhibited both processes (for a review, see Ref. 65). As shown here, DpC and cyathin-R both decreased VDAC1 conductance, whereas DpC inhibited VDAC1 oligomerization and cyathin-R enhanced oligomerization. It seems that most interacting sites for small molecules are in the pore, and their interaction there reduced the channel conductance of current-conducting small ions. This was also shown for tubulin (66), with its tail being inserted into the pore. Other proteins interacting with VDAC1 and reducing its conductance, such as HK, Bcl2, and Bcl-xL (29, 48, 50, 60), interact from the cytoplasmic face and block channel conductance.

The model proposed here (Fig. 10F), in which cyathin-R enhances VDAC1 protein levels, thereby promoting VDAC1 oligomerization and apoptosis, involves a novel mechanism highlighting cyathin-R as a potential lead for an effective anti-cancer drug.

Many cancer cells develop cell survival strategies that implicate anti-apoptotic defense mechanisms. As vital regulators of cell life and death, anti-apoptotic Bcl-2, Bcl-xL (29, 48, 50, 60), interact from the cytoplasmic face and block channel conductance.

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