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Structure–Activity Relationships within a Family of Selectively Cytotoxic Macrolide Natural Products

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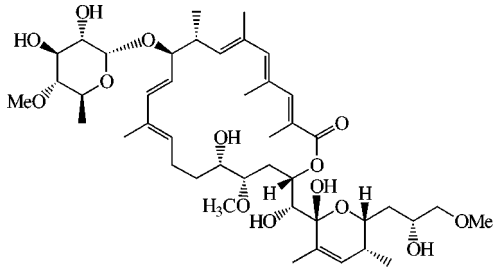
ABSTRACT



We describe a semi-synthetic deglycosylated derivative of apoptolidin that retains considerable activity against the mitochondrial ATPase but has greatly reduced cellular cytotoxicity. We also demonstrate that a related antifungal natural product, cytovaricin, inhibits the same molecular target. Structural comparison of these macrolides provides insights into their conserved features that are presumably important for biological activity and identifies promising avenues at the interface of organic synthesis and biosynthesis for the generation of new selective cytotoxic agents.

Drugs that can selectively sensitize cancer cells to apoptosis induction are crucial. Recently, Seto et al. discovered a novel apoptosis inducer from *Nocardiopsis sp.* named apoptolidin (**1**), which can selectively sensitize cancer cells to apoptosis with considerable potency.[1,2] We have shown that apoptolidin induces cell death by inhibiting the mitochondrial F_0F_1-ATPase.[3,4] Thus, apoptolidin joins a growing family of structurally related macrolide natural products, including the antifungal agents oligomycin and ossamycin, which target the eukaryotic mitochondrial ATPase. Here we describe a semi-synthetic deglycosylated derivative of apoptolidin (**2**) that retains considerable activity against the mitochondrial ATPase but has greatly reduced cellular cytotoxicity. We also demonstrate that a related antifungal natural product, cytovaricin, inhibits the same molecular target. Structural comparison of these macrolides provides insights into their conserved features that are presumably important for biological activity, and identifies promising avenues at the interface of organic synthesis and biosynthesis for the generation of new selective cytotoxic agents.

Treatment of **1** (prepared by fermentation) with 0.2 M HCl for 3 h in methanol delivered **2** in 12% yield. Purification was performed by preparative reversed-phase HPLC with a full linear gradient from 100% water to 100% acetonitrile in 60 min at 15 mL/min on a Beckman C18 column (21.2 mm × 15 cm). The structure of **2** was determined by NMR and mass spectrometry. The linkages of C21−C25 and C9−C1′ were established by HMBC correlations. The macrolide ring and the 6-deoxy-4-O-methyl-glucose were assigned by COSY, HMQC, and HMBC correlations and by comparison

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(1) Kim, J. W.; Adachi, H.; Shin-ya, K.; Hayakawa, Y.; Seto, H. *J. Antibiot.* **1997**, *50*, 628−30.
(2) Hayakawa, Y.; Kim, J. W.; Adachi, H.; Shinya, K.; Fujita, K.; Seto, H. *J. Am. Chem. Soc.* **1998**, *120*, 3524−3525.
(3) Salomon, A. R.; Voehringer, D.; Herzenberg, L. A.; Khosla, C. *Proc. Natl. Acad. Sci. U.S.A.*, in press.
(4) Salomon, A. R.; Voehringer, D.; Herzenberg, L. A.; Khosla, C. *Chem. Biol.*, in press.

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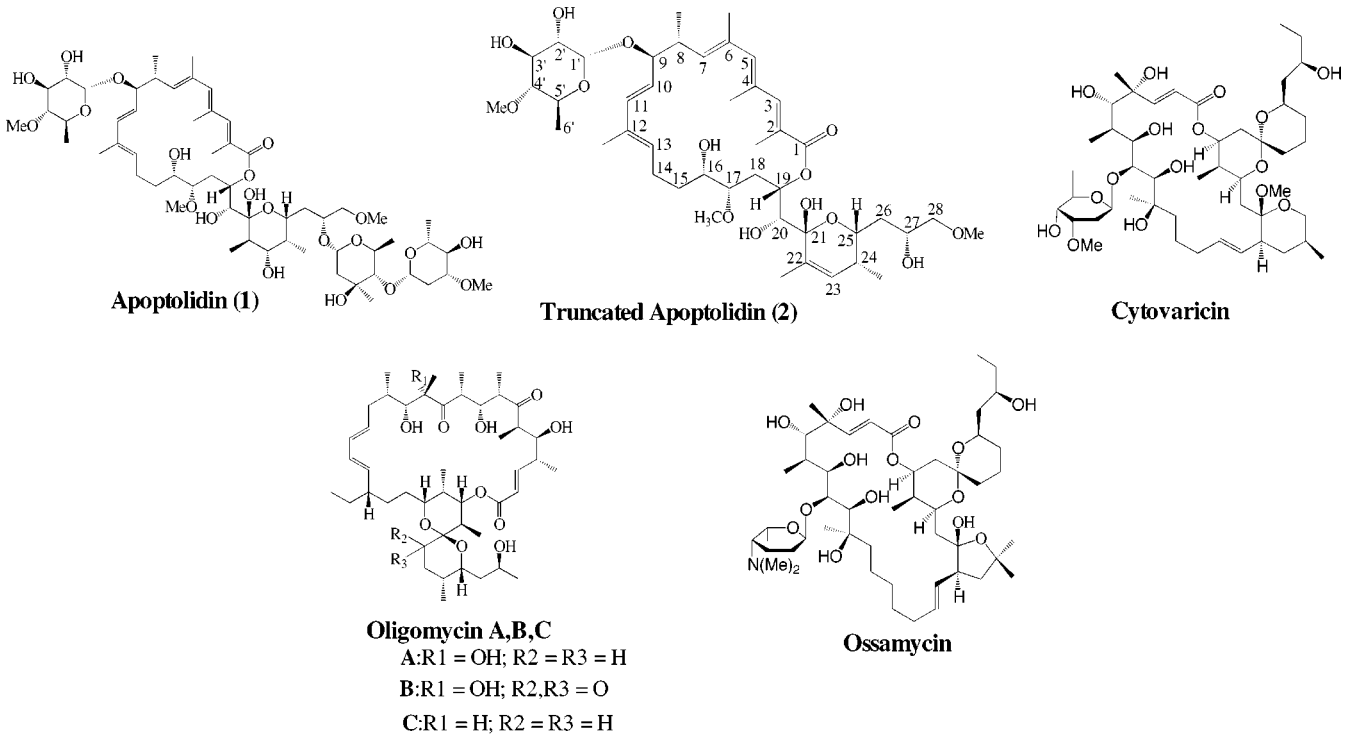

Figure 1. Family of mitochondrial F_0F_1-ATPase inhibitors.

with corresponding correlations observed in **1**. The assignment of the double bond between C22 and C23 was done by HMBC correlations between C21−H(22Me) and C21−H23 as well as correlations between C24−H25, C25−H(24Me), H23−C25, and C23−H25. All methoxy groups were assigned by HMBC correlations with the neighboring carbons. The disappearance of oleandrose and olivomycose was established by the lack of all peaks corresponding to these residues from the COSY and HMQC spectra. The stereochemistry of **2** was assumed to be the same as **1**. The molecular formula of **2** was determined by FAB-MS to be $C_{44}H_{68}O_{13}$ [*m*/*z* 827.4667 $(M + Na)^+$ Δ11.0 mmu] which is consistent with **2** − H_2O. Formation of the C22−C23 olefin in **2** presumably involves dehydration to a glycal, followed by a Ferrier allylic rearrangement[5] to eliminate the C23 hydroxyl group.

As evaluated by an in vitro yeast ATPase assay,[6] the truncated derivative **2** has a K_i of 10 μM while apoptolidin itself has a K_i of 5 μM (Figure 2). In vivo, **2** retains 1% of the activity of the parent molecule against two apoptolidin-sensitive cell lines- a human breast tumor cell line MCF-7 and a murine B cell lymphoma cell line LYas.[7] This decrease in cellular cytotoxicity might be explained by the decrease in hydrophilicity of **2** compared to **1**, which could interfere with cellular transport of the molecule to its mitochondrial

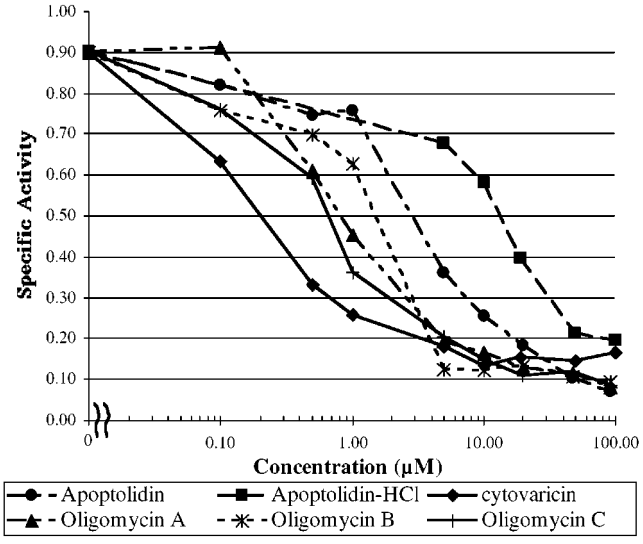

Figure 2. Inhibition of yeast ATPase activity by various polyketides.

(5) Ferrier, R. J.; Prasad, N.; Sankey, G. H. *J. Chem. Soc. C* **1968**, 974−977.

(6) Crude yeast mitochondria were isolated from a lactate grown *Saccharomyces cerevisiae* strain DBY7286 (*matA, ura-/-*) according to published procedures (Glick, B. S.; Pon, L. A. 1995 *Methods Enzymol* **260**, 213−23). Mitochondrial ATPase activity was measured by standard methods (Roberts, H.; Choo, W. M.; Murphy, M.; Marzuki, S.; Lukins, H. B.; Linnane, S. W. *FEBS Lett* **1989**, *108*(2), 501−4). Briefly, 20 μg of yeast mitochondrial protein (as measured by the Lowry method) was added to reaction buffer containing 50 mM Tris (pH 8.0), 1 mM ATP, 0.3 mM NADH, 3.3 mM $MgCl_2$, 2 μg/mL antimycin A, 1 mM phosphoenol pyruvate, 5 U/ml lactate dehydrogenase, and 2.5 U/ml pyruvate kinase at 28 °C. Oxidation of NADH was followed at 360 nm over time. To establish the mitochondrial origin of the ATPase activity, published procedures were used to measure (mitochondrial) cytochrome *c* oxidase activity (Wharton, D. C.; Tzagoloff, A. *Methods Enzymol.* **1967**, *10*, 245−250).

target.[8] Alternatively it might imply a mechanism wherein these natural products induce apoptosis by interfering with a secondary target upon binding to the mitochondrial ATPase. Because **2** retains most of its in vitro activity against the target ATPase, we conclude that the oleandrose and olivomycose sugars in apoptolidin are not absolutely essential to the binding of apoptolidin to this target. Complete deglycosylation of apoptolidin, ideally afforded via inactivation of the glycosyltransferase genes associated with the biosynthetic pathway of this natural product, should establish whether the aglycone is still able to inhibit F_0F_1-ATPase activity. Notwithstanding differences in structures, we noted a striking structural similarity between the polyketide backbones of apoptolidin, cytovaricin, ossamycin, and oligomycin (Figure 3). The presence of the hemiketal ring functionality in apoptolidin is closely mirrored by a spiroketal system in cytovaricin and oligomycin. In cytovaricin and ossamycin the spiroketal is unsubstituted, whereas in oligomycin the spiroketal is variably substituted. Also, two separate unsaturated segments are present in the lactone rings of all molecules. The observed variability in glycosylation patterns within this family of macrolides is consistent with the thesis that deoxysugars are not crucial for ATPase inhibition (vide supra). Figure 2 compares the activities of apoptolidin, semisynthetic analogue **2**, oligomycin A, oligomycin B, oligomycin C, and cytovaricin against yeast mitochondrial ATPase.[5] Of the compounds tested, cytovaricin is the most potent with a $K_i = 0.4\ \mu$M. Previous results have established

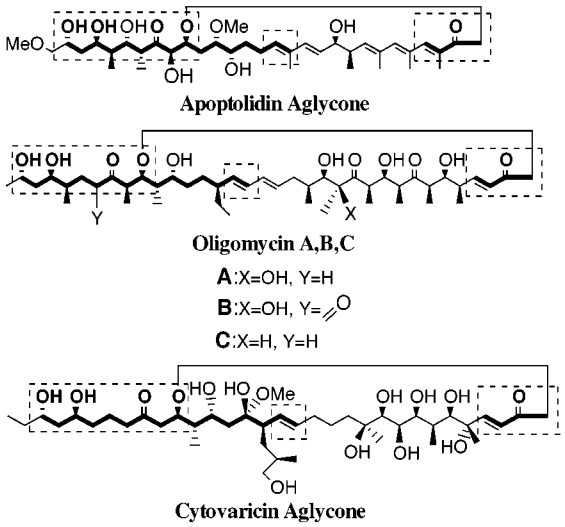

Figure 3. Structural similarities between the polyketide backbones of apoptolidin, cytovaricin, and oligomycin.

that oligomycin and ossamycin bind to subunit 9 of the F0 region of F_0F_1-ATPase.[9] Although the precise binding site of cytovaricin and apoptolidin on this multi-subunit target remains to be established, the above-mentioned structure−activity relationships suggest that the binding sites of all these natural products may be conserved.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC spectra and a table of all peak assignments and correlations observed in the spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Activity was measured by FACS staining for AnnexinV and by MTT assay. In the mouse B cell lymphoma cell line LYas, **1** or **2** were incubated with cells for 6 h and then removed. Cells were stained with AnnexinV-Cy5PE for 15 min and washed three times. Cells were analyzed on the Facscan and the percentage of AnnexinV positive cells was quantified. The IC_{50} of **1** was 100 nM while that of **2** was 10 μM. The breast carcinoma cell line MCF-7 was tested by the MTT assay. Drug dilutions were added to cells in 96 well plates in triplicate for 120 h. MTT was then added to the wells at a final concentration of 0.5 mg/mL. Supernatant was removed and crystals were fully dissolved in 40 mM HCl in 2-propanol. Plates were scanned on a microplate reader at 595 nm. The IC_{50} of **1** was 90 nM and **2** was 10 μM.

(8) The importance of carbohydrates in relation to the bioactivity of natural products has been reviewed elsewhere. See: Weymouth-Wilson, A. *Nat. Prod. Rep.* **1997**, *14*, 99−110.

(9) Galanis, M.; Mattoon, J. R.; Nagley, P. *FEBS Lett* **1989**, *249*, 333−336.