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Apoptolidins B and C: Isolation, Structure Determination, and Biological Activity

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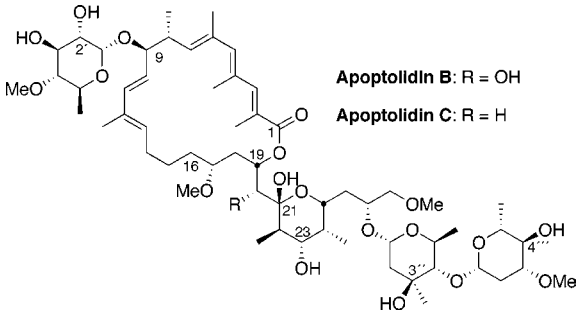
ABSTRACT



Apoptolidin (1) is a promising new therapeutic lead that exhibits remarkable selectivity against cancer cells relative to normal cells. We report the isolation, characterization, solution structure, stability, and biological activity of two new members of this family: apoptolidins B (2) and C (3). These new agents are found to have antiproliferative activity on par with or better than apoptolidin itself in an assay with H292 lung cancer cells.

Apoptolidin (**1**) is a structurally novel macrocyclic lactone that exhibits remarkably selective activity against certain cancer cells. First isolated from the soil bacteria *Nocardiopsis* sp. in 1997 by Seto and co-workers,[1] apoptolidin was shown by NMR to possess a highly unsaturated 20-membered ring core with hydropyranyl appendages attached at positions C-9 and C-19.[2] Of special significance in current efforts to achieve greater selectivity in cancer chemotherapy, apoptolidin shows no cytotoxicity against normal cells even at high concentrations (>1 mM) but induces apoptosis in E1A-transformed rat glia cells at nanomolar concentrations.[1] In studies at the National Cancer Institute, apoptolidin was found to be in the top 0.1% of the most selective agents screened in the NCI-60 cell line assay.[3a] By comparison to activity profiles of other compounds in this assay and through independent studies, apoptolidin was proposed to operate through inhibition of mitochondrial F_0F_1-ATPase.[3]

The promising therapeutic potential of apoptolidin and its exceptional selectivity have stimulated efforts to identify the structural basis for its activity and its mode of action. While total synthesis studies are making important progress toward these goals,[4−6] the ready availability of apoptolidin from its source organism (109 mg/L of fermentation medium) offers

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facile access to apoptolidin as well as to various derivatives through semisynthesis.

Implementation of this "top down" strategy in our laboratory has thus far led to a higher yielding isolation procedure, to the identification of isoapoptolidin (**4**),[7] an isomer of apoptolidin that forms during isolation and assay, and to the synthesis and biological evaluation of numerous semisynthetic analogues[8] which have uncovered new aspects of its mode of action. In the course of these studies, we have also identified several new apoptolidins. We describe herein the isolation, structural characterization, stability, solution structure, and biological evaluation of two new members of this family: apoptolidin B (**2**) and apoptolidin C **(3)**.

During our efforts to obtain apoptolidin A (**1**) (the "A" appended to differentiate members of this now expanding family) through fermentation of *Nocardiopsis* sp., we detected the existence of compounds similar in molecular weight to apoptolidin A but lacking one or two oxygen mass equivalents as determined by MS analysis of the crude ethyl acetate extract of the fermentation medium. While apoptolidin A can be obtained in an improved yield of 130 mg/L of medium in our studies, the newly discovered compounds were present in amounts of only 2−5 mg/L. Isolation of the two new apoptolidins was achieved using flash chromatography (silica gel) for initial purification followed by preparative RP-HPLC for final purification. Both compounds were less polar than apoptolidin A as indicated by TLC R_f values and RP-HPLC retention times.

The molecular formulas of these agents, named apoptolidin B (**2**) and apoptolidin C (**3**) due to their structural relationship to the parent compound, were determined to be $C_{58}H_{96}O_{20}$ [*m*/*z* 1135.6448 $(M + Na)^+$] and $C_{58}H_{96}O_{19}$ respectively [*m*/*z* 1119.6462 $(M + Na)^+$] by high-resolution mass spectrometric analysis of the purified samples. The primary structures of these new apoptolidins (Figure 1) were established by two-dimensional NMR experiments (COSY, TOCSY, HMQC, HMBC) in CD_3OD on a 600 MHz spectrometer. Proton and carbon NMR spectra show extensive similarities to apoptolidin A with respect to chemical shifts and coupling constants (Figure 2). While the overall spin systems for the macrolactone ring, the hemiketal moiety, and the three glycoside linkages remain unchanged according to COSY and HMBC experiments, differences were found in additional

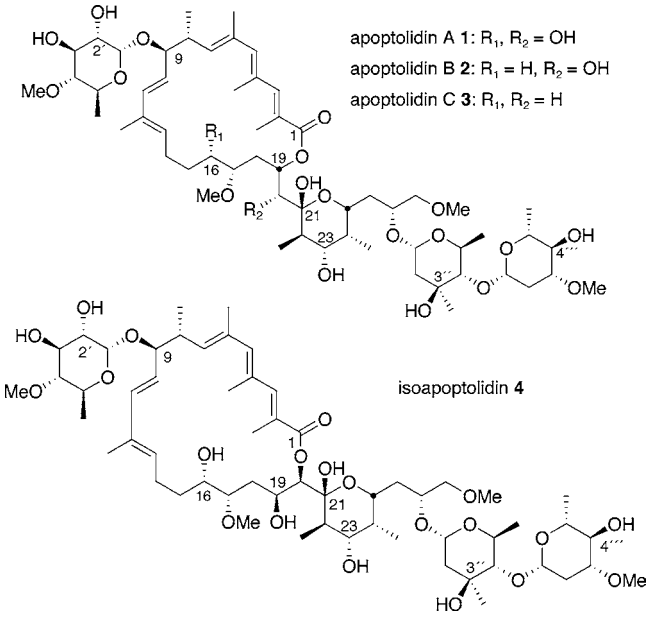

Figure 1. Apoptolidins A−C and isoapoptolidin.

methylene groups within the macrolactone structure. In particular, unlike apoptolidin A, apoptolidin B lacks a hydroxyl group at position C-16 and apoptolidin C lacks hydroxyl groups at positions C-16 and C-20. All three structures are consistent with a polyketide biosynthesis with additional oxygenation arising from monooxygenases.

The secondary structures of apoptolidins B and C were established with ROESY experiments and coupling constants in combination with Monte Carlo conformational search calculations using Macromodel 7.0. In both cases, strong ROESY cross-peaks between H-3 and H-5 as well as H-5 and H-7 indicate that the methyl groups of the triene portion point in the same direction as observed for apoptolidin A. The (*E*)-geometry of all five double bonds of the macrolactone is supported by additional ROESY correlations between H-9 and H-11 and between H-11 and H-13 as well as large vicinal coupling constants for H-11.

The chairlike structure of the hemiketal ring of apoptolidins B and C follows from strong ROESY correlations between 1,3-diaxial substituents (e.g., H-23/H-25 and H-22/Me-24), coupling constants (e.g., $J_{24-25} = 2$ Hz), and further ROE signals with protons outside the hemiketal ring (e.g., H-20 and H-19). The same hemiketal structure and very similar ^1H and ^{13}C chemical shifts were also found for apoptolidin A and isoapoptolidin.[2,7a]

The overall conformation of the macrolactone that includes a turn from C-7 to C-11 and a loop-type region for C-13 to C-16 was established by coupling constants (e.g., for H-9) and transannular ROESY cross-peaks (e.g., between H-7 and H-9 and between H-13 and H-16a). Furthermore, ROE contacts of H-3 with both H-17 and H-18a as well as between H-17 and H-19 indicate a zigzag-type conformation from C-16 to C-19. The stereochemistry of C-17 and C-19 is further supported by coupling constants within the C-17 to

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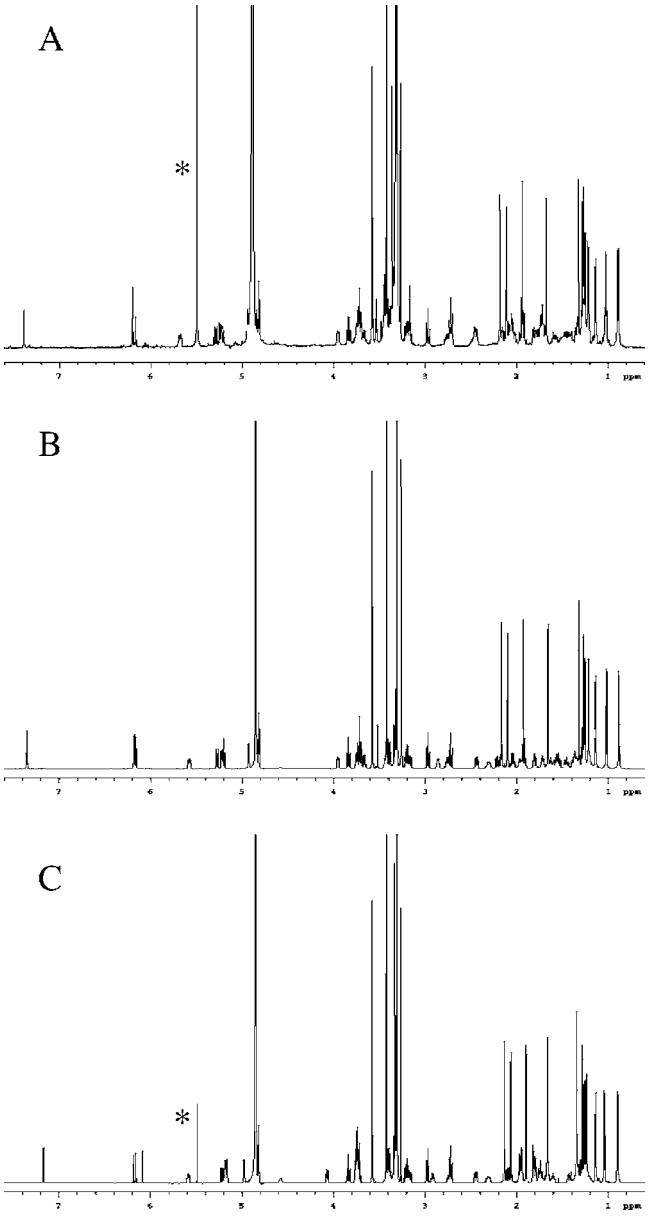

Figure 2. ^{1}H spectra of apoptolidins A (A), B (B), and C (C) (*residual DCM). Expanded ^{1}H and ^{13}C NMR spectra and tabulated data can be found in the Supporting Information.

C-20 moiety and ROESY correlations with hydrogens of the hemi-ketal. Superposition of the macrolactone structures of all three apoptolidin analogues reveals a nearly perfect fit with only minor differences in the C-13 to C-17 moiety due to the missing C-16 hydroxyl group. Finally, coupling constants, chemical shifts and ROE correlations indicate that the hexose structures and stereochemistry of the glycosidic bonds in apoptolidins B and C are the same as those found in apoptolidin A and isoapoptolidin A. Additionally, the C-9 and C-27 stereochemistry are confirmed by transannular ROESY cross-peaks (e.g. H-1′ and H-9, H-1″ and H-25).

The major difference between apoptolidin A/B and apoptolidin C is the conformation from C-19 to C-21. While in apoptolidins A and B the side chain from C-20 to C-28 is closely aligned with the triene moiety of the macrolactone (shown by ROEs, e.g., between Me-2 and H-27), the same side chain in apoptolidin C is flipped toward the methoxy group at C-17. Evidence for the side-chain conformation in apoptolidin C is given by several transannular ROESY correlations between the hemiketal and the C-17 to C-20 moiety (e.g., Me-24 and H-19, Me-24 and H-18b, Me-22 and H-19). In addition, the optical rotation changes quite drastically from +9° for apoptolidin B to −41° for apoptolidin C. The side-chain flip may be a result of the reduction in steric hindrance from removal of the C-20 hydroxyl group. A representative structure of apoptolidin C from the conformational search is depicted in Figure 3 and is fully consistent with data from the ROESY experiment and the coupling constants.

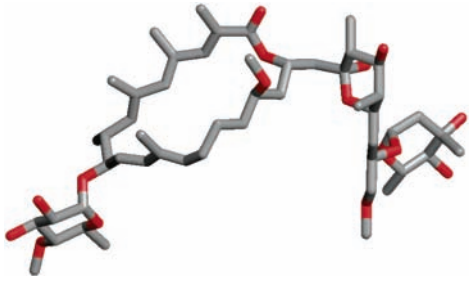

Figure 3. Representative solution structure of apoptolidin C (**3**). Oleandrose moiety and protons not shown.

In addition to expanding the family of naturally occurring apoptolidins, these new compounds are of great interest with respect to the goal of establishing the structural basis for apoptolidin's selectivity and mode of action and for advancing this therapeutic lead. Toward these ends, we were interested in determining the solution stabilities and activities of apoptolidins B and C. We had previously shown that apoptolidin A isomerizes to a new compound, isoapoptolidin, under cell assay conditions by an intramolecular transesterification from C-19 to C-20.[7a]

To determine the stability of apoptolidins B and C under assay conditions, each was evaluated as previously described for apoptolidin A.[7a] Solutions of **2** and **3** in Dulbecco's phosphate-buffered saline (pH 7.4) were prepared and incubated in sealed reaction vessels at 37 °C, and the disappearance of the agents was monitored by RP-HPLC at 254 nM with benzoic acid as an internal standard. The rate constants were obtained by fitting the data to the integrated rate expressions for a simple equilibrium (**2**) or for first-order decomposition (**3**) (Figure 4).

The kinetic data suggests that apoptolidin B forms an equilibrium mixture (6:4) with an isomer[9] similar to that observed for the equilibrium between apoptolidin A and isoapoptolidin A. In the latter case, the time to reach the equilibrium is about two times longer than with apoptolidin

(9) The isomer of apoptolidin B shows slightly lower activity in the H292 cancer cell assay. Structure determination is in progress.

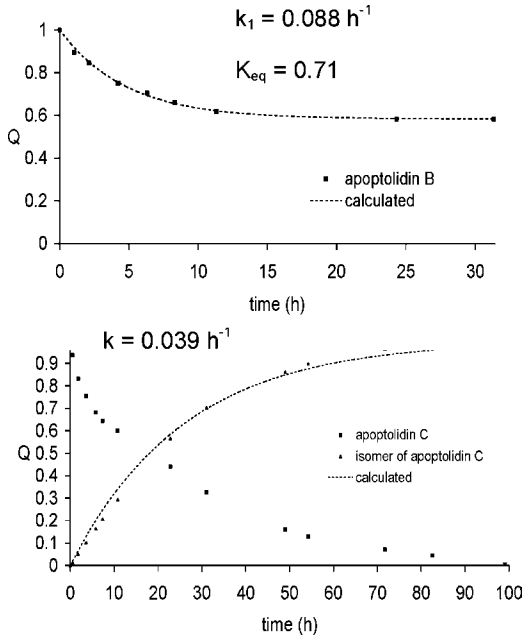

Figure 4. Stabilities of apoptolidins B (**2**) and C (**3**) in PBS buffer at pH 7.4 and 37 °C ($Q = [X]/[X]_{max}$).

B (ca. 20 and 10 h, respectively). This difference is also evident from the rate constants ($k_1 = 0.063$ h^{-1} for apoptolidin A, $k_1 = 0.088$ h^{-1} for apoptolidin B).

The similar stability of apoptolidins A and B is not surprising since the C-16 hydroxyl group is not directly required for isomerization. Interestingly, apoptolidin C shows a completely different chemical behavior. Under the above assay conditions, apoptolidin C isomerizes to a single new compound[10] in 4 days. The formation of the isomer of apoptolidin C follows first-order kinetics ($k = 0.039$ h^{-1}). After 2 days, approximately 15% of apoptolidin C is left in a test solution used for cell-based assays.

To determine the biological activities of apoptolidins A−C, a cell proliferation assay with H292 cancer cells (lung carcinoma) was used. The cells were treated in 96-well plates with different concentrations of the compounds (0.5 nM to 10 μM) for 48 h and stained with Calcein AM.[11] Data points for growth inhibition (GI_{50}) were measured in triplicate. Colchicine was used ($GI_{50} = 5-10$ nM) as a positive control.

The data from these assays (Table 1) indicate that neither the hydroxyl group at C-16 nor at C-20 is essential for activity in this assay. Furthermore, apoptolidin B, which lacks the C-16 hydroxyl group is more potent than apoptolidin A in our cell-based assay (7 nM) despite the slightly lower stability compared to apoptolidin A. This suggests that the C-16 hydroxyl group in apoptolidin A might interfere with binding which is also consistent with our observation that the C-16 acetate of apoptolidin A has reduced biological activity in a cell assay with E1A transformed rat glia cells.[12]

Table 1. Growth Inhibition Assay Results with H292 Cells

	GI_{50} (μM)
apoptolidin A (**1**)	0.032 ± 0.003
apoptolidin B (**2**)	0.007 ± 0.004
apoptolidin C (**3**)	0.024 ± 0.005

Apoptolidin C is less active than B but similar in potency to A. This might be partly due to the transformation of apoptolidin C under the assay conditions or to the conformational differences in the C-20 to C-28 side chain.

Apoptolidins B and C are important new lead structures for the treatment of cancer. Access to these new compounds and importantly their derivatives through semi-synthesis is proving to be of great value in efforts to elucidate the structural basis for the remarkable activity and especially the selectivity of the apoptolidins. Structural characterization of less abundant apoptolidins and their isomers is underway. Apoptolidin C is currently under investigation at the NCI in the NCI-60 cancer cell line assay. These results and protein profiling of these new agents in human primary cells, and related mode of action studies will be reported in due course.

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Supporting Information Available: Procedure for the H292 growth inhibition assay, HPLC methods for purification, and spectroscopic data for **2** and **3**, including ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HMQC, HMBC, ROE distance restraints, IR, and HRMS. This material is available free of charge via the Internet at http://pubs.acs.org.

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