Polyketides

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Fluorescent Probes of the Apoptolidins and their Utility in Cellular Localization Studies**

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Abstract: *Apoptolidin A has been described among the top 0.1 % most-cell-selective cytotoxic agents to be evaluated in the NCI 60 cell line panel. The molecular structure of apoptolidin A consists of a 20-membered macrolide with mono- and disaccharide moieties. In contrast to apoptolidin A, the aglycone (apoptolidinone) shows no cytotoxicity (>10 μM) when evaluated against several tumor cell lines. Apoptolidin H, the C27 deglycosylated analogue of apoptolidin A, displayed submicromolar activity against H292 lung carcinoma cells. Selective esterification of apoptolidins A and H with 5-azidopentanoic acid afforded azido-functionalized derivatives of potency equal to that of the parent macrolide. They also underwent strain-promoted alkyne–azido cycloaddition reactions to provide access to fluorescent and biotin-functionalized probes. Microscopy studies demonstrate apoptolidins A and H localize in the mitochondria of H292 human lung carcinoma cells.*

The apoptolidins are macrocylic natural products produced by an actinomycete (*Nocardiopsis* sp. FU40) soil microbe by way of a type I polyketide synthase biosynthetic pathway.[1] Apoptolidin A (**1**; Figure 1) was reported to induce cell death in E1A-transformed rat glia cells, a model cancer cell phenotype, while not affecting the growth of nontransformed glia cells.[2] The described selective cytotoxicity of apoptolidin A stimulated interest in its total synthesis and mechanism of induced cell death.[3] Salomon and Khosla employed a pharmacological approach to define the mechanism of cell death using LYas mouse lymphoma cells and concluded cell death proceeded by way of the mitochondria-mediated apoptotic pathway (intrinsic pathway).[4] The same investigators suggested F_OF_1 ATPase as a potential target although inhibition potency (K_i = 4–5 μM) against yeast F_OF_1 ATPase in a biochemical assay did not correlate well with observed cytotoxicity in cell culture (EC_{50} 0.2 μM), thus leaving open the possibility of alternative cellular targets.

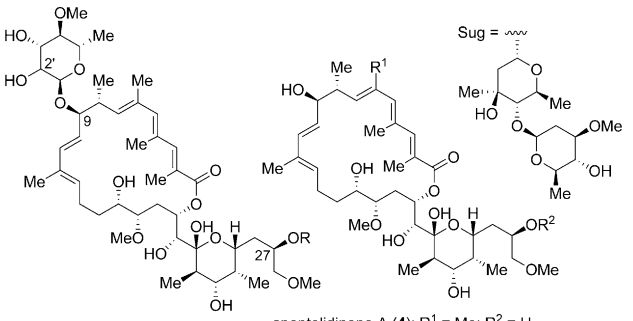

Figure 1. Members of the apoptolidin family of macrolides.

Following the reported isolation of apoptolidin A (**1**), other structural variants have been described either as minor microbial metabolites,[5] products of isomerization,[6] or semi-[7] and total synthesis (Figure 1).[8] When evaluated for cytotoxicity against tumor cells, these apoptolidins reveal considerable tolerance of structural modifications within the macrolide core including deoxygenation (apoptolidins B and C),[5c] demethylation (apoptolidin D),[5a] and C2-C3 double-bond isomerization (apoptolidin G)[6c] without significant loss of cytotoxicity (sub-micromolar).

In contrast to structural changes within the core macrolides, removal of the deoxy sugars resulted in complete loss of activity with EC_{50} values of apoptolidinones A (**4**) and C (**5**) reported to be greater than 10 μmol against several tumor cell lines in cell viability assays.[8c,9] The observed loss in activity upon exhausitive deglycosylation of the core macrolide presented an opportunity to develop a series of apoptolidin-derived probes to support studies on the mechanism of action. We report herein methods to access apoptolidins of varying states of glycosylation (tri-, di-, mono-, and nonglycosylated) and preliminary studies on their use as cellular probes.

Apoptolidin A (**1**) is readily obtained by fermentation of the actinomycete *Nocardiopsis* sp. FU40 with a production of 50–100 mg per liter.[2,10] We previously described the identification and expression of the apoptolidin gene cluster which

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provided an opportunity to access glycovariants of **1** by targeted gene deletion.[10] Three genes encoding for glycosyl transferases (*ApoGT1*, *ApoGT2*, and *ApoGT3*) were identified. To date, targeted gene deletion of *ApoGT2* by double crossover homologous recombination resulted in a *Nocardiopsis* variant producing a previously unreported glycovariant of **1**. In this case fermentation provided (50–100 mg per liter) a new apoptolidin analogue lacking the C27 disaccharide and was identified as apoptolidin H (**3**). The groups of Nicolaou[8a] and Koert[8b] prepared **3** by total synthesis and the group of Wender[5b] reported the isolation of a structurally related minor metabolite termed apoptolidin F (**2**; < 5 mg per liter) which was epimeric at C2′.[11]

Employing a standard cell viability assay using H292 human lung cancer cells, **1** induced cell-growth arrest without any indication of cell death. In this experiment, cells at about 20% confluency were treated with **1** and after 48 hours assayed for cell viability. Even treatment of cells with **1** for as long as 5 days resulted in only the observed antiproliferative effect but no loss of cell integrity. In contrast, cells grown to high confluency (ca. 70%) prior to treatment with **1**, resulted in greater than 95% cell death after 4 days with a calculated EC_{50} value of 20–30 nM. To standardize this assay, cells were systematically plated in a 96-well format (10, 15, 20, and 25 thousand cells per well), allowed to attach (16 h), treated with **1**, and assayed for viability after 4 days. As shown in Figure 2A, 25000 cells per well resulted in a reproducible cytotoxic effect (EC_{50} 16 nM) against human lung (H292) as well as several other tumor cell lines (HCT116 colorectal, MDB MB321 breast, 1483 head and neck squamous). The results summarized in Figure 2A also illustrate the antiproliferative activity of apoptolidin A (EC_{50} < 100 nM) against lower confluency cells (10–20 K cells). In separate experiments we observed that the cytotoxicity of apoptolidin A is potentiated by using cell culture media formulations of increasingly reduced glucose (Figure 2B). Notably, such nutrient starvation conditions have been proposed to mimic poorly vascularized cells seen in solid tumors.[12] We hypothesize that high and low confluency cells differ in metabolic flux with low confluency cells primarily utilizing the Embden-Meyerhof glycolytic pathway (Warburg effect) and high-density cells using the more energetic oxidative phosphorylation (OXPHOS) manifold.[13] These results are in agreement with the results of Salomon and co-workers who demonstrated glycolytic (apoptolidin unresponsive) cells were sensitized to apoptolidin by the addition of 2-deoxyglucose or oxamate, small molecules known to channel carbon flux from the Embden-Meyerhof to OXPHOS pathway.[4a]

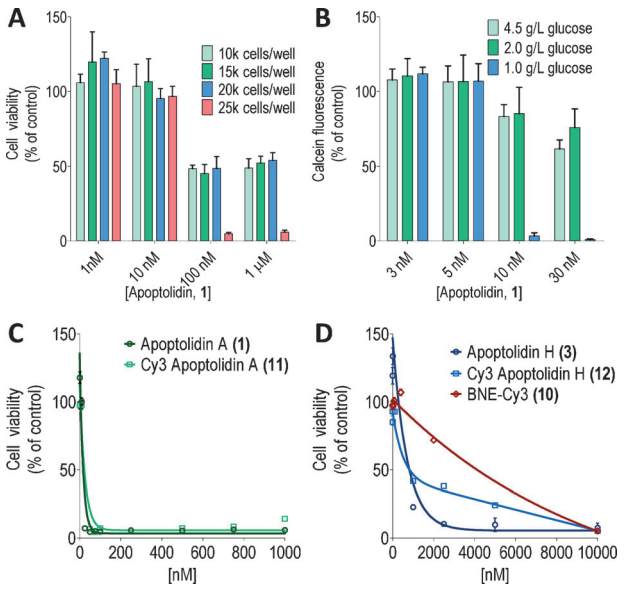

Figure 2. Analysis of apoptolidin toxicity in H292 cell line. A) Dependence of cytotoxic and cytostatic effects of **1** on cell confluency as measured by initial number of cells per well on a 96 well plate. B) Effect of cell growth media of varying glucose concentrations on **1** cytotoxicity. C) Cell viability assay for **1** and **11**. D) Cell viability assay for **3**, **12**, and **10** evaluated using optimized conditions.

The macrolactones apoptolidinone A (**4**) and D (**5**), which are devoid of all three deoxy sugars common to **1**, were evaluated under the apoptolidin A-sensitive high confluency H292 cell culture conditions and shown to be neither cytostatic or cytotoxic at concentrations of apoptolidinones to 10 μM.[8c] However apoptolidin H (**3**), bearing the C9 deoxy sugar, was cytotoxic against H292 cells under the same conditions with an EC_{50} value of 810 nM (ca. 50 times less potent then apoptolidin A) and apoptolidinone D disaccharide (**6**),[8c] lacking the C9 deoxysugar, demonstrated an EC_{50} value of 200 nM against the same cell line. Thus mono- or diglycoslyated variants of apoptolidin are sufficient to restore partial cell cytotoxicity. Finally, we note when assayed against yeast-derived F_OF_1 ATPase, **1** and **3** showed modest and comparable inhibition with K_i values of 4.9 and 13.7 μM, respectively, thus suggesting that the pharmacological importance of the C27 disaccharide is in large part decoupled from the observed activity of apotolidins against F_OF_1 ATPase.

To initiate studies on chemical probes we required the introduction of a azido functional group within the apoptolidin core to enable conjugation to either fluorescent or affinity tags using strain-promoted alkyne–azido cycloaddition (SPAAC) chemistry.[14] To this end we took advantage of a report by the Wender group describing the selective benzolylation of the C2′ hydroxy group of the C9 sugar.[7a] And were pleased to observe selective acylation of the C2′ hydroxy group of **1** and **3** using 5-azidopentanoic acid to afford the azido derivatives **7** and **8**, respectively, in 30–40% yield (Figure 3). Importantly, when evaluated in the cell viability assay, the azido analogues **13** and **14** maintained activity comparable to that of their parent substrates (EC_{50} 19 and 350 nM, respectively). As partner alkyne tags we selected the cyanine dye biotin (**9**) and Cy-3 (**10**) PEG-tethered to click-ready bicycle[6.1.0]nonynes.[15] Coupling of BNE-Cy-3 (**10**) with azido apoptolidins **7** and **8** proceeded smoothly in methanol at room temperature (4 h) to give fluorescently labeled apoptolidins **11** (39%) and **12** (32%), respectively. In addition biotin-BNE (**9**) reacted with **1** under identical reaction conditions to give biotinylated apoptolidin A **13** in 29% yield. Supporting their potential utility in functional imaging studies, the conjugates **11**–**13** also maintained activity relative to their parent macrolides (**11**: EC_{50} 22 nM and **12**:

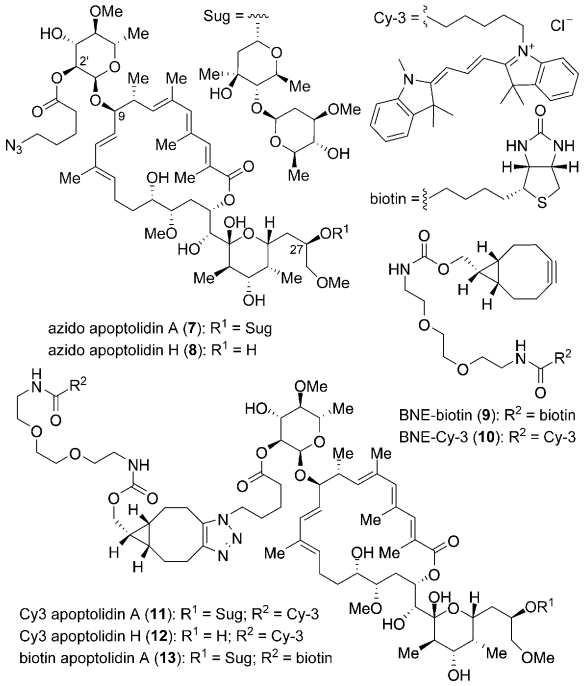

Figure 3. Azido apoptolidins, strained alkyne tagging reagents, and derived probes.

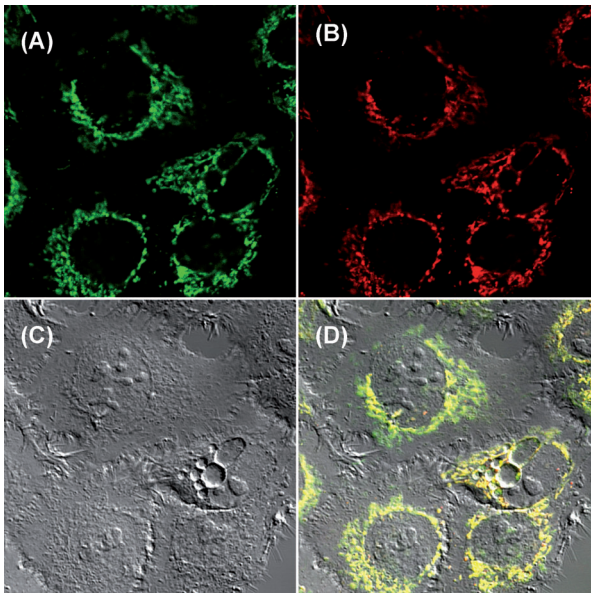

Figure 4. Co-staining Cy3 apoptolidin A and MitoTracker in H292 cells. Fluorescence images of MitoTracker (A), **11** (B), DIC image (C), and merged image (D) are shown.

EC_{50} 820 nM, Figure 2C and D) when evaluated in the H292 cell assay, although the activity of **13** fell to an EC_{50} value of 151 nM.

Confocal microscopy studies were conducted with the Cy-3 apoptolidin conjugates **11** and **12** at concentrations of 200 nM applied to H292 human lung cancer cells. In these experiments compound treatment for 15 minutes was followed by a 60 minute washout to dilute nonspecific binding. Cellular images from experiments using **11** are shown in Figure 4. Staining of washed cells with Mitotracker Green FM (Figure 4A) was conducted to evaluate whether **11** (Figure 4B) was localized within the mitochondria. Inspection of the merged image (Figure 4D) confirmed colocalization of **11** with the Mitotracker stain. Colocalization was further quantified by Costes' analysis which showed excellent overlap with a Pearson's coefficient of 0.89. An identical set of experiments using **12** also demonstrated localization of **12** within the mitochondria.

We do note cationic dyes such as cyanine-3 tend to localize in the mitochondria,[16] and bicyclononyne BNE-Cy-3 (**10**) localizes in the mitochondria but is not cytotoxic against H292 cells (EC_{50} 4.6 μM) in the standard cell viability assay (see Figure 2D). Significantly, regardless of the mechanism enabling localization of the apoptolidin A analogues, mitochondrial localization did not reduce their activity. To more effectively judge whether the bioactivity enabled by glycosylation of the apoptolidins is due to enabling localization within the mitochondria we are now in the process of examining noncationic dyes conjugated to apoptolidins and the nontoxic aglycone (apoptolidinone) in microscopy experiments.

The cell cytotoxicity and observed mitochondrial localization of Cy-3 conjugates **11** and **12** in human lung cancer cells H292 supports earlier conclusions that the apoptolidins act on a mitochondrial target. Further progress on defining the role of sugars to imparting activity against tumor cells and identification of cellular targets of the apoptolidins will be reported in due course.

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Communications

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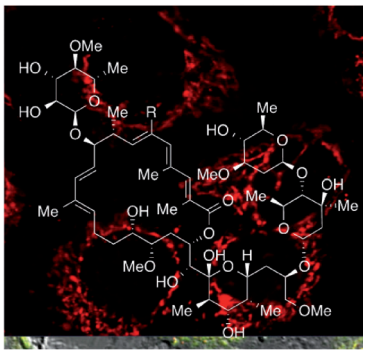

Pop in: Apoptolidins have been described among the top 0.1% most-cell-selective cytotoxic agents to be evaluated in the NCI 60 cell line panel. Combined methods of genetic deletion, to manipulate the biosynthetic machinery, and chemical synthesis provides access to new fluorescent derivatives of the apoptolidins for biological study.