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# Discovery of Incednine as a Potent Modulator of the Anti-apoptotic Function of Bcl-xL from Microbial Origin

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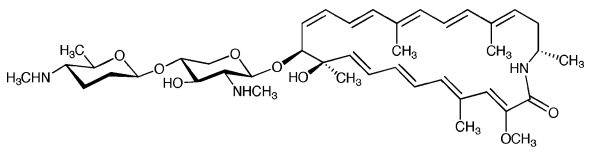
Anti-apoptotic oncoproteins Bcl-2 and Bcl-xL are overexpressed in many cancers,[1] resulting in the expansion of a transformed population and the advancement of the multidrug-resistant stage. Consequently, Bcl-2/Bcl-xL have stood out among molecular targets in oncology, and the functional blockade of these proteins will be an aid to novel anti-tumor therapies. Since these proteins are known to show an anti-apoptotic effect partly through forming a heterodimer with pro-apoptotic Bcl-2 members, such as Bax and Bak,[2] several researchers have rationally designed and synthesized compounds that target their binding pocket and have reported several compounds such as HA14-1 and ABT-737 as Bcl-2/Bcl-xL inhibitors.[3] Currently, several lines of evidence indicate that Bcl-2/Bcl-xL clearly have other functions related to their abilities to interact physically with many other proteins; however, the underlying mechanisms for the regulation of apoptosis by Bcl-2/Bcl-xL through interacting with such proteins still remain unclear.[4] For further understanding of the regulation of apoptosis by Bcl-2/Bcl-xL, the development of a new class of chemical tools is required. Cell-based chemical−genetic screens have been used to help discover small, cell-permeable bioactive molecules that induce phenotypic changes, and through subsequent identification of their target proteins, they can contribute to reveal the molecular basis of biological processes. Therefore, we constructed a cell-based chemical−genetic screening system to discover small molecules that induce apoptosis in Bcl-xL-overexpressing human small cell lung carcinoma Ms-1 cells when combined with anti-tumor drugs. In the course of our screening, we isolated a structurally and functionally unique compound, named *incednine* (**1**), from the culture broth of *Streptomyces* sp. ML694-90F3. Here, we describe the isolation, structure elucidation, and biological activities of **1**.



Incednine (**1**)

Incednine (**1**) was obtained (18.9 mg/L) as a pale-yellow powder from the cultured broth of the producing strain by centrifugal liquid−liquid partition chromatography for two reasons: (1) this compound decomposes easily under acidic conditions or when exposed to light, and (2) the isolation using a solid carrier such as silica gel was inefficient. The molecular formula of **1** was found to be $C_{42}H_{63}N_3O_8$ by HRESIMS. The characteristic UV absorption ($\lambda_{max}$ = 294.5, 309.5, 322.5, 356.0 nm) was indicative a polyene moiety. The positive color reaction to GL reagent, negative reaction to ninhydrin, and the typical IR absorption at 1650, 1510 $cm^{-1}$ suggested the presence of an amido group.

The assignable NMR spectra were obtained when the sample was dissolved in $CD_3OH/H_2O$ (3:1) and measured at −5 °C. The $^{13}C$ NMR and DEPT spectra revealed that **1** contained 42 carbons, including one carbonyl, four quaternary $sp^2$, one quaternary $sp^3$, fourteen $sp^2$ methines, nine $sp^3$ methines, four methylenes, and nine methyl carbons. One carbonyl signal and eighteen $sp^2$ carbons require the presence of three rings from the unsaturation numbers of the formula of **1**.

Partial structures of **1** were determined by $^1H-^1H$ COSY, TOCSY, and HMBC (Figure 1). A detailed analysis of the COSY spectrum of **1** revealed five partial proton−proton connectivities: (a) H-5 to H-9 and methyl group H-24, (b) from H-11 to H-13, (c) from H-21 to NH and methyl group H-27 and H-28 and (A) from H-1′ to H-5′, and (B) H-1″ to H-6″. The linkage of the partial structures between (a) and (b) was established by HMBC correlations from H-25 to C-9, C-10, and C-11, which were bridged by the quaternary oxygenated carbon of C-10. The overlapping olefin region ($\delta$ 6.1−6.3) was assigned by the following experiments. The connectivities from H-13 to H-15, H-17 to H-19 in TOCSY spectra and those from H-26 to C-15, C-16, and C-17 and from H-27 to C-19, C-20, and C-21 in HMBC clarified the linkage of the partial structures (b) and (c) to a pentaene structure. The correlations from the NH proton in the partial structure (c) to C-1, from H-3 to C-1 and C-2, from H-24 to C-3, C-4, and C-5, and from H-29 to C-2 constructed a 24-membered macrocyclic lactam ring. The remaining partial structures (A) and (B) were deduced from HMBC experiments. The correlations from H-5′ to C-1′, from H-5″ to C-1″, and from the *N*-methyl group of H-6′ to C-2′ and H-7″ to C-4″ established the presence of two types of aminosugars: a pentopyranose (A) and a hexopyranose (B), respectively. Furthermore, the HMBC correlations from H-1′ to C-11 and from H-1″ to C-4′ revealed the planar structure of **1**: the linkage of aminosugar (A) and the macrocyclic lactam ring at C-11 and the glycoside linkage of C-1″ of aminosugar (B) and C-4′ of aminosugar (A).

The stereochemistry of incednine aglycon was elucidated as follows. The geometry of the double bonds was determined by the proton coupling constants and NOEs (Figure S8). The coupling constants of $J_{6,7}$ = 14.4 Hz, $J_{8,9}$ = 15.8 Hz, $J_{12,13}$ = 9.0 Hz revealed 6*E*, 8*E*, and 12*Z*, respectively. NOEs observed between H-24 and H-29, H-6 and H-24, H-18 and H-26, H-18 and H-27, and H-19 and H-21 showed 2*Z*, 4*E*, 16*E*, 18*E*, and 20*E*, respectively. The geometry of H-14 was inferred from $^1H-^1H$ *J*-resolved spectroscopy to be 14*E* ($J_{14,15}$ = 14.0 Hz, Figure S9). The relative

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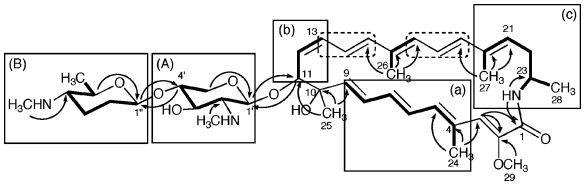


***Figure 1.*** Gross structure of **1** determined by 2D-NMR spectra (bold lines, COSY; arrows, selected HMBC correlations; dashed squares, TOCSY connectivities; single squares, partial structures labeled as indicated).

stereochemistry of three chiral centers was established by NOE experiments and computations. The geometry of double bonds in aglycon and NOEs observed surrounding 4, 16, and 20-methyl groups allowed four types of the ring conformers. The coupling constant of $J_{11,12} = 9.0$ Hz and NOEs observed between H-8 and H-25, H-25 and H-11, and H-11 and H-14 indicated the relative stereochemistry of C-10 and C-11 to be *R** and *S**, respectively. The remaining asymmetric center, C-23, was elucidated by NOEs observed surrounding the C-23 position to be *S**, which was supported by Discovery III programs (Figure S10).

The absolute configuration of the C-11 position of the aglycon (**2**)[5] was determined by the application of a modified Mosher's method.[6] Both the (±)-MTPA esters (**3**, **4**) of **2** were prepared and subjected to $^1$H NMR analysis. The $\Delta\delta$ values of the protons allowed the absolute configuration at C-11 to be *S*; thus, the stereochemistry of the three asymmetric centers of the aglycon was 10*R*, 11*S*, and 23*S* (Figure S12).

The stereochemistry for two aminosugars was deduced as follows. Large coupling constants of $J_{1',2'ax} = 7.0$ Hz, $J_{2',3'} = 7.0$ Hz, $J_{3',4'} = 8.0$ Hz, $J_{4',5'ax} = 8.0$ Hz, $J_{1'',2''ax} = 9.1$ Hz, and $J_{4'',5''} = 9.2$ Hz indicated that the connected carbon, nitrogen, and oxygen atoms were all equatorial. Moreover, the absolute stereochemistry of H-1′ was elucidated to be *S* by NOEs observed between H-1′ and H-11, H-1′ and H-25, and H-6′ and H-25 (Figure S13). Thus, the aminosugar (A) was 2-deoxy-2-methylamino-$\beta$-D-xylopyranose.[7] To determine the only remaining absolute stereochemistry, the 4-*N*-4-bromobenzoyl derivative of the aminosugar (B) was prepared. Methanolysis of the 4″-*N*-4-bromobenzoyl derivative of **1**[8] gave the corresponding $\alpha$ and $\beta$ (**5**) methyl glycoside. Compound **5** was recrystallized with hexane/acetone to give a colorless platelet.[9] X-ray crystallography for **5** elucidated the aminosugar (B) as 2,3,4,6-tetradeoxy-4-methylamino-$\beta$-D-*erythro*-hexopyranose (*N*-monodemethyl-D-forosamine, Figure S14).[10] Thus, incednine (**1**) consists of a novel skeletal structure, enol-ether amide in the 24-membered macrolactam core, with two aminosugars.

Incednine was tested for its suppressive activity against the anti-apoptotic function of Bcl-2/Bcl-xL. Bcl-xL-overexpressing Ms-1 cells displayed resistance to various types of anti-tumor agents, such as adriamycin (Figure S15). However, this resistance was overcome by the sequential combination of anti-tumor agents and 100 nM incednine, whereas incednine alone did not induce apoptosis in these cells (Figure 2, S16). The same results were obtained when Bcl-2-overexpressing cells were used (data not shown). These findings suggest that incednine significantly sensitizes Bcl-2/Bcl-xL-overexpressing cells to chemotherapeutic treatment most likely through dysfunction of Bcl-2/Bcl-xL.

Most anti-tumor agents reportedly induce apoptosis through activation of pro-apoptotic Bcl-2 family proteins such as Bax or Bak.[11] Indeed, overexpression of either Bax or Bak induced

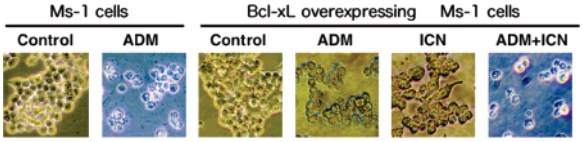


***Figure 2.*** Incednine overcame the anti-apoptotic function of Bcl-xL against adriamycin-induced apoptosis. Cells were treated with drugs as indicated (ADM, adriamycin; 0.3 $\mu$g/mL, ICN, incedenine; 100 nM). After 48 h, cells were observed under a phase-contrast microscope.

apoptosis in HEK293T cells, and it was inhibited by coexpression of Bcl-xL (Figure S17). Upon treatment with 60 nM of incednine, HEK293T cells overexpressing both Bax/Bak and Bcl-xL underwent apoptosis, indicating that incednine restored the pro-apoptotic ability of Bax or Bak that was inhibited by Bcl-xL. However, incednine did not inhibit the binding capacity of Bcl-xL to Bax (Figure S18). In addition, incednine did not decrease the expression levels of Bcl-xL (data not shown). These results clearly suggest that incednine can inactivate the anti-apoptotic function of Bcl-2/Bcl-xL by a distinct mode of action from other inhibitors that recognize the surface pocket of Bcl-2/Bcl-xL.

In conclusion, we isolated a structurally unique compound, incednine, as a modulator of Bcl-xL function through a cell-based chemical−genetic screening from microbial origin. Because this compound inhibits the anti-apoptotic function of Bcl-2/Bcl-xL without affecting its binding to pro-apoptotic Bcl-2 family proteins, it may target other proteins associated with the Bcl-2/Bcl-xL-regulated apoptotic pathway. Therefore, incednine may be a useful tool for further study of Bcl-xL function, and the identification of its target protein could provide a new insight into the anti-apoptotic mechanism of Bcl-2 family proteins.

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**Supporting Information Available:** Experimental procedures, NMR spectra data, crystallographic information, and results of Bcl-xL inhibitory activities for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Crystal data for **5**: $C_{15}H_{20}NO_3Br$, $M_r = 342.23$, monoclinic, $P2_1$, $a = 8.41004$ (15) Å, $b = 10.48964$ (19) Å, $c = 9.31450$ (17) Å, $\beta = 107.8149$ (8)°, $V = 782.31$ (2) Å$^3$, $Z = 2$, $T = 296$ K, R1 = 0.0362, GOF = 1.205. See details in Supporting Information.
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