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Interaction of kendomycin and semi-synthetic analogues with the anti-apoptotic protein Bcl-xl

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ABSTRACT

The cytotoxic macrolide kendomycin was identified as a ligand of Bcl-xl, an anti-apoptotic member of the Bcl-2 protein family. Hydrolysis-stable and protonable semi-synthetic analogues have been obtained that retain cytotoxicity and Bcl-xl binding.

The Bcl-2 homology (BH) family of proteins, encompassing both pro- and anti-apoptotic members, plays a crucial role in the regulation of cell death.[1] Cancer cells with an elevated level of Bcl-2 expression show a broad resistance to cytotoxic agents. A correlation of expression of Bcl-xl, a prominent anti-apoptotic member of this family, with the sensitivity of 60 cell lines in the National Cancer Institute anti-cancer screen to chemotherapeutics has been reported.[2] Small-molecule inhibitors of Bcl-xl function have been discovered from diverse structural classes using both rational drug design as well as high-throughput screening (HTS) approaches.[3] Pre-clinical studies with small-molecule inhibitors indicate that therapies targeted at these apoptotic pathways could be effective anti-cancer treatments[4] and a synthetic inhibitor of the Bcl-2 family has recently entered clinical development.[5,6]

Natural products cover a molecular diversity that is often complementary to that found in synthetic libraries.[7] Their undisputable success rate in drug research is reflected by the large number of natural products and their semi-synthetic analogues in clinical development.[8] In order to identify natural compounds that disrupt the interaction between Bcl-xl and the docking site of the Bak peptide, one of the pro-apoptotic members from the Bcl-2 protein family, we have undertaken a large-scale HTS screening campaign of 124,535 natural product extracts using a fluorescence polarization (FP) assay measuring displacement of a fluorescently labeled Bak peptide from a GST–Bcl-xl fusion protein.[9] The biological activity of a benzophenanthridine alkaloid—chelerythrine—has been reported by us previously.[9] Here, we report the inhibitory activity of kendomycin (**1**), a structurally novel macrocyclic polyketide produced by several species of Streptomyces. The compound was originally described in the patent literature as a potent endothelin receptor antagonist[10,11] and anti-osteoporotic compound.[12] The absolute stereochemistry has been determined and anti-bacterial activity (e.g., MIC = 3.9 μM against the methicillin-resistant *Staphylococcus aureus* strain STA MU50) as well as cytotoxic activity (e.g., GI_{50} < 0.1 μM against HEP G2) has been described previously.[13] Fueled by the interest as a possible lead structure, advances have been made towards revealing its mode of action. Proteasome inhibition has been proposed as a contributing factor in the cytotoxicity against leukemic monocyte lymphoma (U-937) cells.[14] Additionally, the same authors describe an activation of caspase 8 (an enzyme involved in apoptosis) after treatment of U-937 with kendomycin.

In the course of our investigation we noticed instability of kendomycin in aqueous solvents and methanol. There is evidence for a nucleophilic attack on the C-20 followed by ring-opening since the purple quinone **2** could be identified as the primary degradation product (Scheme 1). Stability studies were undertaken at 37 °C in

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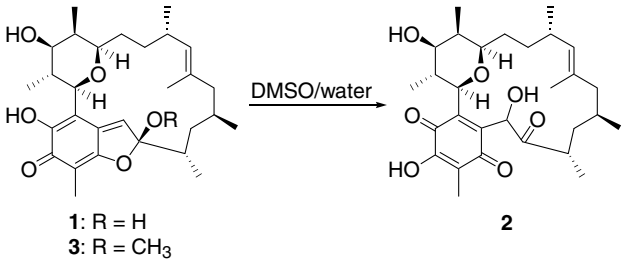


Scheme 1. Hydrolysis of the hemiketal group in kendomycin (**1**).

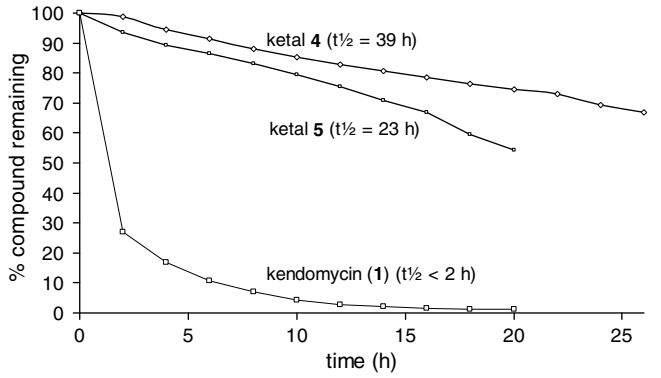


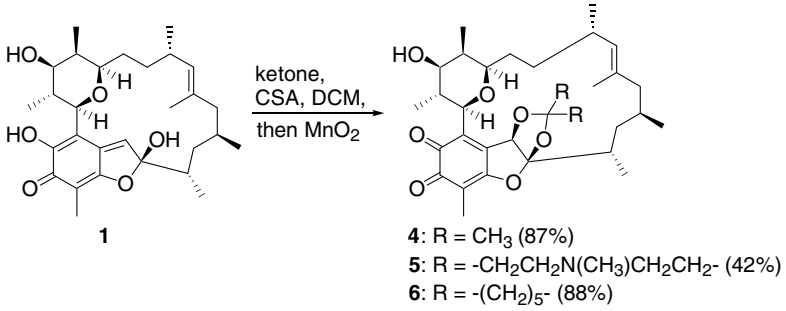
Figure 1. Stability of kendomycin (**1**), ketal **4** and ketal **5** in 12.5% aq DMSO at 37 °C. The amount of compound remaining was determined by HPLC on a Waters XTerra MS C18 column and is based on the UV absorption at 310, 235, and 265 nm, respectively (gradient: 15 min, 25–100% acetonitrile with 0.1% formic acid in the eluents). $t_{½}$ for **4** and **5** were extrapolated.

a 12.5% aqueous DMSO solution because of the insolubility of kendomycin in pure water. The half-life of kendomycin was determined to be less than 2 h under these conditions (Fig. 1).

To address the problem of instability, analogues were prepared in which the hemi-ketal functionality was converted to a ketal. Thus, methyl ketal **3** was prepared according to a previously described procedure.[11] The formation of the acetonide **4** has also been reported earlier.[13] Employing an improved two-step procedure, compound **4** could be obtained in 87% yield. The ketal formation was facilitated using (±)-camphor-10-sulfonic acid followed by oxidation of the intermediate catechol to the *ortho*-quinone with manganese oxide (Scheme 2). Likewise, analogue **5** was prepared from *N*-methyl-4-piperidone, as was analogue **6** from cyclohexanone.[15] The scope of this reaction appears to be quite limited since five-membered cyclic ketones (e.g., cyclopentanone) as well as some non-cyclic ketones (e.g., diethanolamine) failed to yield the corresponding ketals. As anticipated, the stability of both tested diketals, compounds **4** and **5**, showed great improvement compared to kendomycin with estimated half-lives of 39 and 23 h, respectively.

Kendomycin (**1**) inhibited Bak–Bcl-xl interaction with an IC_{50} of 12.3 μM (Table 1) when tested in the Bak–GST–Bcl-xl FP assay. Hydrolysis to **2** or introduction of a methyl group at the hydroxyl group in position 19 in compound **3**, led to greatly reduced activity. On the other hand, conversion of the aromatic ring into an *ortho*-quinone system, which constitutes a relatively complex structural change, was tolerated. Acetonide **4** and the piperidone ketal **5** both inhibited Bak–Bcl-xl interaction (IC_{50} = 9.5 and 5.0 μM, respectively). Surprisingly, the structurally very similar cyclohexyl derivative **6** was inactive in this assay.

It should be noted that kendomycin and the piperidine derivative **5** only achieve a maximal effect (E_{max}) in the FP assay of 70% and 66%, respectively. An E_{max} of only 22% has even been found for the acetonide **4**. This finding may indicate that these compounds bind to an allosteric site on Bcl-xl thereby altering the peptide binding affinity but without causing its complete displacement. To confirm binding of compounds to Bcl-xl, surface plasmon resonance (SPR) studies were performed with immobilised protein using the Biacore system, as described.[16,17] Experiments were performed with both the GST fusion protein, used in the FP assay, as well as native Bcl-xl protein, without the GST tag



Scheme 2. Synthesis of diketals **4**–**6** from kendomycin (**1**). Yields are given in parenthesis.

Table 1
Effects of compounds on Bcl-xl inhibition, A549 cytotoxicity and direct measurement of Bcl-xl binding using SPR

Compounds	Bcl-xl inhibition		Cytotoxicity[a] (A549)	SPR by Biacore	
	IC_{50} (μM)[a]	E_{max} (%)[a]	IC_{50} (μM)	GST–Bcl-xl K_d (M)	Bcl-xl K_d (M)
1	12.3	70	16	$5.28e^{-4}$	$1.83e^{-3}$
2	230	35	25	$1.08e^{-2}$	nb
3	na	—	9	—	—
4	9.5	22	14	—	—
5	5.0	66	14	$3.77e^{-4}$	$3.99e^{-4}$
6	na	—	24	—	—
Gossypol	—	—	—	$1.71e^{-6}$	$3.38e^{-4}$

[a] Values are means of three experiments (na, not active; nb, no binding).

(Table 1). These results confirmed binding of **1** and **5** to the GST–Bcl-xl protein, with much weaker binding observed for the hydrolyzed molecule **2**, in accordance with the FP results. However, binding to the native protein was weaker for **1**, indicating that the addition of the GST moiety may contribute to the observed in vitro interaction between **1** and Bcl-xl. This was also the case for the known Bcl-xl inhibitor gossypol[18], which showed better binding to the GST-tagged protein (Table 1). Interestingly, **5** bound with equal affinity to both the GST-fused and native protein with a K_d equivalent to gossypol. These studies emphasize the importance of confirming preliminary screening data by means of a direct orthogonal assay of compound–target interaction.

Kendomycin and all analogues tested exhibited a cytotoxic effect in the same range (IC_{50} = 9–25 μM) against A549 cells. Hence, there is no apparent evidence for a correlation between in vitro inhibition of Bcl-xl and cytotoxicity. This finding implies that an alternative mechanism is responsible for kendomycin-mediated cell killing in A549 cells and remains to be explored.

In summary, we have shown that kendomycin disrupts the protein–protein interaction between Bcl-xl and Bak, although the affinity for native protein, as measured using Biacore, appears to be weak. Two active semi-synthetic analogues of kendomycin, with clearly improved stability in aqueous media, have been synthesized which may be useful for exploring the mechanism of action of this series.

Acknowledgments

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15. Standard procedure for the diketal formation, synthesis of the cyclohexanone ketal **6**: A solution of 20 mg (0.041 mmol) kendomycin and 424 μl (4.10 mmol) cyclohexanone in 1.5 mL DCM was stirred with 48 mg (0.21 mmol) (±)-camphor-10-sulfonic acid at room temperature for 18 h. To this solution, 30 mg (0.35 mmol) manganese dioxide was added and stirring continued for another 30 min. The mixture was filtered through a pad of Celite and the volatiles were removed in vacuo. The residue was purified by flash chromatography (DCM/MeOH 100:1) to yield 21 mg (0.036 mmol, 88%) of a red foam. TLC (DCM/MeOH = 20:1), R_f 0.60. ^{1}H NMR ($CDCl_3$): 5.92 (1H, s, 20-H), 4.64 (1H, d, *J* = 9.7 Hz, 13-H), 4.27 (1H, d, *J* = 10.2 Hz, 5-H), 3.54 (1H, dd, *J* = 10.5 and 4.6 Hz, 7-H), 3.43 (1H, dd, *J* = 9.4 and 1.9 Hz, 9-H), 2.47 (1H, m, 18-H), 2.32 (1H, m, 12-H), 2.16 (1H, d, *J* = 17.3, 11^b-H), 1.99 (1H, m, 16-H), 1.89 (3H, s, 2-CH_3), 1.80–1.88 (2H, m, 6-H, 8-H), 1.49–1.72 (11H, m, 10^b-H, 11^a-H, 15^b-H, 17-CH_2, 6× cyclohexyl-H), 1.60 (3H, s, 14-CH_3), 1.18–1.48 (6H, m, 10-H, 15-H, 4× cyclohexyl-H), 0.95 (3H, d, *J* = 7.0 Hz, 16-CH_3), 0.94 (3H, d, *J* = 7.2 Hz, 8-CH_3), 0.90 (3H, d, *J* = 6.6 Hz, 12-CH_3), 0.86 (3H, d, *J* = 7.0 Hz, 18-CH_3), 0.82 (3H, d, *J* = 6.5 Hz, 6-CH_3). HRMS ($C_{35}H_{51}O_7$) $[M+H]^+$: found *m/z* 583.3620, calcd *m/z* 583.3629.
16. Surface plasmon resonance (SPR) by Biacore—interactions between a protein immobilized on a biosensor chip and compound flowed over the surface are monitored as a change in SPR in resonance units (RU). GST–Bcl-xl and Bcl-xl were immobilized onto CM5 sensorchip by using amine-coupling chemistry.[17] Kendomycin and its analogues at concentrations of 6.25, 12.5, 25, 50, and 100 μM were injected into the sensorchip at 20 μl/min. 2% DMSO were used as a blank. A typical sample injection cycle consisted of a 150-s sample injection and 300-s of buffer flow (dissociation phase). Residual bound compounds were desorbed with 100 mM HCl, followed by two washes with running buffer. Binding kinetics was obtained using BIA evaluation software. Steady state affinity (K_d) was obtained using the 1:1 Langmuir binding model ($R_{eq} = (C.R_{max})/(K_d + Cn)$), where *C* is the analyte concentration, R_{max} the theoretical binding capacity, and *n* is the number of binding sites of the ligand. K_d is calculated using the equation $K_d = k_d/k_a$ (association rate constant k_a [M^{-1} s^{-1}]; dissociation rate constant k_d [s^{-1}]).
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