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Original article

A new assay for the discovery of Bcl-XL inhibitors

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Abstract

The Bcl-2 family of antiapoptotic proteins is commonly over expressed in many types of human cancer and remains one of the few validated targets. Antiapoptotic family proteins such as Bcl-2 and Bcl-XL function, at least in part, by binding proapoptotic members such as Bax and Bak and thereby prevent release of the apoptotic cascade of events. "BH3-only" members of the family disrupt this interaction by binding, via their BH3 domain, to a hydrophobic pocket on the surface of the antiapoptotic members. Disruption of heterodimerization could be used to modulate cell death reinstating apoptosis in cancer cells. An affinity displacement assay based on Bcl-XL/BH3 interaction has been developed. This assay makes use of soluble His-tagged Bcl-XL and fluorescein tagged BH3. Binding is measured as fluorescence associated with magnetic beads. The assay was miniaturized to 96-well microtiter plates and can be employed in high throughput screening (HTS), in addition it is robust enough to be applied to microbial fermentation extracts.

Keywords: Bcl-Xl; Apoptosis; Screening

1. Introduction

Cell death plays an important role in many physiological and pathological conditions. Cells die by either of two mechanisms, necrosis or apoptosis, also known as programmed cell death (PCD). Apoptosis is a genetically controlled form of cell suicide regulated by specific gene products acting within dying cells. Defective regulation of PCD plays a pivotal role in tumorigenesis, immune disorders, and neurodegenerative pathologies [1–3]. Moreover, it has been recently pointed out that, in a wide panel of human cancer cell lines, there is an inverse correlation between sensitivity to antineoplastic drugs and the expression of an antiapoptotic protein, namely Bcl-XL [4].

A cell is led into apoptosis in presence of a disruption of the normal balance between positive and negative signals [5]. Indeed many different classes of proteins, involved in the regulation and in the execution of apoptosis, are conserved through evolution and seem to play very standardized roles. The Bcl-2 family of proteins, which is constituted by both pro- and antiapoptotic members, possesses a very high position in the hierarchy of the apoptotic pathway [6].

Members of the Bcl-2 family of proteins, including Bcl-2 and Bcl-XL, inhibit apoptosis by preventing caspases from becoming activated. The Bcl-2 family proteins are localized on the external surface of mitochondria where antiapoptotic proteins make contact with proapoptotic ones and, possibly, hetero-dimerize/polymerize. Much of the discussion on the regulation of apoptosis by Bcl-2 family members has concentrated on the relative importance of homodimerization and heterodimerization of these proteins and of pore formation by multimers of them [6].

Because of their strong prosurvival function the Bcl-2 family of antiapoptotic proteins remains one of the few validated and most promising cancer targets identified to date. Bcl-2 inhibitors might find utility not only as primary treat-

Abbreviations: ADA, affinity displacement assay; BSA, bovine serum albumin; CV, coefficient of variability; DMSO, dimethyl sulfoxide; HTS, high-throughput screening; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PBS, phosphate-buffered saline; PDC, programmed cell death; SD, standard deviation; RFU, relative fluorescence unit.

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ments of many cancer types, but also as adjuvants to current therapeutic regimes of poor prognosis outcomes.

Bcl-2 related proteins share homology in four regions, designated as the Bcl-2 homology domains BH1, BH2, BH3 and BH4. Deletion analysis and site directed mutagenesis [6] have shown that the BH3 domain in Bak and Bax is critical for the induction of apoptosis through the formation of heterodimers with Bcl-2 and Bcl-XL. NMR spectroscopy [5] of the complex formed by Bcl-XL and a 16-residue peptide encompassing the Bak BH3 domain showed that the short peptide binds to the hydrophobic cleft formed by the BH1, BH2, BH3 domains of Bcl-XL. After heterodimerization, the prosurvival functions of Bcl-XL or Bcl-2 seem to be inhibited, thus allowing apoptosis progression [5,6]. It has been proposed that inhibitors could bind to this hydrophobic pocket in a manner similar to that of the BH3 peptides [7,8]. Indeed, a potent inhibitor of BH3 peptide binding to Bcl-XL was developed based on mimicry of the α-helix [9]. Virtual screening has also led to the identification of molecules that bind to the hydrophobic cleft [7]. Previous studies have described the use of fluorescence polarization [8] or enzyme-linked immunosorbent assay-based techniques [10] to measure binding of BH3 peptides to antiapoptotic Bcl-2 proteins. Recently, other formats such as scintillation proximity assay (SPA) and homogeneous time-resolved fluorescence (HTRF) have been used to detect Bcl-2 dimerization protein–protein interactions [11]. Dimers are detected either directly by conjugating fluorophores to protein, peptides, etc. or indirectly by using commercially available fluorophore-conjugated reagents that bind to protein tags.

Several new classes of small-molecule inhibitors of Bcl-2 or Bcl-XL were discovered [12], among them it is worth noting the identification of the 2-methoxy derivative of antimycin A3 as an inhibitor of Bcl-2/Bcl-XL. This molecule is produced by *Streptoverticillium blastmyceticum* (*Streptomyces blastmyceticus*); it is inactive as an inhibitor of cellular respiration but still retains toxicity for Bcl-XL + cells and mitochondria with an IC_{50} value of 2 μM [13].

Taken together, all these results strongly indicate that it is possible to discover/design small-molecule inhibitors that block the interactions between Bcl-2/Bcl-XL and proapoptotic proteins (peptides) such as Bak, Bad, and Bax, and inhibit the biological function of Bcl-2/Bcl-XL.

In this paper we describe a miniaturized assay based on affinity displacement (ADA) from the functional binding "pocket" of Bcl-XL of a fluoresceinated peptide (BH3*), resembling the BH3 domain of Bak. The ADA system was employed to screen a microbial extract bank with the aim of selecting molecules that could inhibit the Bcl-XL dimerization and modulate apoptosis.

2. Experimental procedures

2.1. Plasmid construction

The human Bcl-XL cDNA (Accession No. Z23115.1), without the sequences coding for its carboxyl hydrophobic domain (TM), was amplified by PCR using specific adaptor primers containing the recognition sequences for a NdeI, at the ATG start codon, and BamHI, at the added TAA stop codon. The PCR product was inserted in the pCRII vector (TA cloning system; InVitrogen, San Diego, CA, USA) and then subcloned in the bacterial expression vector pET-19b(+)-His-TAG (Novagen; Darmstad, Germany) between the NdeI and BamHI restriction sites yielding pET-19b-Bcl-XL. Plasmid identity was confirmed by DNA sequencing with an ABI PRISM® 310 DNA Sequencer (Applied Biosystem, Foster City, CA, USA).

2.2. Peptides

The sequence corresponding to the BH3 region of the human protein NH_2-GQVGRQLAIIGADINR–COOH was produced by FMOC synthesis [14]. The N-terminus of the native (BH3) or fluoresceinated (BH3*) peptides were protected with amidation. Identity and purity were confirmed, by MS and HPLC analysis. Peptides were dissolved at 0.3 mM in H_2O at pH 7.

5-(6) Carboxyfluorescein (Sigma–Aldrich, St Louis, MO, USA) was used to label the C-terminus of BH3 according to manufacturer's instructions [15].

2.3. Protein expression and purification

His-tagged Bcl-XL protein (His-Bcl-XL) was purified as follows. A 3 l culture of *Escherichia coli* BL21 (pLys) transformed with the pET-19b-Bcl-XL expression vector was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 22 °C overnight. Cells were pelleted and then lysed by sonication in PBS buffer pH 7.6 (137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4) containing 0.25% (v/v) Tween 20, 20 mM imidazole and protease inhibitors mix (1 mM phenylmethylsulphonylfluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 μg/ml leupeptin), and 0.02 μg DNAase. Cells debris was pelleted by centrifugation at 100,000 g for 1 h at 4 °C and the supernatant was kept at –20 °C until use.

The resulting protein extract, diluted 1:5 with buffer A (0.5 M NaCl, 20 mM imidazole, 20 mM sodium phosphate, pH 7.6) was applied to a Ni^{2+}-chelate resin (5 ml). After washing with buffer A, the recombinant protein was eluted using a gradient of buffer B (0.5 M NaCl, 500 mM imidazole, 20 mM sodium phosphate, pH 7.6).

Fractions containing His-Bcl-XL were pooled and then dialyzed extensively against PBS at 4 °C. The identity of the dialyzed protein was confirmed by western blotting with monoclonal anti-Bcl-XL antibody (BD Transduction Laboratories, Lexington, Kentucky, USA) and anti-His antibody (Qiagen N.V., Venlo, The Netherlands).

2.4. Microbial extract bank

Vicuron Pharmaceuticals microbial extract bank was the sample source; its composition, preparation and quality con-