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Robust lanthanide-based assays for the detection of anti-apoptotic Bcl-2-family protein antagonists

Michele F. Rega, John C. Reed, Maurizio Pellecchia *

Burnham Institute for Medical Research, 10901 North Torrey Pines Rd., La Jolla, CA 92037, USA

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Abstract

Anti-apoptotic Bcl-2-family proteins (Bcl-2, Bcl-x_L, Bfl-1, Mcl-1, Bcl-W and Bcl-B) have been recently validated as drug discovery targets for cancer, owed to their ability to confer tumor resistance to chemotherapy or radiation. The anti-apoptotic activity of Bcl-2 proteins is due to their ability to heterodimerize with their pro-apoptotic counterparts (proteins such as Bad, Bim or Bid) via a conserved peptide region termed BH3. Thus, molecules that mimic pro-apoptotic BH3 domains represent a direct approach to overcoming the protective effects of anti-apoptotic proteins such as Bcl-2 and Bcl-x_L. Here, we report on the development and evaluation of two novel Lanthanide-based assays that are formatted for high-throughput screening of small molecules capable of antagonizing BH3–Bcl-2 interactions. The assay conditions, robustness and reproducibility (Z' factors) are described. These assays represent useful tools to enable further studies in the search for novel, safe and effective anti-cancer agents targeting Bcl-2-family proteins.

Keywords: Bcl-2; FRET; Bcl-x_L; Apoptosis; BH3

1. Introduction

Programmed cell death (apoptosis) plays an essential role in normal tissue homeostasis, ensuring a proper balance of cell production and cell loss. However, defective apoptosis promotes tumorigenesis, and also contributes significantly to chemoresistance [1,2].

* Corresponding author. Fax: +1 858 7139925.
E-mail address: mpellecchia@burnham.org (M. Pellecchia).

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Bcl-2-family proteins are central regulators of apoptosis. Over-expression of anti-apoptotic Bcl-2-family proteins, such as Bcl-2, Bcl-x_L, Mcl-1, Bfl-1, Bcl-W and Bcl-B, occurs during the pathogenesis or progression of most cancers and leukemias [3–5]. In particular, Bcl-2 is over-expressed in 80% of B-cell lymphomas, 30–60% of prostate cancers, 90% of colorectal adenocarcinomas and a wide variety of other cancers, while Bcl-x_L is overexpressed in breast and lung cancers [6,7]. These observations have inspired several recent studies involving a variety of chemical approaches aimed at the discovery of compounds against anti-apoptotic Bcl-2-family proteins as potential therapeutic agents [8–17], underlining the importance of these proteins as novel drug targets.

Currently, the method of choice for the characterization of the antagonist activity of these compounds has been the fluorescence polarization assay (FPA) [18]. FPA is a homogeneous assay that we have used successfully for BH3–Bcl-2 proteins interactions [16,17,19]. Briefly, a FITC-labeled-BH3 peptide is used and its polarization measured at increasing protein concentrations. Upon binding, the initially rapid rotational correlation time of the FITC-BH3 will approach that of the macromolecule with concomitant increase of emitted polarized light. Subsequently, test compounds can be monitored for their ability to bind to the BH3 binding pocket of Bcl-2 proteins by detecting decrease of polarization due to the displacement of bound FITC-BH3 [19]. While this assay is relatively robust, it does not measure directly the binding (or the displacement) of the peptide to (from) Bcl-2, but rather relies on the general rotational correlation time differences induced by the binding (or the displacement) [19]. In addition, the dynamic range of such an assay is rather low with differences in polarization values (measured in mp) ranging from 180-200 for a fully bound peptide to 50–80 for the peptide free in solution [19]. Non-specific interactions of the peptide with the assay components, peptide aggregation and fluorescent compounds are common sources of inaccurate results with this assay. Given the pivotal importance of anti-apoptotic Bcl-2-family proteins and the great interest in the development of Bcl-2 antagonists, we sought here to develop novel and robust assays that would provide direct measurement of the binding and displacement of BH3 peptides, thus enabling further research in this area.

2. Materials and methods

2.1. DELFIA (dissociation enhanced lanthanide fluoro-immuno assay)

To each well of 96-wells streptavidin coated plates (Perkin-Elmer),100 μL of a variable concentration of biotin-labeled BH3 peptide (Biotin-*lc*-G-G-G-Q-V-G-R-Q-L-A-I-I-G-D-D-I-N-R; where *lc* indicates a hydrocarbon chain of 6 $-CH_2-$ groups) is added (concentration range from 5 to 100 ng/ml; 2 to 40 nM). After incubation for 1 h (15 min to 1 h), unbound Biotin-BH3 peptide is eliminated with 3 washing steps. Subsequently, to each well are added 89 μL solution of anti-His Eu-antibody conjugate (100 ng/well; 7.2 nM), 1 μL DMSO solution containing a test compound, and 10 μL solution containing His6-Bcl-x_L (or any other anti-apoptotic His6-Bcl-2-family protein) at concentrations of (1–150 nM). After 1 h of incubation, each well is washed five times to eliminate unbound protein (and so the Eu-antibody if displaced by the test compound). The assay buffer from Perkin-Elmer was used in each step. Subsequently, to each well, 200 μL of enhancement solution (Perkin-Elmer) is added, and fluorescence is measured after 30 min incubation (excitation wavelength, 340 nm; emission wavelength, 615 nm). Note that measurements

are made in time-resolved mode given the relaxation properties of Eu. Controls include unlabeled peptide and blanks receiving no compounds. Note that the DELFIA could also be obtained by using GST-fusion Bcl-2 proteins and anti-GST Eu-antibody (Perkin-Elmer). Z' factor measurements [20] were obtained by repeating the experiments (positive and negative controls) multiple times.

2.2. TR-FRET (time-resolved fluorescence resonance energy transfer) assay

To each well of 96-wells (or 384) black plates (Perkin-Elmer), 29 μL of Detection Buffer (Perkin-Elmer), 5 μL of a variable concentration of biotin-labeled BH3 peptide (Biotin-*lc*-G-G-G-Q-V-G-R-Q-L-A-I-I-G-D-D-I-N-R) is added (concentration range from 2 to 5 nM) together with 5 μL of a solution containing the anti-apoptotic Bcl-2 protein (from 1 to 200 nM) and 1 μL DMSO solution containing a test compound. After incubation for 30 min (15 min to 1 h), to each well are added 5 μL solution of Eu-antibody conjugate (from 3 to 25 nM), and 5 μL solution containing APC-streptavidin (from 1 to 4 nM). The Detection buffer from Perkin-Elmer was used. Measurements are taken after 10 min incubation but more robust results are obtained after 2 h (excitation wavelength, 340 nm; emission wavelength, 665 nm). Controls include unlabeled peptide and blanks receiving no compounds. Z' factor measurements were obtained by repeating the experiments (positive and negative controls) multiple times. Note that TR-FRET, as the DELFIA, could also be obtained by using GST-fusion Bcl-2 proteins and anti-GST Eu-antibody (Perkin-Elmer).

3. Results and discussion

Our first choice for the development of a robust assay for BH3 mimics was the DELFIA (dissociation enhanced lanthanide fluoro-immuno assay) assay. DELFIA is a heterogeneous assay similar to ELISA (enzyme linked immunosorbent assay) that has been applied with success using various assay formats in the detection of receptor ligand interactions. In our implementation, a biotin-labeled BH3 peptide (Biotin-BH3) from the protein Bak is bound to streptavidin coated 96-well plates. Then His6-Bcl-x_L (or any of the anti-apoptotic Bcl-2 proteins) and solution containing a Eu-labeled anti-His6 antibody (Perkin-Elmer) are added together with a test inhibitor and then incubated for binding to Biotin-BH3. After washing steps to eliminate unbound Bcl-x_L, an enhancing solution is added to deliver the lanthanide from the antibody to the solution. Residual Eu fluorescence is subsequently detected with an excitation at 340 nm and emission at 615 nm. If the test compound is capable of dissociating Bcl-x_L from the biotinylated-Biotin-BH3 peptide, the antibody carrying the label (Eu) will be washed out and reduced fluorescence will be detected (Fig. 1).

During the implementation of the DELFIA for BH3–Bcl-2 proteins interactions, we have evaluated its reproducibility at several experimental conditions including, Biotin-BH3 (Anaspec) concentration (from 1 to 100 ng/ml; from 0.4 to 40 nM), His6-Bcl-x_L concentration (from 1 to 150 nM); incubation times 1 h (from 30 min to 2 h), and number of washing steps 5 (from 2 to 7). Fig. 2A shows a typical binding curve of Biotin-BH3 as function of the concentration of Bcl-x_L that provides the dissociation constant for the complex. A typical displacement curve by using the non-biotinylated BH3 peptide as test inhibitor is reported in Fig. 2B.

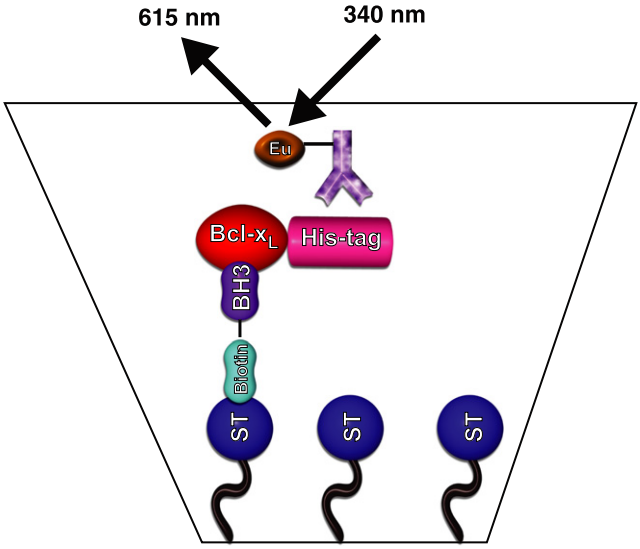

Fig. 1. Schematic representation of the DELFIA method to detect BH3–Bcl-2 interactions.

We obtained K_d and IC_{50} values of 11.8 and 53.4 nM, from the curves in Fig. 2A and B, respectively. These affinities appear greater than previously reported by FPA [21] or by direct Trp-fluorescence quenching [22], obtained with a shorter Bak peptide and Bcl-x_L. However, most of the reported K_d values for various BH3 peptides and anti-apoptotic Bcl-2 proteins fall within the low nanomolar range [19,21].

The DELFIA assay presents several advantages over the fluorescence polarization assay (FPA). First, the sensitivity of Eu is superior to that of most other fluorescent labels, providing a dynamic range of the assay that is several folds higher than provided by FPA. Second, because of the relaxation properties of Eu, detection can be accomplished also in time-resolved mode (TRF) therefore free from possible interferences by test compounds, matrices, plates, etc.

Most importantly, the method directly quantifies the amount of displaced protein (unbound), which is more robust and reliable than measuring the general rotational correlation times of a BH3 peptide (as in the FPA).

To assess the robustness and reproducibility of the assay, we also calculated its Z' values [20]. The Z' factor is designed to reflect both the assay signal noise ratio and the variation associated with the signal measurements. Hence, the Z' factors are commonly used to evaluate the reproducibility of assays for use in high-throughput screening campaigns. In an ideal assay, Z' is close to 1. In practical terms, assays with Z' values above 0.6–0.7 are considered excellent, whereas those with Z' values close or above 0.5 are considered good. Typical values from multiple measurements are reported in Fig. 2C, indicating a very robust assay ($Z' = 0.67$).

The limitations of the DELFIA, however, are the costs associated with the labeled antibodies and coated plates, in addition to the washing steps and the relatively long incubation times, which render the assay relatively laborious. Moreover, the non-homogeneous coating of the plates also leads to some variability in the absolute scale of fluorescence,

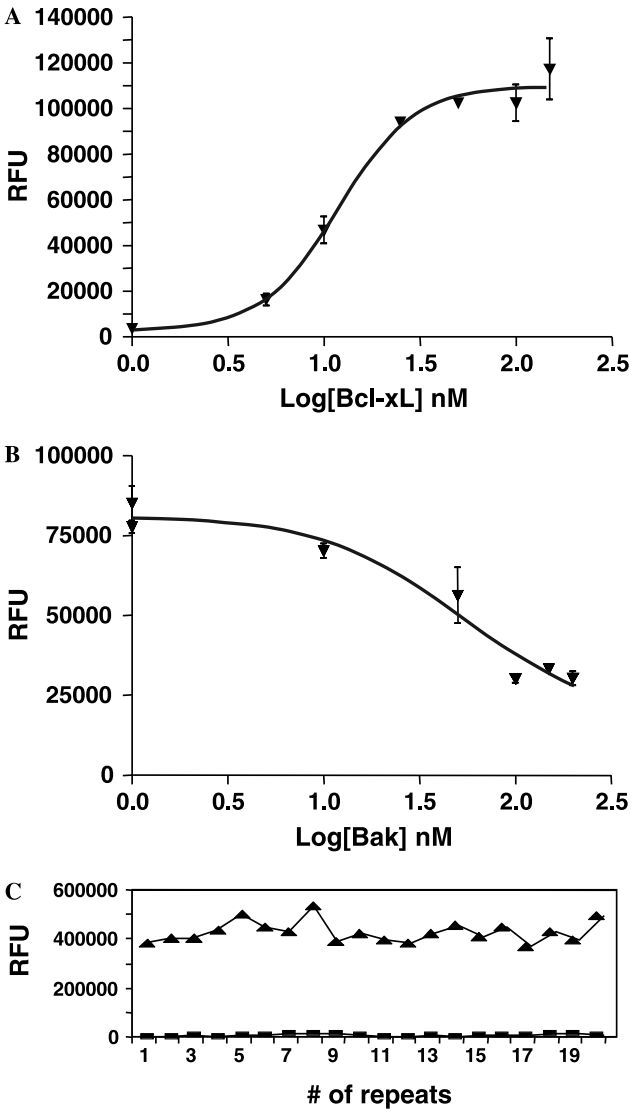

Fig. 2. Results of the DELFIA assay. (A) Dose–response curve as function of Bcl-x_L concentration, K_d value is 11.8 nM. (B) Displacement curve of a non-biotinylated BH3 peptide (Ac-G-G-G-Q-V-G-R-Q-L-A-I-I-G-D-D-I-N-R). IC_{50} for the peptide is 53.4 nM. These assays were performed by using the following conditions: total [Bcl-x_L] = 12 nM; total [Biotin-BH3] = 4 nM; for the other conditions see Section 2. (C) Representative multiple measurement data used to calculate the Z' factor for the assay.

although this problem is relatively minor given the extremely large dynamic range of the assay (Fig. 2).

Nevertheless, we sought to develop a homogeneous version of the assay based on TR-FRET (time-resolved fluorescence resonance energy transfer). In our implementation, a fluorescence transfer is detected between an Allophycocianin (APC)-labeled streptavidin

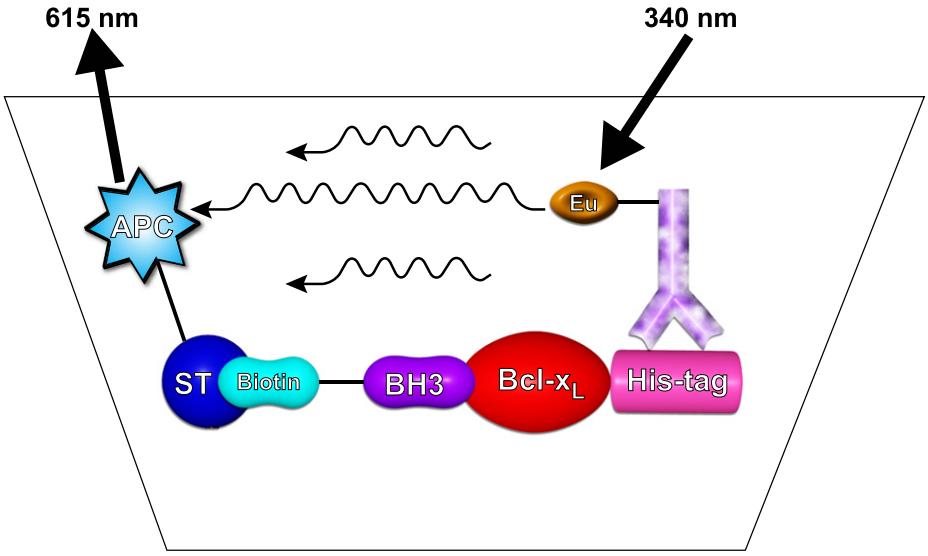

Fig. 3. Schematic representation of the TR-FRET method to detect BH3–Bcl-2 interactions.

(Perkin-Elmer) and the anti-His6 Eu-antibody. Hence, the binding of the Biotin-BH3 to an anti-apoptotic His6-tagged Bcl-2 protein can be detected by the development of a TR-FRET in presence of APC-streptavidin and anti-His6 Eu-antibody (Fig. 3). We have tested the TR-FRET assay at various concentrations of Biotin-BH3 (from 2 to 5 nM), APC-streptavidin (from 1 to 4 nM), Anti-His6-Eu-Antibody (from 3 to 25 nM) and His6-Bcl-x_L (from 1 to 200 nM). We also tested the effect of incubation times (from 10 min to 4 h) prior the final measurement. Fig. 4A shows a typical binding curve of Biotin-BH3 as a function of concentration of Bcl-x_L, demonstrating a K_d value of 8.2 nM, which is in remarkably good agreement with the values found by our DELFIA (Fig. 2A). As done for the DELFIA, we then fixed the concentration of Bcl-x_L around its K_d value (~8 nM) and monitored the displacement of the binding by a non-biotinylated BH3 peptide, functioning as test ligand. As showing in Fig. 4B, the BH3 peptide displaces the Biotin-BH3 with an IC_{50} value of 16.8 nM. We also show, in Fig. 4C, the displacement curve of a known Bcl-x_L antagonist, Gossypol, leading to IC_{50} values of 0.69 μM, again remarkably close to published data [16]. The reproducibility of the assay has been tested by comparing multiple measurements collected at different times (1–4 h). Typical Z' values data are reported in Fig. 4D, indicating excellent assay performance ($Z' = 0.86$).

In order to derive K_i values for such competitive binding assay, the basic assumptions made in the derivation of the Cheng–Prusoff equation [23] could not be used [24]. However, Wang and co-workers [24] have recently reported a modified equation that is used to derive K_i values in FP displacement assays which is therefore applicable also to the TR-FRET.

4. Conclusions

Despite the great relevance of Bcl-2 proteins in drug development, it is quite surprising that assays that monitor direct interactions between the protein components were not yet

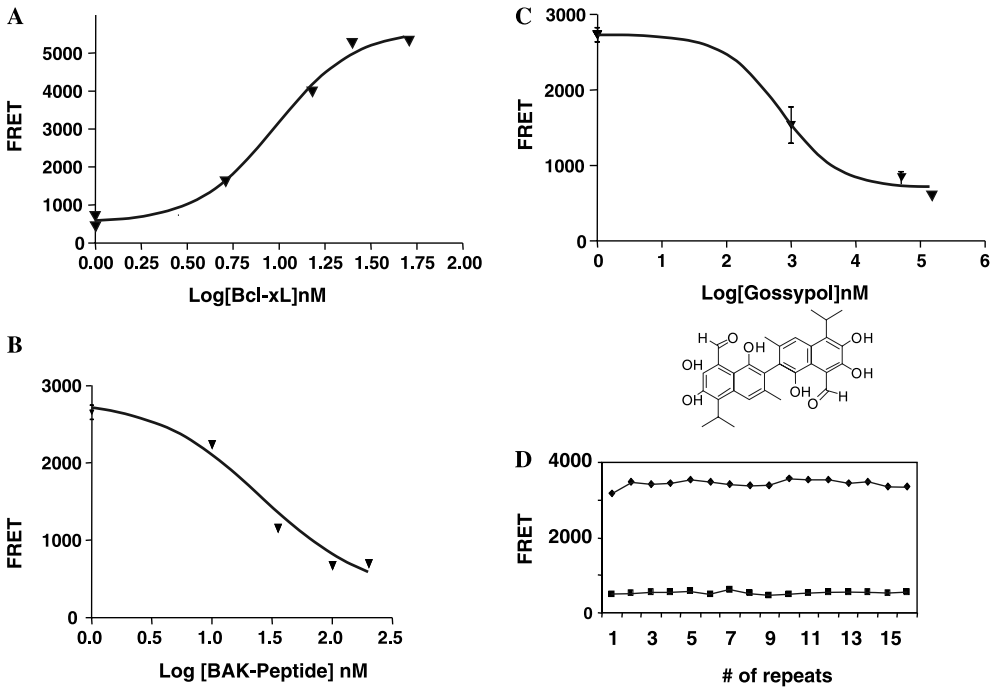

Fig. 4. Results of the TR-FRET assay. (A) Dose–response curve as function of Bcl-x_L concentration, K_d value is 8.2 nM. (B) Displacement curve of a non-biotinylated BH3 peptide (Ac-G-G-G-Q-V-G-R-Q-L-A-I-I-G-D-D-I-N-R). IC_{50} for the peptide is 16.8 nM. (C) Displacement curve for the known BH3 mimic compound Gossypol ($K_i = 0.7$ μM). These assays were obtained by using the following conditions: total [Bcl-x_L] = 8 nM; total [Biotin-BH3] = 4 nM; for the other conditions see Section 2. (D) Representative multiple measurement data used to calculate the Z' factor for the assay.

available. The assays we present here provide a rapid and robust measurement of the direct interactions between BH3 peptides and anti-apoptotic Bcl-2-family proteins, and could therefore prove very useful in the discovery and further development of novel anti cancer agents targeting Bcl-2 proteins. We suggest using the TR-FRET assay as a rapid and cost-effective method for primary screens in high-throughput mode, leaving the DELFIA and/or the FPA assay as secondary, confirmative tests. We are currently screening a large library of compounds with these methods.

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