

Inhibitors of Bcl-2 have a unique profile of inducing the apoptotic cascade downstream of the mitochondria

Abstract #148

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Abstract

The Bcl-2 family of proteins are key regulators of apoptosis and Bcl-2 itself is overexpressed in several cancers and thought to play a key role in tumor survival and resistance to therapy. Antagonizing the function of Bcl-2 therefore is predicted to have considerable therapeutic potential and multiple efforts have been undertaken to discover effective Bcl-2 antagonists. While multiple small molecule series have been discovered that bind to Bcl-2 and also kill cells, one major hurdle in the development of such molecules is the question of whether these compounds kill cells through the antagonism of Bcl-2, i.e. 'on target'. To help in the development of such molecules, we have undertaken to study in detail, the cellular consequences of Bcl-2 inhibition in cells that depend on Bcl-2 for survival. We first used RNAi against Bcl-2 to identify cell lines that need high levels of Bcl-2 for survival; we then studied the time dependency of induction of apoptotic markers downstream of the mitochondria. This analysis reveals a unique, time-dependent apoptotic marker profile for Bcl-2 antagonism that is different from that induced by chemotherapeutics. Surprisingly, when this analysis is applied to published Bcl-2 binders, most of them do not cause cell death through antagonism of Bcl-2. This analysis will be an important tool for the development of targeted therapies directed against the Bcl-2 family of proteins.

Introduction

Resistance to apoptosis is a hallmark of cancer. One mechanism by which cancer cells resist apoptotic signals is through the overexpression of anti-apoptotic members of the Bcl2 family. Although there are a number of compounds published to bind to Bcl-2, it is unclear whether their mechanism of cell killing is Bcl2 mediated. Using RNAi, we first identify cell lines that depend on Bcl-2 for survival, and then define the time profile of induction of apoptosis markers when a Bcl-2 dependent cell line dies by Bcl-2 depletion. We then used this time-dependent "apoptotic profile" to ask which of the published Bcl-2 binders elicit a profile consistent with causing cell death through Bcl-2 antagonism.

Results

	Ki (μM) Bcl-2
ABT-737	<0.001
GX015-070	>1
HA14-1	50
Gossypol	35
BH3I-1	3.6
Compound 6	29

Figure 1. Structures and affinities of small molecules reported in the literature to bind Bcl-2 (1,2,3)

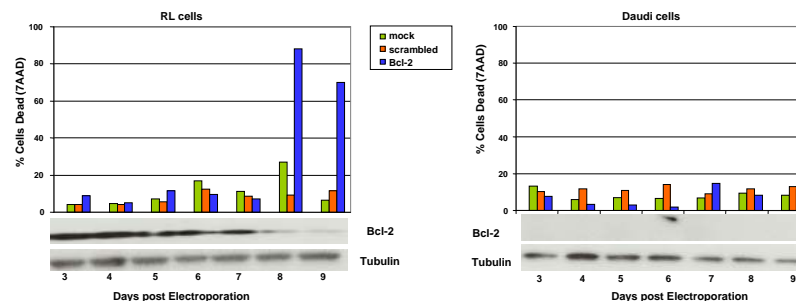


Figure 2. RL cells are dependent on Bcl-2 for survival. RNAi was used to identify cell lines dependent on Bcl-2 for survival. Bcl-2 or control RNAi constructs were delivered to cells by electroporation, knockdown of Bcl-2 confirmed and the consequences on cell viability determined. The RL cell line is derived from a follicular lymphoma patient and contains the t(14;18) translocation. Daudi cells originated from a patient with Burkitt's lymphoma, do not express detectable levels of Bcl-2 and as assessed by RNAi are not dependent on Bcl-2 for survival.

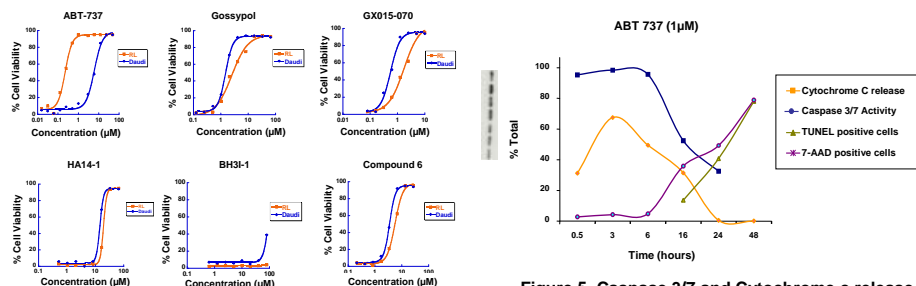


Figure 3. Published Bcl-2 binders vary in their ability to specifically kill the Bcl-2 dependent cell line RL. Increasing concentration of compounds were incubated with RL and Daudi cells and viability was quantified by alamar blue. The cell viability data was independently confirmed using the cell impermeable dye, 7AAD (data not shown)

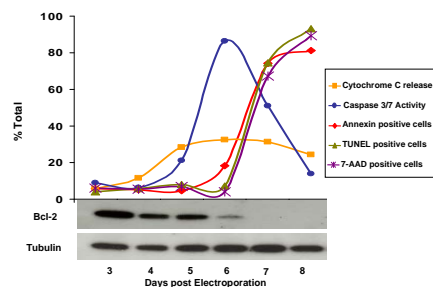


Figure 4. Silencing of Bcl-2 induces apoptosis in RL Cells. Knockdown of Bcl-2 by RNAi was used to determine the timing of the appearance of different apoptosis markers after Bcl-2 depletion. The earliest measurable response was cytochrome C release into the cytoplasm and Caspase 3/7 activation.

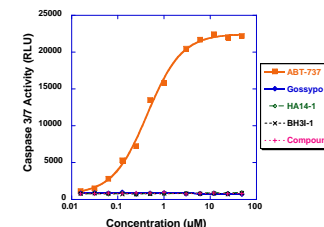


Figure 7. Most published Bcl-2 binding compounds are unable to rapidly activate caspase 3/7 in RL cells. Despite the inability of many of the Bcl-2 binders to specifically kill RL cells over Daudi cells, we assessed their ability to activate caspase 3/7 in RL cells after 1 hour. Consistent with the RL and Daudi cell kill data, ABT-737 was the only compound that activated caspase 3/7 after 1 hour.

Summary

- RL cells are dependent on Bcl-2 for survival; Daudi cells are not
- Comparisons of RL versus Daudi cytotoxicity can therefore be used to distinguish on-target cell killing from off-target effects
- The molecular consequences of inhibiting Bcl-2 in a cell line that depends on Bcl-2 for survival is a unique profile of time dependent induction of downstream apoptosis markers
- The most striking event is rapid activation of Caspase 3, which is specific for Bcl-2 antagonists when compared to chemotherapeutics that also activate apoptosis
- Of the published Bcl-2 antagonists tested, ABT-737 is the only compound that appears to act on mechanism

Materials and Methods

Apoptosis Assays
Caspase 3/7 (Caspase Glo 3/7 assay, Promega), Annexin (Guava Technologies), DNA fragmentation (TUNEL assay, Guava Technologies) and cell death assay (Viacount assay, Guava Technologies) were performed according to the manufacturer's instructions

Cytochrome C release
Cells were treated with compound for the appropriate time points. After treatment, cells were pelleted at 2000rpm for 10 minutes, the supernatant removed and buffer containing 0.005% digitonin added to the cell pellets. The cells were incubated at room temperature for five minutes, pelleted by centrifugation at 2000rpm for 10 minutes and the supernatants transferred to a 1.5ml eppendorf for ELISA analysis. ELISA analysis was performed using a Cyto C Quantokine kit (R&D Systems) according to the manufacturer's instructions.

RNAi
RL and Daudi cells were resuspended at a density of 5 x10⁴ cells/100ul in Amaxa nucleofector solution (Amaxa Biosystems) and 5μg Bcl-2 shRNA plasmid or non-silencing control shRNA plasmid added. The electroporation was performed according to the manufacturer's instructions at a voltage of 140V for RL cells and 180V for Daudi cells. Electroporated cells were immediately transferred to fresh media and returned to the incubator. The Bcl-2 shRNA hairpin sequence used was:

5'AGATAGTGATGAAGTACATTCAGAGAAGATGTACTTCATCATCTACTCT-3'

References

1. Reed, J.C., Pellecchia, M (2005) Apoptosis-based therapies for hematologic malignancies *Blood* 106, 408-418
2. Oltersdorf, T, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 435, 677-681
3. Van Delft, M.F. et al. (2006) The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl1 is neutralized *Cancer Cell* 10, 389-399

Figure 5. Caspase 3/7 and Cytochrome c release are rapidly induced in RL cells by the Bcl-2 inhibitor, ABT-737. ABT-737 was the only published Bcl-2 inhibitor tested that specifically killed RL cells. The timing of apoptotic marker activation upon treatment of RL cells with ABT-737 was investigated and found to be similar to that observed after Bcl-2 depletion using RNAi. As early as 30 minutes after treatment, significant levels of cytochrome c release and caspase 3/7 activation could be detected.

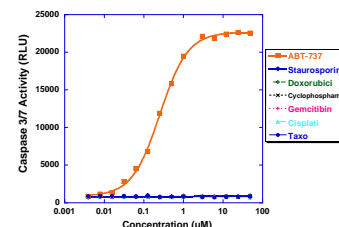


Figure 6. The rapid activation of Caspase 3 is specific for Bcl-2 antagonists. To determine how specific the rapid activation of caspase 3/7 in RL cells is to Bcl-2 inhibition, a panel of cytotoxic agents were assessed for their ability to activate caspase 3/7 activity in RL cells after 1 hour. Of the compounds tested, only the small molecule Bcl-2 antagonist, ABT-737 activated Caspase 3/7 after 1 hour.