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A Unique Apoptosis Marker Profile for Antagonists of Bcl-2

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ABSTRACT

The Bcl-2 family of proteins are key regulators of cell survival. The anti-apoptotic member, Bcl-2, is overexpressed in several cancers and is thought to play a key role in tumor survival and resistance to therapy. Antagonizing the function of Bcl-2 is predicted to have considerable therapeutic potential. To aid in the development of small molecule inhibitors of Bcl-2, we have used RNAi against Bcl-2 to identify cell lines that are, or are not dependent on Bcl-2 for survival. Using published small molecule Bcl-2 binders, we then studied the consequences of Bcl-2 inhibition in a cell line that is dependent on Bcl-2 for survival. We reveal a time-dependent profile of apoptosis markers downstream of the mitochondria that is unique for the inhibition of Bcl-2 and easily distinguishable from chemotherapeutic agents. This unique profile makes it possible to discriminate between Bcl-2 binding molecules that kill through Bcl-2 antagonism versus molecules that, while binding to Bcl-2 in a biochemical assay, kill cells by an off target mechanism. Using these new assays, we investigate a number of previously published Bcl-2 binding small molecules. Remarkably, in our hands, only very few of these compounds affect cell death through Bcl-2 antagonism.

INTRODUCTION

Bcl-2 is a well validated and attractive target for cancer therapy. 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 defined the time profile of induction of apoptosis markers when a Bcl-2 dependent cell line dies by Bcl-2 depletion. We 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.

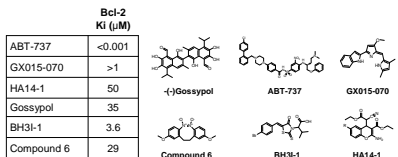


Figure 1. Structures and affinities of small molecules reported in the literature to bind Bcl-2^{1,2,3}

RESULTS

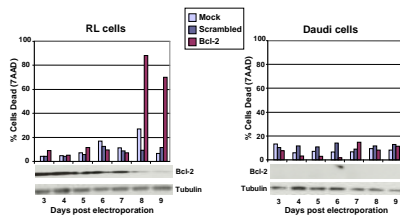


Figure 2. RL cells are dependent on Bcl-2 for survival. RNAi was used to identify cell lines dependent on Bcl-2 for survival. Bcl-2 or control RNAi constructs were delivered to cells by electroporation, knockdown of Bcl-2 confirmed and the consequences on cell viability determined.

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.

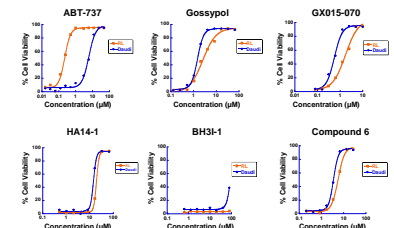


Figure 3. Published Bcl-2 binders vary in their ability to specifically kill the Bcl-2 dependent cell line RL. Increasing concentration of compounds were incubated with RL and Daudi cells and viability was quantified by alamar blue. The cell viability data was independently confirmed using the cell impermeable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

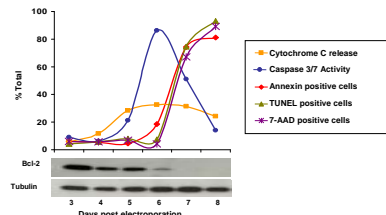


Figure 4. Silencing of Bcl-2 induces apoptosis in RL Cells. Knockdown of Bcl-2 by RNAi was used to determine the timing of the appearance of different apoptosis markers after Bcl-2 depletion. The earliest measurable response was cytochrome C release into the cytoplasm and Caspase 3/7 activation.

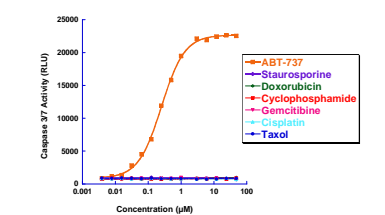


Figure 6. The rapid activation of Caspase 3 is specific for Bcl-2 antagonists. To determine how specific the rapid activation of caspase 3/7 in RL cells is to Bcl-2 inhibition, a panel of cytotoxic agents were assessed for their ability to activate caspase 3/7 activity in RL cells after 1 hour. Of the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

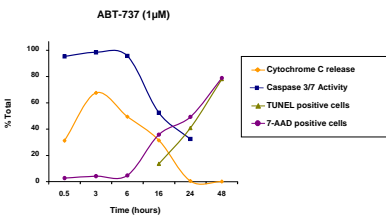


Figure 5. Caspase 3/7 and Cytochrome c release are rapidly induced in RL cells by the Bcl-2 inhibitor, ABT-737. ABT-737 was the only published Bcl-2 inhibitor tested that specifically killed RL cells. The timing of apoptotic marker activation upon treatment of RL cells with ABT-737 was investigated and 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.

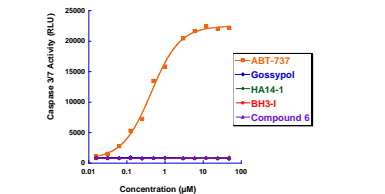


Figure 7. Most published Bcl-2 binding compounds are unable to rapidly activate caspase 3/7 in RL cells. Despite the inability of many of the Bcl-2 binders to specifically kill RL cells over Daudi cells, we assessed their ability to activate caspase 3/7 in RL cells after 1 hour. Consistent with the RL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

CONCLUSION

- 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 & METHODS

Apoptosis Assays

Caspase 3/7 (Caspase 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 CytoC Quantokine kit (R&D Systems) according to the manufacturer's instructions.

RNAi

RL and Daudi cells were resuspended at a density of 5×10^6 cells/100ml in Amasa nucleofector solution (Amasa Biosystems) and Sup 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'-AGATAGTATGAAGTACATTTCAAGAAAGTGTACTTCTACATCT-3'

REFERENCES

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