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COMMENT

Challenges in small-molecule target identification: a commentary on "BDA-366, a putative Bcl-2 BH4 domain antagonist, induces apoptosis independently of Bcl-2 in a variety of cancer cell models"

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Over 30 years ago BCL2 was shown to prevent cells dying [1]. Since then our understanding of the BCL2 family proteins and how they control apoptosis has progressed significantly. Venetoclax, a specific BCL2 inhibitor, is now an FDA-approved treatment for chronic lymphocytic leukemia (CLL) [2, 3]. This discovery shows the power in targeting apoptotic pathways as a potential cancer treatment. In 2015, Han et al. described the small-molecule BCL2-BH4 domain-antagonist, BDA-366 [4]. BDA-366 suppressed growth of lung cancer xenografts offering a promising new agent for targeting apoptosis. The mechanism attributed to BDA-366's action was unique. Venetoclax and other BCL2 targeting molecules block the BCL2 binding-groove preventing BCL2 binding the proapoptotic BH3-only proteins and BAX, thus allowing these target molecules to promote apoptosis [3, 5, 6]. In contrast, BDA-366 was thought to convert BCL2 into a proapoptotic protein through interactions with its BH4 domain, one of four BCL2 homology (BH) sequence motifs common to BCL2 family proteins [4]. This was a surprising finding. Han et al. presented a series of in vitro and in silico experiments to justify this mechanism; however, there were some peculiarities in the authors experiments that raised concerns for the proposed action mechanism.

First, BDA-366 was identified from a 300,000-compound library using an in silico docking screen against the BCL2 BH4 domain. In silico screens provide powerful tools to screen compounds on established binding sites. However, in this case there is no established BH4 site for BCL-2. Consequently, the results should have been treated with caution as in silico simulations will always provide an answer, whether or not it is relatable to meaningful physical binding. To overcome this limitation, the authors used a fluorescence polarization assay to assess BDA-366 interactions with BCL2. This assay was based on indirect competition between BDA-366 and a labeled BAK-BH3 peptide for BCL-2 binding. Most literature agrees that the BAK BH3 peptide used by Han et al. does not bind to BCL2 or, at least, that the interaction is very weak [7]. Furthermore, no positive or negative control compounds were used to validate their use of this assay. These oversights raise concerns with the fluorescence polarization assay and interpretations that led to the controversial BDA-366 action mechanism. However, Han et al. did show in cells that quadruple mutation of the BCL2 BH4 domain confers selective resistance to BDA-366 and not venetoclax. This indicates that changes to the BCL-2 N-terminal region could convey resistance to BDA-366's activity in cells. Furthermore, BDA-366 did show promising activity as a potential anti-cancer compound that worked by promoting apoptosis.

In this issue of Cell Death and Disease, Vervloessem et al. [8] have convincingly challenged the original hypothesis that BDA-366 targets BCL2. They show that patient CLL cells have a high variability in response to BDA-366, with only half the population showing BDA-366 sensitivity and with the remaining population insensitive to treatment. CLL cells have high BCL2 levels so the entire population should be sensitive to BDA-366 treatment [9]. To understand this finding further they tested BDA-366 on multiple diffuse large B-cell lymphoma (DLBCL) cell lines and showed that there was no correlation between BCL2 expression levels and BDA-366 activity. Furthermore, cells with very-low BCL2 levels, which one predicts would be

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resistant to BDA-366 if it truly binds BCL2, were sensitive to BDA-366. Another intriguing result was CLL and DLBCL cells that were resistant to venetoclax, which specifically targets BCL2, were sensitive to BDA-366. This made the authors question if BDA-366 targeted BCL2 or if another mechanism was involved.

Using BAX/BAK double knockout cells, Vervloessem et al. showed that BDA-366 activity was reduced in these cells confirming the observations of Han et al. that BDA-366 does induce BAX/BAK-dependent apoptosis. To ascertain which members of the BCL2 family proteins were targeted by BDA-366 they utilized a series of rigorous in vitro liposome assays. These assays probe interactions of BCL2 family proteins in a simplified system, which can be challenging in cells due to the complex expression of multiple family members. The assays convincingly show that BDA-366 does not activate BAX on its own or in the presence of BCL2. BAX can be activated by the proapoptotic protein BIM in these assays [10] and the inclusion of BCL2 restrains the BIM activity preventing BAX activation. If BDA-366 showed BCL2 binding it should displace BIM and activate BAX; this concept was the basis for the fluorescence polarization assays used by Han et al. Using venetoclax as a control, Vervloessem et al. show that venetoclax could displace BIM from BCL2 and promote BAX activation in this assay, but BDA-366 could not. Combined with the cellular experiments this data convincingly shows BDA-366 activity is independent of BCL2 and that it must function through an alternate mechanism.

This left the question, how does BDA-366 promote BAX/BAK-mediated apoptosis? The authors noted that BDA-366 has an anthraquinone core and that anthraquinones are known PI3K/AKT pathway inhibitors [11]. They investigated that this showing BDA-366 reduces the levels of phospho-AKT, but not total AKT levels, indicating disruption of the pathway. The PI3K/AKT pathway regulates MCL1 levels, by decreasing expression via mTOR and increasing proteasomal degradation through GSK3 [12, 13]. They show that total MCL1 protein levels rapidly decrease upon incubation with BDA-366. In addition, Vervloessem et al. show a loss of BCL2 phosphorylation at Ser70 upon treatment with BDA-366. This may partially explain the discrepancies between their results and those reported in the original paper. These changes in MCL1 and BCL2 will trigger apoptosis in a BAX-dependent manner. This provides a more conventional explanation for how BDA-366 promotes apoptosis. This is potentially relevant in a therapeutic context as MCL1 over expression and BCL2 Ser70 phosphorylation can reduce venetoclax efficacy [14, 15]. This suggests that BDA-366 could be used in combination with Venetoclax to improve efficacy.

Apoptotic signaling involves a complicated network of multiply redundant interactions that integrate to determine cell fate. The BCL2 family members are established targets for small molecule therapy. However, establishing small molecule targets and mechanisms from in silico screens can be challenging. The conclusions from Han et al. were based on a series of experiments that, superficially, made sense. However, a detailed understanding of the interactions between BCL2 family proteins and how these lead to apoptotic cell death may have steered the original observations in a different direction. Vervloessem et al. should be commended for their scientific rigor and dedication to correct a questionable hypothesis. Their data clearly show that BDA-366 does not directly interact with BCL2 to modulate its activity. This correction will improve the apoptosis literature and lead to more appropriate interpretation of experiments using BDA-366 as a tool in future work. Furthermore, this has implications beyond the apoptosis research community for it changes how BDA-366 is assessed for therapy. Understanding how BDA-366 directly influences the PI3K/AKT pathway is now important as it will inform on potential resistance mechanisms and treatment strategies. This changes the direction of BDA-366 research to explore its exciting potential for cancer therapy.

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Compliance with ethical standards

Conflict of interest RWB is an employee of the Walter and Eliza Hall Institute, which has an agreement with Genentech and AbbVie and receives milestone and royalty payments related to venetoclax.

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