Title: Further insights into the effects of pre-organizing the BimBH3 helix

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Our recent publication[1] tested the activity of stapled Bim BH3 peptides (BimSAHB) previously reported and characterized by the Walensky group[2]. We note the queries raised in their accompanying counterpoint article[3], and take this opportunity to address some of these concerns. Principally, these question the validity of our conclusions drawn from experiments using specific peptides in our original publication[3]. New data we now provide clarifies and confirms the major conclusions from our original studies - that stabilizing the BimBH3 helix through stapling does not necessarily enhance binding affinity or biological activity.

Gavathiotis *et al.*[2] reported the use of two stapled peptides: one (Bim 20-mer, residues 145-164) was used extensively for biophysical studies whereas another (Bim 21-mer, residues 146-166; $SAHB_A$), was used for cell-based biological assays. In that publication, the latter peptide at high concentrations was shown to kill (and was presumably taken up by) $Bax^{-/-}Bak^{-/-}$ MEF cells reconstituted with wild-type Bax (Figure 5c of Gavathiotis 2008[2]). We instead observed no killing with an identical peptide in MEFs (supporting information from[1]). We had also tested the 20-mer peptide and found it to be inert in bioassays. We are aware that the Walensky group subsequently reported that MEFs are insensitive to this stapled Bim peptide whilst other cell lines are sensitive[4].

To provide some clarity, we have now also re-tested the 21-mer $BimSAHB_A$ in cell lines used in that publication[4], namely K562 and U937. We confirm that these cell lines are sensitive to the 21-mer $BimSAHB_A$ (Figure 1). In contrast, the colon cancer cell line HCT-116 was insensitive (data not shown), suggesting that susceptibility to the 21-mer $BimSAHB_A$ is not a general feature of all tumor cell lines. Why some cell lines (K562, U937), but not others (MEFs, HCT-116), are sensitive to the 21-mer $BimSAHB_A$ is currently unclear.

Although the control wild-type (linear) 21-mer BimBH3 peptide was inactive, as anticipated[4], we unexpectedly found that the unclosed linear equivalent (unstapled 21-mer peptide containing 2 pentylalanine residues but not subjected to ring-closing metathesis, Table 1a) was as active as the closed stapled $BimSAHB_A$ peptide (Figure 1). These results strongly indicate that the cellular activity is not imparted by the staple *per se*, (see also [5]), but is rather due to specific sequence characteristics, emphasizing the need to further study and fully characterize these interesting reagents before broad conclusions can be made about their properties and utility.

Previously, our biophysical studies focused on testing the stapled 20-mer Bim peptide (residues 145-164). We found that stapling reduced affinity for Bcl-x_L, Bcl-w and Bcl-2 in competition assays, and for Bcl-x_L and Mcl-1 in direct binding experiments, compared to the equivalent wild-type linear peptide. The effect on A1 and Mcl-1 affinity was unclear in the competition assays as the K_i for the linear peptide is below the detection limit of the assay. The difference in affinity is a property imparted by the pentylalanine staple and not by peptide length or sequence (except for those residues replaced with pentylalanine). We proposed that loss of stabilizing intra-molecular interactions might account for this reduced affinity[1].

We have now also performed binding assays with the longer 21-mer BimSAHB$_A$ and find that stapling also reduces the affinity of this peptide for Bcl-x$_L$ and Mcl-1 (Table 1b). This is consistent with our proposal that it is important to maintain stabilizing intra-molecular interactions, and confirms that our original observations were not the consequence of only testing the shorter version of BimSAHB$_A$ that had been designed to have a lower affinity for target proteins[2]. Additionally, in our original publication[1], we also tested the role of lysine 21 in Bax activation and found it did not play an essential role. Others have reached similar conclusions[6].

Yours,

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1. Okamoto T, Zobel K, Fedorova A, Quan C, Yang H, et al. (2013) Stabilizing the pro-apoptotic BimBH3 helix (BimSAHB) does not necessarily enhance affinity or biological activity. ACS Chem Biol 8: 297-302.
2. Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, et al. (2008) BAX activation is initiated at a novel interaction site. Nature 455: 1076-1081.
3. Bird GH, Gavathiotis E, LaBelle JL, Katz SG, Walensky LD. (2013) Distinct BimBH3 (BimSAHB) Stapled Peptides for Structural and Cellular Studies. ACS Chem Biol *(in press).*
4. LaBelle JL, Katz SG, Bird GH, Gavathiotis E, Stewart ML, et al. (2012) A stapled BIM peptide overcomes apoptotic resistance in hematologic cancers. J Clin Invest 122: 2018-2031.
5. Giordanetto F, Revell JD, Knerr L, Hostettler M, Paunovic A, et al. (2013) Stapled Vasoactive Intestinal Peptide (VIP) Derivatives Improve VPAC2 Agonism and Glucose-Dependent Insulin Secretion. ACS Med Chem Lett 4: 1163-1168
6. Peng R, Tong JS, Li H, Yue B, Zou F, et al. (2013) Targeting Bax interaction sites reveals that only homo-oligomerization sites are essential for its activation. Cell Death Differ 20: 744-754.

Table 1 (a) Sequences for peptides. Unstapled 21-mer BimSAHB refers to a BimSAHB peptide in which pentylalanine residues have not been linked. (b) Direct affinity measurements for peptides to Bcl-x_L and Mcl-1. Measurements were performed using a BIAcore 4000. Peptides were passed over a sensor chip to which either recombinant Bcl-x_L-GST or Mcl-1-GST fusion proteins had been coupled via an anti-GST antibody. Direct association and dissociation rates were measured at a range of concentrations from which K_D values were calculated. Values are representative of two independent experiments.

(a)

	Sequence	**Note**
21-mer BimSAHB$_A$	Ac-IWIAQELRXIGDXFNAYYARR-NH_2	X represents linked (*S*)-pentenylalanine residues
Unstapled 21-mer BimSAHB	Ac-IWIAQELRXIGDXFNAYYARR-NH_2	X represents unlinked (*S*)-pentenylalanine residues
Wild-type BimBH3	Ac-IWIAQELRRIGDEFNAYYARR-NH_2	

(b)

	Affinity K_D
Bcl-x_L	
21-mer BimSAHB$_A$	20 nM
Unstapled 21-mer BimSAHB	54 nM
Wild-type BimBH3	0.8 nM
Mcl-1	
21-mer BimSAHB$_A$	1.7nM
Unstapled 21-mer BimSAHB	1.5 nM
Wild-type BimBH3	0.16 nM

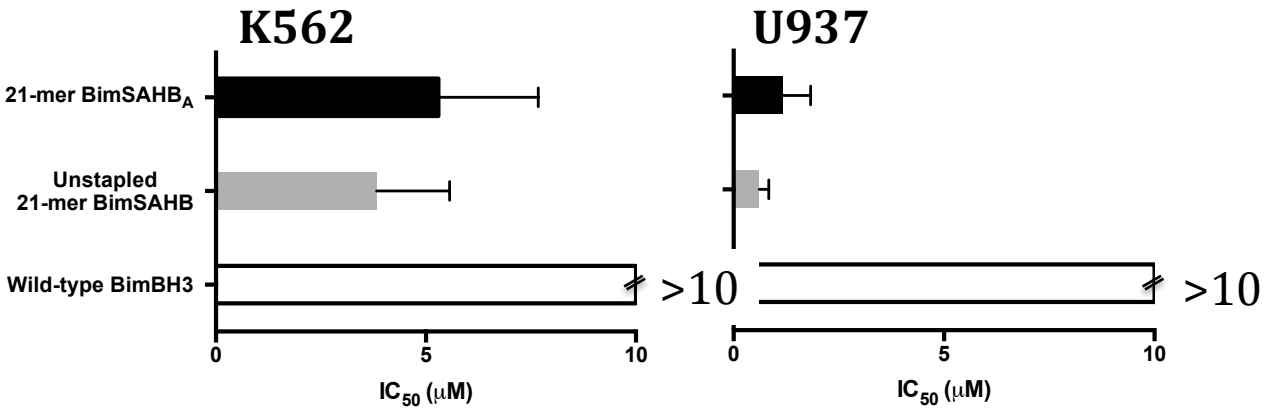

Figure 1. Sensitivity of some cancer cell lines to Bim BH3 peptides. K562 and U937 cells were treated with the indicated peptides for 2h in serum-free RPMI. An equal volume of RPMI with 20% FCS was then added to each well and the cells were incubated for a further 24h at 37°C. The effect on cell proliferation/viability was measured using Cell Titer Glo assay (Promega). Relative fluorescence data were acquired using an Envison plate reader (Perkin Elmer) and normalized to cells treated with vehicle alone (0.1% DMSO in 10% FCS/RPMI). IC_{50} values were calculated using non-linear regression algorithm in Prism Graphpad software. Data shown represent the mean IC_{50} ± 1 SD from 3 independent experiments.