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Correspondence

A transgenic mouse model to inducibly target prosurvival Bcl2 proteins with selective BH3 peptides *in vivo*

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Dear Editor,

BH3-mimetic drugs that antagonize Bcl-2 family prosurvival proteins are effective against some cancers, particularly those with abnormally high expression of the prosurvival protein target.[1] Despite the recent success of BH3-mimetics targeting Bcl-2, Bcl-x_L and Bcl-w, mechanism-based cell killing *in vivo* by targeting other important prosurvival proteins such as Mcl-1 or Bfl-1 has yet to be demonstrated. In the absence of small molecules targeting these prosurvival proteins, BH3-domain peptides, the prototypes for this class of drug, are useful because their binding specificity profile can be manipulated.[2,3] Such peptides have been applied in *in vitro* studies to validate the targeting of particular prosurvival proteins in certain tumor types.[4,5] However, *in vivo* applications require technically challenging chemical modifications of peptides, and while mouse xenograft models of virally-infected BH3 domain-expressing tumor cells can be used, this does not allow evaluation of effects of the ligand on normal tissues.

Here we have generated transgenic mice in which peptide-based BH3 ligands can be inducibly expressed to evaluate their effects *in vivo* and provide proof-of-principle for similar-acting drugs. To achieve this we adapted a previously described strategy allowing FLP-recombinase-mediated insertion of an expression cassette at an frt 'landing pad' at the type I collagen (*Col1a1*) locus in mouse embryonic stem cells.[6] The cassette comprises sequences encoding a BH3-domain protein under control of a tetracycline (tet)-regulated element (TRE) promoter. To develop a system that is broadly applicable to BH3 domains with different specificities, we employed the Bim_S BH3-only protein as a scaffold in which BH3 domains with different specificities could replace the native BH3 sequence (Figure 1a). Bim_S is an intrinsically unstructured protein that tolerates extensive mutation of its BH3 sequence, and Bim_SBH3 chimeras display the prosurvival protein specificity profile of the replacement BH3 domain.[7] In this study we focussed on the BH3 domain of Bad because it targets the prosurvival proteins Bcl-2, Bcl-x_L and Bcl-w.[7] Hence Bim_SBad expression should mimic the BH3-mimetics ABT-737 and ABT-263 that have been extensively studied *in vivo*.[1]

We generated TRE-Bim_SBad transgenic mice and crossed them to mice expressing the rtTA (tet-on) transactivator under the control of the cytomegalovirus (CMV) promoter, which provides high-level expression in many tissues including blood.[8] Bitransgenic mice were then treated with doxycycline (Dox) to induce Bim_SBad expression (Figure 1a). Western blot analysis verified Dox-inducible transgene expression in white blood cells of Bim_SBad; CMV-rtTA bitransgenic mice (Figure 1b). As ABT-737/263 induces thrombocytopenia in mice and humans due to antagonism of Bcl-x_L, we measured platelet levels as a biomarker for functional expression of the ligand.[1] Notably, blood cell analysis of these mice revealed a significant reduction (~65% decrease) in platelet counts (Figure 1c and d), a degree of thrombocytopenia comparable with that seen in patients administered with ABT-263.[9] Importantly, platelet counts rebounded to normal levels following removal of Dox for 7 days (Figure 1d), illustrating the reversibility of the system. We also generated an additional transgenic mouse strain allowing inducible expression of a Bim_SBad construct possessing a BH3 sequence mutation ($Bim_S Bad^{mut}$; Figure 1a) that decreases its affinity for Bcl-x_L by >40-fold (K_D 8.5 nM *versus* <0.2 nM as measured by surface plasmon resonance). Induction of $Bim_S Bad^{mut}$ expression (Figure 1b) did not alter blood cell or platelet counts (Figure 1d), indicating that the thrombocytopenia observed in Bim_SBad mice is due to Bcl-x_L inhibition. These data provide the first evidence that BH3-only proteins can be inducibly expressed in a mouse model, effectively mimicking at least one functional consequence (reduced platelet counts) seen in mice and humans treated with BH3-mimetic drugs of similar specificity. We envisage multiple applications for similarly engineered mice. For example, tet-regulated expression of different Bim_S variants in mice could reveal toxicities associated with neutralization of their prosurvival protein targets (individually or in combination), and tissue-specific effects could be addressed by crossing onto mice where rtTA expression is driven by different promoters. Moreover, mice crossed to different tumor-prone models could provide *in vivo* evidence for the tumor-killing efficacy associated with different BH3 specificities not yet available through small molecule

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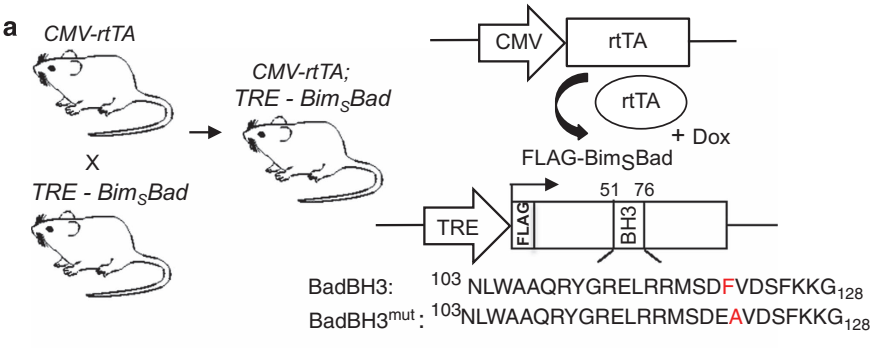

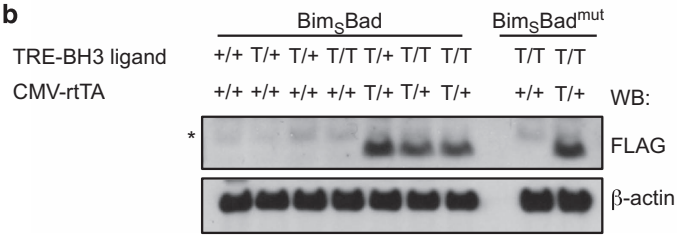

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	Doxycycline 7 days		
	+/+;+/+ (n=7)	TRE-BimSBad (n=15)	TRE-BimSBad; CMV-rtTA (n=17)
Erythrocytes (x10⁶ µL⁻¹)	11.19 ± 0.17	11.32 ± 0.20	11.13 ± 0.13
Hematocrit (%)	54.91 ± 1.05	55.75 ± 0.56	55.48 ± 0.65
MCV (femtolitres)	49.07 ± 0.44	48.46 ± 0.30	49.85 ± 0.34
Leukocytes (x10³ µL⁻¹)	9.81 ± 0.64	10.29 ± 0.53	9.12 ± 0.37
Neutrophils (x10³ µL⁻¹)	0.91 ± 0.20	0.80 ± 0.24	1.19 ± 0.20
Lymphocytes (x10³ µL⁻¹)	8.47 ± 0.55	8.93 ± 1.93	7.12 ± 1.12
Monocytes (x10³ µL⁻¹)	0.17 ± 0.047	0.17 ± 0.058	0.25 ± 0.030
Platelets (x10³ µL⁻¹)	1240 ± 61.82	1200 ± 36.12	439 ± 30.53****
MPV (femtolitres)	6.82 ± 0.23	6.93 ± 0.12	7.42 ± 0.098

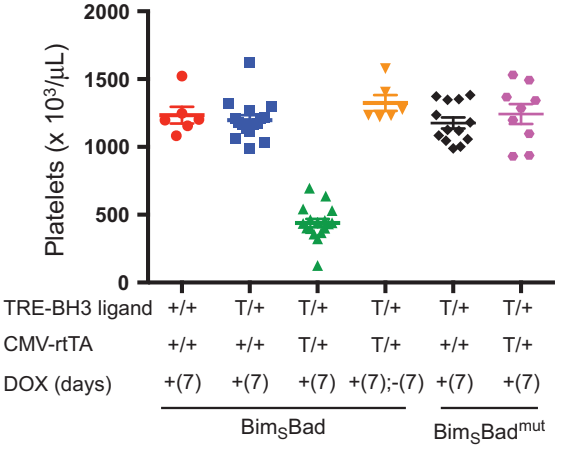

Figure 1 Expression of Bim_SBad reduces platelet levels in mice. (**a**) Schematic of the CMV-rtTA and TRE-Bim_SBad transgenes. Upon Dox treatment of bitransgenic mice, the rtTA (tet-on) protein transactivates the TRE promoter to drive expression of N-terminally FLAG tagged Bim_SBad. Targeting vectors were generated by cloning MluI-flanked FLAG-Bim_SBad/$Bim_S$$Bad^{mut}$ PCR amplicons into the MluI site of a modified version of the pgkATGfrt vector[6] in which the TRE promoter has been replaced with TREtight. BH3 domain numbering refers to the amino acid residue position within the respective Bim_S or Bad protein sequences. The BadBH3 residue in red (F121) was mutated to alanine in the $Bim_S$$Bad^{mut}$ mice. Targeted ES cell clones were injected into C57Bl/6 blastocysts and chimeras crossed to C57Bl/6 female mice. All mouse colonies were maintained by C57Bl/6 backcrossing. (**b**) Western blot of white blood cells isolated from TRE-Bim_SBad/$Bim_S$$Bad^{mut}$; CMV-rtTA bitransgenic mice or wild-type or Bim_SBad/$Bim_S$$Bad^{mut}$ single transgenic control mice following 7 days of Dox food (600 mg/kg). Blood (100 μl) was cleared of red blood cells by 10-fold dilution in red cell lysis buffer for 5 min followed by centrifugation. The white blood cell-containing pellet was then resuspended in lysis buffer (20 mM Tris pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X100 and 10% glycerol) for 1 h on ice, followed by centrifugation. The supernatant was then analyzed by Western blot probed with an anti-FLAG antibody and reprobed with anti-β-actin as loading control. * Indicates a non-specific band (**c**) Blood cell parameters after Dox treatment of bitransgenic and control animals for 7 days. $^{****}P < 0.0001$ (*t*-test) compared with littermate controls (**d**) Platelet levels in TRE-Bim_SBad bitransgenic mice treated with Dox for 7 days rebound to normal levels after 7 days without Dox treatment. No changes in platelet levels were observed in $Bim_S$$Bad^{mut}$ mice following Dox treatment

drugs, and extended to studies on combination therapies with existing drugs.

Conflict of Interest

The authors declare no conflict of interest.

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