### Correspondence

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

EF Lee[1,2], M Takiguchi[1], A Pettikiriarachchi[1], M Evangelista[1], DCS Huang[1,2], RA Dickins*[1,2] and WD Fairlie*[1,2]
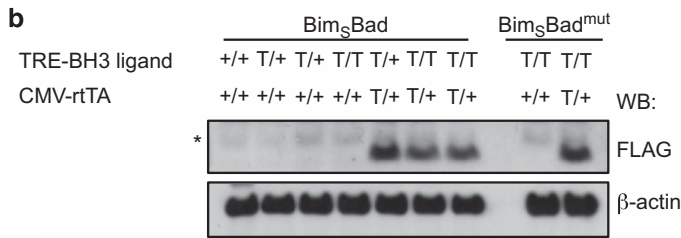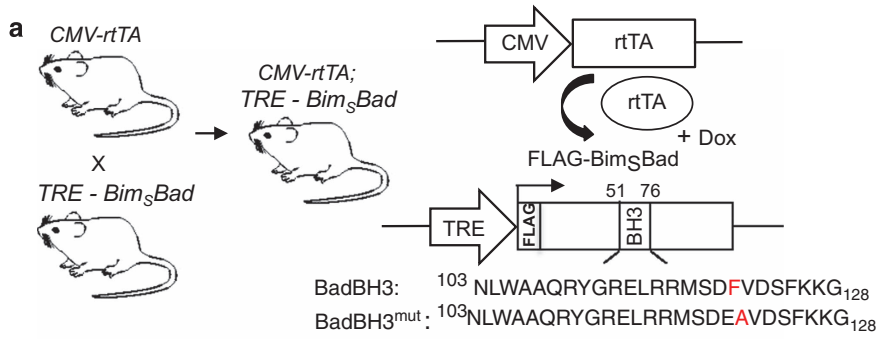
*Dear Editor*,

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 $Bim_S$BH3 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 $Bim_S$Bad expression should mimic the BH3-mimetics ABT-737 and ABT-263 that have been extensively studied *in vivo*.[1]

We generated TRE-$Bim_S$Bad 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 $Bim_S$Bad expression (Figure 1a). Western blot analysis verified Dox-inducible transgene expression in white blood cells of $Bim_S$Bad; 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 $Bim_S$Bad 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 $Bim_S$Bad 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
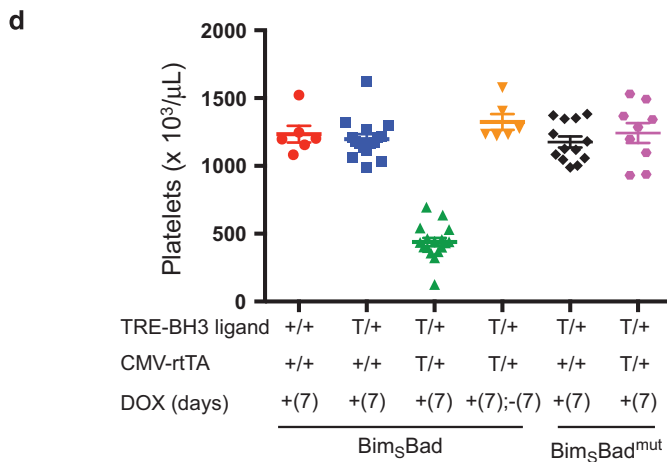
[1]Structural Biology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia and [2]Department of Medical Biology, The University of Melbourne, Parkville, Victoria, Australia
*Corresponding author: WD Fairlie or RA Dickins, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville 3052, Victoria, Australia.
Tel: +61 3 9345 2689 or +61 3 9345 2482; Fax: +61 3 9345 2686; E-mails: fairlie@wehi.edu.au or rdickins@wehi.edu.au

**a**

CMV-rtTA × TRE - Bim_SBad → CMV-rtTA; TRE - Bim_SBad

CMV → rtTA → rtTA + Dox → FLAG-Bim_SBad

TRE → FLAG → BH3 (51 76)

BadBH3: $^{103}$NLWAAQRYGRELRRMSD**F**VDSFKKG$_{128}$

BadBH3$^{mut}$: $^{103}$NLWAAQRYGRELRRMSDE**A**VDSFKKG$_{128}$

**b**

| | Bim_SBad | | | | | | | Bim_SBad$^{mut}$ | |
|---|---|---|---|---|---|---|---|---|---|
| TRE-BH3 ligand | +/+ | T/+ | T/+ | T/T | T/+ | T/T | T/T | T/T | T/T |
| CMV-rtTA | +/+ | +/+ | +/+ | +/+ | T/+ | T/+ | T/+ | +/+ | T/+ |

WB: FLAG, β-actin

**c**

| | Doxycycline 7 days | | |
|---|---|---|---|
| | +/+;+/+ (n=7) | TRE-Bim_SBad (n=15) | TRE-Bim_SBad; CMV-rtTA (n=17) |
| Erythrocytes (x10$^6$ 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 (x10$^3$ µL$^{-1}$) | 9.81 ± 0.64 | 10.29 ± 0.53 | 9.12 ± 0.37 |
| Neutrophils (x10$^3$ µL$^{-1}$) | 0.91 ± 0.20 | 0.80 ± 0.24 | 1.19 ± 0.20 |
| Lymphocytes (x10$^3$ µL$^{-1}$) | 8.47 ± 0.55 | 8.93 ± 1.93 | 7.12 ± 1.12 |
| Monocytes (x10$^3$ µL$^{-1}$) | 0.17 ± 0.047 | 0.17 ± 0.058 | 0.25 ± 0.030 |
| Platelets (x10$^3$ 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 |

**d**



| TRE-BH3 ligand | +/+ | T/+ | T/+ | T/+ | T/+ | T/+ |
|---|---|---|---|---|---|---|
| CMV-rtTA | +/+ | +/+ | T/+ | T/+ | +/+ | T/+ |
| DOX (days) | +(7) | +(7) | +(7) | +(7);-(7) | +(7) | +(7) |

Bim_SBad | Bim_SBad$^{mut}$

◀

**Figure 1** Expression of Bim$_S$Bad reduces platelet levels in mice. (**a**) Schematic of the CMV-rtTA and TRE-Bim$_S$Bad 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$_S$Bad. Targeting vectors were generated by cloning MluI-flanked FLAG-Bim$_S$Bad/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$_S$Bad/Bim$_S$Bad[mut]; CMV-rtTA bitransgenic mice or wild-type or Bim$_S$Bad/Bim$_S$Bad[mut] single transgenic control mice following 7 days of Dox food (600 mg/kg). Blood (100 $\mu$l) 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-$\beta$-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$_S$Bad 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.

1. Roy MJ *et al. Br J Pharmacol* 2014; **171**: 1973–1987.
2. Lee EF *et al. J Cell Biol* 2008; **180**: 341–355.
3. Foight GW *et al. ACS Chem Biol* 2014; **9**: 1962–1968.
4. Glaser SP *et al. Genes Dev* 2012; **26**: 120–125.
5. Del Gaizo Moore V, Letai A. *Cancer Lett* 2013; **332**: 202–205.
6. Beard C *et al. Genesis* 2006; **44**: 23–28.
7. Chen L *et al. Mol Cell* 2005; **17**: 393–403.
8. Takiguchi M *et al. PLoS One* 2013; **8**: e54009.
9. Roberts AW *et al. J Clin Oncol* 2012; **30**: 488–496.

Author/s:
Lee, EF; Takiguchi, M; Pettikiriarachchi, A; Evangelista, M; Huang, DCS; Dickins, RA; Fairlie, WD

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

Date:
2015-03-01

Citation:
Lee, E. F., Takiguchi, M., Pettikiriarachchi, A., Evangelista, M., Huang, D. C. S., Dickins, R. A. & Fairlie, W. D. (2015). A transgenic mouse model to inducibly target prosurvival Bcl2 proteins with selective BH3 peptides in vivo. CELL DEATH & DISEASE, 6 (3), https://doi.org/10.1038/cddis.2015.54.

Persistent Link:
http://hdl.handle.net/11343/260098

File Description:
Published version
License: