BIOLOGIA (PAKISTAN)
June, 2016, 62 (1), 37-43

PKISSN 0006 – 3096 (Print)
ISSN 2313 – 206X (On-Line)

# Identification of Endogenous "Bimbam" A novel GC-induced BH3-Domain containing Isoform through 2D Gel Electrophoresis

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## ABSTRACT

Glucocorticoids (GC), steroid hormones, induce apoptosis and cell cycle arrest in lymphoid tissues and hence widely employed in the therapy of children with acute lymphoblastic leukaemia (ALL). We reported a pro-apoptotic novel BH3-containing transcript, termed Bimbam, from children with ALL and cell lines. Bimbam is similar to Bim in molecular mass and co-migrates on SDS-PAGE as recognized by anti Bim antibody. The current study was carried out to separate the both isoforms and whether Bimbam exist at endogenous level. To separate both splice variant, Bimbam subclone#4 and Bim subclone#17 were resolved on long SDS-PAGE and the western blot was recognized by anti Bim antibody but it could not serve the purpose. Further, we applied 2D Gel Electrophoresis to discriminate Bimbam and Bim. This technique includes; Iso-electric point that resolves the protein on the basis of charges of amino acid in one direction and molecular mass in the other direction. When whole cell proteins from Bim subclone #17, Bimbam subclone # 4 and their mixture were probed with anti Bim antibody on 2D blot, the protein spot corresponding to both proteins could be recognized. To confirm the endogenous existence of Bimbam, dexamethasone treated Pre B cells were subjected to 2D Gel electrophoresis and the blot was subsequently probed with anti Bim antibody. The presence of double spots at the level of 25kd provides convincing evidence regarding the presence of endogenous Bimbam but the conclusive evidence can only be obtained through knowk down by Bimbam siRNA.

**Key Words:** Glucocorticoids, 2D Gel Electrophoresis, Bimbam, apoptosis, Bim

## INTRODUCTION

Glucocorticoids (GCs) being steroid hormones induce apoptosis in immature lymphoblasts and has been involved in the development of the immune repertoire and regulation of immune responses (Cidlowski *et al.,* 1996; Ashwell *et al.,* 2000). This property of GCs is being exploited in the cure of children diagnosed with acute lymphoblastic leukemia (ALL) and other lymphoid malignancies (Pui *et al.,* 2004, 2006). GCs bring about their effect through a complex made up of GC receptor and the ligand which latter on moves into the nucleus and affect the gene expression (Laudet & Gronemeyer, 2002 and Ramamoorthy & Cidlowski, 2013).

Glucocorticoids have been found to regulate a great variety of protein-encoding genes in lymphoid lineage cells (Schmidt *et al.,* 2004) and in other biological systems (Schmidt *et al.,* 2006), but the mechanism of GC-induced cell death and GC effect on immune repertoire is still illusive (Schmidt *et al.,* 2004; Rainer *et al.,* 2012).

The process of apoptosis via GCs is mainly dependent on the varied expression of Bcl-2 family members, the central player in cell death. Bcl-2 family has been grouped into pro-survival proteins such as Bcl-2 and Bcl-xL etc., pro-apoptotic members such as Bax and Bak, and a number of BH3-only proteins, such as Bad, Bid, Bim or Bmf (Gross *et al*., 1999). The BH3-only protein, Bim has been found crucial in T-lymphocyte cell death (Ploner *et al.,* 2008). Bim has been delineated into three functional isoforms namely Bim EL, Bim L and Bim S. These isoforms vary in their potential of cytotoxity based on BH3- only domain and with Bim-S being the most potent (Puthalakath *et al.,* 1999). In recent years, new isoforms of Bim have been reported to understand the role of different members of BCL2 family in Glucocorticoid induced cell death. Mami *et al.* (2001) has defined 6 novel isoforms of Bim which are product of alternative splicing.

Currently we have reported two novel BH3-only transcripts from BCL2L11 locus; Bam and Bimbam in children diagnosed with acute lymphoblastic leukemia and in other biological system (Mansha *et al.,* 2011, 2014). We investigated that Bam may not be translated due to the presence of three inappropriate start codons in the beginning of Bam ORF (Mansha *et al*., 2012) but Bimbam may be translated into protein due to the presence of strong 5' end just like Bim .

Bimbam is the product of alternative splicing from the BCL2L11 locus. The 5' portion of Bimbam up to exon 8 (harbouring BH3-only domain) is similar in sequence to Bim but it encompasses a

**Author's Contribution: M.M**., Developed concept and planned research work; **M.T**., Did experiment and analyzed Data; **M.W**., Wrote manuscript. **Corresponding author: dr.mansha@ue.edu.pk*

unique 3' portion which is not present in any Bim splice variant. Moreover, Bimbam transcript is 27 nucleotide long than that of Bim variants. As both proteins harbour similar 5' portion, they can be recognized by anti Bim antibody. Since both splice variant have similar molecular mass and almost equal in pro-apoptotic potential, there is a need to segregate the tow splice variant on protein level to establish their role in cell death separately.

To distinguish the two isoforms, we applied long conventional SDS-PAGE. As two isoforms were almost similar in molecular weight, this technique could not serve the purpose. Thereafter, we used 2D gel electrophoresis for separation of these proteins basing on their isoelectric point (pI) in the first direction and molecular mass in the second direction as used previously (O'Farrel, 1975). Anderson & Anderson, (1977) used 2D PAGE for separation and identification of proteins of blood plasma. In current work we used this approach to ascertain the existence of endogenous Bimbam.

## MATERIALS AND METHODS

### Cell Lines

The T-ALL and precursor B-cell lines used in the present study were analysed for mycoplasma absence by DNA finger printing (Parson *et al*., 2005).The Suspension cells like CCRF-CEM-C7H2 (Strasser-Wozak *et al*., 1995) and their derivative, CEM-C7H2-2C8 (Löffler *et al.,* 1999) (T-ALL cell lines) having tetracycline-regulated reverse transactivator, rtTA (Gossen *et al.,* 1995) and the pre B-cells were cultured in RPMI 1640 and adherent cell line like HEK 293T in DMEM. Both these media were supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin. Cells were cultured at 37°C, 5% CO2 and saturated humidity.

### Lentivirus Generation and Infection

One $\times 10^6$ packaging cells (HEK 293T) were cultured to ~70% confluence, transfected with 2µg of expression plasmid (e.g., pHR-tetCMV-Bimbam) along with 1µg VSV-G plasmid (vesicular stomatitis virus), plus 1µg pCMV8.91 with 12µl of Metafectene$^{TM}$ . The supernatants enriched with lentivirus were filtered through 0.45µm syringe and supplemented with 4µg/ml polybrene and used to infect $5\times 10^5$ C7H2-2C8 (the target cell line). The infected cells were let to recover for 2 days and then limiting dilution cloning was made. After 3 weeks, the Bimbam subclones were screened with doxycycline, FACS analysis and western blot (Fig., 1).

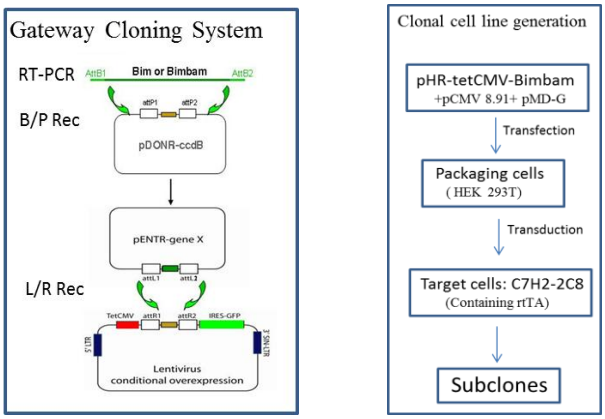



**Fig., 1**: Gateway Cloning System for cell line generation:
(A) The Bimbam PCR product anchored with AttB1 and AttB2 was first cloned in pDONR using B/P recombination to get pENTR-Bimbam. The later plasmid was recombined by LR-recombination to get Lentivirus overexpression vector having Bimbam and tetracyclin responsive promoter. (B)The expression vector of Bimbam along with pCMV8.91 and pMD-G were transfected in to packaging cell line, HEK 293 T. The resultant viral supernatant was used to transduce target cell line, CEM-C7H2-2C8 containing rtTA.

### Quantification of Protein Content (Bradford)

The quantification of proteins in the samples was done by Bradford quantification method (Bradford, 1976). The protein samples were diluted 1:5 and mixed with 200µl Bradford-reagent. After incubation for 5 minutes, absorbance was measured at 595 nm in duplicates. Data obtained thereafter was analyzed in MS excel to achieve the final protein concentrations.

### Antibodies and Immunoblotting

For immunoblotting, 30-50 µg proteins were separated by size using 12.5% polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE). Thereafter the proteins were transferred onto nitrocellulose membranes (0.45µm) by a Semi-Dry blotting apparatus (at constant 1.36 mA per $cm^2$). Ponceau-S-red staining was used to examin the transfer efficiency and location of protein marker on the membranes. A blocking buffer consisting of PBS,1% NP-40, 5% milk powder, was used to block the membranes which were subsequently put for 10-12 hours in blocking buffer suplemented with anti Bim antibody that can recognize both variants. To ensure equal loading of protein samples and to compare expression of Bim and Bimbam, a housekeeping protein α-tubulin was probed using monoclonal antibody against α-tubulin (CP06,

Calbiochem) for one hour. Later on the membranes were given 10 minutes wash in PBS containing 1% NP-40 twicely and kept in the anti-mouse or anti-rabbit HRP-conjugated secondary antibody for 40 minutes. On washing for further 10 minutes, proteins were viewed by applying ECL plus system (enhanced chemiluminescence reagent) and exposure to AGFA Curix X-ray films.

**Two-Dimensional Gel Electrophoresis (2-D Electrophoresis)**

Since Bim and Bimbam proteins possess an almost identical molecular weight and both proteins were detected by the Bim antibody it was not possible to discriminate the two isoforms on conventional Western blots. However, both proteins have substantially different isoelectric point (PI) and therefore can be separated on 2-D gel.

2-D electrophoresis was performed as described by Skvortsov *et al*. (2007). Bim and Bimbam clones were harvested by centrifµging at 4°C for 5 minutes at 100 ×g. The cells were washed in 10 mL PBS containing protease inhibitors and resuspended in 100 µL of homogenization buffer suplemented with protease inhibitors and then sonified. The homogenized cells were centrifuged at 1700 ×g at 4°C (10 minutes) and the supernatant was obtained in a new tube. Proteins were precipitated using methanol/chloroform and subsequently resolubilized in lysis buffer. Proteins were quantified by Bradford quantification method.

200 µg of protein sample was lysed in 200 µL lysis solution and loaded on immobilized 11 cm pH 3-11 NL gradient strips. For first dimension, the sample was put to active rehydration (50 V) at 20°C overnight. Subsequently the samples were subjected to Isoelectric focusing at 250 V for 30 minutes, 500 V for one hour, 2000 V for one hour and finally at 8000 V until 35000 V/hours were reached in total. Afterwards, the protein samples were size fractioned on 16% PAGE for second dimension and electroblotted onto nitrocellulose membranes. Ponceau red staining was used to control protein loading of membranes. The membranes were blocked for 1 hour in blocking buffer (TBS) supplemented with 5% fat free milk and 0.1% Tween 20 and were subsequently kept for twelve hours at room temerature in TBS containing antibody against Bim and Bimbam. After performing washing steps twicely the membranes incubated for one hour with HRP-conjugated anti-rabbit secondary antibody. Proteins were viewed by applying an enhanced chemiluminescence reagent, ECL plus system.

## RESULTS

**Detection of Bimbam by conventional SDS-PAGE**

To address the fundamental question of endogenous existance of Bimbam, a stable Bim subclone #17 (kindly provided by Ploner, 2008) and Bimbam subclone #4 derived from the CCRF-CEM-C7H2-2C8 cell lines were used and characterized by using different doxycycline concentrations (Fig.,2).

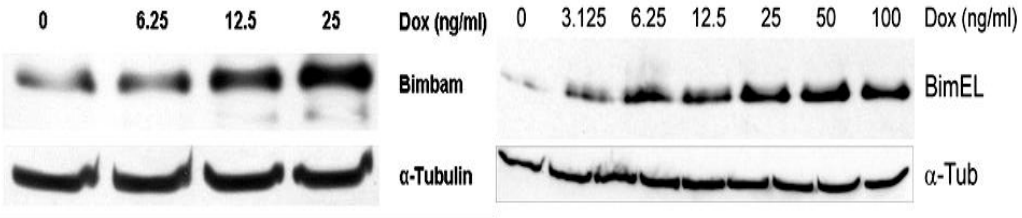



**Fig., 2:** Characterization of T-ALL cell lines. A doxycyclin dose response curve for Bimbam and Bim (Ploner, 2008) protein is shown. α-tubulin was used as loading control.

Bimbam and Bim proteins co-migrate on SDS-PAGE as there is almost negligible difference in their molecular masses. To separates the two proteins, a mixture of Bimbam subclone #4 and Bim subclone # 17 was fractionated on a long conventional SDS-PAGE. The resultant blot that was probed with anti Bim antibody could not resolve the two isoforms on account of same molecular mass (Fig., 3).

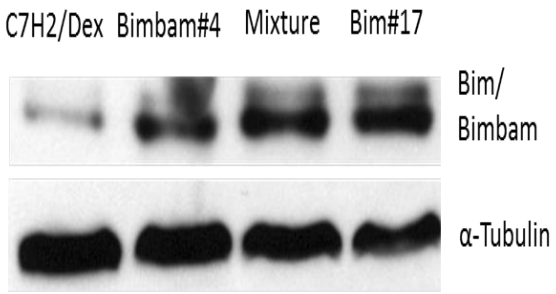



**Fig., 3.** Separation of Bimbam and Bim on Long Conventional SDS-PAGE: The protein samples of C7H2-2C8-Bimbam#4 and C7H2-2C8-Bim#17 were analyzed on long SDS-PAGE with antibody against Bim. α-tubulin, a loading control was probed with anti α-tubulin antibody. 24h Dex treated C7H2 was used as control of experiment.

**Detection of Endogenous Bimbam By 2D Gel Electrophoresis**

Since there was a sharp difference between the isoelectric point (PI) of Bim and Bimbam due to the presence of diverse charged amino acids in the 3′ portion, we opted to resolve the proteins on 2D gel electrophoresis. In the first experiment, the Bimbam subclone #4 and Bim subclone # 17 were analysed on 2D gel and the blots were subsequently probed with anti Bim antibody. The 2-D blot for Bim revealed some spots at around 25 kd level indicating most likely the phosphorylated form of Bim (indicated by blue arrows and numbered as 0,1,2,3,4) whereas on Bimbam blot, five spots at about 25 kd, can be observed reflecting most probably phosphorylated form of this isoform (Fig., 4).

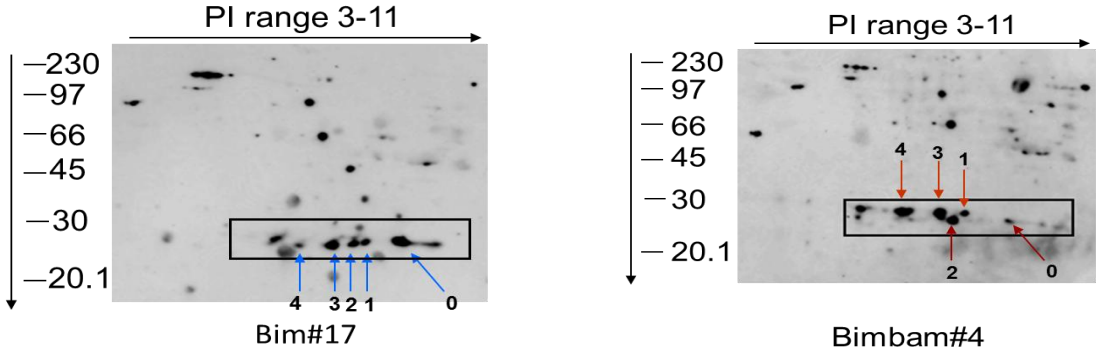
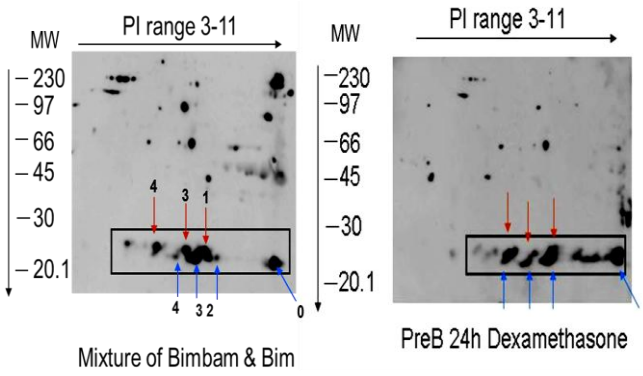



**Fig., 4**: Separation of Bim and Bimbam on 2D blot:

C7H2-2C8-Bim-17 and C7H2-2C8-Bimbam-4 were induced with 25ng doxycycline for 6 hours and extracts were prepared and separated by 2D gel electrophoresis [pI range 3–11] in the horizontal and SDS-PAGE in the vertical dimension) and the isoforms were probed with anti Bim antibody. Phosphorylated spots of BimEL and Bimbam are represented with numbers and arrows in a box.

When the mixture of both these isoforms was subjected to 2-D gel and probed with anti Bim antibody, the spots corresponding to Bim and Bimbam can be distinguished clearly at about 25 kd level as indicated with red and blue arrows (Fig., 5A).




(A) (B)

**Fig., 5**: Presence of endogenous Bim and Bimbam on 2D blot:

A) Mixture of C7H2-2C8-Bim-17 and C7H2-2C8-Bimbam-4 separately induced with 25ng doxycycline for 6 hours and extracts were prepared, mixed in equal and examined by 2D gel electrophoresis [pI range 3–11] in the horizontal and SDS-PAGE in the vertical dimension) and immunoblot was probed with anti Bim antibody. Proteins corresponding to isoforms of BimEL are indicated with numbers and arrows in a box. (B) The protein sample of dex treated Pre-B cells was examined by 2D gel electrophoresis [pI range 3–11] in the horizontal and SDS-PAGE in the vertical dimension). The protein blot was probed with anti Bim antibody. The doublet spots representing the isoforms of Bimbam and BimEL are shown with arrows in a box.

After successful experiments with recombinant Bim and Bimbam, the 2D gel electrophoresis was employed to identify endogenous Bimbam. The whole protein from 24h dexamethasone treated pre B cells was run on 2-D gel and the blot was probed with anti Bim antibody. The presence of doublet spots at about 25 kd level most probably corresponds to Bim and Bimbam. A deep comparison of both blots (endogenous and recombinant mixture) reflects that Bimbam isoform exist endogenously on protein level (Fig., 5B).

## DISCUSSION

### Detection of Recombinant Bimbam by 2D Gel Electrophoresis

We have already reported the existance of Bimbam, a novel Bim varient, at mRNA level as validated by SYBR-green real time RT-PCR (Mansha *et al.,* 2012). Bimbam may be a central player in GC-induced apoptosis, if it exists on protein level on account of its BH3-domain and a translatable 5´ end similar to Bim.

To resolve the phenomena of GC-induced apoptosis, new isoforms of Bim have been discovered and functionally analysed. Different investigators have reported different isoforms from BCL2L11 due to alternative splicing but Bimbam as reported earlier is unique in its C-terminal domain which is not present in any other Bim variant.

Regarding its endogenous existence, the conventional westren blot was porbed with anti Bim antibody (which is specific to N-termianl region) but the result could not differentiate the two isoforms. It is pertinent to mention here that no commercial antibody specific to C-terminal portion of Bimbam is available in the market. Therefore we used “long westren gels” for separation of Bim and Bimbam proteins. This technique could not serve the purpose as the difference in MW of two isoforms was almost negligible (MW of Bim and Bimbam is 22.17 and 22.69 kd, respectively) and corresponding protein bands could not be separated. Regarding endogenous existance of Bimbam and its separation from Bim, we employed 2D gel electrophoresis basing on the difference in isoelectric point (IP for Bim is 8.43 and Bimbam is 6.82). The mixture of recombinant Bim and Bimbam on 2D gel revealed delineation of spots corresponding to phosphorylated forms of both proteins.

### Detection of Endogenous Bimbam by 2D Gel Electrophoresis

To mimic the *in vivo* condition, pre B cell line was treated with dexamethasone for 24h and subjected to 2D gel electrophoresis. The blot explicitly shows doublet spots indicating the presence of most likely the phosphorylated form of Bim and Bimbam proteins. The spots of both isoforms appear to share almost same position of phosphorlation but Bimbam spots are little higher than Bim spots. Our results are well supported by Hubner *et al.,* (2008) where multisite phophorylation of Bim is shown by 2D gel electrophoresis and the location of Bim spots agrees with our findings. Seward et.al., (2003) also indicated the multi site phosphorylation of Bim in lymphocytes where the phosphorylated Bim spots supported our findings.

We are of the view that multisite phosphorylation of both isoforms (Bim and Bimbam) look alike. The doublet spots in preB cell line most probably indicate endogenous presence of both isoform at around 25 kd level. But the conclusive evidence can only be derived by knowk down of doublet spots specifically by Bim and Bimbam siRNA.

**ACKNOWLEDGEMENTS**

We are greatly thankful to Prof. A.villunger, Prof. Reinhard kofler and Prof. Stefen Geley for valuable and thoughtful discussions. We are specially thankful to Dr. Skvortsov for providing support in 2 D gell experimentation. This work has been supported by the Austrian Science Fund (SFB-F021,) and Higher Education Commission of Pakistan (HEC).

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1 **Received: 20-10-2015** **Revised: 16-03-2016** **Accepted: 27-04-2016**