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ROLE OF BCL-2 FAMILY MEMBERS TO PROMOTE GLUCOCORTICOID –INDUCED APOPTOSIS BY MEK INHIBITORS IN LEUKEMIC CELLS

ANILA RAMBAL
Virginia Commonwealth University

Center for Environmental Studies
Virginia Commonwealth University

This is to certify that the thesis prepared by Rambal Anila Ashok entitled ROLE OF BCL-2 FAMILY MEMBERS TO PROMOTE GLUCOCORTICOID –INDUCED APOPTOSIS BY MEK INHIBITORS IN LEUKEMIC CELLS has been approved by her committee as satisfactory completion of the thesis requirement for the degree of Master of Science in Environmental Studies

Dr. Hisashi Harada, Department of Internal Medicine

Dr. Peter L. DeFUR, Center for Environmental Studies

Dr. Rima B. Franklin, Department of Biology

Dr. Greg Garman, Director of Center for Environmental Studies

Dr. Thomas Huff, Vice-Provost for Life Sciences

Dr. F. Douglas Boudinot, Dean of the Graduate School

20th April, 2009

ROLE OF BCL-2 FAMILY MEMBERS TO PROMOTE GLUCOCORTICOID – INDUCED APOPTOSIS BY MEK INHIBITORS IN LEUKEMIC CELLS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science, Environmental Studies at Virginia Commonwealth University.

By

RAMBAL ANILA ASHOK
Master of Philosophy in Environmental Science and Engineering
Pondicherry University, India, 2006

Director: DR. HISASHI HARADA
ASSISTANT PROFESSOR, DEPARTMENT OF INTERNAL MEDICINE

Virginia Commonwealth University
Richmond, Virginia
May 2009

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Table of Contents

Page

Acknowledgement v

List of Figures vi

Abstract vii

Introduction 1

Hypothesis 20

Materials and Methods.....21

Results 24

Discussion 35

Literature cited.....39

Vita 45

List of Figures

Page

Figure 1: Difference between necrosis and apoptosis.....3

Figure 2: Intrinsic Pathway to carry out apoptosis. 6

Figure 3: The three classes of BCL-2 family member proteins. 9

Figure 4: Chemotherapy triggers the intrinsic pathway.....11

Figure 5: Classical and alternative pathway for GC-induced cell death.....14

Figure 6: RAS/REF/MEK/ERK pathway. 19

Figure 7: CCRF-CEM (T-ALL) cells show an increase in apoptosis when co-treated with Dexamethasone and PD184352. 25

Figure 8: Western blot analysis of the BCL-2 family member proteins. 27

Figure 9a: Down-regulation of BIM by shRNA 29

Figure 9b: BIM is required for cell death induced by Dexamethasone and PD184352...29

Figure 10a: Down-regulation of BAD by shRNA. 31

Figure 10b: BAD is dispensable for cell death induced by Dexamethasone and PD184352. 31

Figure 11a: The expression of PUMA in CEM/shPUMA clone 37 and clone 47. 33

Figure 11b: Cell death in CEM/shPUMA clone 37 and clone 47 treated with Dex and/or PD184352. 33

Abstract

ROLE OF BCL-2 FAMILY MEMBERS TO PROMOTE GLUCOCORTICOID – INDUCED APOPTOSIS BY MEK INHIBITORS IN LEUKEMIC CELLS

By Rambal Anila Ashok, M.S.

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Major Director: Dr. Hisashi Harada
Assistant Professor, Department of Internal Medicine

Glucocorticoids (GC) are common components of many chemotherapeutic regimens for lymphoid malignancies. GC-induced apoptosis involves an intrinsic BCL-2 family-regulated pathway. It has been shown that BIM (BCL-2 interacting mediator of cell death), a BH3-only pro-apoptotic protein, is up-regulated by dexamethasone (Dex) treatment in acute lymphoblastic leukemia (ALL) cells. Furthermore, BIM is inactivated by extracellular signal-regulated kinase (ERK)-mediated phosphorylation. We therefore

hypothesized co-treatment with Dex and MEK/ERK inhibitors would promote apoptosis in ALL cells through BIM up-regulation and activation. We show here that a MEK inhibitor, PD184352 synergistically enhances Dex lethality in CCRF-CEM (T-ALL) cells. Co-treatment with Dex and PD184352 results in BIM accumulation. Down-regulation of BIM by short-hairpin RNA in CCRF-CEM cells suppressed apoptosis by Dex/PD184352 co-treatment. In contrast, another BH3-only protein, BAD is dispensable. Thus, BIM is a critical molecule in this regimen, and targeting BIM by drugs combination could be effective on ALL and possibly other malignancies.

Key words: Leukemia, BCL-2, apoptosis, glucocorticoids, MEK inhibitor

Introduction

Cancer and Apoptosis:

Cancer can be defined as a condition in which mutated cells divide without control and are able to spread to adjacent or other tissues. Cancers are of various types depending upon the cells from where they originate.

Carcinoma occurs in cells covering the body surfaces such as breast, colon and is the most frequent type of cancer.

Sarcoma occurs in cells of supporting tissues such as cartilage, fat, muscle and bone.

Lymphoma occurs in the lymph nodes and impairs the body's immune system.

Leukemia occurs in the blood cells of the bone marrow and they circulate and accumulate in the bloodstream.[1]

Cancer cells can spread to other parts of the body through the blood and lymphatic systems via two mechanisms: invasion and metastasis. Invasion refers to the direct migration and penetration by cancer cells into neighboring tissues. Metastasis refers to the ability of cancer cells to penetrate into lymphatic and blood vessels and then invade normal tissues elsewhere in the body via the circulation system.

Cells have a finite lifespan and cell death occurs mainly as a result of passive necrotic processes or due to an active process of programmed cell death termed apoptosis. Necrosis is premature death of cells, caused by external factors like infections or trauma. Necrosis may prove to be fatal and it is in contrast to apoptosis. Figure 1 illustrates the difference between necrosis and apoptosis.

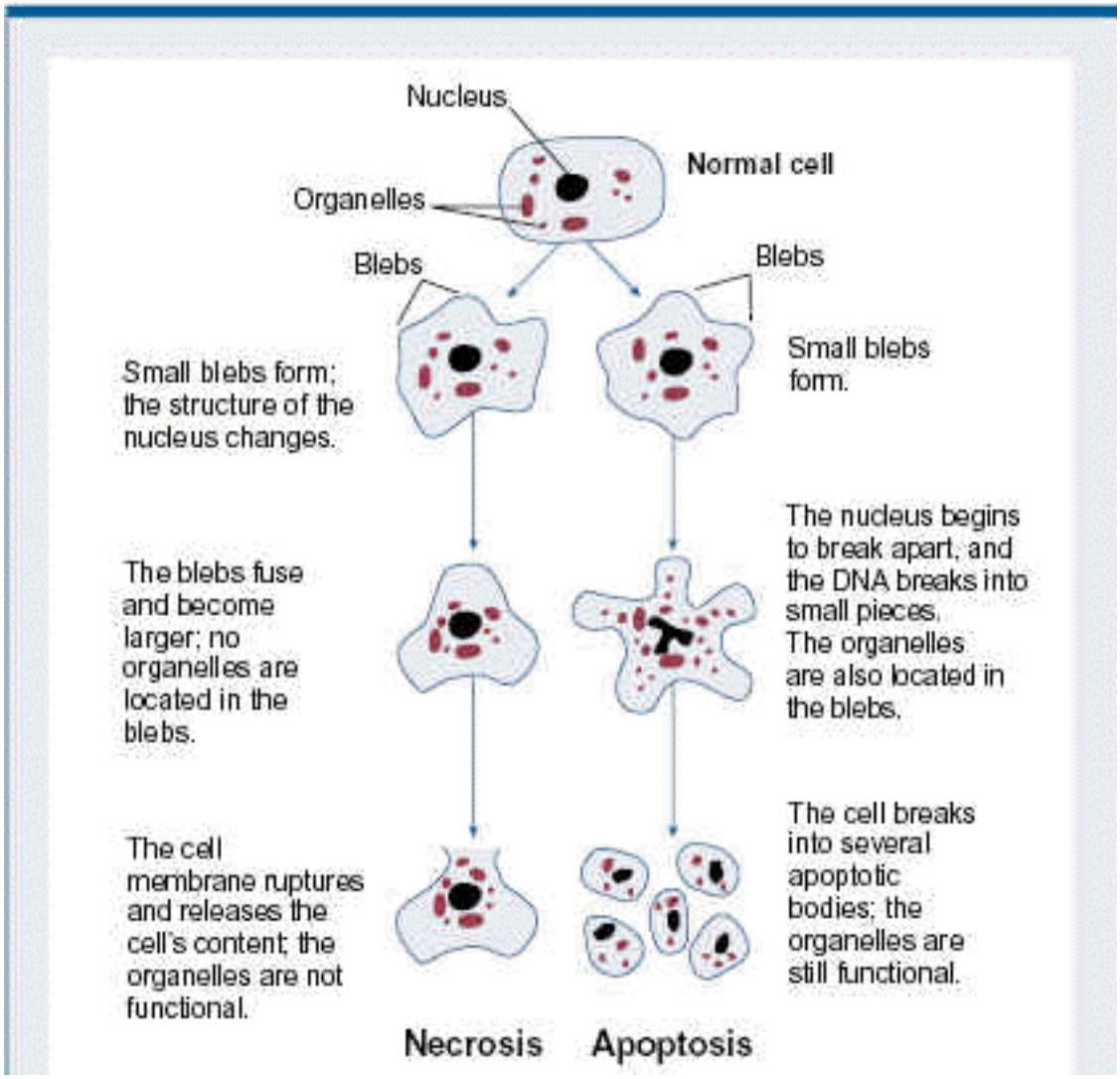

Figure 1: Difference between necrosis and apoptosis. Cells undergoing necrosis initially swell and their internal components or organelles break down. The cells eventually rupture and spill debris that leads to local inflammation. This inflammation can then result in the death of adjacent cells. During apoptosis, the cell breaks up into several smaller bodies that are still surrounded by a membrane. These "apoptotic bodies" then are engulfed and destroyed by scavenging cells. Reference (2): Goodlett RC

Apoptosis plays an important role both in human embryonic development and in adult tissue homeostasis. Apoptosis is the most common mechanism by which the body eliminates damaged or unneeded cells without local inflammation from leakage of cell contents. In normal cells, apoptosis is initiated in response to developmental cues, cell stress, and changes in growth factor signaling. Dysregulation of apoptosis is implicated in a variety of diseases states. Accelerated cell death is implicated in the pathogenesis of a number of diseases, including Alzheimer's disease and acquired immunodeficiency syndrome (AIDS). Conversely, an inappropriately low rate of apoptosis can give rise to cancer or autoimmune disorders.

The balance of cell proliferation and apoptosis genetically controls cell growth; dysregulation of this balance causes tumor development. One group of genes implicated in the development of cancer is "oncogenes." Oncogenes are activated by overexpression and/or mutations, resulting in uncontrolled cell growth. A second group of genes implicated in cancer are the "tumor suppressor genes." Tumor suppressor genes are the genes whose absence can lead to cancer. One example of the tumor suppressor genes called "p53" can normally trigger apoptosis. In cells that have undergone irreversible DNA damage, p53 protein eventually initiates cell suicide, thereby preventing the genetically damaged cell from growing out of control. Thus, activation of oncogenes and inactivation of tumor suppressor genes are the key hallmarks of cancer and are critical for cancer development and tumor cell survival.[3-5]

Apoptosis is executed through two major signaling pathways — the 'intrinsic' and the 'extrinsic' pathways .Figure 2. shows the diagrammatic representation of the intrinsic pathway The intrinsic pathway is triggered within the cell by developmental cues or severe cell stress, such as DNA damage. The extrinsic pathway is activated when a pro-apoptotic ligand, for example, Apo2L/TRAIL (apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand) binds to pro-apoptotic receptors, DR4 and DR5. The extrinsic and intrinsic pathways converge via activation of intracellular enzymes called 'caspases'. The caspase cascade ultimately triggers cell death through the destruction of cellular proteins, which are important for cell viability.

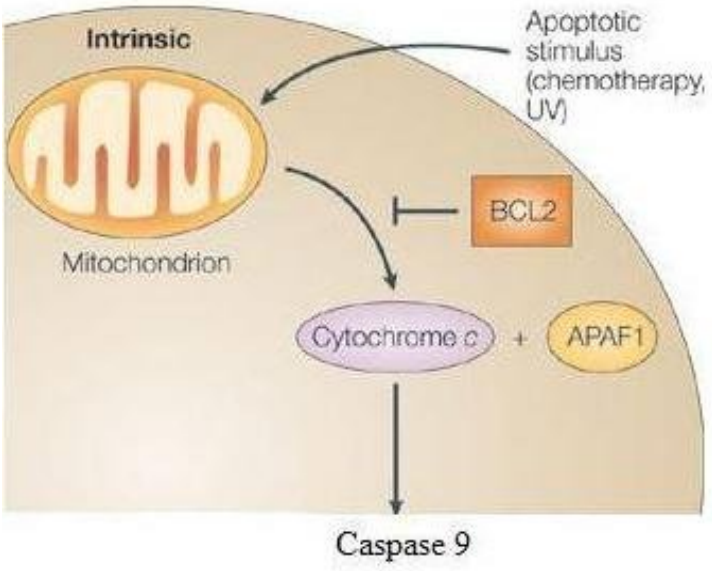

Figure 2: Intrinsic Pathway to carry out apoptosis. External stimuli such as UV or chemotherapeutic treatments trigger apoptosis through the BCL-2 family proteins localized at mitochondria. The release of cytochrome c from mitochondria and subsequent caspase 9 activation leads to cell death. Reference (6): Andersen MH

BCL-2 Family Member Proteins:

The intrinsic pathway relies on the balance between pro- and anti-apoptotic members of the B-cell lymphoma-2 (BCL-2)-family proteins. They play an important role in the regulation of apoptosis as they have the ability to regulate mitochondrial cytochrome *c* release. The BCL-2 family is subdivided into three main groups based on regions of one to four BCL-2 homology (BH1-BH4) domains and function. Multi-domain anti-apoptotic subfamily which contains trans-membrane domains (TM) is typically associated with membranes (e.g., BCL-2, MCL-1, BCL-X_L). Multi-domain pro-apoptotic BAX-like subfamily (e.g. BAX, BAK) lacks BH4 domains and promotes apoptosis by forming pores in mitochondrial outer membranes. BAX was the first death-inducing protein identified as part of the BCL-2 family. BH3-only pro-apoptotic proteins is a structurally diverse group of proteins that only display homology within the small BH3 motif (e.g., BAD, BID, BIM, PUMA). The anti-apoptotic BCL-2 proteins inhibit apoptosis by preventing BH3-only protein-induced oligomerization of BAX and/or BAK at mitochondrial outer membranes, which would otherwise lead to the release of cytochrome *c*. The anti-apoptotic BCL-2 proteins differentially bind to the BH3-only proteins. BH3-only proteins like BID and BIM interact with all anti-apoptotic BCL-2 proteins, whereas others like NOXA interact only with certain BCL-2-family members. To date, over twenty different BCL-2 family members have been identified.[7-12]

BCL-2 family member proteins are essential for normal tissue development and homeostasis. Figure 3 illustrates the BCL-2 family member proteins along with their

molecular weights, BH1-4 regions, and trans-membrane domains. Homo- and hetero-dimerization among the BCL-2 family proteins is one mechanism that regulates the apoptotic activity of these proteins. BH3-only proteins, when over expressed, promote apoptosis and are responsible for activating or suppressing pro- and anti-apoptotic BCL-2 proteins. It is now clear that activation of BH3-only proteins by apoptotic stimuli initiates mitochondria-dependent cell death pathway. The intrinsic pathway begins with cell stress to activate BH3-only proteins, which cause cytochrome c release from mitochondria by activating BAX and/or BAK. The anti-apoptotic BCL-2 family of proteins prevents this process. Cytochrome c released from mitochondria binds the adaptor apoptotic protease activating factor-1 (APAF-1), forming a large multi-protein structure known as the apoptosome. The apoptosome then recruits and activates caspase-9, which in turn activates the downstream effector caspases, including caspase-3, -6, and -7, leading to target protein degradation.

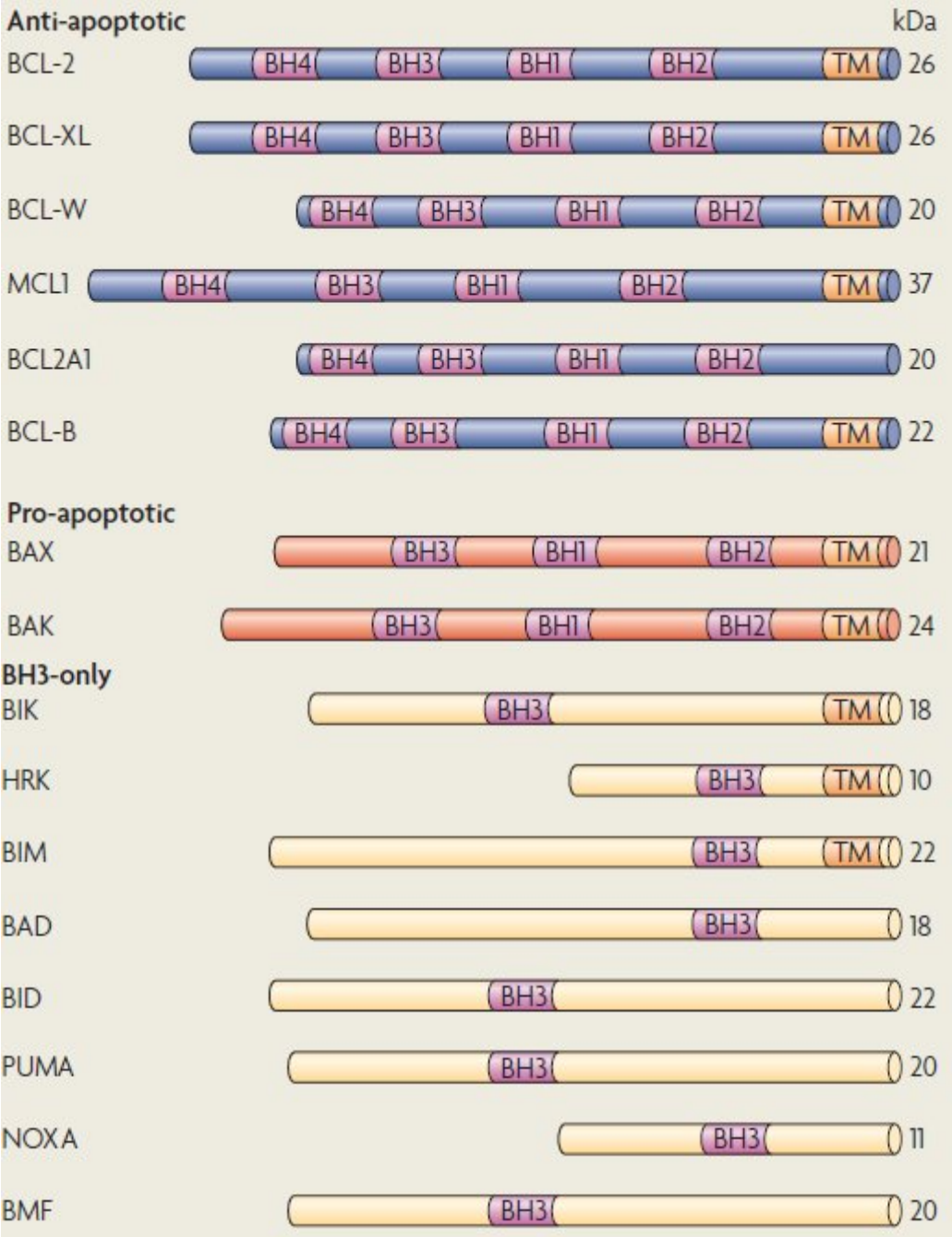

Figure 3: Three classes of BCL-2 family member proteins. They include anti-apoptotic, pro-apoptotic and BH3-only proteins. The figure also shows the trans-membrane domain along with the BH1-4 regions. Reference (13): Taylor, CR

Chemotherapy:

Chemotherapy is the general term for treatments with chemical agents to stop cancer cells from growing. Chemotherapy can eliminate cancer cells at sites great distances from the original cancer. As a result, chemotherapy is considered a *systemic* treatment and causes therapeutic activation of apoptosis in cancer cells. Commonly used anticancer drugs can induce tumor cell death by apoptosis. Altered expression of the BCL-2 family proteins, either overexpression of pro-survival proteins or decreased/altered expression of pro-apoptotic members has been identified in many cancer cell types and may contribute to the resistance of these tumor cells to chemotherapeutic agents. This observation, together with the central role of the BCL-2 family proteins in cell death pathways, makes these proteins attractive targets to kill tumor cells or to sensitize them to death induced by common cytotoxic drugs. Figure 4 illustrates the intrinsic pathway, which gets triggered when DNA damage occurs due to chemotherapeutic drugs Thus, targeting the intrinsic pathway by chemotherapeutic drugs is a strategy to kill tumor cells. In the present study we examine the role of two chemotherapeutic drugs - glucocorticoids and MEK inhibitors.

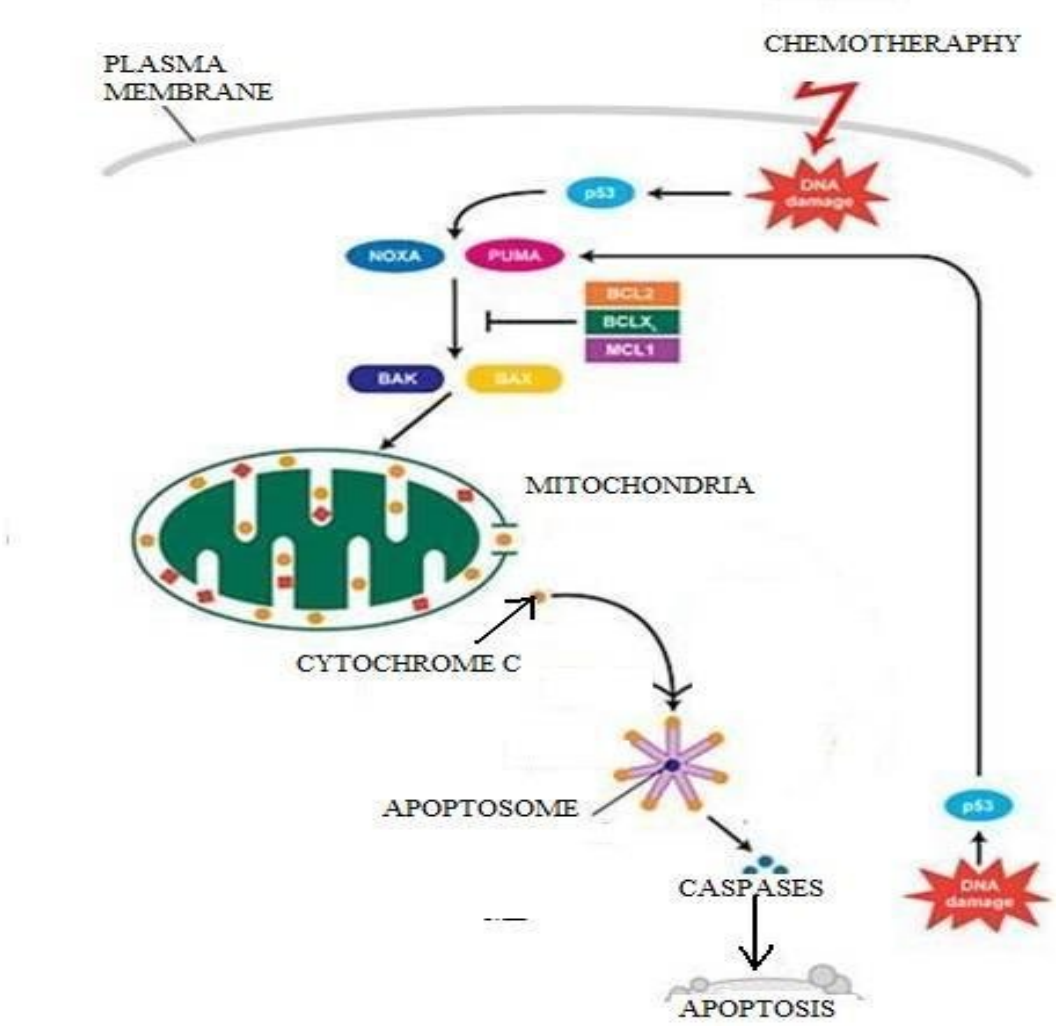

Figure 4: Chemotherapy triggers the intrinsic pathway.

Chemotherapy triggers DNA damage which activates the BCL-2 family member proteins leading to cytochrome c release. Cytochrome c leads to formation of apoptosome, which activates caspases and eventually causes cell death.

Reference: http://www.researchapoptosis.com/apoptosis/pathways/intrinsic/index.m

Glucocorticoids and their interaction with the BCL-2 family member proteins:

Glucocorticoids (GCs): These are a class of steroid hormones produced in the adrenal glands. GC induces apoptosis in certain lymphoid cells and plays an important role in the treatment of childhood acute lymphoblastic leukemia (ALL) and other lymphoid malignancies. Figure 5 explains that GC induces cell death via two possible pathways:

The classic pathway: GC might induce cell death by directly regulating genes controlling cell survival and apoptosis, or via (de)regulating genes or gene networks leading to cellular distress, which in turn constitutes an apoptotic stimulus. In both scenarios, members of pro- and anti-apoptotic BCL-2 family proteins, referred to as the 'BCL-2 rheostat', might be involved either as direct GR targets or as sensors for potentially harmful GC effects. GC-induced intrinsic pathway regulated by the BCL-2 family proteins can be broadly classified into three stages:

- Initiation stage: GC binds to the glucocorticoid receptor (GR), a ligand-activated transcription factor of the nuclear receptor superfamily that resides in the cytoplasm. Then GR translocates into the nucleus and modulates gene expression via binding to specific DNA response elements or by protein-protein interactions with other transcription factors.

- Decision stage: Pro- and anti-apoptotic BCL-2 family proteins regulate the execution of apoptosis at the mitochondrial level. This leads to the release of cytochrome c from the intermembrane space of mitochondria into cytosol.
- Execution stage: The release of cytochrome c triggers the activation of caspases, which cleaves a variety of substrates and also results in the activation of endonucleases leading to DNA cleavage. These events finally lead to cell death

The alternative pathway: Apart from regulating the BCL-2 family members, GC has dramatic effects on metabolism in numerous lymphocytic and non-lymphocytic cells. Thus, anabolic processes (such as glucose uptake, amino acid transport, ATP production, RNA polymerase activity, nucleoside accumulation, protein and nucleic acid biosynthesis) are decreased and catabolic processes (such as protein and RNA degradation) stimulated. In most tissues, GR is subject to negative feedback, making the above (and/or perhaps other) potentially harmful GC-mediated events transient. In cells undergoing GC-induced apoptosis, GR is not repressed - to the contrary, there are several examples where sensitivity is associated with GR auto-induction. In the continuous presence of GR (and even more so in the case of GR auto-induction), the above GC effects will become permanent and, given sufficient time, lead to a state incompatible with cellular survival.[14-20]

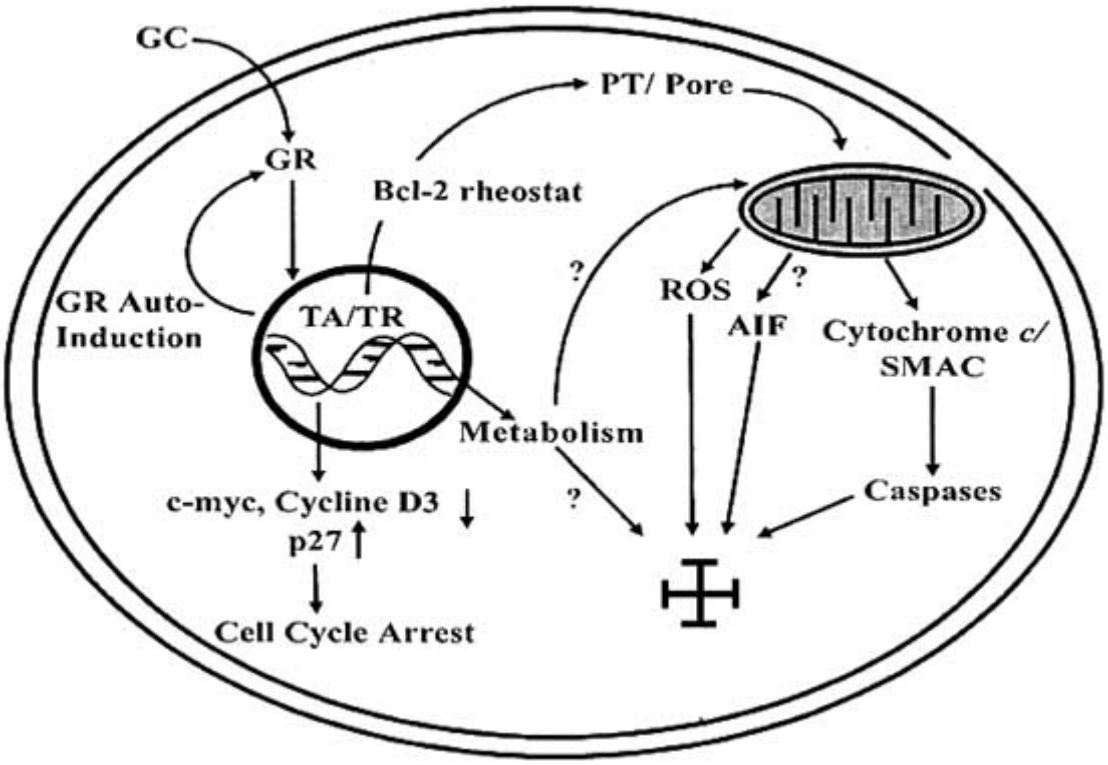

Figure 5: Classical and alternative pathway for GC-induced cell death. Left side of the figure explains rapid apoptosis induced by GC by directly regulating typical apoptosis genes, possibly those controlling the "BCL-2 rheostat". In the presence of (oncogenic) overexpression of anti-apoptotic BCL-2 family members (represented by BCL-2 in the figure), this mechanism may be delayed allowing a second mechanism to take place (right side of the figure) that proposedly critically depends on GR auto-induction ("positive feed-back loop"). In its presence, otherwise transient, detrimental GC effects (catabolic effects with reduced macromolecule biosynthesis and others) may become permanent and thus incompatible with cellular survival. Reference (21): Renner K

Dexamethasone (Dex) is a member of the synthetic glucocorticoid and is used in chemotherapy to treat leukemia/lymphoma. The current understanding of the mechanisms of Dex-induced apoptosis is as follows:

- Dex-induced apoptosis is regulated by the BCL-2 family proteins, which play vital roles in the decision stage.
- It has been shown that BIM (BCL-2 Interacting Mediator of cell death), a BH3-only pro-apoptotic BCL-2 family protein, is induced by Dex in leukemic cells and plays a critical role in Dex-induced apoptosis.[22]

RAS-RAF-MEK-ERK pathway:

The RAS/RAF/MEK (mitogen-activated protein kinase/ERK kinase)/ERK (extracellular signal-regulated kinase) pathway has diverse effects which can regulate cell cycle progression, survival or differentiation**.** Abnormal activation of this pathway occurs in human cancer due to the activation of the upstream molecules by (1) mutations at membrane receptors such as FLT-3, KIT, or FMS, (2) overexpression of wild-type or mutated EGFR, or (3) chromosomal translocations such as BCR-ABL. Furthermore, this cascade also regulates the activity of many proteins involved in apoptosis by the post-translational phosphorylation of apoptotic regulatory molecules including BAD, BIM, MCL-1, caspase-9 and more controversially BCL-2.

RAS is an upstream activator of the RAF/MEK/ERK kinase cascade; it is a small GTP-binding protein. Four RAS proteins have been identified, namely Ha-RAS, N-RAS, Ki-RAS 4A and Ki-RAS 4B. The two isoforms of Ki-RAS are produced from the same gene by alternative splicing. RAS proteins show varying abilities to activate the RAF/MEK/ERK and PI3K/AKT cascades.

The mammalian RAF gene family consists of A-RAF, B-RAF and RAF-1 (C-RAF). RAF is a serine/threonine (S/T) kinase and is normally activated by a complex series of events including: (i) recruitment to the plasma membrane mediated by an interaction with RAS; (ii) dimerization of RAF proteins; (iii) phosphorylation /dephosphorylation on different domains; (iv) disassociation from the RAF kinase

inhibitory protein (RKIP) and (v) association with scaffolding complexes [e.g. kinase suppressor of RAS (KSR)]. RAF activity is further modulated by chaperonin proteins.

MEK is a tyrosine (Y)- and S/T-dual specificity protein kinase. Its activity is positively regulated by phosphorylation on S residues in the catalytic domain mediated by RAF. All three RAF family members are able to phosphorylate and activate MEK but different biochemical potencies have been observed (B-RAF > RAF-1 > A-RAF). The predominant downstream target of MEK is ERK. In contrast, downstream ERK has multiple targets. Thus, therapeutic targeting of MEK is relatively specific.[23-27]

ERK are S/T kinases and their activities are positively regulated by phosphosphorylation mediated by MEK. ERK can directly phosphorylate many transcription factors including ETS-1, c-JUN and c-MYC. ERK can also phosphorylate and activate the 90 kDa ribosomal S6 kinase (p90RSK), which then leads to the activation of the transcription factor CREB. ERK has over 160 downstream targets and activates more then 70 substrates. Figure 6 explains the RAS/REF/MEK/ERK pathway.

MEK inhibitors:

The RAF/MEK/ERK pathway is an important pathway to target for therapeutic intervention. Inhibitors of RAS, RAF, and MEK and some downstream targets have been developed and many are currently in clinical trials.

Blocking ERK activity via small-molecule MEK inhibitors has come to the forefront as an exciting approach in cancer therapeutics. PD98059 was the first specific MEK inhibitor described. It was identified by screening a compound library for inhibitors with an assay that measured phosphorylation of an ERK target protein in the presence of both MEK and ERK. A second MEK inhibitor, U0126 was also identified by screening a compound library using an assay designed to find an inhibitor that could antagonize activator protein-1-driven transcription without blocking the transcription of glucocorticoid response elements. PD184352 (CI-1040), the MEK inhibitor used in our current study, was the first small-molecule MEK inhibitor that proceeded to clinical testing. PD184352 was developed based on compounds and structures identified during the screening that led to the identification of PD98059, but had improved potency and selectivity. [28-31]

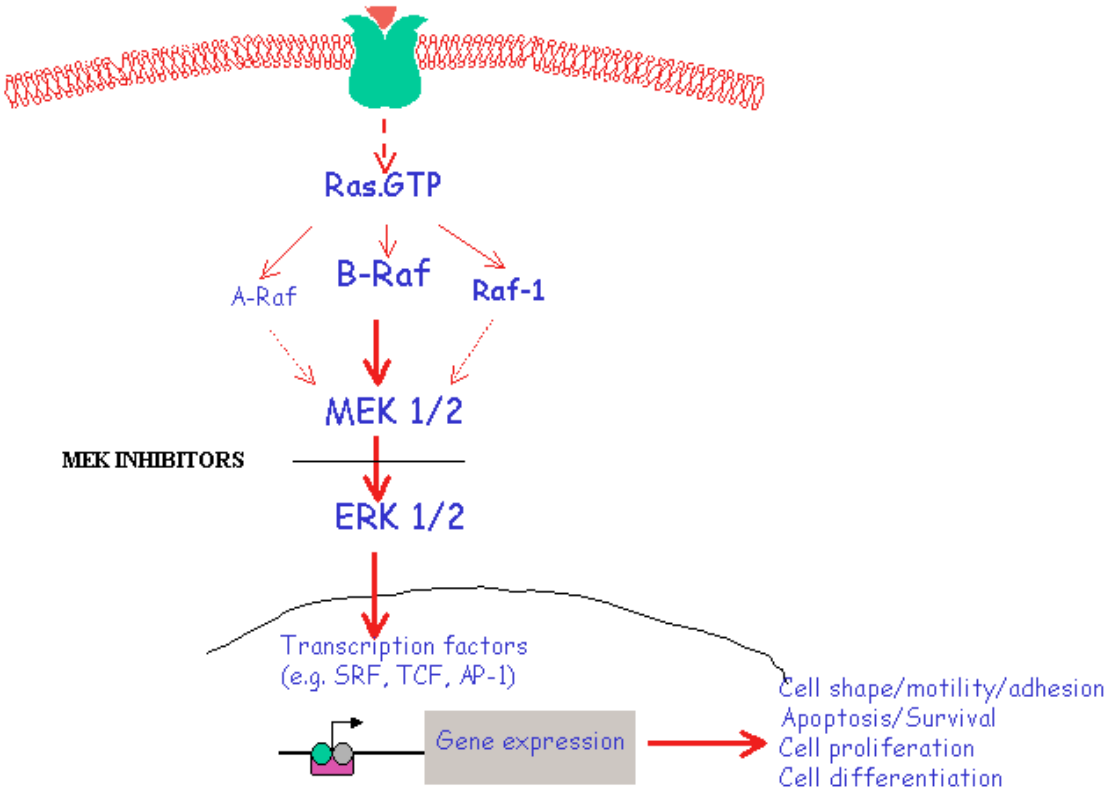

Figure 6: RAS/RAF/MEK/ERK pathway. RAF proteins have been identified as critical signaling intermediates between RAS and ERKs. MEK inhibitors block the pathway, which prevents the activation of transcription factors regulating gene expression for cell proliferation, survival, and differentiation. Reference (32): Pritchard C.

Hypothesis

We hypothesize that co-treatment of Dex and MEK inhibitors will synergize cell death in leukemic cells by the mechanism that a) GCs can up-regulate BIM; and b) pharmacologic MEK inhibitors may further potentiate BIM activation by blocking BIM phosphorylation and degradation.[33] We specifically aim to understand the role of the following BH3-only proteins in the intrinsic pathway.

- BIM: as it is induced by Dex and is activated by MEK inhibitors.
- BAD: as it acts downstream of the MEK/ERK pathway.
- PUMA: as it plays a role in Dex-induced cell death in non-leukemic lymphoid cells.

Materials and Methods

Cell line and culture:

The human T-ALL cell line CCRF-CEM was purchased from the American Tissue Culture Collection. The cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), and streptomycin/penicillin G (Invitrogen) at 37 ^{o}C in a 5% CO_2 incubator.

Chemicals and antibodies:

Dexamethasone and Propidium Iodide (PI) were purchased from Sigma. Antibodies for Western blot were purchased as follows: BIM from Calbiochem; BAX (N-20), β--tubulin, phospho-ERK, and ERK from Santa Cruz Biotechnology; BAK from Upstate/Millipore; BCL-2 from Sigma; MCL-1 from Assay Designs; BAD, PUMA, and BCL-X_L from Cell Signaling Technology; GAPDH from Abcam. A phospho-S65 BIM antibody was developed in our lab as described previously.[33] A MEK inhibitor, PD184352 was kindly provided by Dr. Steven Grant at Virginia Commonwealth University. Annexin V-FITC was purchased from BD-Pharmingen.

Plasmid construction and transfection:

For down-regulation of BIM by short-hairpin RNA (shRNA), pSR-BIM was constructed by inserting the target sequence for human BIM (GenBank AF032457, nucleotide 37-56; GACCGAGAAGGTAGACAATT) into pSUPER.retro.puro (Oligoengine) according to the manufacture's protocol. As control, a scrambled, non-specific sequence (AATTCTCCGAACGTGTCACGT) was inserted into the same vector (pSR-con). For down-regulation of BAD or PUMA by shRNA, microRNA-adapted shRNA construct sdesigned against human *Bad* (5'-ACGTGCTCACTACCAAATGTTA-3') or human *puma* (5'-ACCATTGCATAGGTTTAGAGAG-3') were purchased from Open Biosystems. Transfection was performed by electroporation using a Bio-Rad electroporator. The CEM cells were suspended in RPMI 1640 (4×10^6/400 µl) with 10 µg of DNA and electroporated in 0.4 cm cuvettes at 300 V, 500 µF. Puromycin (2 µg/ml) selection to establish stable clones began 24 hrs after electroporation.

Western Blot analysis:

Cells were pelleted by centrifugation (5000 rpm for 1 min), washed with PBS, resuspended in lysis buffer {20 mM Tris (pH7.4)/ 137 mM NaCl/ 1 mM DTT/ 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate/ 20 mM NaF/ 10 mM β-glycerophosphate, and a protease inhibitor mixture (1:200 dilution; Sigma)}, and kept on ice for 20 mins. They were then centrifuged at 13,000 rpm for 5 mins in cold room and the supernatant was used as whole cell lysate. Protein concentration was determined by

the Lowry method (Bio-Rad). Equal amounts of proteins were loaded on SDS/PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblotting.

Cell Viability assay:

Cells were pelleted by centrifugation, washed with PBS twice, and resuspended in 100 µl of binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC (3 µl) and Propidium Iodide (10 µl) were added to the cells and incubated at room temperature in dark for 15 mins. Four-hundred µl of binding buffer was then added to the sample followed by flow cytometric analysis with FACScan (Becton Dickinson).

Statistical Analysis:

For flow cytometric analyses of Annexin-V/PI, values represent the means ± SD for three separate experiments. The significance of differences between experimental variables was determined using the Student's t-test. Values were considered statistically significant at $P < 0.05$.

Results and Conclusions

We first tested our hypothesis that up-regulation of BIM (by Dex) together with diminished phosphorylation of BIM (by MEK inhibitors) enhances cell death in CCRF-CEM (CEM), an acute lymphoblastic leukemia (ALL) cell line. A MEK inhibitor, PD184352 was used throughout this study. Treatment with PD184352 or Dex alone shows apoptosis of about 8% and 17% respectively. However, when the cells are co-treated with PD184352 and Dex, apoptosis increases to about 60% (Fig. 7). Treatment of as low as 30 nM Dex significantly increases cell death in combination with 5 μM of PD184352. The PD184352 dose-response curve reveals that as low as 1 μM PD184352 significantly increases the toxicity of 100 nM Dex. Time course analysis indicates that simultaneous exposure of 100 nM Dex and 5 μM PD184352 results in little apoptosis after 24 hrs, but extensive at later time points (data not shown). We also observe Dex and PD184352 interactions in several other ALL cell lines. These results indicate that minimally toxic concentrations of MEK inhibitors markedly potentiate the lethality of low concentrations of dexamethasone in ALL cells.

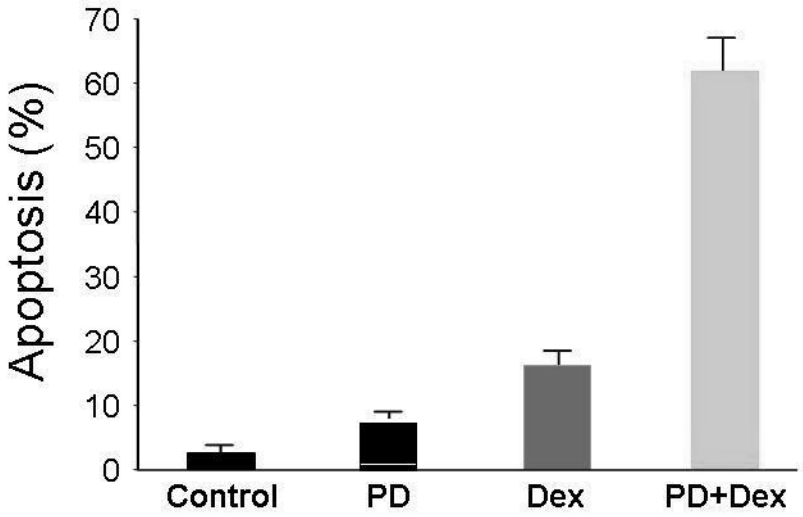

Figure 7: CCRF-CEM (T-ALL) cells show an increase in apoptosis when co-treated with Dexamethasone and PD184352.
CEM cells were exposed to 5 μM PD184352 (PD) and 100 nM dexamethasone (Dex) alone or in combination for 48 hrs. Cell death was quantified by Annexin-V-fluorochrome conjugates (Annexin-V-FITC) and propidium iodide staining followed by flow cytometry analysis.

Protein expression of the BCL-2 family members was determined by Western blots (Fig. 8). In CEM cells, BIM_{EL} (extra-long) is the most abundant BIM isoform derived by alternative splicing [BIM_L (long) and BIM_S (short) are the other two isoforms]. We observe the expression of BIM_{EL} in cells co-treated with PD184352 and Dex is much more than that in cells treated with PD184352 or Dex alone. Expression of BAD, PUMA, BAK, BAX, BCL-2, BCL-X_L and ERK in the cells remains unchanged under all four treatment conditions. MCL-1 shows reduced expression when cells are treated with PD184352 and increased expression with Dex treatment. When cells are co-treated with PD184352 and Dex, MCL-1 shows similar expression as control. Expression of phosphorlyated form of ERK (pERK) is totally abrogated by PD184352 regardless of Dex treatment, indicating that the ERK activity is inhibited by PD184352 independent of Dex. GAPDH is used as a loading control. Hence the expression of BIM is correlated with the percentage of cell death observed in Fig. 7. In contrast, the expression of other members of the BCL-2 family proteins is not much altered under all four treatment conditions.

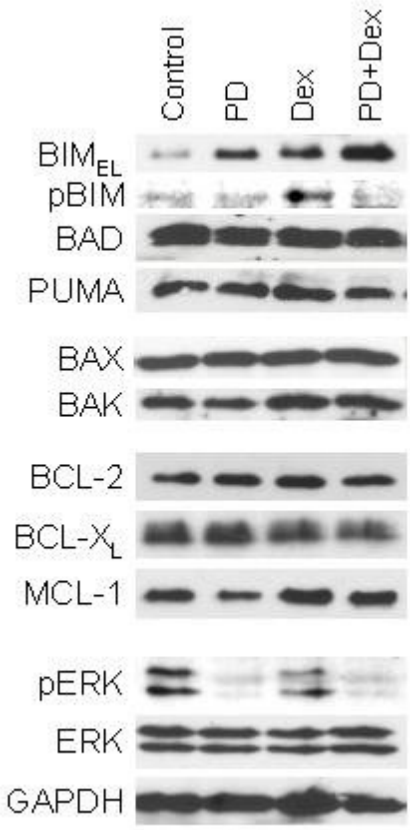

Figure 8: Western blot analysis of the BCL-2 family member proteins.

CEM cells were treated with 100 nM Dex and/or 5 μM PD184352 for 24 hrs. Equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

To examine the significance of BIM, we established the CEM cell clones that express shRNA for *Bim* to reduce the expression of endogenous BIM. As control, a scrambled, non-specific shRNA construct was transfected. Short hairpin RNA (shRNA) is used to silence gene expression via RNA interference. BIM_{EL} is highly induced in the transfected control cells when co-treated with PD184352 and Dex (Fig. 9a), which is also observed in the parental cells (Fig. 8). The induction of BIM_{EL} in shBIM clone 2 cells is completely inhibited under all four treatment conditions. In shBIM clone 15 cells, the induction of BIM_{EL} is partially inhibited in the four treatment conditions. Expression of pERK in control, shBIM clone 2, and clone 15 cells is nil with PD184352 treatment. In the case of treatment with Dex, the expression of pERK in all three clones is similar to the untreated control. Thus, the expression of BIM_{EL} in shBIM clone 2 and 15 is completely and partially down-regulated, respectively, without affecting the expression of pERK. The cell death assay (Fig. 9b) illustrates that apoptosis in control cells with co-treatment is around 45% and it is around 8% and 20% in shBIM clone 2 and clone 15 cells respectively. Hence cell death induced by Dex/PD184352 treatment corresponds to the BIM expression; i.e. apoptosis is completely inhibited in shBIM clone 2 cells and is partially inhibited in shBIM clone 15 cells as compared to the transfected control clone.

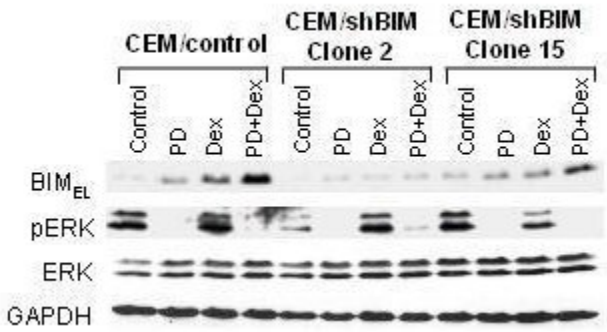

Figure 9a: Down-regulation of BIM by shRNA.
shBIM clones 2 and 15 were established to down-regulate BIM by shRNA. Cells were treated with 100 nM Dex and/or 5 μM PD184352 for 24 hrs. Equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

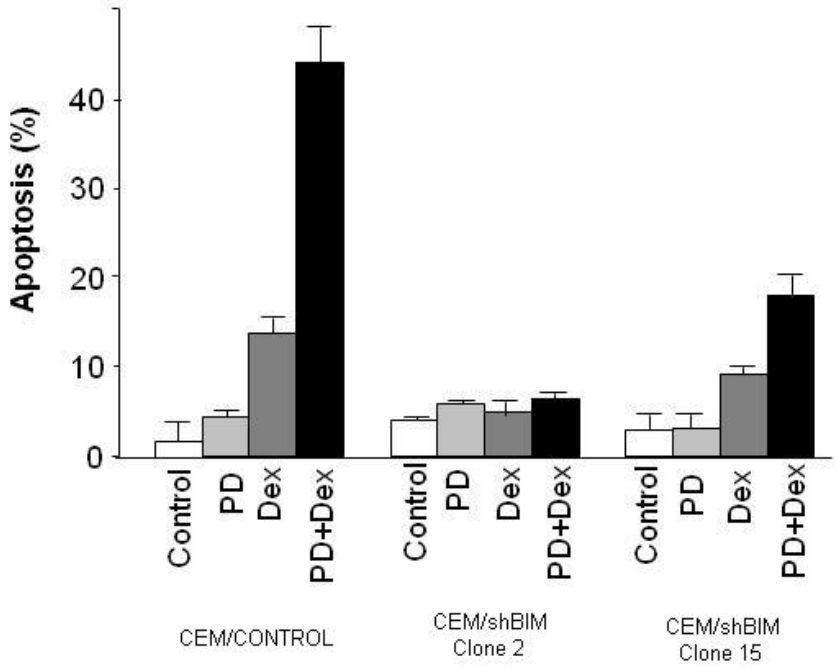

Figure 9b: BIM is required for cell death induced by Dexamethasone and PD184352.
CEM clones in Fig. 7a were exposed to 5 μM PD184352 (PD) and 100 nM dexamethasone (Dex) alone or in combination for 48 hrs. Cell death was quantified by Annexin-V-FITC/PI staining followed by flow cytometry analysis.

We next examined the significance of BAD using the CEM clones that express shRNA for *Bad*. We observe that expression of BAD in untreated clone 7 and clone 12 cells is completely abrogated by the introduction of shBAD (Fig. 10a, left panel). However, the expression of BIM_{EL} is similar among the transfected control and shBAD clone cells. When cells are treated with PD184352 and/or Dex, the expression of BAD is still totally inhibited in shBAD clone 7 cells (Fig. 10a, right panel). In contrast, similar expression of BIM_{EL} is observed in control and shBAD clone 7 cells. Tubulin is used as a loading control. These results indicate that down-regulation of BAD does not affect BIM expression. The cell death assay (Fig. 10b) shows around 50% of apoptosis in all four clones co-treated with PD184352 and Dex. This assay confirms that BAD is dispensable in cell death induced by co-treatment with PD184352 and Dex.

Figure 10a: Down-regulation of BAD by shRNA.
shBAD clone 7 and 12 were established to down-regulate BAD by shRNA. Cells were treated with 100 nM Dex and/or 5 μM PD184352 for 24 hrs. Equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

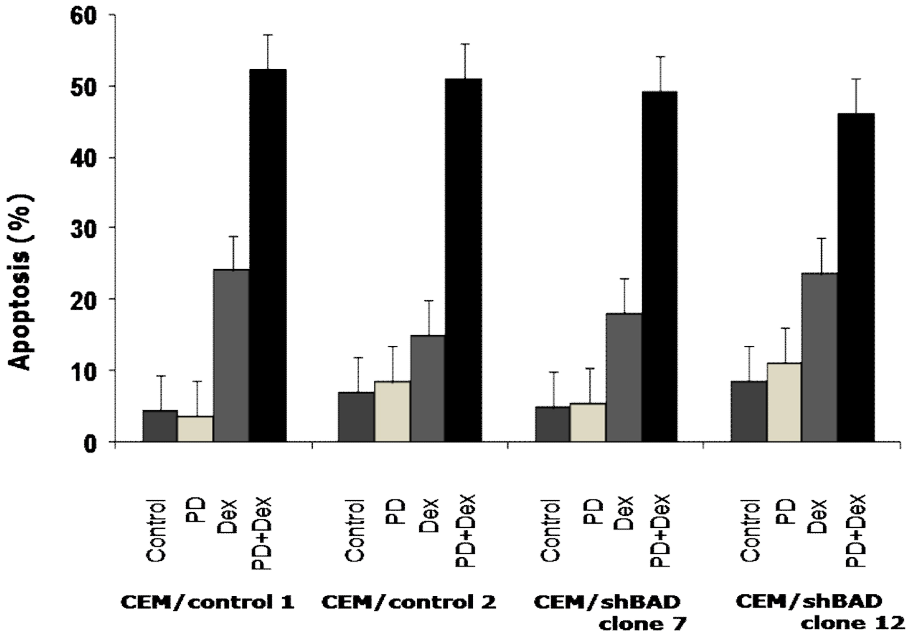

Figure 10b: BAD is dispensable for cell death induced by Dexamethasone and PD184352.CEM clones in Fig. 8a were exposed to 5 μM PD184352 (PD) and 100 nM dexamethasone (Dex) alone or in combination for 48 hrs. Cell death was quantified by Annexin-V-FITC/PI staining followed by flow cytometry analysis.

Finally, we examined the significance of PUMA using the CEM clones that express shRNA for *PUMA* to down-regulate PUMA. Since the shRNA plasmid construct harbors puromycin-resistant gene, we selected two puromycine-resistant CEM clones (shPUMA clone 37 and clone 47). The expression of PUMA in control, shPUMA clone 37, and clone 47 was determined when cells were treated with PD184352 and Dex alone or in combination (Fig. 11a). Tubulin is used as a loading control. We observe that the expression of PUMA under all conditions is constant. The cell death assay (Fig. 11b) shows similar amounts of apoptosis induced by Dex and/or PD184352 in between control, clone 37, and clone 47. These results indicate that the shPUMA construct used is not working as desired. Thus, this experiment needs to be performed using a different construct.

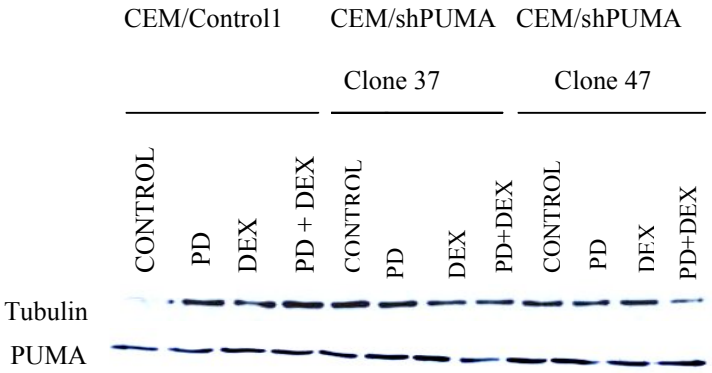

Figure 11a: The expression of PUMA in CEM/shPUMA clone 37 and clone 47.
Cells were treated with 100 nM Dex and/or 5 μM PD184352 for 24 hrs. Equal amounts of total cell extracts were subjected to Western blotting with the indicated antibodies.

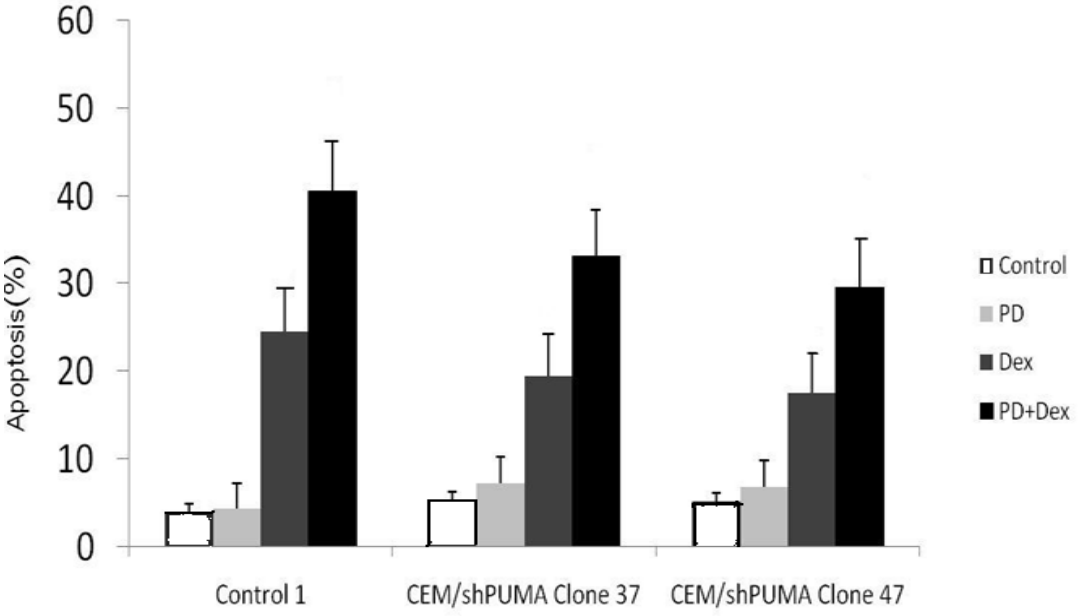

Figure 11b: Cell death in CEM/shPUMA clone 37 and clone 47 treated with Dex and/or PD184352.
CEM clones were exposed to 5 μM PD184352 (PD) and 100 nM dexamethasone (Dex) alone or in combination for 48 hrs. Cell death was quantified by Annexin-V-FITC/PI staining followed by flow cytometry analysis.

Based on the above results, we conclude that:

- Combination of dexamethasone and PD184352 synergistically induces apoptosis in ALL cells.
- BIM plays a significant role in apoptosis induced by co-treatment with PD184352 and Dex.
- BAD knock-down experiments confirm that BAD is dispensable for cell death in this treatment.
- Future work needs to be carried out on PUMA using a new construct to confirm whether it has any significant role in apoptosis induced by Dex and MEK inhibitors in ALL cells.

Discussion

Glucocorticoids have been used in chemotherapy for leukemia, lymphoma, and myeloma for decades. Although they are effective in the initial stages, resistance often emerges, and the molecular mechanisms of sensitivity/resistance to this agent are still not fully understood. We and others have demonstrated that BIM, a BH3-only pro-apoptotic BCL-2 family, is transcriptionally induced by Dex treatment in various cell types and plays a critical role in Dex-induced cell death. The activity of BIM is regulated not only by transcription, but also by post-translational mechanisms. Among these, ERK-mediated phosphorylation, ubiquitylation, and subsequent protein degradation has been demonstrated in a variety of cells and MEK inhibitors abrogate such regulation.[12, 14-16] Thus, we hypothesized combined treatment with Dex and MEK inhibitors might act synergistically in their cell killing activity. We demonstrate here that Dex interacts in a highly synergistic manner with a clinically relevant MEK inhibitor (i.e. PD184352) to induce apoptosis in CCRF-CEM T-ALL cells.

The observation that the inhibition of cell death correlated well with the reduction of BIM expression by shRNA in clone 2 and clone 15 (Fig. 9a and 9b) strongly suggests

that BIM is a central regulator in this regimen at least in CCRF-CEM T-ALL cells. Recent results involving epithelial breast cancer cells suggest that BAD phosphorylation status represents the primary integrator of cell death following interruption of the AKT and ERK pathways. However, down-regulation of BAD with shRNA, in marked contrast to BIM, failed to protect CEM cells from Dex/PD184352-mediated lethality (Figure 10 a, 10b), suggesting that BAD is not a critical molecule in lethality in this setting. Differences between current and previous reports may therefore reflect cell type-specific roles of BAD in integrating death signals following concomitant interruption of the MEK/ERK and AKT pathways.

PUMA seems to be dispensable for normal development and health, as *puma*-deficient mice are born with normal appearance and body weight, and they also exhibit normal cellularity and composition of hemopoietic organs. It has been shown that the level of *puma* mRNA increases in primary murine thymocytes exposed to Dex and non-malignant thymocytes from PUMA-deficient mice are resistant to Dex-induced apoptosis.[34] However, the expression of PUMA in CEM cells were not changed by Dex-treatment in CEM cells (Fig. 7), and it has been recently reported that *puma* mRNA is not regulated in Dex-treated ALL patients. Thus, the human *puma* gene is not a transcriptional target of the GR, and if Puma contributes to GC-induced apoptosis in human ALL, it does so in a transcription-independent manner. Hence, PUMA might play a minor role in the apoptosis induced by co-treatment with Dex and MEK inhibitors.

It is widely recognized that the RAS/RAF/MEK/ERK signaling pathway mediates survival signaling in diverse transformed cell types. The implication of the present findings is that in ALL cells, phosphorylation/degradation of BIM mediated by ERK may represent a pro-survival mechanism by which such cells escape the lethal consequences of GC treatment. A corollary of this hypothesis is that MEK inhibition may potentiate the lethal effects of Dex and potentially other novel agents by preventing BIM phosphorylation/degradation.[35-41] Thus, BIM phosphorylation/expression status may represent a determinant of the activity of such strategies. If validated, this concept could have implications for the development of novel anti-leukemia regimens involving the combined administration of clinically relevant agents targeting at the RAS/RAF/MEK/ERK pathway (e.g. MEK inhibitors, farnesyltransferase inhibitors, HMG CoA-reductase inhibitors) and GC. A recent study has demonstrated that the receptor tyrosine kinase inhibitor, SU11657 (potentially inactivating the RAS pathway) interacts synergistically with Dex to modulate signaling through BIM and to induce apoptosis in a highly GC-resistant ALL xenograft model.[42] BIM also plays an important role in cell death induced by other chemotherapeutic drugs such as STI571 (imatinib mesylate)[43-45] and histone deacetylase inhibitors (HDACI).[46,47] In these cases, BIM is transcriptionally induced through FOXO and E2F, respectively. If our hypothesis that combination of BIM up-regulation and stabilization synergistically promotes cell death is validated, it will be interesting to test whether combinations of STI571 or HDACI with MEK inhibitors interacts synergistically in ALL cells. In fact, it has been shown that this

is the case in BCR/ABL^{+} leukemia cells and other adherent malignant cells.[48,49] To date, several pharmacological MEK inhibitors including PD184352 (or CI-1040), PD0325901, and AZD6244 (ARRY142886) have been developed clinically.[28-30] Results of early clinical trials indicate that it is feasible to achieve the desired pharmacodynamic effect (e.g. ERK inactivation) at well-tolerated doses of MEK inhibitors. Collectively, our findings could have implications for understanding the mechanisms underlying synergistic interactions between MEK inhibitors and other targeted agents in ALL and potentially other hematologic malignancies.

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VITA

Anila Rambal was born in Mumbai, India on December 27, 1981 and is an Indian citizen. She graduated from Wilson College, University of Mumbai, India in 2002 with Bachelors in Microbiology. She then attended University of Pune and Pondicherry University, in India, for her Master's and M.Phil in Environmental Science.

She joined Virginia Commonwealth University in Fall-2007. She spent a year working in Dr. Hisashi Harada's laboratory as a Research Assistant for her Master of Science in Environmental Science and completed her degree in May of 2009.

She has one publication to her credit:

Rambal AA, Panaguiton Z, Kramer L, Grant S, Harada H. MEK inhibitors potentiate dexamethasone lethality in acute lymphoblastic leukemia cells through the pro-apoptotic molecule BIM. *Leukemia* 2009; advance online publication 30 April 2009; doi: 10.1038/leu.2009.80