

## Original Article

# Novel piperazine induces apoptosis in U937 cells

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**Abstract:** The effect of 1,4-bis-(4-(1H-benzo[d]imidazol-2-yl-phenyl)) piperazine (BIPP), a newly synthesized piperazine derivative, on U937 leukemia cell viability was investigated. We show that BIPP induces dose-responsive apoptotic cell death in U937 cells by intrinsic mechanisms of apoptosis. Maximum apoptotic effect of BIPP on U937 cells was observed at 12.8 $\mu$ M. BIPP-induced apoptosis was evident by characteristics such as altered annexin-V binding, caspase activation, loss of mitochondrial membrane potential (MMP) and cytochrome c release. BIPP also differentially activates initiator and effector caspases combined with the loss of MMP strongly suggesting that BIPP causes an intrinsic apoptosis in U937 leukemia cells. Due to our observations that BIPP induces leukemia cell death without significantly affecting normal cells, our data suggests that it may be a potential therapeutic agent for human myeloid leukemia.

**Keywords:** Piperazine, apoptosis, cancer, caspase, mitochondria, membrane potential

## Introduction

The evasion of apoptosis has been characterized as one of the six major hallmarks of cancer [1]. In cancerous cells, the normal programming to undergo apoptosis may not be activated due to the nonreception of proapoptotic signals, the decrease or lack of synthesis of proapoptotic signals, the increase in the synthesis of antiapoptotic signals or a combination of these [2]. As multiple gain- or loss-of-function mutations, from spontaneous, cytotoxic, or UV-induced mutation, viral infection or uncorrected misreading during transcription, are incorporated into the defective genomes, these evasive cells may subsequently acquire invasive and/or metastatic ability, ultimately leading to cancer and the possible demise of the host.

Strategies have been ongoing in many laboratories to look for new agents, such as piperazine derivatives, which can function as anti-cancer drugs [1, 3]. Historically, most piperazine derivatives have been used as antihelminthics or antibiotics [4-11]. However, it has been recently demonstrated that some piperazine de-

rivatives are capable of inducing apoptosis in some cancer cells [10]. This report provides novel evidence that 1,4-bis-(4-(1H-benzo[d]imidazol-2-yl-phenyl)) piperazine (BIPP) (**Figure 1A**) induces apoptosis in leukemia cells without affecting normal monocytes. We further characterize that BIPP utilizes intrinsic apoptotic signaling mechanisms to induce leukemia cell death.

## Materials and methods

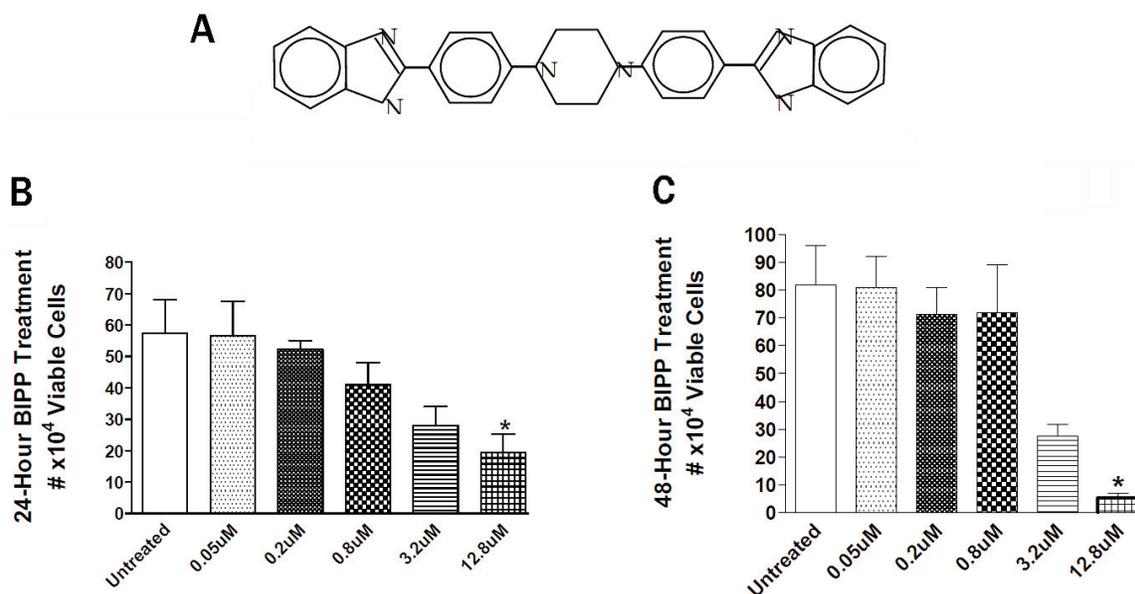
### Cell culture

U937 myeloid leukemia cells and K562 erythro-leukemia cells were acquired from ATCC, grown in RPMI 1640 media (Cellgro, Mediatech, Inc.) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals) and 1% Penicillin-Streptomycin (Gibco, Invitrogen Corporation) and maintained at 37°C, 5% CO<sub>2</sub>.

### Ficoll monocyte extraction

Ficoll-Paque PREMIUM reagents were obtained from GE Healthcare and monocytes isolation

## Novel piperazine induces apoptosis in U937 cells



**Figure 1.** Molecular structure of BIPP and its effect on leukemia cell viability. **A.** Molecular structure of BIPP. **B.** 24-hour BIPP dose response effect on U937 leukemia cell viability.  $5 \times 10^5$  cells/mL U937 cells were either untreated or treated with different doses of BIPP for 24 hr and viability assessed by trypan blue exclusion. Data represents mean and SD of 3 experiments. **C.** 48-hour BIPP-induced U937 cell leukemia cell death. Experimental conditions were identical to those of Figure 1B. Data represents mean and SD of 3 experiments.

protocol was followed. Primary monocytes (peripheral blood mononuclear cells) were isolated from whole blood obtained from New York Blood Center. Using the Ficoll-Paque extraction technique, the isolated monocytes were maintained in RPMI -1640 culture medium in the presence of 10 % FBS and 1% pen/strep antibiotic and allowed an hour to distress and recover. Subsequently, the cells were either untreated or treated with 12.8 $\mu$ M BIPP and followed for viability and caspase activation.

### Preparation of BIPP

BIPP was synthesized in the laboratories of Drs. Isaac Donkor and Tien L Huang and solubilized in 14.1M DMSO from Sigma and stored at a stock concentration of 10 $\mu$ g/ml at -20°C.

### Trypan blue exclusion cell viability assay

U937 cells, K562 cells, or primary isolated monocytes were treated with 12.8 $\mu$ M BIPP as specified under figure legend and the ability of the compound to induce apoptosis was evaluated at various time points. Untreated cells were maintained in an identical manner and

used as a control. 10 $\mu$ L of cell culture was added to 90 $\mu$ L of trypan blue exclusion dye and examined under microscope. Viable cell population was estimated by hemocytometer.

### DNA ladder detection

Apoptosis was induced in  $1 \times 10^6$  cells/mL U937 leukemia cells by treating with various concentrations of BIPP for desired time points. Samples were prepared according to Quick Apoptotic DNA Ladder Detection Kit protocol from MBL International Corporation. Next, 1% agarose gel electrophoresis at 5V for 2 hours was used for DNA separation. DNA ladder or bands were visualized under UV light and photographed.

### Colorimetric caspase assays

All caspase assay kits were acquired from MBL International Corporation and the manufacturer's protocols were followed to detect caspase activity. U937 cells or monocytes were untreated or treated with various concentrations of BIPP for desired time points. Cells were counted and pelleted to achieve a density of

## Novel piperazine induces apoptosis in U937 cells

2x10<sup>6</sup> cells/mL. Cells were then suspended in 50µl of chilled lysis buffer provided in the assay kit from MBL and incubated on ice for 10 minutes. Protein concentration was performed by BCA Protein Assay from Pierce Protein Products (now Thermo Fisher Scientific) and 150µg of protein was diluted in 50µl of cell lysis buffer. Next, 50µl of 2x reaction buffer (containing 10mM DTT) was then added to each sample and followed by the addition of 5µl of 4mM x-pNA substrate. The mixture was incubated at 37°C for 2 hours after which the samples were read at 405nm by plate reader.

### *Annexin apoptosis detection assay*

Assay kit was acquired from MBL International Corporation and the manufacturer's protocol was followed. Specifically, 2x10<sup>5</sup> U937 cells were either untreated or treated with 12.8µM BIPP for desired time points. Annexin binding data was collected using fluorescence microscopy and plate reader.

### *Reactive oxygen species (ROS) assay*

Image-iT Live Green Reactive Oxygen Species Detection Kit from Invitrogen Detection Technologies was acquired and protocol followed. About 2x10<sup>5</sup> U937 cells were either untreated or treated with various concentrations of BIPP for desired time points. Samples were washed three times with PBS and fluorescence recorded at 495/529nm for estimation of ROS levels.

### *Nitric oxide assay*

Nitric Oxide Colorimetric Assay Kit from MBL International Corporation was acquired and protocol followed. About 2x10<sup>5</sup> U937 cells were either untreated or treated with various concentrations of BIPP for desired time points and absorbance was read at 540nm for estimation of NO levels.

### *Mitochondrial membrane potential (MMP) assay*

Mitochondrial Membrane Potential Detection Kit from Stratagene (now Agilent Technologies, Santa Clara, CA) was acquired and protocol followed. About 1x10<sup>6</sup> U937 cells were either untreated or treated with various concentrations of BIPP for desired time points. Fluorescence measured red - 585/590nm, green -

510/527nm using fluorescence plate reader.

### *Cytochrome c assay*

Cytochrome c ELISA Kit from MBL International Corporation was acquired and protocol followed. Isolation of cytosolic and mitochondrial fractions was done according to the protocols provided in the kit and certified by the vendor. Briefly, about 5x10<sup>6</sup> U937 cells were either untreated or treated with various concentrations of BIPP for desired time points. The cells were collected, washed in cold PBS and homogenized in a homogenizing buffer provided with the kit. The homogenate was centrifuged at 10,000 x g for 60 minutes at 4° C and the supernatant was collected and used as cytosolic fraction. The pellet was resuspended in ice cold buffer-2 (also provided by the kit) and sonicated with Ultra sonicator (BRANSON; SONIFER), for three times (20 second each) on ice. The homogenate was centrifuged at 10,000 for 30 minutes at 4°C. The supernatant was collected and used as mitochondria fraction. The samples (cytosolic and mitochondrial fractions) were assayed separately for Cytochrome C using the Cytochrome C ELISA kit provided by the vendor and samples were read on plate reader at 450nm.

### *Luminescent cell viability assay*

CellTiter-Glo Luminescent Cell Viability Assay from Promega was acquired and protocol followed. 5x10<sup>6</sup> U937 cells were either untreated or treated with various concentrations of BIPP for desired time points. Cell viability was assessed as outlined in the protocol.

### *ADP/ATP ratio assay*

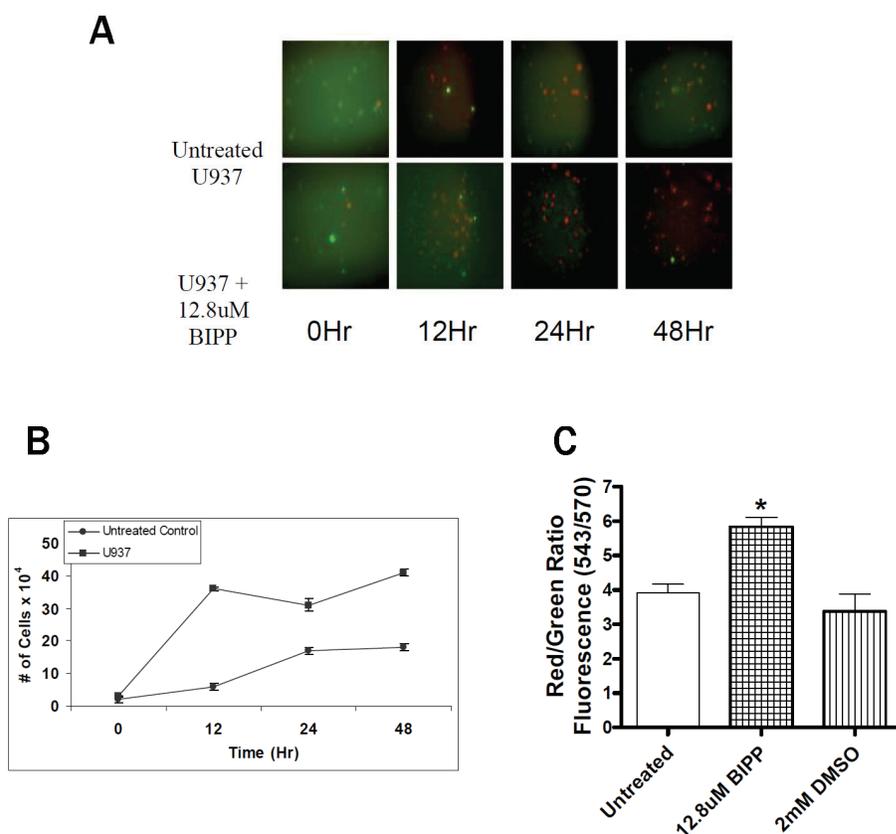
ApoGlow Assay Kit from Cambrex Bio Science Rockland, Inc. was acquired and protocol followed. 5x10<sup>6</sup> U937 cells were untreated or treated with various concentrations of BIPP for desired time points and ADP/ATP ratio measured. Data from three experiments were averaged for each experiment.

## **Result**

### *BIPP decreases cell viability*

In this experiment, the ability of BIPP to cause reduction of the viable U937 leukemia cell populations *in vitro* was examined. As shown in

## Novel piperazine induces apoptosis in U937 cells



**Figure 2.** Time Course of BIPP-induced Annexin V binding during apoptosis.  $2 \times 10^5$  cells/mL U937 leukemia cells were either untreated or treated with  $12.8\mu\text{M}$  BIPP for up to 48-hours. **A.** BIPP induces apoptosis but not necrosis. Cells were untreated or treated with  $12.8\mu\text{M}$  BIPP for up to 48 hours. Red stain indicates apoptotic cells while green stain indicates necrotic cells. **B.** Shows tallied increase in apoptotic cells after 48-hour treatment. **C.** Demonstrates amount of red/green fluorescence ratio measured during 48-hr period. Data represents a mean plus SD of 3 experiments.

**Figures 1B and C,** we investigated the effects of various concentrations of BIPP on cell viability over several time points using the viability of untreated cells as control in order to find the inhibitory concentration of BIPP that produces a  $\geq 50\%$  (IC<sub>50</sub>) decrease in U937 cell viability. The data in **Figure 1B** shows that BIPP induces a dose-responsive decline in U937 leukemia cell viability and as seen in **Figure 1C**, BIPP induces dose-dependent decline in U937 cell viability during a 48-hour treatment. Again, maximum effect of BIPP is seen at  $12.8\mu\text{M}$ . Similar results were seen in K562 erythroleukemia cells (data not shown). The data also shows that at BIPP concentration of  $12.8\mu\text{M}$  produced maximum reduction in cell viability and that the reduction is sustained through a 96 hour time period exemplified by an IC<sub>50</sub> in cell population density.

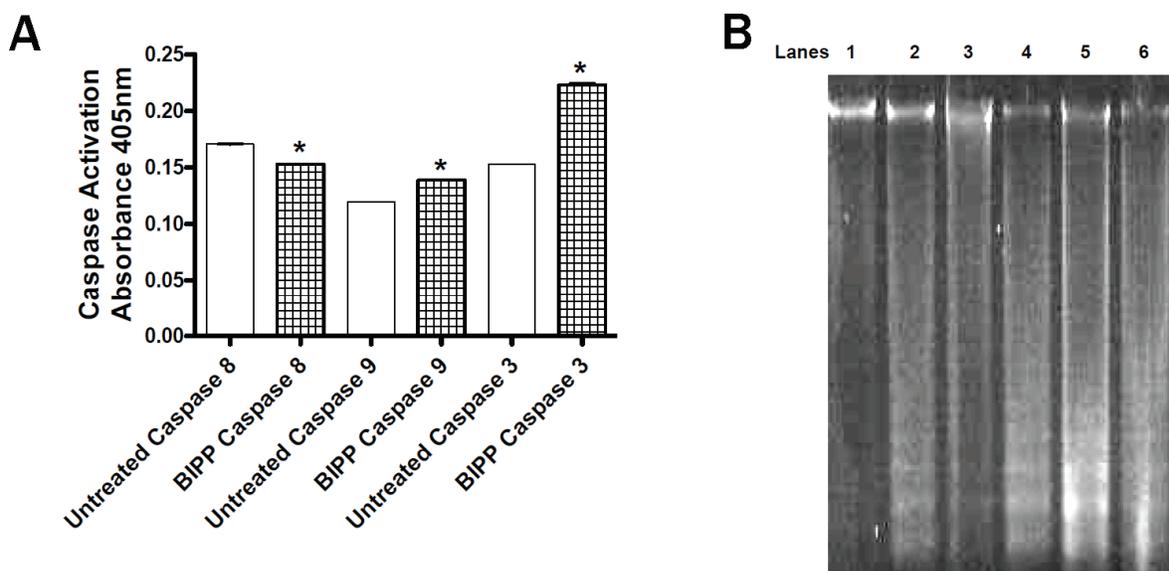
### *BIPP induces annexin-V binding*

One of the defining characteristics of apoptosis is Annexin-V binding [12, 13]. We used SYTOX

green fluorescent dye from MBL International Corporation to stain the treated cells. This allowed us to discriminate apoptotic cells from necrotic cells. We stimulated U937 cells with  $12.8\mu\text{M}$  BIPP to induce cell death and compared the results to those in untreated cells. At the 48-hr time point, cells were analyzed for apoptotic death by this differential fluorescent detection. As shown in **Figures 2A**, apoptotic cells fluoresce red while necrotic cells fluoresce green. The ratio of the red/green fluorescence, **Figure 2B and C**, shows an approximate two-fold increase in annexin-V binding in BIPP-treated cells compared to vehicle treated cells suggesting BIPP induces apoptosis in the U937 cells.

### *BIPP induces DNA fragmentation*

Next, to provide further evidence that BIPP induces apoptosis, we probed for another characteristic of apoptosis, DNA fragmentation. DNA is fragmented during apoptosis due to the activation of various downstream protease activity such as caspase-6 and caspase-activated DNase (CAD)



**Figure 3.** BIPP-induced caspase activation and DNA fragmentation. **A.** BIPP induces differential activation of caspases.  $2 \times 10^6$  cells/mL U937 leukemia cells were untreated or treated with  $12.8\mu\text{M}$  BIPP and caspase activity was measured as indicated in the *Materials and Methods*. **B.** BIPP causes DNA fragmentation.  $1 \times 10^6$  cells/mL U937 leukemia cells were either untreated or treated with varying doses (Lanes: 1 - untreated; 2 -  $0.05\mu\text{M}$ ; 3- $0.2\mu\text{M}$ ; 4 -  $0.8\mu\text{M}$ , 5 -  $3.2\mu\text{M}$ , 6 -  $12.8\mu\text{M}$ ) of BIPP. DNA fragmentation as evidence for apoptosis is shown by the stained bands on 1% agarose gel electrophoregram.

[14, 15]. These degrading enzymes are activated downstream by the prior activation of caspase-3. Upon their activation, they translocate to the nucleus where they cut the linker DNA between nucleosomes into approximately 200bp segments [16]. As shown in **Figure 3A**, apoptosis was induced in U937 leukemia cells and DNA fragmentation was seen at each concentration ( $5\text{nM}$  –  $12.8\mu\text{M}$ ) of BIPP tested.

#### *BIPP induces caspase activation*

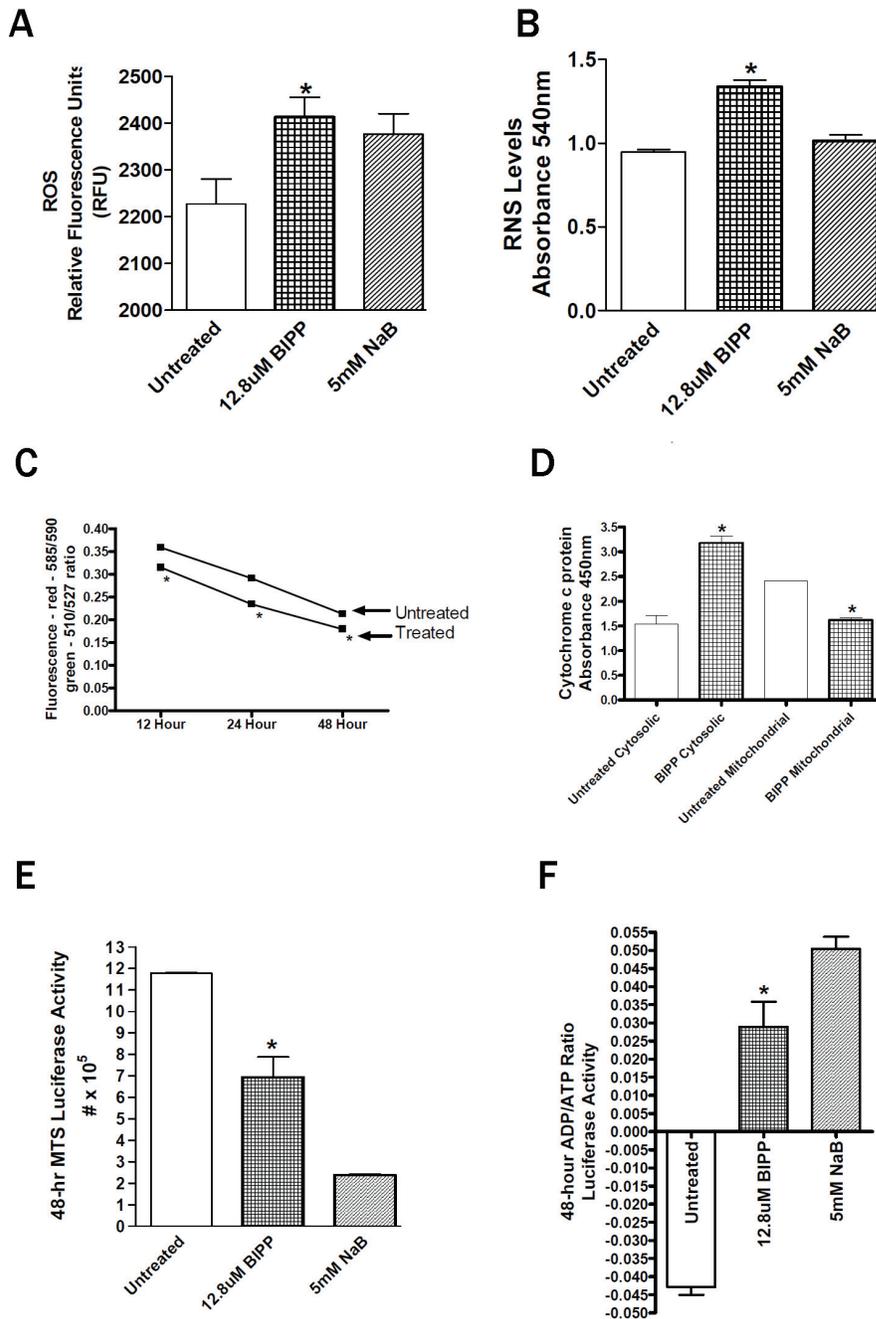
These experiments were performed to determine which, if any, of the various caspases in the cell are being activated in response to stimulation with BIPP further supporting apoptotic activation. In **Figure 3A**, U937 cells were either untreated or treated with  $12.8\mu\text{M}$  BIPP for 96 hours and then analyzed for caspase activation. BIPP significantly increased activation of the initiator caspase-9 and the effector caspase-3. However, there was as slight decrease in caspase-8 activity (**Figure 3A**) but no change in the activities of in initiator caspases-2 and -10 (data not shown), which would ordinarily be activated due to some receptor-ligand binding initiating the caspase signal cascade

from outside the cell. The activation of the effector caspase-3 without activation of the initiator caspases aforementioned is strongly suggestive of intrinsic apoptosis [17].

#### *BIPP stimulates generation of reactive oxygen and nitrogen species*

After establishing that BIPP triggers an intrinsic apoptotic response, we next decided to investigate whether BIPP treatment leads to generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which may contribute to apoptosis. The rationale is that during intrinsic apoptosis, genomic damage from UV or g-induced radiation or the presence of a cytotoxic drug results in the emergence of a proapoptotic signal that is sent to the mitochondria causing the disruption of the outer membrane integrity. One of the initial post-BIPP treatment events noticed, was the accumulation of radical species: reactive oxygen and nitrogen. The charged radicals may then cause oxidative damage to many subcellular structures [18 - 20]. As seen in **Figures 4A** and **B**, cells were treated with  $12.8\mu\text{M}$  BIPP for 48 hours followed by measurement of the accumulated ROS and

## Novel piperazine induces apoptosis in U937 cells



**Figure 4.** BIPP-induced changes in mitochondrial microenvironment. U937 leukemia cells were either untreated or treated with 12.8μM BIPP for 48 hours and ROS, RNS, mitochondrial membrane potential, cytochrome c release and ADP/ATP ratios levels were all measured as indicated under Material and Methods.  $2 \times 10^5$  cells/mL were used to measure ROS and RNS.  $1 \times 10^6$  cells/mL were used for MMP.  $5 \times 10^6$  cells/mL were used for MTS, cytochrome c and ADP/ATP ratio. Data represents mean plus SD of 3 experiments. **A.** BIPP-induced ROS generation. **B.** BIPP-induced RNS generation. **C.** BIPP-induced decline in mitochondrial membrane potential. **D.** BIPP-induced mitochondrial cytochrome c release. **E.** BIPP-induced inhibition of ATP synthesis. **F.** BIPP-induced increase in ADP/ATP ratio attesting to decline in ATP synthesis.

RNS. An untreated sample was used as negative control while sodium butyrate-treated cells were used as a positive control for comparison [21]. Clearly, significant increase in both radical species (ROS and RNS) was apparent in BIPP treated cells when compared to the untreated U937 sample.

### *BIPP causes loss of MMP*

The buildup of oxygen radicals triggers series of events that cause the disruption of outer mitochondrial membrane (OMM) and a subsequent loss of components necessary for the maintenance of MMP [19, 20, 22]. This further accen-

tuates the apoptotic response as the cells ability to make ATP is further jeopardized and the decrease in MMP is thought to begin stimulating the release of cytochrome c from the mitochondria which is used to form apoptosomes [19]. In **Figure 4C**, cells were untreated or treated for 48 hours with 12.8 $\mu$ M BIPP, then stained to measure MMP levels. Results in treated samples were compared to those in untreated samples. Clearly, BIPP causes significant decline in MMP in these cells.

### *BIPP stimulates mitochondria cytochrome c protein release*

For the apoptotic response to continue, a necessary component of the apoptosome, cytochrome c, must be released from the mitochondria into the cytoplasm [19, 20]. Decline in mitochondrial membrane potential and disruption of the OMM leads to cytochrome c release to the cytoplasm and then utilized in apoptosome formation. In **Figure 4D**, after 12.8 $\mu$ M BIPP treatment for 48 hours there is a significant release in cytochrome c from the mitochondria to the cytosol.

### *BIPP reduces ATP synthesis*

All of the aforementioned changes to the mitochondrial microenvironment ultimately affect the cell's ability to synthesize ATP. The depletion of ATP hinders the cell's ability to undergo metabolic processes and exacerbates the apoptotic response. To obtain data to support this, cells were untreated or treated with 12.8 $\mu$ M BIPP for 48hrs. Since only viable and proliferation cells with intact oxidative phosphorylation systems synthesize ATP, we employed a luminescent cell viability assay (MTS) from Promega to indirectly assess ATP level. A direct ADP/ATP ratio was measured using an adenylate nucleotide ratio assay obtained from Cambrex Bio Science. The results from both experiments demonstrate that ATP synthesis is markedly decreased after treatment with BIPP, shown in **Figures 4E** and **F**.

### *BIPP fails to induce cell viability changes and apoptosis in human monocytes*

Because U937 leukemia cells are cancerous it was important to investigate the effect of BIPP on normal human cells to ascertain whether BIPP has similar or dissimilar effects in normal and transformed cells. To achieve that goal we

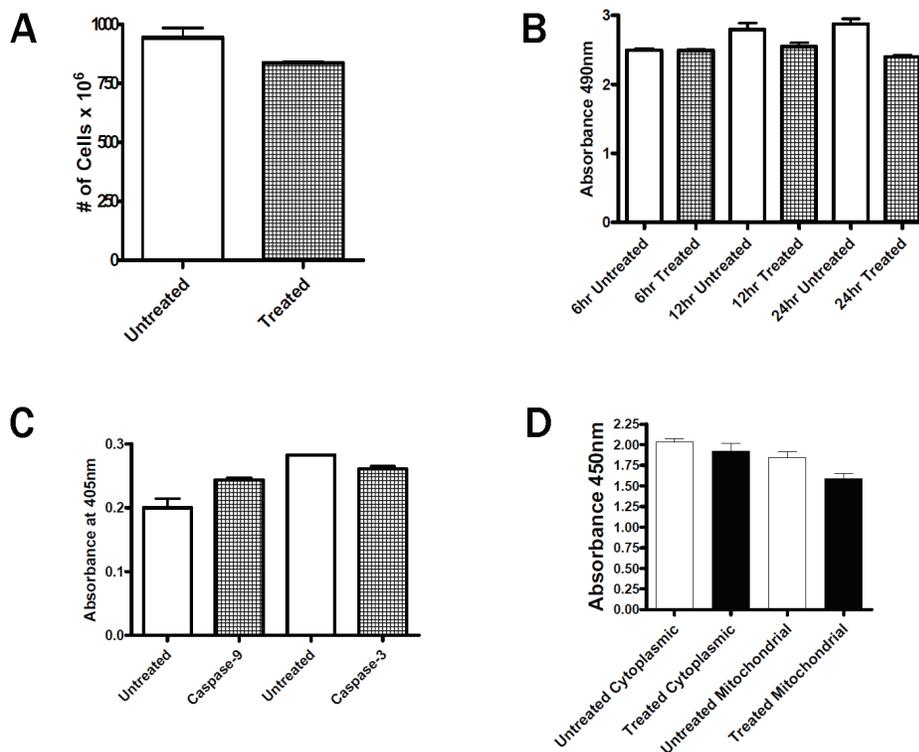
examined the effect of BIPP on primary human monocytes (PBMC). Primary monocytes were isolated from whole blood and either untreated or treated with 12.8 $\mu$ M BIPP for 24-hours. Based on results from trypan blue exclusion assay (**Figure 5A**), it was concluded that there was no significant loss in monocytic cell viability in response to BIPP treatment. This data was further supported by the outcome of MTS assay for monocytes viability (**Figure 5B**). Similarly, BIPP failed to induce significant change in both caspases-9 and -3 activities (**Figure 5C**). Taken together, the results in **Figure 5** further suggest that the killing/apoptotic effect of BIPP is more prominent in cancerous cells than noncancerous cells. The apparent reasons why normal cells were less sensitive to BIPP remains unknown at this point and require future investigation.

## Discussion

Piperazine-based compounds have recently been receiving increasing attention due to the chemical advancements they provide in drug delivery. Knowing that the evasion of apoptosis is one of the predominant ways that cells become oncogenic [23] and that this characteristic is one of the most difficult targets in designing an appropriate cancer treatment [18], we wanted to stimulate U937 cells into eliciting an apoptotic response by treating them with BIPP, a newly synthesized piperazine derivative. We expect the drug to exhibit toxicity primarily to cancerous cells based on previously published data [4 – 8, 11]. Concerns of toxicity have been a problem in designing an efficient and permissible drug [3, 4]. Our rationale for using BIPP, being that the drug will induce DNA damage in cancerous and noncancerous cells but noncancerous cells should be less susceptible and more capable of correcting or resisting the damage. This is because they are still able to halt the cell cycle to analyze its condition or initialize the apoptotic response. Cancerous cells, with their increased mitotic rate, however, would be more susceptible to the induced DNA damage as they often do not halt the cell cycle to correct DNA damage and thus be more susceptible to apoptosis.

We determined that BIPP has the ability to induce apoptosis in U937 leukemia cells. We demonstrate that there is a dose-dependent decrease in viable cells at each time point ex-

## Novel piperazine induces apoptosis in U937 cells



**Figure 5.** Lack of BIPP-induced apoptosis in primary monocytes. Primary monocytes were isolated from whole blood using ficoll extraction.  $3 \times 10^6$  cells/mL of isolated primary monocytes were either untreated or treated with  $12.8 \mu\text{M}$  BIPP for 24 hours and examined for viability, using trypan blue exclusion dye (A) or MTS assay (B), caspase-9 and -3 activation assay (C), and cytochrome c release (D) as described under Materials and Methods.

aminated. We observed that at 24 hours there is a linear concentration-dependent sensitivity of the cells to the drug as the drug concentration used increased (Fig 1B) and that the effect of the drug corresponding to an  $\text{IC}_{50}$  was achieved using  $12.8 \mu\text{M}$  concentration. To determine the mechanism by which BIPP kills U937 cells, we conducted several experiments to determine whether the type of death seen was apoptosis as opposed to other forms of cell death; most notably necrosis.

Caspase activation is one of the defining characteristics of apoptosis [1]. Caspases are normally inactive zymogens inside the cell until some stimulus is received which causes a series of events leading to their eventual activation. Once activated, these proteases act to activate other caspases, to expand their effect, and to dismantle the cell in an orderly fashion to be lastly discarded by activated phagocytic cells [16]. Extrinsic apoptosis involves the binding of a ligand to its cognate receptor triggering the cytoplasmic end of the receptor to recruit adaptor proteins and certain initiator caspases most notably caspase-8, ultimately forming a death-inducing signaling complex (DISC) [24, 25]. Since the drug is quite lipophilic, we believe that

it passively diffuses into the cell through the plasma membrane. After 96-hour BIPP treatment of U937 cells, there was a noticeable increase in the activation of caspase-3 and -9 (Figure 3A) but not caspase-2, -8, and -10 (data not shown). This suggests an intrinsic mitochondrial involvement in the induction of apoptosis of these cells following BIPP treatment. The activation of the effector caspase-3 without activation of the initiator caspases is strongly suggestive of intrinsic apoptosis [17]. Thus, our data suggests that BIPP triggers intrinsic apoptosis in U937 leukemia cells.

This lack of extrinsic initiation of apoptosis, directs us toward investigating an intrinsic activation of apoptosis. Intrinsic apoptosis normally begins with DNA damage that is caused by UV or  $\gamma$ -irradiation or loss of inner mitochondrial membrane integrity [22, 26], spontaneous mutation or cytotoxic drug treatment. Whilst it is currently unknown whether BIPP induces DNA damage or affect the DNA repair mechanisms in the leukemia cells once inside the cell this drug signals the cell to initiate the apoptotic response. Bands are visualized that correspond to the production of DNA fragments in each of the induced samples. This damage results in a

signal sent forth to the cytoplasmic portion of the cell to begin apoptosis. The fact that BIPP induces DNA fragmentation is evidenced by the presence of DNA laddering, which occurred at all of the BIPP concentrations that were investigated (**Figure 3B**).

To verify that the reduction in viable cells was due to apoptosis and not necrosis we looked for another characteristics of apoptosis, namely, annexin-V binding. During apoptosis, enzymes are activated that are responsible for externalizing phosphatidyl serine (PS) from the cytoplasmic side of the plasma membrane to its extracellular surface [12]. PS residues can be marked and visualized by using a fluorescent annexin-V conjugate. Our data indicates that treatment of U937 cells with 12.8 $\mu$ M BIPP results in an increase in the amount of annexin-V binding signifying the induction of apoptotic cell death (**Figures 2A, B and C**). Similar results were obtained when K562 cells were studied (data not shown).

We also probed for the generation of ROS and RNS since these radicals serve as some of the internal signals for initiation of the apoptotic process. Even though their production is inherent in normal metabolic function, their generation is increased due to some cellular stressing event. Further implication of a buildup of the reactive radicals is the reduction of MMP associated with a decrease in ATP synthesis. Treatment of the U937 cells with 12.8 $\mu$ M BIPP for 48 hours resulted in a significant increase in ROS and RNS radical formation (**Figure 4A and B**). Next, we studied the effect of BIPP on MMP. As expected, after a 48-hour treatment with 12.8 $\mu$ M BIPP a marked decrease in MMP was observed (**Figure 4C**).

Further evidence of the sequential effect of BIPP-induced apoptosis in U937 cells was obtained when the ATP and ADP content of the cells post-BIPP treatment was measured. Using both indirect and direct approaches as indicated under methods we measured ATP levels. It was determined that the amount of ATP generated by BIPP treated U937 cells was significantly lower than the amount of ATP generated by untreated cells. Next, the amount of ADP in the cells post BIPP treatment was directly quantified. A dramatic increase in ADP buildup after a 48-hour treatment of the cells with BIPP was observed compared to untreated cells (**Figure**

**4F**). This is consistent with the fact that BIPP-treated cells display decreases in oxidative phosphorylation and MMP.

Cytochrome c is a peripheral protein loosely associated with the outer side of the inner mitochondrial membrane. Its release requires permeabilization of the OMM. The free cytochrome c becomes part of a functioning apoptosome after combining with Apaf-1 and caspase-9. BIPP treatment enhanced the release of cytochrome c into cytosol (**Figure 4D**). Taken together, our data strongly suggest that BIPP induces apoptosis in U937 cells and that the apoptotic response is intrinsic in nature.

In order to determine whether BIPP will elicit similar effects on noncancerous cells, we examined the effects of the drug on primary human monocytes by assessed changes in cell viability, caspase activation, and cytochrome c release in untreated and BIPP-treated monocytes. As shown in **Figure 5**, BIPP causes little yet no significant induction of cell killing or apoptosis in the normal cells. The results taken from these experiments; no change in viable cells as seen with trypan blue exclusion, no significant change in proliferation seen through MTS, no significant caspase-9 or -3 activation, and no significant cytochrome c release from the mitochondria to the cytoplasm clearly suggest that BIPP may differentially target leukemia cells while sparing normal hematopoietic cells.

Clearly, decline in mitochondrial membrane potential associated with cytochrome c release and caspases-3 activation is triggered by BIPP during apoptosis in U937 leukemia cells. In conclusion, we have shown that BIPP induces U937 leukemia cell death by intrinsic apoptosis mechanisms and that BIPP has little yet no significant effect on viability of human monocytes. Therefore, we propose that BIPP may be a novel piperazine derivative that may be an effective anticancer agent against myeloid monocytic leukemia cells without cytotoxic effects on normal monocytes.

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## Novel piperazine induces apoptosis in U937 cells

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