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Vulnerability of HL-60 Cells towards TGHQ-Mediated Apoptosis during the Cell Cycle

Hashimul Ehsan
Department of Biology, University of Houston-Victoria, Victoria, TX-77901
ehsanh@uhv.edu

ABSTRACT

TGHQ, a hematotoxic metabolite of benzene, depletes intracellular glutathione and induces rapid ROS-dependent apoptosis in HL-60 cells. To determine the influence of the cell cycle on TGHQ induced apoptosis, we investigated synchronized and asynchronous cells by biochemical and flow cytometric analysis. Synchronized HL-60 cells were more sensitive to TGHQ induced apoptosis than their asynchronous counterparts. Synchronized cells underwent TGHQ- induced apoptosis in as little as 2 hours, compared to 6 hours in asynchronous cell culture. S-phase cells exposed to TGHQ initiate apoptosis as early as 40 minutes after treatment and G2-M cells were less sensitive to PS externalization compared to S-phase cells. The proteosome inhibitor MG 132 induce apoptosis but did not potentate TGHQ mediated apoptosis in ROS induced HL-60 cells.

Keywords: *PS externalization, TGHQ, FCM analysis, checkpoint, apoptosis*

1. INTRODUCTION

Programmed cell death, or apoptosis, is highly regulated process, deregulation of which contributes to various diseases, including autoimmune and immunodeficiency diseases, neurodegenerative disorders, and cancer [1, 2] Apoptosis is also essential for life; for example, selective deletion of cells is necessary for the correct development of the nervous system, during which approximately 50 percent of nerve cells die by apoptosis [3]. This genetically controlled process of cell death is influenced by diverse stimuli, including DNA damage, the withdrawal or addition of hormones or growth factors, viral infections, and drug or chemical exposure [4]. Two main pathways of apoptosis exist: the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. Downstream (early) events of both apoptotic pathways require the activation of initiator and effectors caspases, leading to DNA fragmentation and phosphatidyl serine (PS) externalization. The biochemical and morphological features of this process include rearrangements in the lipid bilayer at the cell surface, membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation, and DNA degradation [5-7]. Mitochondria play an important role in the regulation of apoptosis induced by DNA damage and oxidative stress.

Cellular response to DNA damage depend on the extent of the damage [8]. Although reactive oxygen species (ROS) can stimulate cell growth, higher concentration ROS induce lipid per oxidation, the oxidation of proteins and nucleic acids, and cell death via both apoptosis and necrosis/oncosis [9]. 2,3,5-tris-(glutathion-S-yl)-hydroquinone (TGHQ), a metabolite of hydroquinone (HQ), generates reactive oxygen species (ROS) and induces oncotic/necrotic cell death of renal epithelial cells but induces apoptotic cell death in HL-60 cells [10, 11]. Several recent studies have investigated ROS and the cell cycle. ROS induced or inhibited some cell cycle regulatory proteins—such as MAPK kinase, CDK/cyclin, and CDC25—in a dose dependent manner [9]. Alteration of cellular redox state by depletion of GSH (increased ROS) resulted in delayed progression through G1 and S phases, as well as G2 arrest [12]. Functional activity of cyclin E/Cdk2 during the DNA replication is controlled by ROS treatment [13]. Cyclin B1/Cdk1 delays its activation in G2/M checkpoint upon ROS treatment, possibly via the inhibitory phosphorylation of Cdc25c by Chk1 kinase [13]. Inhibitors of Cdks, such as p21, are induced upon ROS (nitric oxide) treatment and as a result of cell cycle blockage at G1/S transition via inactivation of Cdk/cyclin complexes [14]. After DNA damage, phosphorylation of Chk1 leads to the phosphorylation of specific protein substrates that direct the cell towards the repair mechanisms or cell cycle halt. Recent studies in human cells (A549 cells) have shown the degradation of Chk1 by the ubiquitin-proteasome pathway upon genotoxic stress [15]. Another deactivation method may involve the phosphatase PPM1D, which interacts with Chk1, and de-phosphorylated its Ser-345, thereby deactivating it [16, 17]. Depletion of Chk1 leads to premature activation of Cdc2-cyclin B complexes [18]

Thus the degradation or depletion of an important protein such as Chk1 might have an effect towards cell death. Abnormal proteins may be exported to the cytosol and degrades mainly by the proteosomes machinery. However, ER stress could be induced by many other stimuli so; application of proteosome inhibitor may not rescue the effect of protein degradation. In addition, it has been well documented that the proteosome inhibitors can induce

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apoptosis in various cancer cells [19, 20]. Since the apoptosis and cell cycle signaling machinery are ultimately connected and since ROS can interfere with later and induces the former the present study were conducted to determine the role of the cell cycle with response of HL-60 cells to TGHQ- induced ROS. Here, we report the mechanism of apoptosis, TGHQ sensitivity in synchronous versus asynchronous cultures, and proteosome inhibitor MG 132 does induce apoptosis but do not potentate TGHQ mediated apoptosis through the ROS production in HL-60 cells.

2. MATERIALS AND METHODS

2.1 Reagents

a. Caution

TGHQ is nephrotoxic and nephrocarcinogenic in rats and therefore must be handled with protective clothing and in a ventilated hood.

b. Chemicals

Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were of the highest grade commercially available. TGHQ was synthesized in the laboratory according to established methodology [10].

c. Antibody and all Other Assay Kit

Anti PARP was purchased from Cell Signaling Technology (San Diego, CA). ROS assay kit and z-VAD-FMK purchased from Invitrogen, USA. Apo active caspase assay kit purchased from Cell Technology, CA. Annexin/PI kit from BD bioscience, USA. DNA fragmentation kit was purchased from Clonetech, USA.

d. Cell Culture Conditions and Synchronization

HL-60 cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) and maintained in a regulated incubator at 37ºC with constant 5% CO_2 supply. Exponentially growing cells were seeded at a cell density of 1.0 x 10^6 cells/mL and incubated for overnight. For active synchronization experiment, aphidicolin (5µg/mL) was added after 18 hours of growth in a complete medium for 12 hours followed 3 times extensive washing with the same medium except FBS. Cells were then released into new pre-warm fresh medium, collected samples (3 x 10^6 cells) and return to the incubator for the following time point collection. For each time point of apoptosis analysis, approximately same amount of cells were collected and prepared for FCM analysis. Cell synchronization was monitored by FCM analysis. In brief, cells were collected at hourly intervals, washed twice with cold PBS (approximately 10 volumes of PBS) and centrifuged for 5 min at 1200 rpm. Cells were then fixed with 70% cold ethanol for at least 30 minutes at -20^{o}C for further analysis by flow cytometer. Cells were washed twice with PBS and filtered (20 µM) to yield cell suspensions which were subsequently treated with RNAse A and stained with propidium iodide (50 µg/ml). Cell cycle analysis was performed using a FACS scan flow cytometer (Beckman Coulter) followed by Wincycle software.

e. ROS, Annexin, PS-externalization, DNA Fragmentation, Caspase and Mitochondrial Membrane Potential Assay and Brdu Incorporation

The percentage of apoptotic cells was determined according to the manufacturer's protocol using an annexin V FITC kit and analyzed by FACS scan flow cytometer (Beckman Coulter) followed by CXP software (Beckman Coulter) analysis for PS-externalization. DNA fragmentation assay were done according to manufacturer's protocol. Active caspase assay was done according to manufacturer's instruction. ROS measurements were performed according to Yang et. al. [11] using carboxy-H2DCFDA.

e. **Western Blot Analysis**

After specified treatments, HL-60 cells were washed twice with phosphate buffered saline (PBS) and homogenized in ice-cold lysis buffer (Cell Signaling Technology, Inc.- 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1µg/ml leupeptin). After 3 x freeze thaw cycle, homogenates were centrifuged at 10,000 g for 10 min at 4^{o}C. Supernatants were collected, and 50 µg of total protein electrophoresed on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. After blocking in TBS-T containing 5% dried milk or 5% BSA, membranes were incubated with appropriate primary antibodies (1:1000 dilution) overnight at 4^{o}C. After Washing in TBS-T, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilutions) (Santa Cruz Biotechnology). After thoroughly washing in TBS-T, bound antibodies were visualized using standard chemilumiscence on autoradiographic film.

3. RESULTS

3.1 TGHQ-Mediated Apoptosis in HL-60 Cells

TGHQ stimulates the production of ROS within 30 minutes and production persists for at least 90 minutes (Fig. 1A). A biochemical hallmark of apoptotic cell death is the translocation of PS from the cytoplasmic side of the cell membrane to the external cell surface. Analysis of cells using FITC-conjugated annexin V in combination with PI,

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revealed that 53% of cells treated with TGHQ (200 µM) for 6 hours, exhibited PS externalization some of which remained in early apoptosis (PI -, Annexin +), whilist others were in "late" apoptotis (PI +, Annexin +) (Fig. 1B). Cleavage of PARP from the native 116 kDa to the 85 kDa form represents another hallmark of apoptosis, and occurred 2 hours after treatment of HL-60 cells with 200 µM TGHQ (Fig. 1C). Asynchronous TGHQ treated HL-60 cells also exhibited typical DNA fragmentation 4 hours after TGHQ treatment (Fig. 1D). TGHQ (100 and 200 µM) treated HL-60 cells undergoing apoptosis also contain activated caspases, as revealed by increases fluorescence intensity compared to control cells (Fig. 2A). z-VAD-FMK, a caspase inhibitor effectively inhibited caspase-3 activity at a concentration of 50 µM whereas 20 µM has less inhibition (Fig. 2B).

3.2 TGHQ Sensitivity in Synchronized S-phase and G2-M Checkpoint

To elucidate the mechanism(s) by which apoptosis is regulated in a synchronous environment, we first synchronized the HL-60 cells by using aphidicolin as an S-phase blocker. Two FCM analyses were used to assess the degree of synchronization. When aphidicolin was removed from the culture, more than 50% of cells proceeded from S-phase to the next phase of the cycle (Fig. 3A). As the cell cycle proceeded further, G2/M phase (4n) showed a lower degree of synchronization (Fig. 3B: right panel); this is not unexpected, as a population of cells will normally lose its synchrony with time. We then compared apoptosis induction between synchronous and asynchronous cultures after TGHQ treatment. In asynchronous culture, cells treated with TGHQ (200 µM) showed apoptosis after 4 hours (Fig. 3C). In contrast, synchronous cultures, apoptotic cell death was observed within 1 hour after TGHQ treatment. We then monitor and compare PS externalization in S phase and G2/M phase of the cycle. Flow cytometric analysis for annexin-V reveals that synchronously-inducible apoptosis in S-phase starts as early as 30 minutes after TGHQ treatment (Fig. 4A). Between 60 – 120 minutes, PS externalization after TGHQ treatment shows 60 – 80% of cells are apoptotic, whereas untreated cells maintain baseline levels (~20%). This baseline level of apoptosis may be due to aphidicolin treatment, since cell-synchronizing drugs induce a certain degree of apoptosis. Next, aphidicolin-synchronized HL-60 cells were released into S-phase and were monitored thorough the G2/M phase by flow cytometry (data not shown). TGHQ then was subsequently applied to the cell cultures at the beginning of G2 phase (i.e., 4 hours after aphidicolin removal), and PS externalization monitored at 30-minute intervals. The data revealed that TGHQ also promoted PS externalization during the G2/M phase of the cycle (Fig. 4B) although these cells appeared in less susceptible than S-phase cells. Taken together, the results indicate that S-phase HL-60 cells are more sensitive to TGHQ.

3.3 MG 132 Induced Apoptosis do not potentiate Towards ROS Mediated Apoptosis

An increasing amount of evidence shows that that the proteosome inhibitors MG 132 can induce apoptosis in various cancer cells [19, 20]. And we wanted to see whether TGHQ mediated cell death increases after treated cells with MG132. In other words, we wanted to see whether MG132 follow the downstream events of ROS production. We therefore study the effect of MG 132 from nanomolar concentration to micromolar concentration in order to see at what concentration cell starts PS externalization. Flow cytometric analysis show that the level of PS externalization increases from above 200 nM concentration of MG 132 (Fig. 5). The significant increases apoptosis with the micromolar concentration of MG 132 (1-10 µM) shows a drastic PS externalization and therefore excluded for further studies (Fig. 5). To further establish the critical role for apoptosis induction, we monitor the caspase actvtity with the 400 nM concentration of MG 132. In contrast to the control, 400 nM MG 132 induces 11% caspase activation as control cells shows less than 1% of caspase activity (Fig. 5 lower two right panel).

To better understand the critical role of caspase activation with the MG 132 treatment, we monitor the possibilities of potentiation towards ROS mediated apoptosis like the TGHQ does. Therefore, in subsequent studies, we lower the concentration of TGHQ to 50 µM and MG 132 to 300 nM for the experiment. Under our condition, MG 132 did not potentate the TGHQ mediated apoptosis induction as it 12% with MG 132 treatment and 20% with TGHQ treatment and 32% in combo treatment. (Fig. 6).

4. DISCUSSION

Although the ROS mediated apoptosis in HL-60 cells are well documented but the mechanism towards cell death during the cell cycle is somehow unknown. In an early study, we have demonstrated TGHQ mediated ROS generation toward cell death followed by characterization of apoptosis inducing machinery [11, 21]. To support this notion, we described the cell cycle profile upon TGHQ mediated apoptosis in HL-60 cells. In this study, we first well characterized the TGHQ mediated apoptosis induction with the recent advanced methodology such as apo-active caspase assay in addition to the traditional assay. To understand the impact of TGHQ at various points in the cell cycle, synchronized cultures are necessary. Cell cycle stages can be manipulated in cultures by many procedures, including serum starvation, contact inhibition, and/or chemical treatments. Few reports are available on cell cycle

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studies in synchronized HL-60 cell lines. Therefore, it was necessary to establish a good synchronized culture.

The genetically controlled process of apoptosis requires suppression and expression of several genes in series of processes which can be separated into two phases. The commitment phase, where cells enter to the cell death pathway, may last from a few hours to several days after exposure to an apoptotic stimulus; the execution step is faster (1 hour). In recent years, the checkpoint signaling pathway has attracted increasing attention in the context of the regulation of cell death [15, 16, 18]. Both Chk1 and Chk2 protein kinases can be activated upon exposure to DNA damage [22]. Human Chk2 is a stable protein expressed throughout the cell cycle and seems to be largely inactive in the absence of DNA damage [23, 24]. Several studies have been reported the necessity of Chk1 during the cell cycle progression; for example, Chk1 induced in late G1 phase is active even in unperturbed cell cycles [23, 25]. Upon genotoxic stress, Chk1 protein also plays a central role in delaying the cell cycle progression through S or G2 phases by inhibiting phosphatase proteins Cdc25A and Cdc25c [26, 28]. Partial reduction of Chk1 causes spontaneous S-phase damage in developing mammary epithelium in vivo [29]. In response to DNA-damaging agents, two predominant signaling pathways have been described, which regulate cell cycle progression and apoptosis [30]. The ataxia telangiectasia mutated (ATM)/checkpoint kinase (Chk2) and ataxia telangiectasia related (ATR)/Chk1 pathways elicit control at the different cell cycle checkpoints [31, 32]. ATR is the well-established upstream kinase that phosphorylates and activates Chk1 in response to replicative stress [23, 33]. Perhaps the degradation of some proteins is critical for intracellular signaling including cell proliferation and cell death. In this context, we used the proteosome inhibitor MG 132 followed by the analysis of PS-externalization. We showed that MG 132 do not potentate TGHQ mediated apoptosis but promote apoptosis. An independent investigation using a proteosome inhibitor Bortezomib induces mitotic catastrophe in some B-cell lymphoma cells [34]. Therefore, it is also possible that mitotic catastrophe may be contributing to MG 132-induced apoptotic cell death. Castedo et al. linked apoptosis to cell cycle and mitotic catastrophe [35].

Figure 1:

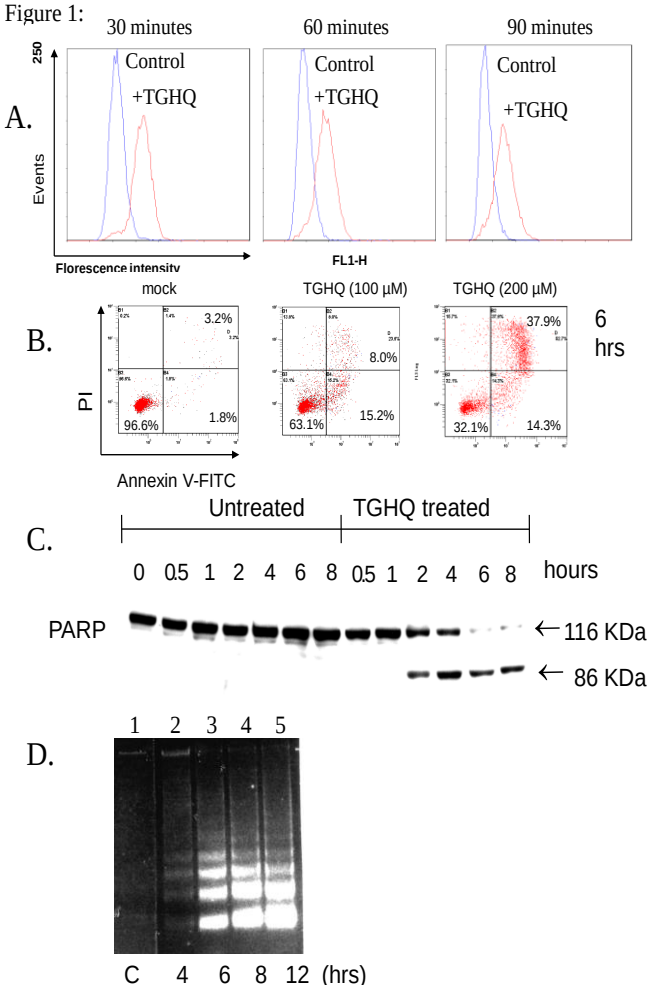

Fig 1: Induction of apoptosis in HL-60 cells after TGHQ treatment. (A) Induction of ROS upon TGHQ treatment

After 200 µM TGHQ treatment, the cells were collected and resuspended with PBS at each time point. Carboxy-DCF fluorescence was measured in an Beeckman Coulter FC-500 flow cytometer with excitation (495 nm) and emission (525 nm). Blue curves represent control cells and orange curves represent TGHQ-treated cells. (B) apoptosis induced by TGHQ is accompanied by PS exposure. Six hours after TGHQ (100 and 200 µM) treatment, cells were analyzed by flow cytometry with FITC-labeled annexin V and propidium iodide (PI). Viable cells are at the bottom left (annexin V-, PI-), early apoptotic cells are bottom right (annexin V+, PI-), late apoptotic cells are at top right (annexin V+, PI+). (C) Proteolytic cleavage of PARP by the activated caspase upon TGHQ (200 µM) treatment. PARP cleavage produced ~85 kDa fragments, which are the classic

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feature of apoptosis. (D) DNA fragmentation assay after TGHQ treatment reveals the DNA laddering pattern that is characteristic of apoptosis. In the TGHQ-treated cells, apoptotic cell death was observed as early as 6 hours, and increased with time.

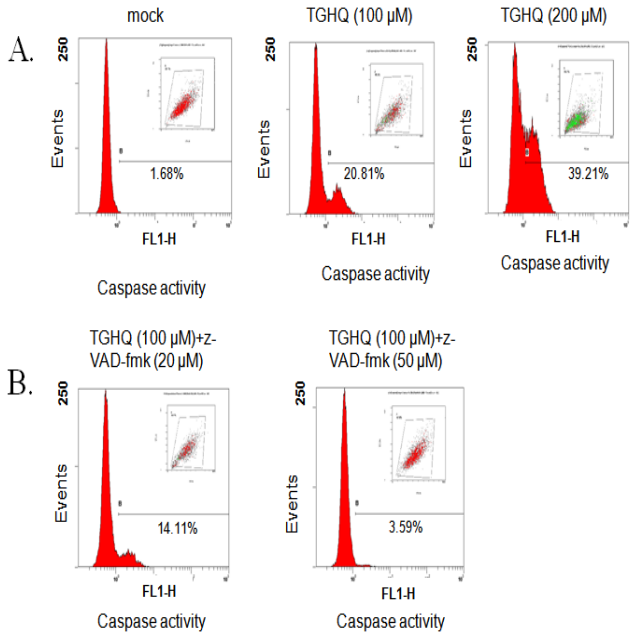

Fig 2: TGHQ induces caspase 3 activity towards apoptosis mediated cell death.

(A) Ab rose against 163-175 of caspase 3 and this epitope is present on the p18 subunit of cleaved caspase 3. Upon TGHQ treatment, cells were fixed, stained with Ab followed by FITC lebeled 2º Ab. (B) Inhibition of TGHQ (100 µM) mediated apoptosis by caspase inhibitor z_VAD-FMK in asynchronous HL-60 cells upon 6 hrs treatment.

Figure 3:

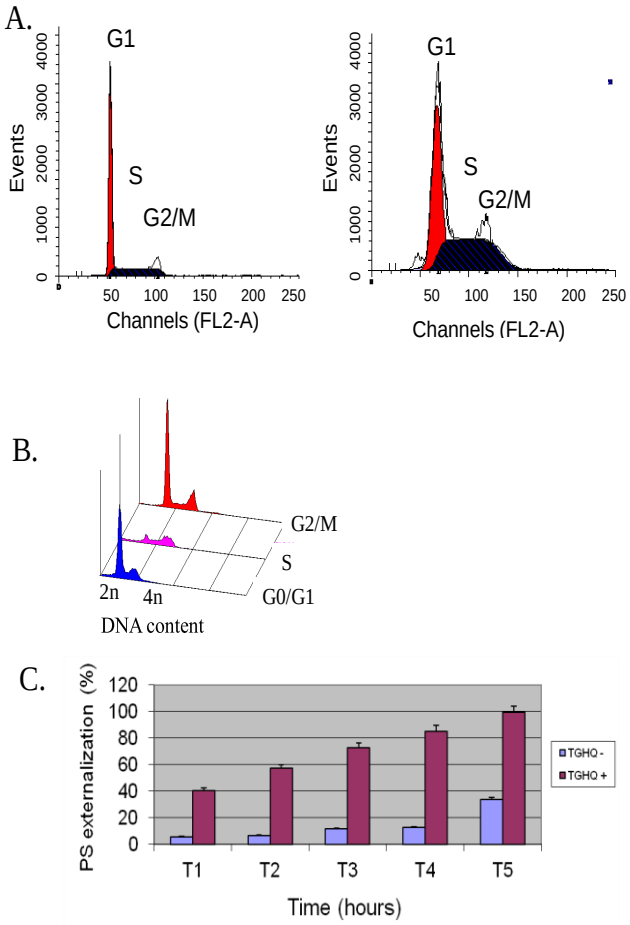

Fig 3: Flow cytometric cell cycle analysis of synchronized HL-60 cells:

(A) Asynchronous culture (left), and synchronized S-phase cells (right) shows 52% of cells are in DNA replication phase. (B) 3-D overlay images of the DNA cell cycle histograms. (C) Asynchronous HL-60 cells are less sensitive towards apoptosis mediated cell death than synchronous counterpart. Time course analysis of apoptosis in asynchronous HL-60 cells after 200 µM TGHQ treatment (versus controls), using annexin V staining and flow cytometric analysis (see materials and methods). Cell death was observed as early as 4 hours after TGHQ treatment in asynchronous cells. In comparison to synchronized cells, S-phase cells undergo apoptosis as early as 1 hour.

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Figure 4:

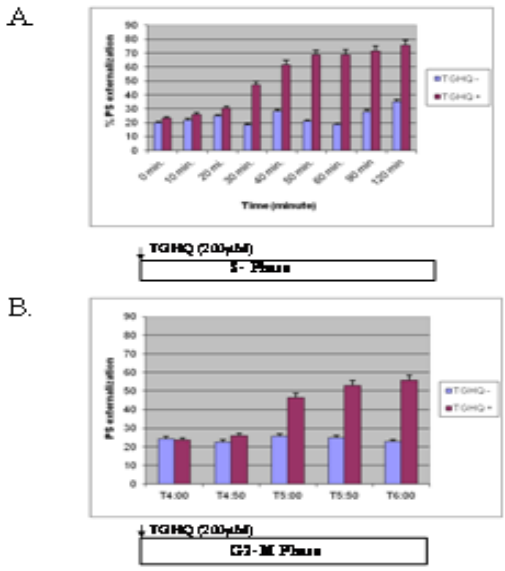

Fig 4: S-phase cells are more vulnerable than G2/M cells upon TGHQ treatment.

Aphidicolin-synchronized, S-phase released cells were treated with/without 200 µM TGHQ, followed by annexin V staining and flow cytometric analysis (see Materials and Methods). (A) Synchronized cultures were monitored every 10 minutes and showed apoptotic cell death within 60 minutes after TGHQ treatment. After two hours of treatments at S-phase cells, 76% of the cells show PS externalization. (B) Aphidicolin-synchronized S-phase released cells were allowed to reach G2-M phase (4 h after release), and then were treated with/without 200 µM of TGHQ. These cells were analyzed by annexin V staining and flow cytometric analysis. Cells were collected at 30 minute intervals; increased cell death was observed after two hours of treatment. At this time point of G2-M phase cells, only 57% of the cells shows PS externalization, compare to 76% in S-phase cells.

Figure 5:

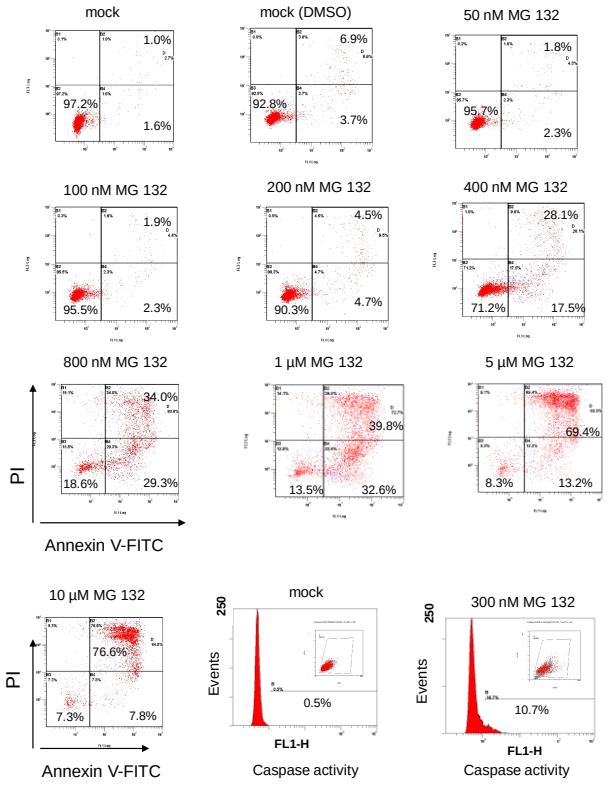

Fig 5: Induction of apoptosis by proteosome inhibitor MG 132.

Asynchronous cells were treated with different concentration of MG 132 together with control (DMSO) for 12 hours. Cells were analyzed for PS externalization with the flow cytometer upon FITC-labeled annexin V and propidium iodide (PI) staining. Cell death was observed at a concentration of 400 nM MG 132 treatment in asynchronous cells after 12 hours. Subsequent higher concentration of MG 132 (micromolar range) gradually increases the apoptotic mediated cell death. Bottom two right panel: induction of caspase activity upon 400 nM MG 132.

Figure 6:

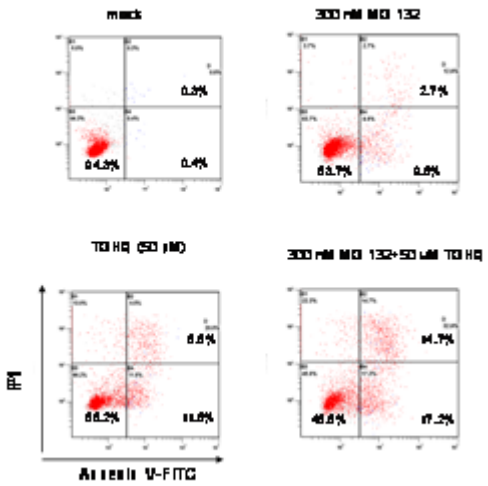

Fig 6: MG 132 does not potentiate TGHQ mediated apoptosis induction in HL-60 cells.

Asynchronous cells were treated with 300 nM concentration of MG 132 together with control (DMSO) for 12 hours. TGHQ also applied to the cells at the same condition with or without (50uM) MG 132, cells were then analyzed for PS externalization with the flow cytometer upon FITC-labeled annexin V and propidium iodide (PI) staining. Cell death was observed at a concentration of 300 nM MG 132 treatment (13%) in asynchronous cells after 12 hours and 21% in TGHQ treated cells. Together with TGHQ and MG 132 treated cells, only 32% cells shows PS externalization.

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ABBREVIATIONS

PS: Phosphatidylserine
PI: Propidium Iodide
PARP: Poly ADP ribose polymerase
ROS: Reactive oxygen species
TGHQ: 2, 3, 5-tris (Glutathion-S-yl) hydroquinone