The Role of Oxidative Stress in Koenimbine-Induced DNA Damage and Heat Shock Protein Modulation in HepG2 Cells

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Abstract

Background. Murraya koenigii (L.) Spreng, is a significant herb of traditional Ayurvedic system of medicine. Koenimbine, a carbazole alkaloid isolated from this plant holds antiproliferative and apoptotic effects. The aim of this study was to assess koenimbine-induced DNA damage and to clarify the role of free radicals in cell death mechanisms, using HepG2 cells. *Methods.* The level of cytotoxicity was assayed by MTT assay. To elucidate the role of glutathione (GSH), the intracellular GSH level was analyzed. The effect of koenimbine in the cell mitochondria was evaluated using mitochondrial membrane potential (MMP) changes. Single cell gel electrophoresis assay was used to examine the level of DNA damage. Heat shock proteins, Hsp 70 and Hsp 90 expressions were checked at mRNA and protein level. Ascorbic acid and catalase were used as control antioxidants. *Results.* It was observed that koenimbine considerably increased DNA damage in HepG2 cells at subcytotoxic concentrations. Koenimbine induced the increased levels of reactive oxygen species (ROS) and reduction of GSH level in HepG2 cells, together with time-dependent loss of MMP. In addition, results clearly showed that koenimbine encouraged cells to express Hsp 70 and Hsp 90 in a concentration-dependent manner up to a concentration of 100 μM and a time-dependent manner at 24-hour incubation both at transcriptional and translational levels. The antioxidant capacity of ascorbic acid was found to be not as prominent as to catalase throughout the study. *Conclusion.* Based on these data it can be concluded that koenimbine causes DNA strand breaks in HepG2 cells, probably through oxidative stress. Moreover, the oxidative stress induced was closely associated with MMP reduction and GSH depletion associated with HSP modulation at subcytotoxic concentration.

Keywords

oxidative stress, cell death, apoptosis, koenimbine, heat shock proteins

Background

Cell death is an inevitable action in drug-induced cancer therapy. Even though many forms of cell death exist, apoptosis is the preferred one because of its ability to kill the cancer cells by programmed pathways rather than inducing inflammation-mediated death.[1] Hence this form of cell death is physiologically different, to a great extent, from other forms of cell death like necrosis. Most important, the apoptotic form of cell death is seldom altered in cancer cells.[2] Although, the introduction of external stimuli such as radiation or chemical agents is able to induce apoptosis, understanding the way in which it executes this killing of cells is of great importance. It unfolds the physiological change which happens within the cancer cell.

Reactive oxygen species (ROS) such as hydroxyl radical, superoxide anions, and hydrogen peroxide and peroxyl radicals has been shown to play both beneficial as well as deleterious roles to biological systems.[3] Very low concentrations of ROS act as second messenger in signal transduction; however, excess concentrations of ROS can cause damage to DNA and other vital cyto-components.[4,5] To counterbalance the harmful effect of ROS, cells are

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harmonized with production of heat shock protein (HSP) or antioxidant defense system alteration that comprises SOD (superoxide dismutase), catalases, glutathione, and others as a defensive system. Although the antioxidant system and ROS complement each other to keep balance under normal circumstances, even mild disturbance in symmetry leads to generation of ROS, called oxidative stress.[6,7] The major consequences of these stresses are lipid peroxidation and shutdown of mitochondrial functions, reduction in mitochondrial membrane potential (MMP), energy supply, and DNA damage.[8,9] Hence these triggers and other proliferative responses alone or collectively lead to cell death.

Chemotherapy is the best available tool for the managemt of cancer. For decades, scientists tried to understand the physiological implications of new drug candidates from synthetic and natural sources.[10] It is important to know the mechansim of these candidates' genetic regulation within cancers. *Murraya koenigii*, a sigificant herb of the Indian subcontinent, has been used for centuries in the complementray and traditional Ayurvedic system of medicine.[11] It is a rich source of phytochemicals, including carbazole alakaloids. Many studies have shown the capacity of carbazole alkaloids isolated from *M koenigii* to induce apoptosis.[12,13] Koenimbine is a carbazole alakaloid that exhibits antiproliferatve and proapoptotic effects in cancer cells.[14-16] Its ability in cancer cell death is similar to other carbazole alkaloids.[12,17] But its ability to kill the cancer cells via fress radical generation is not known. Recently, anticancer agents such as 5-fluorouracil, tamoxifen, and paclitaxel have been found to eliminate cancer cells effectively by continually maintaining cellular ROS at a threshold level.[18,19] Since oxidative stress level also can be detected in the cells after cell death, it is important to measure the role of ROS in inducing cell death at subcytotoxic concentrations. Hence in the current investigation, the ability of koenimbine to induce significant oxidative stress that can lead to DNA damage and cell death has been thoroughly investigated using HepG2 cell as an in vitro model.

Materials and Methods

Materials

Koenimbine was kindly gifted to this research by Dr Syam Mohan, Jazan University. The yield of koenimbine was 2 mg per 400 g leaves. The purity of the compound was checked and found to be 98.5%, which was in full agreement with previous reports.[20]

Cell Viability Assay

HepG2 and WRL-68 cells were purchased from American Tissue Culture Collection (ATCC). The cells (1×10^5 cells/mL) were plated out into 96-well microtiter plates. Koenimbine

was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was 0.1% (v/v). Different concentrations of the sample were prepared with serial dilution. DMSO (0.1%) was used as a control.

Cytotoxic activity of koenimbine on HepG2 cells was determined colorimetrically using MTT assay. The cells were treated with various concentrations of koenimbine for 3 and 24 hours. Untreated cells were used as control. The absorbance was measured in a microplate reader at a wavelength of 560 nm with background subtractionation 690 nm. The inhibitory rate of cell proliferation was calculated by the following formula: growth inhibition = $[(OD_{control} - OD_{treated})/OD_{control}] \times 100$, where OD is optical density.

Koenimbine Treatment on HepG2

HepG2 cells were seeded at a density of 1×10^5 cells/mL in culture flask and incubated. After 24 hours, the cells were treated with koenimbine with or without catalase (2400 U/mL) and ascorbic acid (100 mM) for 3 and 24 hours. Reduction of HepG2 cells' viability by koenimbine at concentrations up to 50 μM did not exceed 15% after 3 hours of incubation with koenimbine. These subcytotoxic concentrations were chosen for subsequent experiments to investigate the oxidative DNA damage during treatment by the compound. The koenimbine concentrations used for the 3-hour treatment were 10, 20, 50, and 100 μM. For 24-hour treatment, concentrations used were 50 and 100 μM. Apart from this, a 30-minute pretreatment with antioxidants was done along with 50 and 100 μM at 3 and 24 hours.

Measurement of Intracellular Reactive Oxygen Species (ROS)

The ability of koenimbine to induce intracellular ROS formation was determined using a fluorescent probe, DCF-DA. The treatment procedure was same as mentioned earlier. At the end of designed reaction time, DCF-DA (5 μM) was added 30 minutes before the termination of koenimbine treatment in dark. The cells were then washed with PBS (phosphate buffered saline), trypsinized, and resuspended in 3 mL of PBS, and the intensity of green fluorescence was immediately read in a specrtrofluorometer at 485 nm. Intracellular ROS level was expressed as percentage relative to control fluorescence (assuming control ROS level as 100%).

Determination of Intracellular Glutathione (GSH)

The intracellular GSH levels were measured using a fluorogenic probe, monochlorobimane (MCB). After treatment, the 1×10^5 HepG2 cells were washed once with ice-cold wash buffer. Then prechilled cell lysis buffer was added to lyse the cells for 10 minutes on ice. Cells and debris suspensions were transferred to microcentrifuge tubes. After

centrifugation at 12 000 × *g* for 10 minutes, the supernatant was collected as cell lysate. Ninety microliters of each lysate was added to a 96-well plate followed by addition of 50 μM MCB solution. After incubation at room temperature for 2 hours, the plate was read in a fluorescence microplate reader using a 380/460 nm filter set. The GSH level in the test sample was expressed as percentage fluorescence of control.[21]

Determination of Mitochondrial Membrane Potential (MMP)

Rhodamine 123 (Rh123) was prepared in ethanol as a 5 mg/mL stock solution. At the end of reaction time, cells were harvested and washed twice in cold PBS, then resuspended in Rh123 (2 μg/mL) for 30 minutes in dark. Rh123 staining intensity was measured by flow cytometry (BD FACSCanto-II, BD Biosciences, Franklin Lakes, NJ, USA) with an excitation wavelength of 485 nm. Intensity of Rh123 is directly related to mitochondrial membrane potential. The percentage of rhodamine negative cells implicates percentage collapse of MMP.

Single-Cell Gel Electrophoresis (SCGE, the Comet Assay)

The modified comet assay using formamidopyrimidine glycosylase (FPG) and endonuclease III (Endo III) was used to determine oxidative DNA breaks. Alkaline comet assays were performed using a Comet Assay kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Preparation and reconstitution of reagents used for this assay are according to the methods explained earlier.[22] The cells were gently scraped from the culture plates, washed once with PBS. Subsequently the 1×10^5 cells were transfered into a prewarmed microcentrifuge tube, resuspended in ice-cold PBS (Ca-Mg-free) and incubated at 37°C. Meanwhile, low melting point (LM) agarose was melted for 5 minutes in a beaker in boiling water. Molten LM agarose was placed in 37°C waterbath for 20 minutes to cool. Molten LM agarose (140 μL) and 1×10^5 cells/mL (40 μL) were combined at a ratio 10:1 (v/v). Immediately 75 μL sample of this mixture was pipetted into a 20-well comet slide (provided with kit). Using the tip of the pipette, the cells in agarose were gently spread into the well and the slide was placed at 4°C in dark. After 30 minutes, the slide was immersed in prechilled lysis solution (provided with kit) to remove cellular proteins and membranes and reincubated at 4°C overnight. The slides were then washed 3 times with prechilled enzyme buffer (40 mM HEPES, 0,1 M KCl, 0.5 mM ethylenediaminetetraacetic acid [EDTA], 0.2 mg/mL bovine serum albumin, pH adjusted to 8.0 with KOH). After the last wash, excess buffer was drained on to a tissue paper. The slides were then

incubated for 45 minutes at 37°C with Endo III, FPG, or with buffer alone. The slides were then placed in a horizontal electrophoresis tank containing alkaline unwinding solution (200 mM NaOH, 1 mM EDTA) for 60 minutes at room temperature in dark. The slides were then placed in prechilled alkaline electrophoresis solution (200 mM NaOH, 1 mM EDTA) in electorphoresis slide, covered and electrophoresed with slide tray overlay. A constant power of 21 volts was applied for 30 minutes. Excess electrophoresis solution was drained and the slides were then immersed twice in distilled water for 5 minutes each time. The slides were then washed for 5 minutes with 70% ethanol. The slides were dried at ≤45°C for 15 minutes and stored overnight at room temperature with dessicant. The following day, the slides were stained with 50 μL SYBR Green dye and allowed to stain for 5 minutes at 4°C. The slides were viewed under confocal micrscope and a total of 100 comets were scored according to the procedure discussed earlier.[23]

Induction of Hsp 70 and Hsp 90 mRNA

The total extraction of RNA was done using Neasy Mini Kit (Qiagen, Germantown, MD, USA) as described by the manufacturer. RNA quality, quantity, and integrity were determined via the A260/A280 ratio using nanodrop. Two micrograms of total RNA from each sample were subjected to first strand cDNA synthesis in a total volume of 20 mL. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using an ABI 7700 Prism Sequence Detection System and TaqMan primer probes (Applied Biosystems, Foster City, CA, USA).

Reverse transcription reaction were performed at activation at 95°C for 30 seconds, 40 cycles of denaturation at 95°C for 5 seconds, and then annealing and extension at 60°C for 30 seconds. Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as an internal control for each sample. PCR products were detected using gel electrophoresis. The sequences of the primers sets used for this analysis are as follows: HSP70, forward: 5′-ATCGACCTGGGCACCACCTA-3′, reverse: 5′-CAGCACCATGGACGAGATCT-3′; HSP90, forward: 5′-ATTGCCCAGTTGATGTCATTGA-3′, reverse: 5′-ATGCATCTGATGAATTTGAAATGAG-3′; GAPDH, forward: 5′-GGATTTGGTCGTATTGGG-3′, reverse: 5′-TCGCTCCTGGAAGATGG-3′. Data were analyzed according to the comparative fold increase or decrease in gene expression determined by Ct values and normalized by GAPDH expression in each sample.

Induction of Hsp 70 and Hsp 90 Proteins

After treatment, the cells were washed once with ice-cold wash buffer. Prechilled cell lysis buffer was added to lyse the cells for 10 minutes on ice. Cells and debris suspension

were collected and transferred to a microcentrifuge tube. After centrifugation at 12 000 × *g* for 10 minutes, the supernatant was collected as cell lysate. Ten microliters supernatant were allocated for total protein content assay by the Bradford method (Bio-Rad, Hercules, CA, USA).

The assay was performed using the ELISA kit (Uscn Life Science Inc, China). Briefly, 100 μl each of standard, blank, and samples were added to the 96-well plate. The plate was sealed with plate sealer and incubated at 37°C. After 2 hours of incubation, the liquid in the wells was replaced with 100 μL of detection reagent A (supplied with kit) and incubated for 1 hour at 37°C. The reagent was removed and the plate washed thrice with 350 μL of wash solution (supplied with kit) using a multichanel pipet and allowed to dry for 1 to 2 minutes. One hundred microliters of detection reagent B (supplied with kit) was added to the wells, and the plate incubated for 30 minutes at 37°C. After incubation, reagent was removed and the washing process was repeated 5 times. The plate was allowed to dry for 1 to 2 minutes. To each well, 90 μL of substrate solution was added. The plate was covered and incubated at 37°C in dark. The reaction was stopped by adding 50 μL of stop solution into each well. The concentrations of Hsp 70 and Hsp 90 were determined at 450 nm immediately in a microplate reader. The optical density of treatment was compared with the optical density of control and the value was expressed as percentage difference compared to control.

Results

Survival Rate of HepG2 Cells Was Decreased

The cytotoxicity assay (MTT) performed in this study revealed that HepG2 had shown a selective dose-dependent effect in HepG2 cells compared with the WRL-68 normal cell. The IC_{50} value of koenimbine on the viability of HepG2 cells has been found to be 68 ± 5.1 μM, while it could only kill the WRL-68 cells with IC_{50} of 110 ± 7 μM. The antiproliferative effect of koenimbine on HepG2 cells was less significant at 3-hour incubation thus further emphasizing time dependency of the inhibition toward cellular proliferation. Reduction of HepG2 cells viability by koenimbine at concentrations up to 50 μM did not exceed 15% after 3 hours of incubation (Figure 1). Thus, these subcytotoxic concentrations were chosen for subsequent experiments to investigate the oxidative DNA damage during treatment by the compound.

The ROS Production Significantly Increased With Koenimbine Treatment

As shown in Figure 2A, the intensity of DCF fluorescence increased significantly with koenimbine treatment of HepG2 cells in a dose-dependent manner. The level of intracellular
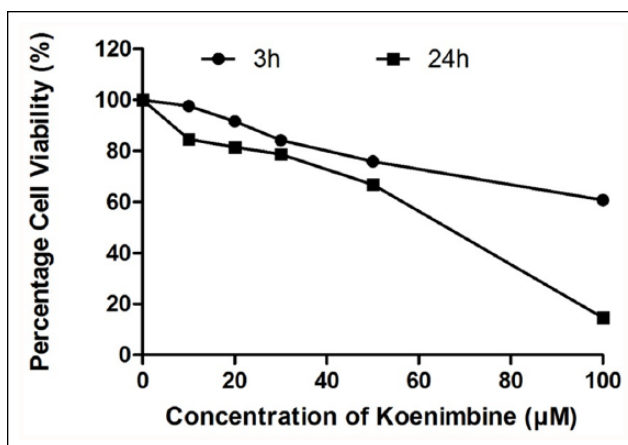

Figure 1. Growth curve for HepG2 cells treated with koenimbine at 3 and 24 hours determined by MTT assay.

ROS of HepG2 cells at 3 hours was increased from 101.20% ± 6.3% at control to 190.4% ±14.1% at 100 μM. Meanwhile, at 24 hours, the maximum concentration (100 μM) showed remarkable elevation in ROS level (210.2% ± 17.2%). The current study further investigated the role of antioxidants in koenimbine-induced ROS production by HepG2 cells. The antioxidants apparently alleviated koenimbine-induced ROS overproduction. The level of ROS after 3 hours of incubation using catalase with 50 and 100 μM koenimbine was 147.3% ± 6.28% and 172.10% ± 6.44%, respectively. After 24 hours, the level of ROS using catalase with 50 and 100 μM koenimbine increased substantially to 176.11% ± 3.54% and 200.7% ± 7.11%, respectively. In the case of using ascorbic acid as antioxidant, the percentage of ROS level showed insignificant changes.

Effect of Koenimbine on Intracellular Level of Glutathione

As shown in Figure 2B, koenimbine treatment caused an initial increase and later decrease in HepG2 intracellular GSH. In this respect, after 20 μM koenimbine treatment for 3 hours, the glutathione level increased by 40%. Further increase in koenimbine concentration, however, depleted the initial elevation of GSH level. The GSH level was greatly preserved even after pretreatment with antioxidants with only minimal decrease as compared to control. However, at 24 hours, when GSH level was 86.44% ± 9% on exposure to 50 μM koenimbine, pretreatment with catalase reduced this level to 79.50% ± 4.2%; whereas, pretreatment with ascorbic acid could only reduce GSH level to 85.65% ± 5.09% (Figure 2B). This trend was apparently observed when HepG2 cells were pretreated either with catalase or ascorbic acid. Thus, though no notable decrease were observed on antioxidant pretreatment, catalase acted slightly better than ascorbic acid.
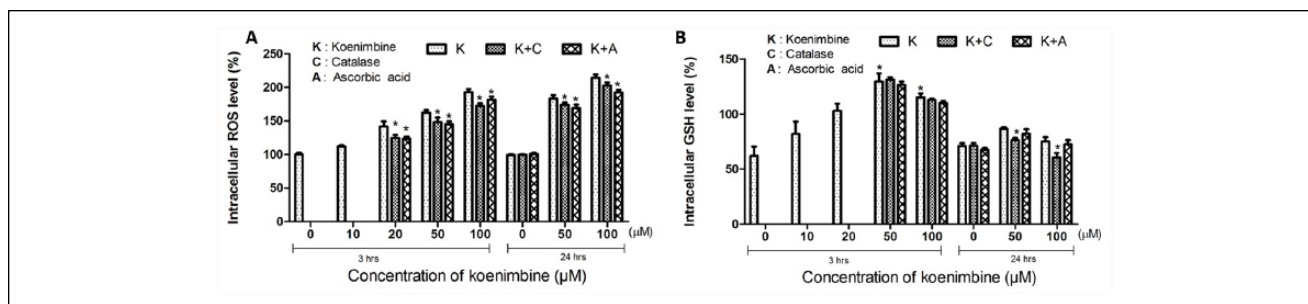
Figure 2. Effects of koenimbine on HepG2 cells reactive oxygen species (ROS) generation (A) and glutathione (GSH) level (B). Values are mean ± SD from 3 independent experiments. Triplicates of each treatment group were used in each independent experiment. The statistical significance is expressed as *P < .05.
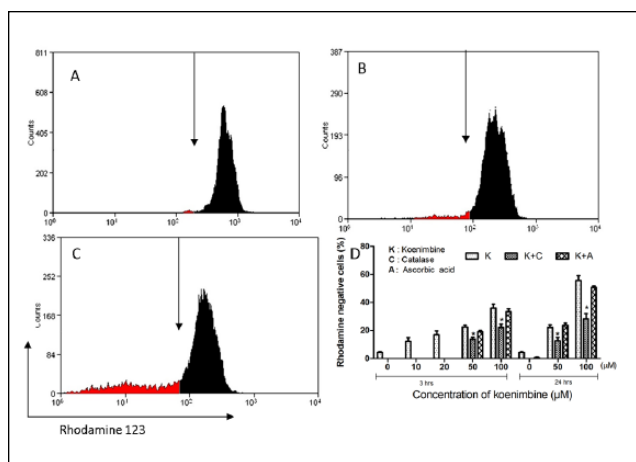

Figure 3. Effects of koenimbine on HepG2 cells mitochondrial membrane potential (MMP) reduction. (A-C) Flow cytometry histograms for 0, 10, and 100 µM treatment, respectively. Values are mean ± SD from 3 independent experiments (D). Triplicates of each treatment group were used in each independent experiment. The statistical significance is expressed as *P < .05.

Figure 4. Induction of DNA damage in HepG2 cells by koenimbine was analyzed using Comet assay. (A-C) Representative figures of comet formed from 0, 10, and 100 µM treatments, respectively. Data obtained were analyzed separately for 3 hours (D) and 24 hours (E). Values are mean ± SD from 3 independent experiments. Triplicates of each treatment group were used in each independent experiment. The statistical significance is expressed as *P < .05.

Koenimbine Significantly Reduced the Mitochondrial Membrane Potential

As shown in Figure 3A-C, loss of MMP was time-dependent. For example, control cells with decreased mitochondrial membrane potential had levels of 4.2%, while koenimbine treatment decreased to the same to a maximum of 35.8% at 3 hours. In order to further evaluate the relationship between ROS generation and koenimbine-induced decline of MMP, HepG2 cells were preincubated with catalase or ascorbic acid for 30 minutes. This was then followed by incubation with either 50 or 100 µM koenimbine for 3 and 24 hours. As shown in Figure 3D, pretreatment with catalase prevented HepG2 cells from koenimbine-induced MMP decline. Ascorbic acid pretreatment did not provide protection agaist koenimbine-induced MMP collapse.
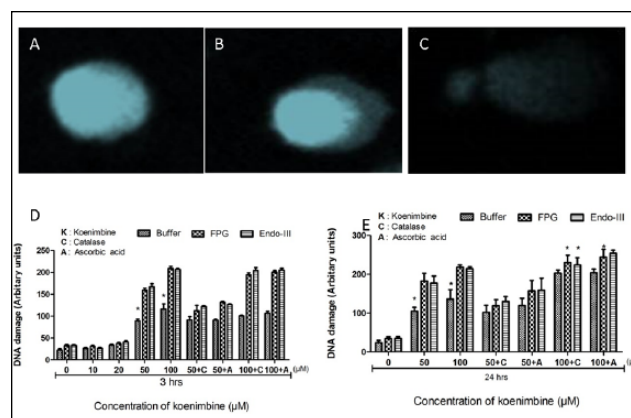
Koenimbine Induced Oxidative DNA Damage

Results of the visual scoring of DNA damage included FPG and Endo III sensitive sites induced by koenimbine as illustrated in Figure 4. DNA damage was significantly increased in a concentration-dependent manner at concentrations up to 100 µM. As compared with control, there was a significant (P < .05) 2-fold increase in the basic DNA damage on exposure to 100 µM koenimbine after 3 hours of incubation. Meanwhile, oxidative DNA damage, however, showed a significant (P < .05) 5-fold increase over the control on exposure to 100 µM koenimbine after 3 hours of incubation. Further demonstrating time dependency, the basic DNA damage showed significant (P < .05) 5-fold increase on treatment with 100 µM koenimbine at 24 hours of incubation (Figure 4B). Meanwhile, DNA damage was decreased (about 4- and 4.2-fold DNA damage with catalase and
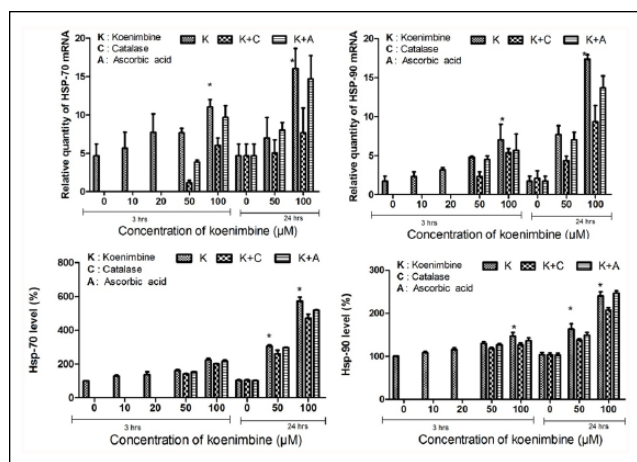
Figure 5. Hsp 70 and Hsp 90 mRNA and protein expression after 3 and 24 hours in HepG2 exposure to either vehicle or koenimbine. Values are mean ± SD from 3 independent experiments. Triplicates of each treatment group were used in each independent experiment. The statistical significance is expressed as *$P < .05$.

ascorbic acid, respectively, as compared with control) in HepG2 cells on pretreatment with antioxidants. Moreover, oxidative DNA damage (ie, difference between total DNA damage and DNA damage with antioxidant treatment) was markedly increased by koenimbine treatment.

DNA Dammage Was Significantly Associated With Both Hsp 70 and Hsp 90

Hsp 70 and Hsp 90 expression after HepG2 exposure to either vehicle consisting of media or different concentrations of koenimbine are shown as in Figure 5. The results clearly showed that koenimbine induces Hsp 70 and Hsp 90 expression in a dose-dependant manner up to a concentration of 100 μM and in a time-dependent manner over 24 hours of incubation both at the transcriptional and translational levels. Treatment with 100 μM koenimbine caused HepG2 cells Hsp 70 mRNA level to increase by 3-fold over control. While Hsp 90 mRNA level was not significantly greater at 3 hours of treatment, it rose up to 3-fold at 24 hours. The same results were observed on protein detection of both Hsp 70 and Hsp 90.

To correlate the relationship between ROS generation and koenimbine-induced Hsp induction, HepG2 cells were preincubated either with catalase or ascorbic acid. The result showed evidence that both antioxidants were capable in reducing Hsp 70 level, especially catalase. The HepG2 cells Hsp 70 level at 24-hour exposure to 100 μM koenimbine decreased substantially by 100% on catalase pretreatment whereas with ascorbic acid pretreatment, the decrease was 24%. Ascorbic acid pretreatment did not cause as much reduction in HepG2 cells Hsp 90 level, either. However,

catalase pretreatment decreased Hsp 90 level by 15% when treated with 100 μM koenimbine for 24 hours with concurrent decrease of 5% reduction in mRNA HSP-90 level.

Discussion

Free radicals, particularly ROS, have been proposed as common mediators for apoptosis.[24] Many agents that induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. Conversely, many inhibitors of apoptosis have antioxidant activities or enhanced cellular antioxidant defenses.[25] Previous studies have indicated that cells used reactive oxygen species as part of the signaling process responsible for activating an important mechanism for eliminating cancer cells, apoptosis.[26,27] It is now clear that several biological molecules, which are involved in cell signaling and gene regulation systems, are very sensitive to the redox status of the cell.

The generation of highly ROS has been shown to induce apoptosis in different cell types. Numerous studies have demonstrated that exogenous ROS or oxidants can induce apoptosis.[28,29] Antioxidants such as catalase and ascorbic acid treatment could eliminate the oxidative effect leading to apoptosis.[30,31] In the current investigation, the apoptotic effect of koenimbine on HepG2 cells was associated with an early elevation in intracellular ROS level. At 24 hours of treatment with a high concentration of koenimbine, ROS level was increased to reach almost 54%. A concentration of 10 μM koenimbine was capable of elevating the ROS level by 5% after 3 hours and 3-fold by 20 μM; indicating that the use of subcytotoxic concentrations at the early stages can induces ROS generation within the cells. Thus, on prolonged exposure at increasing concentrations of koenimbine, ROS generation had increased substantially, hence increasing oxidative stress. An increase in oxidative stress could exhaust the cellular antioxidant capacity and push the ROS stress level beyond a "threshold," which would cause cell apoptosis.[32] The generation of ROS was in agreement with the formation of GSH that was observed on treatment. GSH is the main nonprotein antioxidant in the cell, which is able to remove intracellular ROS.[33] In the cell, GSH is consumed by conjugation via glutathione *S*-transferases (GST) or by GSSG (glutathione disulfide) formation when GSH reacts with ROS.[34] In this current investigation, formation of intracellular ROS in HepG2 cells occurred after 3 hours of exposure to koenimbine. However, in HepG2 cells, GSH tended to decrease after 50 μM koenimbine exposure for 3 hours and more substantially after100 μM koenimbine exposure for 24 hours. One possible explanation for this observation is that, at higher concentrations of the koenimbine, more GSH was consumed, resulting insignificant reduction of intracellular GSH on HepG2 cells.

Some cytoproteins function to protect cells against oxidative stress. They are known as heat shock proteins (HSPs),

and play a vital role in cellular protection as well as repair. HSPs are classified according to their molecular weight.[35] In the eukaryotic cell, they may contribute to the resistant cancer cell phenotype by stabilizing the mutated and overexpressed ontogenesis that can overcome stresses. It has been observed that even at nonsignificant oxidative damage and toxicity levels in cells, the HSPs can be induced by oxidative or chemical stress. Thus, the induction of HSP proteins is considered as an early biomarker of oxidative stress.[36,37] The current study showed that koenimbine induced sharp elevation in the expression level of Hsp 70 and Hsp 90 at concentrations ranging from noncytotoxic to sublethal (subcytotoxic concentration) at both the translational and transcriptional levels. The increasing levels of main inducible HSP70 expression, may be due to the oxidative effect of koenimbine, which upregulated the expression of Hsp70 as a defence system. The current results are in good agreement with the data published elsewhere, which report the oxidative stress mediated upregulation of the Hsp70.[38] In addition, it has been recognized that Hsp also protects against oxidative stress-induced apoptosis by functioning at multiple sites in the apoptotic-signalling pathway.[39]

Since the mitochondrion is the most important organelle in cytostatic and cytotoxic studies[40] and mitochondria are the main source of ROS generation,[41] the current investigation attempted to characterize the relationship between ROS production and changes in MMP. The effect of koenimbine on MMP was studied by using the mitochondrial-specific probe Rh123. Rh123 is a cationic dye, which is selectively retained in mitochondria to an extent that is directly proportional to the MMP.[41] The flow cytometric assessment of mitochondrial depolarization showed that this parameter was also affected by treatment with koenimbine which caused a decrease in MMP. Theoretically, as a consequence of ROS generation in cells, mitochondrial dysfunction should occur.[42] The current investigation revealed significant decline in mitochondrial activity and dissipation of MMP, collectively providing evidence of mitochondrial dysfunction. The catastrophic loss of MMP may be due to the opening of permeability transition pore induced by oxidative pressure[43] and depletion of GSH level. In addition, concerning the time course of ROS burst and MMP depolarization, the ROS burst occurred before intracellular MMP depolarization. This assessment was supported by current results obtained from HepG2 co-incubated with koenimbine and the antioxidants catalase or ascorbic acid. Both catalase and ascorbic acid attenuated the MMP depolarization to cause primarily by koenimbine. These results demonstrated that the ROS burst was a prerequisite for MMP collapse and cell death induced by koenimbine.

In the current investigation, koenimbine-induced DNA damage in HepG2 cells with regards to oxidative stress was examined using alkaline single cell gel electrophoresis (SCGE, comet assay). Though the comet assay is one of the standard methods used for assessing DNA damage including single- and double-strand DNA breaks and alkali-labile sites, the introduction of lesion-specific endonucleases allows detection of oxidized bases and specific measurement of oxidative DNA damage.[44] In this way, Endo III is used to detect oxidized pyrimidines and FPG to detect the major purine oxidation product, 8-oxoguanine as well as other altered purines.[45] The additional strand breaks (breaks formed other than with buffer) induced by incorporation of FPG and Endo III to the standard comet assay is due exclusively to oxidative bases. The current results revealed that koenimbine-induced basic DNA damage in the HepG2 cells at concentrations of 10 to 100 μM caused less than 15% cell mortality, after 3 hours of incubation, suggesting that koenimbine induced only slight increase in basic DNA damage in HepG2 cells. This DNA damage, however, was more pronounced after 24 hours of incubation of the cells with koenimbine. This finding clearly indicates that koenimbine is able to induce DNA strand breaks in human liver cancer cells. The current results further revealed that koenimbine-induced DNA strand breaks increased significantly when both repair endonucleases FPG and Endo III were incorporated in the assay. The specific oxidative DNA damage markedly increased in a dose-dependent manner and was more intense than basic DNA damage after 3 hours of incubation. Collectively, the results confirmed koenimbine's ability to induce oxidative DNA damage in the human cancer cell line HepG2.

The results obtained in the current study revealed that there was an increase in ROS which was followed by depletion of GSH, Hsp induction, DNA damage, and MMP collapse in HepG2 cells treated with koenimbine and antioxidant in combination. On the contrary, the antioxidant catalase could potentially inhibit koenimbine's oxidative stress related effects. The antioxidant capacity of ascorbic acid, a nonenzymatic system, is not as prominent as catalase, probably because ascorbic acid may form H_2O_2 by reacting with superoxides.[46] Depletion of intracellular GSH levels may not induce apoptosis but instead may probably render cells more susceptible to apoptosis induced by subsequent stimuli. This would suggest that the redox alteration is a component of an apoptotic signaling, which is necessary for the cell death program, but it is not sufficient to drive apoptosis. It is well-known that mitochondria play a central role in energy metabolism within the cell by the mitochondrial respiration chain located on the inner-mitochondrial membrane. Therefore, MMP, ROS, and apoptosis are all interrelated.[47] Collectively, the study conducted had shown that koenimbine caused the MMP collapse in HepG2 cells and this effect could in turn be attenuated by catalase and ascorbic acid. Thus, it can be concluded that the collapse of MMP and subsequent cell death was triggered by ROS generation, which resulted in the depletion of intracellular GSH in HepG2 cells after treatment with koenimbine.

These results suggest that the presence of koenimbine in *M koenigii* leaves may be responsible for induction of DNA dammage in cancer cells, which can be studied further in depth to exploit its role as a promising adjuvant in cancer therapy.

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