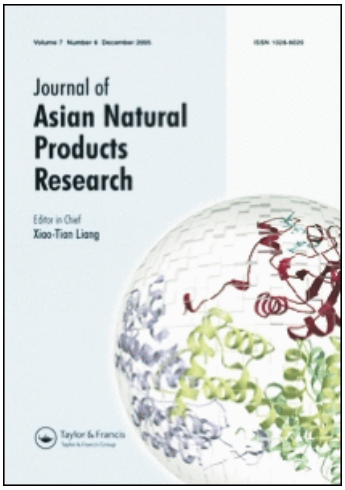

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DNA damage induced by shikonin in the presence of Cu(II) ions: potential mechanism of its activity to apoptotic cell death

Hong-Ming Cheng[a]; Ying-Kun Qiu[b]; Zhen Wu[ab]; Yu-Fen Zhao[ab]
[a] Department of Chemistry and Key Laboratory for Chemical Biology of Fujian Province, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen, China [b] Department of Pharmaceutical Science, Medical College of Xiamen University, Xiamen, China

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Hong-Ming Cheng[a], Ying-Kun Qiu[b], Zhen Wu[ab]* and Yu-Fen Zhao[ab]

[a]*Department of Chemistry and Key Laboratory for Chemical Biology of Fujian Province, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China;* [b]*Department of Pharmaceutical Science, Medical College of Xiamen University, Xiamen 361005, China*

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Shikonin (β-alkannin), a naphthazarin derivative, has shown a variety of abilities such as anti-inflammatory, antitumoral, cytotoxic, and antimicrobial activities. In the presence of Cu(II), shikonin caused breakage of supercoiled plasmid pBR322 DNA. Other metal ions tested [Mg(II), Ca(II), and Ni(II)] were ineffective and only Fe(II) has the same ability in the DNA breakage reaction. The involvement of active oxygen in the reaction was established by the inhibition of DNA breakage by superoxide dismutase, catalase, thiourea, sodium azide, potassium iodide, and sodium benzoate. Cu(I) was shown to be an essential intermediate using the Cu(I)-specific sequestering reagent neocuproine. Shikonin induced HeLa cell apoptosis involved in the mechanism of increasing intracellular reactive oxygen species (ROS). It was suggested that shikonin generated ROS as a pro-oxidant in the presence of Cu(II), and ROS resulted in DNA damage and apoptotic cell death in cells.

Keywords: shikonin; apoptosis; DNA cleavage; Cu(II) reduction; reactive oxygen species

1. Introduction

The roots of *Arnebia euchrona* (Royle) Johnst as a medical herb have been officially documented in Chinese and Japanese pharmacopeia [1]. Pharmacological studies revealed that the active components are a series of naphthoquinone compounds, of which shikonin (**1**) (Figure 1) presents multiple activities *in vivo* and *in vitro,* such as anti-inflammatory [2], antitumoral [2], cytotoxic [3], and antimicrobial activities [3,4]. Shikonin ointment also has the ability to accelerate wound healing [5] via an anti-inflammatory effect involving reactive oxygen species (ROS) [6].

Quinones are widely distributed in nature, and many clinically important chemotherapeutic drugs have a quinone moiety in their structures. For example, menadione (2-methyl-1,4-naphthoquinone) is cytotoxic, and mutagenic, which in turn causes DNA damage *in vitro* mediated via formation of ROS [7,8].

Several of the biological antioxidants have been shown to be capable of generating ROS in the presence of transition metals such as copper ions [9,10]. According to the structure of shikonin, it is suggested that shikonin exerts strong antioxidative activities against various types of ROS, and our experiment proved that it also had this pro-oxidant property.

As already mentioned, copper ions occur naturally in chromatin and can be

*Corresponding author. Email: wuzhen@xmu.edu.cn

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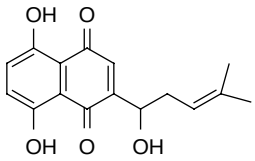

Figure 1. Structure of shikonin (**1**).

mobilized by metal-chelating agents [11]. The concentration of copper in various tissues ranges from 13 to 56 μM [12]. Furthermore, serum, tissue, and cellular concentrations of copper are known to be considerably elevated in various malignancies [13,14].

It was reported that the ROS got involved in the apoptosis of human cells induced by shikonin [15,16]. The results in our laboratory demonstrated that activated p53 mediated cell-cycle arrest and apoptosis in shikonin-induced DNA damage in A375-S2 cells, and phosphorylated ERK contributed to p53 expression in shikonin-induced apoptosis in HeLa cells [17–19].

The present study demonstrated that shikonin induced DNA breakage that was mediated by free radical in the presence of Cu(II) and induced HeLa cell apoptosis by increasing ROS. DNA damage caused by ROS generated from shikonin/Cu(II) system was involved in the potential mechanism of apoptotic cell death induced by shikonin in HeLa cells.

2. Results and discussion

2.1 Strand breaks of plasmid DNA

Double-stranded supercoiled structure of plasmid pBR322 DNA with a relatively high electrophoretic mobility is disrupted upon the formation of strand breaks, resulting in an open-circular conformation with a reduced electrophoretic mobility in agarose. Linear DNA, formed either by double-strand breaks or closely opposed single-strand breaks, has a mobility intermediate between that of the supercoiled and open-circular conformations of plasmid DNA. The effect of **1** on the DNA conformation was investigated. Treatment of plasmid with **1** and copper produced a major band of open-circular DNA, and the increase in the concentration of **1** raised the ratio of open-circular form of DNA (Figure 2). Figure 3 showed an electrophoretic profile, where several metal ions were tested to complement **1** in the DNA degradation reaction [20]. Only Fe(II) showed the conversion of supercoiled DNA to relaxed circular form.

In order to analyze the role of ROS in the 1/copper-dependent DNA breakage, several specific scavengers of activated oxygen were applied and agarose gel electrophoresis was performed. Superoxide dismutase (SD) and catalase (CA) remove superoxide and H_2O_2, respectively; potassium iodide (PI), thiourea (TU), and sodium benzoate (SB) are scavengers of ·OH and sodium azide

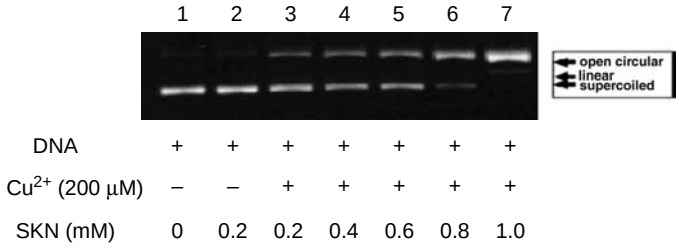

Figure 2. Cleavage of supercoiled plasmid DNA by **1** and Cu(II). One microgram of pBR322 plasmid DNA, 200 μM Cu^{2+}, and **1** in Tris–HCl (10 mM, pH 7.4) were incubated for 2 h at 37°C. Agarose gel electrophoresis was performed (lane 1, DNA control; lane 2, DNA + 0.2 mM SKN; lanes 3–7, DNA + Cu^{2+} + SKN). Example shown was repeated in $n = 3$ individual experiments.

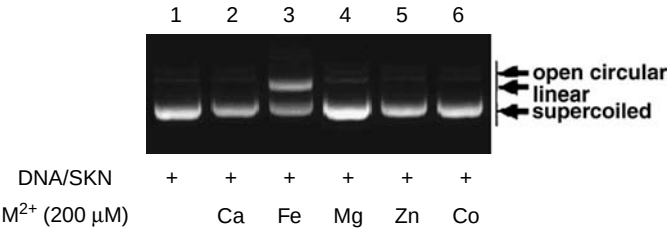

Figure 3. The effects of different metal ions on the shikonin-mediated degradation of DNA. One microgram of pBR322 plasmid DNA, 0.6 mM **1**, and 0.2 mM M^{2+} in Tris–HCl (10 mM, pH 7.4) were incubated for 2 h at 37°C. Agarose gel electrophoresis was performed (lane 1, SKN + DNA; lane 2, SKN + DNA + Ca^{2+}; lane 3, SKN + DNA + Fe^{2+}; lane 4, SKN + DNA + Mg^{2+}; lane 5, SKN + DNA + Zn^{2+}; lane 6, SKN + DNA + Co^{2+}). Example shown was repeated in $n = 3$ individual experiments.

(SA) is scavenger of $^1O_2$ [20]. As shown in Figures 4 and 5, SD, CA, TU, SA, PI, and SB effectively protected plasmid DNA from the 1/copper-dependent fragmentation. These results indicated that **1** induced DNA breakage involved in the mechanism of generation of free radical.

2.2 Production of active oxygen species by shikonin

In order to confirm shikonin-induced DNA breakage involved in the mechanism of generation of free radical, electron spin resonance spectroscopy (ESR) assay and the assessment assay of hydroxyl radicals and superoxide anion were applied. Firstly, after the reaction mixture containing nitroblue tetrazolium (NBT) and Cu(II) was treated with 0, 5, 10, 20, 40, and 80 μM of **1** for 30 min, respectively, the proportion of superoxide anion from solutions of **1** by incorporating NBT in the reaction medium increased in a dose-dependent manner, and 100 μg/ml of SD inhibited markedly the reaction (Figure 6). Secondly, hydroxyl radicals generated by **1** and Cu(II) were found to be dependent on the concentration of **1**, and 50 mM of TU significantly inhibited **1**/Cu(II)-generated free radicals (Figure 7). Finally, as shown in Figure 8, after Cu(II) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were treated with 40 μM of **1** for 30 min,

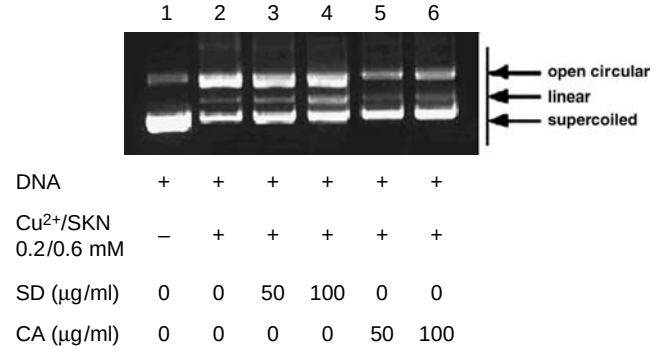

Figure 4. The effects of SD and CA on the shikonin-mediated degradation of DNA. One microgram of pBR322 plasmid DNA, 0.6 mM **1**, and 0.2 mM Cu^{2+} in Tris–HCl (10 mM, pH 7.4) were incubated with or without SD or CA for 1 h at 37°C. Agarose gel electrophoresis was performed (lane 1, DNA control; lane 2, DNA + Cu^{2+} + SKN; lanes 3–4, DNA + Cu^{2+} + SKN + SD; lanes 5–6, DNA + Cu^{2+} + SKN + CA). Example shown was repeated in $n = 3$ individual experiments.

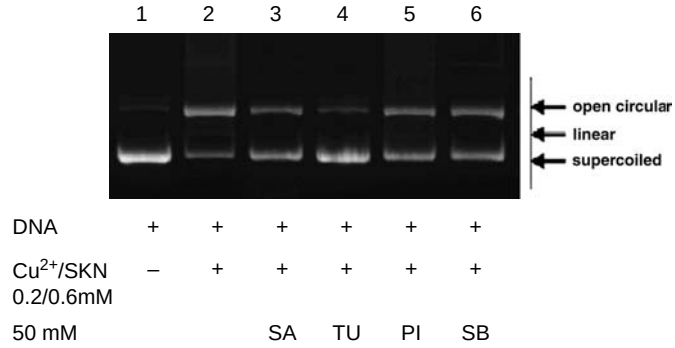

Figure 5. Specific scavengers of activated oxygen reversed shikonin-mediated degradation of DNA. One microgram of pBR322 plasmid DNA, 0.6 mM **1**, and 0.2 mM Cu^{2+} in Tris–HCl (10 mM, pH 7.4) were incubated with or without 50 mM additives for 1 h at 37°C. Agarose gel electrophoresis was performed [lane 1, DNA control; lane 2, DNA + Cu^{2+} + SKN; lane 3, DNA + Cu^{2+} + SKN + SA; lane 4, DNA + Cu^{2+} + SKN + TU; lane 5, DNA + Cu^{2+} + SKN + PI; lane 6, DNA + Cu^{2+} + SKN + SB]. Example shown was repeated in $n = 3$ individual experiments.

the four-line spectrum of typical DMPO spin adducts of hydroxyl radicals (DMPO-OH) and the 12-line signal of DMPO spin adducts of superoxide anion (DMPO-OOH) were observed [21]. These results demonstrated that hydroxyl radicals and superoxide anion generated from **1** and the Cu(II) system play a crucial role in **1**/Cu(II)-induced DNA damage.

2.3 Reduction of copper ion

Growing evidence indicates a central role for Cu(II) in **1**/Cu(II)-generated free radicals. To investigate the role of Cu(II) in **1**-induced DNA damage, the neocuproine-Cu(I) complex analysis was performed. After treatment with increasing concentrations of **1**, Cu(II) was reduced to Cu(I) in a dose-dependent manner, suggesting Cu(I) to be an essential intermediate in this cleavage reaction (Figure 9). The result indicated that **1** (0–250 μM) has a potent reducing activity in the reaction of **1** with Cu(II).

2.4 ROS-mediated cell apoptosis by shikonin in HeLa cells

It was reported that cell apoptosis induced by **1** was mediated by ROS/JNK in Bcr/Abl-positive chronic myelogenous leukemia cells [15]. To further confirm whether the production of ROS by **1** plays

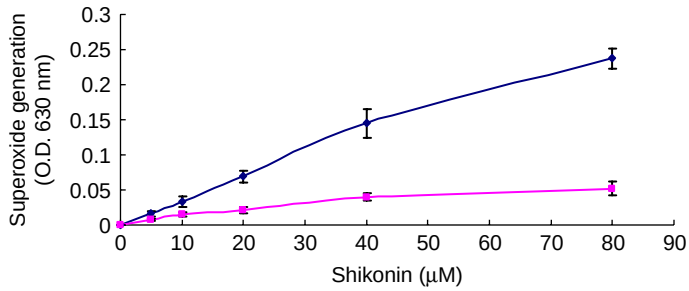

Figure 6. Effect of concentrations of **1** on generation of superoxide anion. Generation of superoxide anion by increasing concentration of **1** (5–80 μM) incubation in the presence (■) and absence of (□) SD (100 μg/ml). Each point represents the mean ± SD of three experiments.

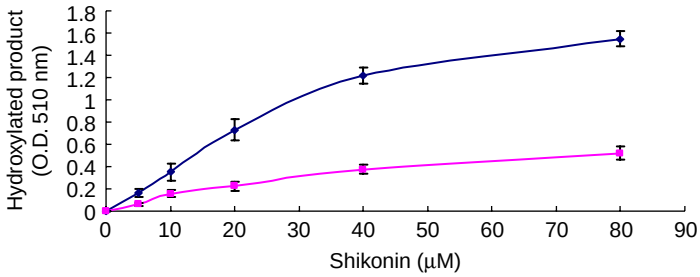

Figure 7. Effect of concentrations of **1** on generation of hydroxyl radicals. Generation of hydroxyl radicals by increasing concentrations of **1** (5–80 μM) incubation in the presence (■) and absence of (□) TU (50 mM). Each point represents the mean ± SD of three experiments.

a key role in cell apoptosis, the assessment of ROS generation and assay of apoptosis in **1**-induced HeLa cell death were performed. Intracellular production of ROS was monitored by fluorescence in HeLa cells treated with **1**. 2′,7′-Dichlorofluorescein diacetate (DCF-DA) can be oxidized intracellularly to 2′,7′-dichlorofluorescein (DCF) which is fluorescent, and generation of ROS was estimated by increasing the fluorescence of DCF-loaded cells. The data showed that **1** increased dependently the intracellular ROS in 1-induced HeLa cell death (Figure 10).

Effect of **1** on the viability of HeLa cells was examined by flow cytometry analysis. Treatment of HeLa cells with **1** of 10 μM induced a significant percentage of cells to undergo apoptosis (20.23 ± 1.23%) (Figure 11).

These results suggested that 1-mediated apoptosis of HeLa cells was closely related to the increase in the concentrations of ROS in cells. Shikonin can act as a pro-oxidant by activating molecular oxygen through the reduction of Cu(II) in cells, resulting in the DNA damage and induction of apoptotic cell

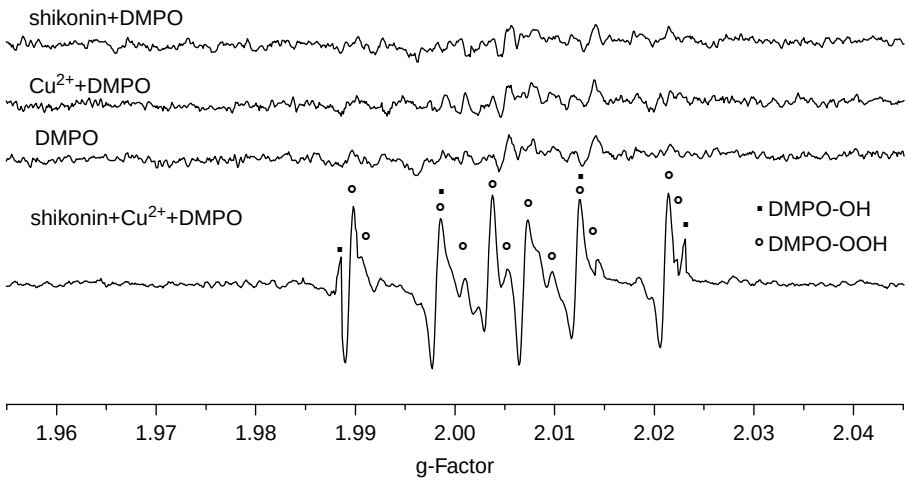

Figure 8. ESR spectra of DMPO spin adducts of oxygen-free radicals generated from **1** (40 μM) and copper (200 μM). Open circles and closed circles indicate DMPO spin adducts of superoxide anion (DMPO-OOH) and hydroxyl radicals (DMPO-OH), respectively. The average relative intensity of the strongest signal was 305783.3 ± 68992.8 (mean ± SD, $n = 3$), and the representative spectra was shown from triplicate measurements.

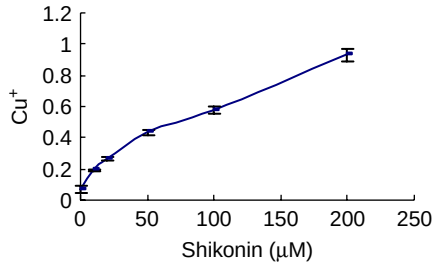

Figure 9. Reduction of copper ion by **1**. Copper reduction was followed by determining the cuprous ion concentration with neocuproine. Shikonin (6.25–200 μM) was mixed with Tris–HCl buffer (pH 7.5) containing neocuproine and $CuSO_4$, respectively, and the mixture was incubated and measured at room temperature. Each point represents the mean ± SD of three experiments.

death. DNA damage induced by **1** in the presence of Cu(II) ions may be the potential mechanism of apoptotic cell death.

3. Experimental

3.1 Materials

Shikonin was obtained from the Beijing Institute of Biological Products (98%, Beijing, China); calf thymus DNA, DCF-DA, neocuproine, SD (bovine erythrocyte), CA, NBT, DMPO, salicylate, and agarose were obtained from Sigma-Aldrich China (Shanghai, China); double-stranded supercoiled plasmid pBR322 was obtained from Takara Biomedicals (Shiga, Japan); Fluorescein isothiocyanate (FITC)-labeled Annexin V (Annexin V-FITC) solution was obtained from BD Biosciences (San Diego, CA, USA).

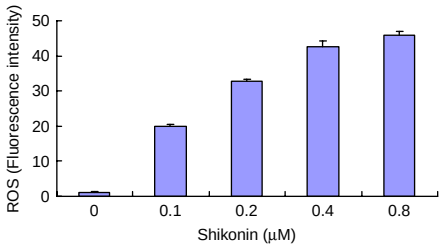

Figure 10. Shikonin increased the intracellular ROS in HeLa cells. After treatment with 300 mM DCF-DA for 1 h, HeLa cells were washed with PBS and treated with **1** (0.1–0.8 μM) for 2 h. The fluorescence of the cells was determined with a spectrofluorometer. Each column represents the mean ± SD of three experiments.

3.2 Reaction of shikonin with plasmid pBR322 DNA

The plasmid pBR322 was treated with **1** in the presence of $CuSO_4$. Ten microliters of the mixture contained 1 μg of pBR322, 0.2 mM $CuSO_4$, and various concentrations of **1** in 10 mM of Tris–HCl buffer (pH 7.4). The mixture was incubated at 37°C for 2 h, and was analyzed by 0.8% agarose gel electrophoresis stained with ethidium bromide.

3.3 Assay of active oxygen species

Superoxide anion was detected by the reduction of NBT to a formazan. The reaction mixture containing 10 mM of Tris–HCl buffer (pH 7.4), 0.1% NBT, 200 μM Cu^{2+}, **1**, 100 μg/ml SD, and other components was incubated for 30 min at room temperature [22]. Hydroxyl radicals were assayed by incubating a solution containing 10 mM of Tris–HCl buffer (pH 7.4), 2 mM of salicylate, 0.1 mM of EDTA, 50 mM of TU, 200 μM of Cu^{2+}, **1**, and other components in a total volume of 2 ml for 1 h at room temperature. Salicylate was converted to hydroxylated products, which were extracted and determined colourimetrically [23].

3.4 ESR spectroscopy and spin trapping

ESR spectroscopy assay was performed at room temperature using an ER 320 spectrometer (Bruker, Karlsruhe, Germany). Spectra were obtained using 9.8 GHz of microwave frequency, 1.96 mW of microwave power, 100 kHz

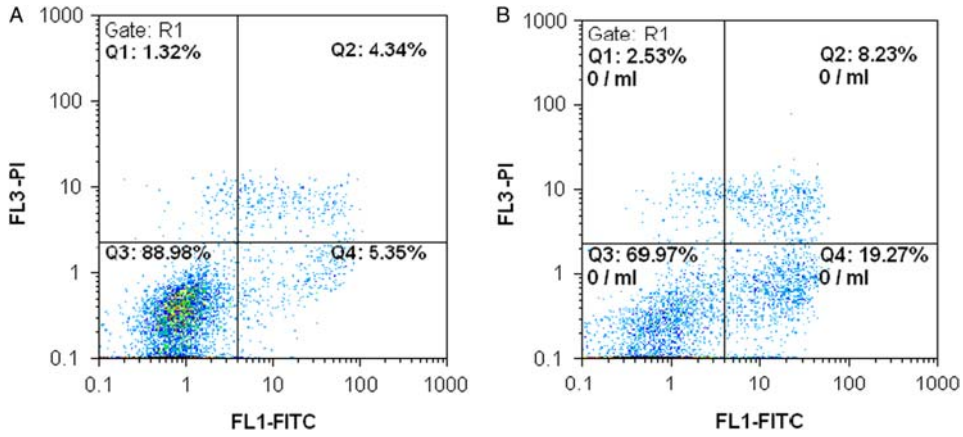

Figure 11. Shikonin induced apoptotic cell death in HeLa cells. HeLa cells were treated with or without 10 μM **1** for 4 h, and after stained with Annexin V-FITC and PI, DNA content was analyzed by fluorescence flow cytometry. (A) Control and (B) treated with 10 μM **1**. Example shown was repeated in $n = 3$ individual experiments.

of modulation frequency, 1 G of modulation amplitude, a field set of 3515 G with a scan range of 100 G, a 5.43 s sweep time, and a 1.28 ms constant time.

Sample solutions for analysis containing 10 mM of Tris–HCl buffer, 40 μM of **1**, 200 μM of Cu^{2+}, and 200 mM of DMPO in a total volume of 1 ml were incubated for 30 min at room temperature.

3.5 Rate of Cu(II) reduction by shikonin

Shikonin in 10 mM of Tris–HCl buffer (pH 7.4) was mixed with $CuSO_4$ and a final neocuproine concentration of 0.4 mM in a total volume of 1.0 ml. The neocuproine-Cu(I) complex was determined by measuring the absorbance at 450 nm immediately after mixing the reagents.

3.6 Cell culture and assessment of ROS generation

Human cervical cancer cells (HeLa #CCL-2) was obtained from the American Type Culture Collection (ATCC Manassas, VA, USA). The cells were cultured in RPMI 1640 (Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum, 2 mM of L-glutamine, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. For the measurement of intracellular ROS, 2′,7′-DCF-DA of 300 mM was administered to the HeLa cells in the medium [24]. The DCF-loaded cells were cultured at 37°C for 1 h and washed twice with phosphate-buffered saline (PBS). HeLa cells were further treated with 0, 0.1, 0.2, 0.4, and 0.8 μM of **1**, respectively, for 2 h. Fluorescence of the cells was determined with the fluorescence plate reader set with the excitation wavelength at 475 nm and the emission wavelength at 525 nm (Spectra Max M2 microplate reader, Molecular Devices, Sunnyvale, CA, USA).

3.7 Annexin V-FITC staining for evaluation of apoptosis

The HeLa cells (5×10^5 cells in RPMI-1640 cell culture medium with 10% FBS) exposed to **1** for different periods of time were centrifuged at 1000*g* for 5 min and the pellets were washed twice in cold PBS and cold binding buffer (10 ml of Hepes/NaOH pH 7.4, 140 mM of NaCl, and 2.5 mM of $CaCl_2$). The pellets were suspended in 100 μl of binding buffer

and stained with 2 μl of Annexin V-FITC solution and 10 μl PI solution for 15 min at room temperature in the dark. Then, the samples were diluted with 400 μl of binding buffer and analyzed by flow cytometry within 30 min. A minimum of 50,000 cells were maintained for all the samples. The samples were analyzed as per the protocol provided by the manufacturer (BD Biosciences).

4. Conclusions

In the present study, **1** induced ROS-mediated DNA breakage and cell apoptosis in HeLa cells and caused copper-dependent plasmid DNA cleavage. Cu(II) plays an important role in **1**/Cu(II)-induced DNA damage. These biochemical events were possibly associated with free radical. In the presence of Cu(II), **1** caused cleavage in DNA and such degradation was mediated by ROS. Cu(II) was reduced by **1**, and Cu(I) appeared to be an essential intermediate in this reaction. ROS induced by **1** may result in DNA damage and apoptotic cell death in HeLa cells.

Acknowledgements

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