Induction of apoptosis by Xiakemycin A in human hepatoma HepG2 cells

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Abstract
Xiakemycin A (XKA), a new antibiotic in the pyranonaphthoquinone family, shows antitumor activity. However, the type of cell death induced by XKA remains elusive. In this study, we aim to investigate the type of death induced by XKA in hepatic cancer.

The apoptotic features, such as chromatic agglutination, reactive oxygen species generation and membrane potential of mitochondria, in HepG2 cells treated by XKA were measured by Hoescht 33342 staining and flow cytometry. Apoptosis of HepG2 cells treated with XKA was determined by Annexin V-FITC/propidium iodide double staining and Western blot analysis, respectively.

XKA had a significant dose-dependent elevation of chromatic agglutination, reactive oxygen species generation, Annexin V and propidium iodide staining, decrease of membrane potential. Meanwhile, in apoptotic HepG2 cells induced by XKA, robust increment was noticed in p33 expression, cleavage of PARP, caspase-3, and caspase-9.

XKA showed potent inhibitory effects on the proliferation of HepG2 cells. Such phenomenon may be related to activation of the apoptotic pathway.

Abbreviations: PI = propidium iodide, PNQ = pyranonaphthoquinone, PS = phosphatidylserine, ROS = reactive oxygen species, XKA = Xiakemycin A.

Keywords: apoptosis, cancer, HepG2 cells, Xiakemycin A

1. Introduction

Pyranonaphthoquinone (PNQ) antibiotics have the basic skeleton of naphtho[2,3- c]pyran-5, 10-dione ring system and are reported to be closely associated with a series of biological activities, including inhibitory activities against tumor, fungi, bacteria, insect, virus, and mycoplasma.[1] The inhibitory effects of PNQ members on cancer have been mainly focused on inhibition of DNA topoisomerase, AKT, and protein kinase A.[2] However, the mechanisms underlying the signaling pathway and tumor cell death induced by PNQ antibiotics remain unclear.

Xiakemycin A (XKA), a new member of the PNQ family, was firstly reported by our team as a type of antitumor antibiotic.[3] Our previous study indicated that XKA demonstrated weak inhibition on the AKT kinase activity in vitro.[4] Besides, the number of propidium iodide (PI)-stained cells showed increase in a dose dependent manner after XKA treatment. Furthermore, degradation of apoptosis-related proteins (eg, p53 and PARP-1) was noticed in XKA-treated PC-3 cells. However, the type of death induced by XKA in tumor cells remain unclear. In this study, we determined the type of death induced by XKA in hepatic cancer. The results showed that activation of apoptotic caspase pathway contributed to its action towards HepG2 cells.

2. Materials and methods

2.1. Chemicals and reagents

XKA extracted from the fermentation broth of Streptomyces sp. was purified according to the previous description.[3] HPLC indicated the purity was more than 95% as previously described. XKA was diluted into a final concentration of 5 mM using methanol. The solution was maintained at a temperature of –20°C before usage. Dimethyl sulfoxide and PI were provided by Sigma-Aldrich (CA). H2DCFDA was purchased from Invitrogen (MI). This study was approved by the Ethical Committee of the Jiujiang University.

2.2. Cell culture

HepG2 cells were cultured in RPMI-1640 medium. All of the medium were supplemented with 10% fetal bovine serum.
(Thermo, CA), and 2 mM glutamine. HepG2 cells were cultured at 37°C in humidified atmosphere with 5% CO₂.

2.3. Hoechst 33342 staining

Cells were treated using 0.2% methanol (v/v, served as the vehicle group), 1 μM, 2 μM, and 5 μM XKA for 48 hours, and only 5 μM XKA, followed by staining with Hoechst 33342 for 10 minutes. Cells received no XKA exposure were set as control. The images were observed under a fluorescent microscope (IX83, Olympus, Tokyo, Japan). Hoechst 33342 staining was performed according to the previous description.[5] Briefly, cells (1 × 10⁵) were placed on 12 wells at 37°C in 5% CO₂. After culture for 24 hours, the HepG2 cells were treated by XKA for 48 hours, and washed using PBS twice, followed by Hoechst 33342 staining for 10 minutes. Finally, the cells were washed using PBS and observed under a microscope.

2.4. Determination of reactive oxygen species (ROS) generation

About 1 × 10⁵ cells were counted for each group treated using 0.2 μM, 0.5 μM, and 1 μM XKA. Cells with no treatment served as control. Then the cells were stained with 5 mM H2DFFDA, at 37°C for at least 1 hour. Flow cytometry was given after cell harvest under excitation and emission settings of 488 nm and 530 nm, respectively.

2.5. Evaluation of membrane potential

To determine the changes of membrane potential in mitochondria, Rhodamine 123 staining was conducted in the HepG2 cells in different groups including no XKA treatment, 0.2 μM, 0.5 μM, and 1 μM XKA after treating for 24 hours. Determination of membrane potential was determined as previously described.[6]

2.6. Annexin V-FITC/PI double staining

To quantify apoptosis, cells treated by 0.5 μM, 1 μM, 2 μM, and 5 μM XKA and 0.2% methanol (v/v) for 48 hours were subject to Annexin V-FITC/PI Apoptosis kit (BD Biosciences, CA), according to the manufacturer’s instructions. The fluorescence intensity was measured using a BD FACSCalibur flow cytometer. Cells treated with nothing served as control.

2.7. Western blot analysis

Lysis buffer containing protease inhibitors was utilized to treat HepG2 cells. Proteins were subject to electrophoresis using SDS-PAGE in groups treated with 0.5 μM, 1 μM, 2 μM, and 5 μM XKA, as well as the cells treated with no XKA and methanol. Then they were transferred onto a polyvinylidene fluoride membrane, followed by blocking with 5% nonfat milk. Then the membrane was incubated with primary antibodies for PRAP-1, p53, Caspase-3, Caspase-9, cleaved Caspase-3, and Caspase-9 for 2 hours at room temperature and was incubated with an appropriate peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using the ECL Plus Western Blotting Detection System (Piscataway, NJ). The level of β-actin for each sample was used as a loading control. The antibodies against Caspase-3, Caspase-9, cleaved caspase-3, and caspase-9 were purchased from Cell Signaling (MA). The antibodies against PRAP-1, p53, and β-actin were from Santa Cruz (CA).

3. Results

3.1. Induction of DNA damage by XKA in HepG2 cells

The typical features for apoptosis included chromatic agglutination and formation of apoptotic body. As a blue fluorochrome, Hoechst 33342 could penetrate the cellular membrane and bind with the minor groove of double stranded DNA, especially the DNA rich in A/T.

In the control group and methanol group, no significant changes were noticed in the nucleus. The staining in the nucleus was even (Figs. 1 and 2). No fluorescence was conducted in the XKA group. In contrast, chromatic agglutination and apoptotic body was noticed in the groups treated by XKA, which was in a dose-dependent manner.

3.2. Accumulation of ROS in hepG2 cells treated by XKA

ROS, a product of oxygen metabolism, played crucial roles in the signaling transmission and redox equilibrium. In cases of external stimuli, the ROS may increase, which then triggered injury of cellular structure and final apoptosis. H2DCFDA showed no fluorescence at first. It was hydrolyzed by esterase, and then was oxidated by ROS that presented fluorescence. Compared with the blank control, the elevation of fluorescence density was about 167%, 882%, and 167% in the cells treated with XKA with a concentration of 0.2 μM, 0.5 μM, and 1 μM, respectively. Cells treated with 1 μM XKA showed decline of ROS, which was caused by cellular membrane injury.

This implied that XKA induced significant elevation in ROS of HepG2 cells (Fig. 3). Therefore, it was reasonable to assume that XKA triggered the formation of ROS in HepG2 cells during the apoptosis, while ROS may involve in the XKA-induced apoptosis.

3.3. Disruption of mitochondrial membrane potential in XKA-treated apoptosis

At the early stage of cellular apoptosis, the most typical changes in mitochondria were permeability of mitochondrial membrane,
which resulted in decrease of transmembrane potential. Rhodamine 123 could aggregate in the mitochondria after penetrating the cell membrane, and the fluorescence intensity was positively related to the membrane potential. A decrease of 27%, 46%, and 63% was noticed in the membrane potential after treating with 0.2 μM, 0.5 μM, and 1 μM XKA, respectively. The decrease of mitochondrial membrane potential was in a dose-dependent manner. This indicated that...
Figure 4. XKA triggered decrease of mitochondrial membrane potential in HepG2 cells. (A) Blank control; (B) cells treated with 0.2 mM XKA; (C) cells treated with 0.5 mM XKA; (D) cells treated with 1 mM XKA. XKA = Xiakemycin A.

Figure 5. Apoptosis of HepG2 cells after XKA treatment as revealed by flow cytometry. Cells treated using XKA for 48 h were subject to flow cytometry. Cells were stained with Annexin V-FITC/PI for 10 min. (a) Methanol group; (b) blank control; (c) cells treated with 0.5 mM XKA; (d) cells treated with 1 mM XKA; (e) cells treated with 2 mM XKA; (f) cells treated with 5 mM XKA. PI = propidium iodide, XKA = Xiakemycin A.
XKA could induce decrease of membrane potential when triggering the HepG2 apoptosis (Fig. 4).

3.4. XKA induced apoptosis of hepatic cancer cells

To investigate the roles of XKA in inducing apoptosis of hepatocarcinoma cells, double staining was performed using Annexin V-FITC/PI. Annexin V was a Ca$^{2+}$-dependent annexin, which could specifically bind with phosphatidylserine (PS) in a high affinity. Normally, PS was mainly distributed in the internal cell membrane. At the early stage of apoptosis, the PS on the cell membrane would migrate on the cellular surface and then bind with Annexin V. As a dye for nucleic acid, PI could not penetrate the cell membrane, but it could bind with the nucleus and present in a red color after penetrating the cells during the middle and late stages of apoptosis.

Compared with the blank control, significant increase was noticed in the apoptosis of cells treated using XKA, which was shown in a dose-dependent manner especially treated by a concentration of >2 μM. Therefore, we concluded that XKA could induce HepG2 cell apoptosis in a dose-dependent manner (Fig. 5).

3.5. XKA modulated the apoptosis-related protein expression in HepG2 cells

To further confirm the roles of XKA in the apoptosis of HepG2 cells, Western blotting analysis was carried out after treating with XKA for 48 hours. Partial cleavage of PARP-1 was observed in cells treated using 0.5 μM, 1 μM, and 2 μM, respectively. Complete cleavage was noticed after treating with 5 μM XKA. The cleavage was in a dose-dependent manner. With the elevation of XKA, the expression of P53 protein showed increase in a dose-dependent manner. Meanwhile, Caspase-3 and Caspase-9 protein were gradually degraded after treating with XKA. The concentration of cleaved Caspase-3 and Caspase-9 showed significant increase with the increase of XKA concentration ($P < .05$, Fig. 6). These demonstrated that XKA contributed to the increase of ROS and decrease of membrane potential, which finally triggered the apoptosis of HepG2 cells.

4. Discussion

Apoptosis played a crucial role in cancer therapy.[7] XKA initially extracted by our team was reported to show antitumor effects. In this study, we investigated the potential roles of XKA in the apoptosis of HepG2 cells, and the possible mechanisms for it by determining the chromatic agglutination, ROS generation, alternations of mitochondrial membrane potential, and apoptosis-related protein expression.

XKA, as a member of PNQ, showed anticancer effects and may inhibit the growth of cancer cells through the following pathways:

1. decreased the topoisomerase II activity and interfered the DNA replication/transcription;
2. inhibited the maturation of 45sRNA precursor of ribosome and induced cell arrest into G2 phase; or triggered DNA breakdown.

In a previous study, *Scutellaria barbata* extract was reported to induce typical morphological changes of apoptotic H22 cells including chromatic agglutination and fragmentation of nuclei, mitochondria swelling, as well as formation of apoptotic body.[8]

In this study, XKA induced chromatic agglutination in HepG2 cells in addition to formation of apoptotic body, which were the typical features of cellular apoptosis. On this basis, XKA may trigger apoptosis in HepG2 cells, but additional studies are required to illustrate the exact mechanism for it.

ROS has been widely generated in biological systems. Intracellular production of ROS is closely related to the arrest of cellular proliferation.[9] Similarly, ROS production in response to external stimuli is implicated in the activation of transcription factors and triggering of apoptosis.[10] In addition, some antitumor agents were reported to increase ROS production and DNA damage, as well as inhibiting tumor promotion.[11] Abnormal mitochondrial function was reported to trigger apoptosis in vivo and in vitro.[12] Meanwhile, it has been considered to play crucial role in the apoptotic pathway. It has been well acknowledged that the opening of the mitochondrial permeability transition pore could result in depolarization of the transmembrane potential.[13] In this study, XKA resulted in ROS accumulation and decrease of mitochondrial membrane potential, which were the typical features of apoptosis. We concluded that XKA induced apoptosis in vitro. For the mechanism, it may be related to the generation of ROS and decrease of membrane potential on mitochondria.

P53 protein has been commonly regarded as a marker for cancer.[14,15] Our previous study indicated that XKA-induced p53 activation may contribute to the cleavage of AKT mediated by caspase and the self-degradation of AKT, which finally resulted in the cell death.[4,16] Previously, AKT activation was closely related to cell apoptosis as it contributed to the phosphorylation of Ser at 166 and 188 on the Mdm2, which subsequently induced degradation of proteasomes through p53.
ubiquitination.\textsuperscript{17} In this study, degradation was observed in the apoptosis related protein including PARP-1, p53, caspase-9, and caspase-3 after XKA interference. Meanwhile, cleavage was noticed in PARP-1, caspase-9, and caspase-3 after treating with XKA. In future, further studies are needed to investigate the potential mechanism.

Indeed, there are some limitations for this study. In this study, we only focused on the roles of XKA in inducing apoptosis, and the signaling pathways were still not well defined. Unlike our previous study showing that XKA could not induce necrotic apoptosis, this study indicated that XKA induced apoptosis. The potential reasons are still illusive. Moreover, during the determination of nucleus staining, ROS, mitochondrial membrane potential and apoptosis related protein, the concentration of XKA was not completely the same, but an obvious trend was noticed.

In a word, XKA showed antitumor effects in HepG2 cells in vitro, and it could induce the apoptosis of HepG2 cells. Such process may be related to the modulation of apoptosis-related proteins. In future, the mechanism and in vivo study are required to investigate the potential efficiency of XKA against cancer.

**Author contributions**

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