8-Methoxypsoralen Induces Intrinsic Apoptosis in HepG2 Cells: Involvement of Reactive Oxygen Species Generation and ERK1/2 Pathway Inhibition

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Key Words
8-Methoxypsoralen • Reactive oxygen species • ERK • Intrinsic apoptosis pathway

Abstract
Background/Aims: 8-Methoxypsoralen (8-MOP), a formerly considered photosensitizing agent, induces apoptosis when used alone. On this basis, the present study was designed to explore the effects and mechanisms of 8-MOP-induced apoptosis in human hepatocellular carcinoma HepG2 cells, independent of its photoactivation. Methods: We analyzed the cell viability with MTT assay. Flow cytometry was used to examine the apoptosis rate, mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) generation after specific staining. The expression and location of apoptosis-associated protein as well as the activation status of cell signaling pathway were determined by Western blot analysis. Results: 8-MOP significantly decreased cell viability and induced cell apoptosis through mitochondrial apoptotic pathway, as demonstrated by increased Bax/Bcl-2 ratio, collapsed MMP, and induced cytochrome c release (Cyt c) and apoptosis-inducing factor (AIF) transposition. ROS generation was significantly increased by 8-MOP and the eradication of ROS significantly abolished 8-MOP-induced apoptosis. In addition, the activation of ERK1/2 was drastically decreased by 8-MOP as ERK inhibitor PD98059, indicating a role of ERK1/2 signaling pathway in 8-MOP-induced cell apoptosis. Conclusion: 8-MOP induces intrinsic apoptosis by increasing ROS generation and inhibiting ERK1/2 pathway in HepG2 cells. The findings are important in substantiating the anti-tumor role of 8-MOP in cancer therapy.

H. Yang and J. Xiong contributed equally to this work.
Introduction

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer and also represents the third leading cause of cancer death worldwide [1]. Despite surgical management and employment of non-surgical therapeutic modalities, the incidence and recurrence of HCC is still on the rise [2]. High recurrence rate after resection renders most patients not eligible for surgery [3]. Moreover, conventional chemotherapeutic treatments are always accompanied by serious adverse reactions and frequent resistance. Therefore, to develop effective agents with minimal side effects and to understand their molecular mechanisms will contribute greatly for improving HCC treatment [4-6].

8-Methoxypsoralen (8-MOP) is a naturally occurring furanocoumarin drug, as well as a classic photochemotherapeutic agent [7]. The combination of 8-MOP and UVA irradiation, a therapy referred to as psoralen plus ultraviolet A (PUVA), represents a useful approach in the treatment of such hyperproliferative skin conditions as psoriasis, vitiligo and eczema [8-9]. The planar structure of 8-MOP is activated by UVA irradiation to form the photoadducts with pyrimidines in cellular DNA and further reactphotochemically with a pyrimidine base on the DNA thus leading to an interstrand cross-link. It is believed to be the primary cause of PUVA-induced cell killing[10]. Cell destruction resulted from PUVA treatment is associated with apoptosis, cell cycle arrest and cell viability inhibition [11-13]. Moreover, mitochondrial dysfunction caused by the opening of the permeability transition pore and the generation of reactive oxygen species (ROS) are reported to get involved in PUVA-induced oxidative DNA damage and apoptosis [14-15]. When used at dark, 8-MOP preferentially binds to A–T base pairs with no major perturbation in DNA double helix conformation [16]. In our previous study, 8-mop increases the ratio of Bax/Bcl-2 and decreased the viability in HepG2 cells when used alone [17]. Although many aspects of PUVA therapy have been extensively studied, the effects and mechanisms of 8-MOP, independent of the exposure to UV, on cell proliferation and cytotoxicity in HCC cells still require full elucidation.

The intrinsic apoptosis pathway, triggered by mitochondrial membrane depolarization, is one of the two established signaling pathways that result in cell apoptosis. Mitochondrialdependent apoptosis is mainly regulated by the Bcl-2 protein family. Bax is a central pro-apoptotic member of Bcl-2 family proteins, which regulates the balance between cell survival and death [18]. In response to apoptotic signals, Bax transforms into a lethal mitochondrial oligomer and becomes activated to cause mitochondrial damage, a key step for the intrinsic pathway to apoptosis [19-20]. With the involvement of Bcl-2 family, the loss of mitochondrial integrity leads to the translocation of various apoptotic proteins, including apoptosis inducing factor (AIF) and cytochrome c (Cyt c) that trigger either caspase-activated or caspase-independent death pathways [21].

In the present study, it is demonstrated that 8-MOP induces apoptosis in HCC HepG2 cells in a mitochondria-dependent way. Further investigations are designed to explore
the pro-apoptotic role and mechanisms of 8-MOP by focusing on the oncogenesis-related intracellular signaling transduction and the accumulation of ROS. The findings not only help to clarify the molecular mechanism of the apoptosis induction by 8-MOP, but also contribute to build more of a basis for its anti-tumor role in HCC chemotherapy.

Materials and Methods

Materials

8-MOP (purity>99%) was purchased from sigma (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO) to 100 mM, and stored at -20°C. The concentrations used in the study were 25, 50 or 100 μM, and freshly diluted with RPMI-1640 (Gibco, Carlsbad, CA) to final concentrations. Controls were always treated with the same amount of DMSO (0.1%) as used in the corresponding experiments. MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyterazolium-bromide] was purchased from SunShine Biotechnology (Nanjing, China). N-Acetyl-L-cysteine NAC was from Beyotime (Shanghai, China). Erk1/2 inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and PI3K inibitor (LY294002) were from sigma (St. Louis, MO, USA). RPMI-1640 and fetal bovine serum (FBS) were from Gibco (Gaithersburg, MD, USA). The suppliers and dilution of primary antibodies used in the present study were shown as follows: Bcl-2 (1:1000), Bax (1:1000), Cyt-c (1:1000), AIF (1:1000), GADPH (1:5000), ERK (1:1000) and phospho-ERK (Thr202/Tyr204) (1:1000) were from Bioworld, St. Louis Park, USA; Cox IV (1:1000) was from Proteintech, Wuhan, China; anti-Akt (1:1000) and anti-phospho-Akt (Ser473) (1:1000) were from Abgent, San Diego, USA. All other reagents were of analytical grade and commercially available.

Cell culture

The human hepatocarcinoma (HepG2) cell line, HCT-116 (human colorectal carcinoma cell line), BGC-823 (human gastric cell line), SGC-7902 (human gastric carcinoma cell line) and MCF-7 (human breast cell line) are from the cell bank of Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Cell viability inhibition assay

MTT assay was used to determine the cell viability. Cells were seeded (2 × 10⁴ cells/well) in 96-well plates at 37 °C with 5% CO₂ for overnight incubation and treated with various concentrations of 8-MOP for 24 or 48 h. The cells were then added 20 µl of 5 mg/ml MTT, and were incubated at 37 °C for another 4 h. The culture medium was discarded and the formazan crystals formed were dissolved in 150 µl DMSO. The absorbance was measured at 570 nm using an Automated Microplated Reader ELx800 (BioTek).

Cell cycle analysis

HepG2 Cells were seeded in 6-well plates and incubated for 24 h to resume exponential growth. The cells were treated with different concentrations of 8-MOP for 24 h, thereafter, fixed gently by putting 70% ethanol in ice overnight and then resuspended in PBS containing 50 mg/mL propidium iodide and 0.05 mg/ml RNase for 30 min at 37 °C in the dark. The cells were analyzed on a Becton Dickinson FACS Calibur flow cytometer equipped with an argon ion laser at 488 nm wavelength. Finally, the cells in sub-G1 phase were determined as a percentage of the total number of cells.

Annexin V/PI Apoptosis Assay

Apoptosis rates were determined by using a FITC-labeled Annexin V/propidium iodide (PI) apoptosis detection kit (KeyGen Biotech, Nanjing, China) according to the manufacturer’s instructions. Briefly, the cells were harvested and washed twice with PBS, resuspended in 500 µl binding buffer, and incubated with annexin V-FITC/PI in dark for 10~15 min. Analysis was performed with a Becton Dickinson FACS Calibur flow cytometer at Ex./Em.-488/530 nm using Flowjo software. The cells in early stages of apoptosis were Annexin V positive and PI negative, whereas the cells in the late stages of apoptosis were both Annexin V and PI positive.
Measurement of mitochondrial membrane potential (MMP)

The electrical potential difference across inner mitochondrial membrane (Δψm) was monitored using the Δψm specific fluorescent probe JC-1. JC-1 is a cationic dye that can be used as an indicator of mitochondrial potential. It exhibits mitochondrial potential-dependent accumulation, which can be detected by a fluorescence emission shift from green to red [26]. Cells were continuously incubated with 10 mM JC-1 for an additional 30 min in dark. Relative fluorescence intensity was monitored using the flow cytometry (FACS Calibur, Becton Dickinson), and analyzed by software Modfit and Flowjo with settings of FL1 (green) at 530 nm and FL2 (red) at 585 nm.

Measurement of ROS generation

According to the method described previously [27], the level of intracellular ROS was detected using fluorescent dye 20, 70-dichlorfluoresceindiacetate (DCFH-DA, Sigma, USA). The HepG2 cells were treated with different concentrations of 8-MOP for 6 h, 12 h or 24 h. The cells were collected and incubated with 100 mM DCFH-DA dissolved in serum-free medium for 30 min at 37 °C in the dark. The fluorescence intensity was measured by FACS Calibur flow cytometry (Becton Dickinson) at Ex./Em.-488/525 nm.

Preparation of phosphoprotein and extraction of subcellular fractions

At the end of treatment, cells were harvested and washed with ice-cold PBS. The cells used for phosphoproteins were lysed with lysis buffer enriched with protease and phosphatase inhibitors cocktail. Subcellular fractionation was performed using the mitochondria isolation kit and nuclei isolation kit for cultured cells (KeyGen Biotech, Nanjing, China) according to the manufacturer’s instructions. Isolated subcellular fractions were then checked for purity by Western analysis of the respective markers for cytoplasmic, mitochondrial or nuclear compartments. An anti-GAPDH antibody was used as a loading control for whole-cell lysates and the cytosolic fraction, an anti-COX-IV antibody for the mitochondrial fraction, and an anti-H4 antibody for the nuclear fraction.

Western blot analysis

Protein concentrations were determined with BCA protein assay based on the albumin standard (Pears, Rockford, IL, USA). Equal amounts of protein were separated on a 10% SDS-polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline/0.1% Tween 20 for 2 h, subsequently blotted with respective primary antibodies overnight, and then blotted with horseradish peroxidase-conjugated secondary antibody for 1 h. The protein bands were visualized with enhanced chemiluminescence detection system. Protein levels were quantified by density analysis using Image J software (NIH), and expressed as interest protein/internal control.

Statistical analysis

The experimental results were expressed as the mean ± SEM of at least three separate experiments. Statistical analysis was performed with a one-way ANOVA, followed by Duncan’s multiple comparison test. The differences were considered statistically significant when p < 0.05. All statistical analyses were performed using SPSS17.0 analysis software.

Results

8-MOP inhibits the viability of HepG2 cells

Firstly, the effects of 8-MOP on cell viability were investigated with different concentrations. The IC50 values of 8-MOP on cell viability were determined by different concentrations. The IC50 values of 8-MOP for 48 h were 121.15 ± 3.81 μM, 69.66 ± 6.50 μM, 162.91 ± 4.93 μM, 203.95 ± 9.43 μM or 153.85 ± 5.75 μM, respectively (Fig. 1A). In addition, the viability of HepG2 cells was inhibited in a time- and concentration-dependent manner by 8-MOP, as IC50 values for 24 h and 48 h were 124.07 ± 6.89 μM and 69.66 ± 6.50 μM respectively (Fig. 1B). According to the results, the human hepatocarcinoma cell line HepG2 cells were sensitive to 8-MOP to the largest extent, therefore, it was chosen for all the following experiments with 25, 50 or 100 μM of 8-MOP treatment for 48 h.
8-MOP induces apoptosis in HepG2 cells

To investigate whether 8-MOP induced growth inhibitory effect due to cell apoptosis, cytometric cell cycle assay and cytometric apoptosis assay were applied. Propidium iodide staining was used to analyze cell cycle and cell apoptosis. Exposure of the cells to 8-MOP remarkably increased the sub-G1 hypodiploid cell population in a dose-dependent manner (Fig. 2B). The increased percentage of sub-G1 hypodiploid cells (genomic DNA fragmentation) indicated that 8-MOP-induced cytotoxicity was due to the promotion of cell apoptosis. In addition, cell apoptosis induced by 8-MOP was further confirmed by AnnexinV/PI staining assay and cell apoptosis rate was determined as the percentage of the early and the late apoptotic cells. As shown in Fig. 2C and 2D, increased apoptosis rate was detected after the treatment with 8-MOP for 48 h. Compared with untreated cells, the apoptosis rate was increased from 4.49 ± 0.40% to 15.64 ± 0.97%, 20.3 ± 0.83% or 31.45 ± 1.53% with the treatment of 25, 50 or 100 μM 8-MOP respectively. Therefore, the results indicated that 8-MOP induced cell apoptosis in HepG2 cells.

8-MOP activates the mitochondrial apoptosis pathway in HepG2 cells

To ascertain whether 8-MOP-induced apoptosis was mediated by a mitochondria-dependent pathway, we analyzed the effect of 8-MOP on MMP by flow cytometry using the mitochondrial cationic dye, JC-1. It was shown that with the treatment of 8-MOP, cell MMP was decreased in a dose-dependent way (Fig. 3A and 3B). It was about 30% decrement of MMP with the treatment of 100 μM 8-MOP, compared with the untreated cells. The mitochondrial apoptosis related proteins such as Bcl-2 and Bax were investigated by Western blot in the meanwhile. As shown in Fig. 3C and 3D, after 48 h treatment, there was a decrease of Bcl-2 expression and an increase of Bax in a dose-dependent manner. As shown in the quantification data, the Bax/Bcl-2 ratio was significantly increased by the treatment of 8-MOP (Fig. 3D). Furthermore, the amount of cytochrome C significantly decreased in mitochondria but increased in cytosol (Fig. 3E and 3F), whereas the level of AIF decreased in mitochondria but increased in nucleus when the cells were exposed to increasing concentrations of 8-MOP (25, 50 or 100 μM) for 48 h (Fig. 3G and 3H). The translocation of AIF and cytochrome C from the mitochondria indicated that 8-MOP induced the dysfunction of mitochondria and activated intrinsic mitochondrial apoptosis pathway, subsequently leading to the cell apoptosis.
Induction of intracellular ROS generation by 8-MOP plays in the upstream of cell mitochondrial apoptosis pathway activation

Cellular endogenous ROS is implicated in genotoxicity, tumor initiation and progression. A high level of ROS induces cell death via apoptosis and/or necrosis [27-29], and is associated with the induction of apoptosis by several phytochemicals [30-31]. To examine the importance of ROS in 8-MOP-induced apoptosis in HepG2 cells, intracellular ROS levels were examined by flow cytometry with a ROS-sensitive fluorescent probe, DCFH-DA. As shown in Fig. 4A, the exposure of cells to 8-MOP for 6 h, 12 h or 24 h significantly induced the generation of ROS. Compared with untreated cells, nearly 2-fold ROS was generated in the cells treated with 100 μM 8-MOP for each period. To further confirm the role of ROS in 8-MOP induced cell apoptosis, NAC, an antioxidant, was used to inhibit the generation...
of ROS. It was shown that the apoptosis rate in 8-MOP-treated cells was attenuated in the presence of NAC (10 mM) (Fig. 4B). In the meanwhile, NAC also abolished 8-MOP-induced decrease of Bcl-2 and increase of Bax (Fig. 4C). The data illustrated that 8-MOP induced ROS generation and subsequently caused the deterioration of cell mitochondria, which resulted in cell apoptosis.
Inhibition of ERK pathway potentiates the pro-apoptotic effect of 8-MOP in HepG2 cells

To further elucidate the underlying mechanisms, it was investigated whether 8-MOP-induced apoptosis was connected with the modulation of intracellular signaling pathways such as MAPKs and Akt in HepG2 cells. Firstly, the specific inhibitors of respective signaling pathways were used prior to the treatment of 8-MOP (100 μM), in order to screen the related pathways in 8-MOP-induced apoptosis. The classical Annexin V/PI double staining method was used. It was shown that the addition of p38 MAPK inhibitor (SB203580) (10 μM) or PI3K inhibitor (LY294002) (10 μM) didn’t change the performance of 8-MOP on cell apoptosis in HepG2 cells (Fig. 5A and 5B). Furthermore, cell phospho-lysates were prepared and the activation of respective signaling pathway was detected by Western blotting assays.
It was shown that the activation of PI3K/Akt (Fig. 5C and 5D) and p38 MAPK (Fig. 5E and 5F) signaling pathways wasn’t altered by 8-MOP, as evidenced by the constant phosphorylation status with the treatment of 8-MOP (100 μM). Nevertheless, 8-MOP significantly suppressed ERK1/2 phosphorylation after the incubation lasting for 1 min and the suppression was perseverative (Fig. 6A and 6B). The suppression of ERK1/2 signaling by the treatment of 8-MOP (100 μM) for 48 h was comparable with the inhibitory effect of classical ERK1/2 inhibitor PD98059 (Fig. 6C and 6D). It indicated that 8-MOP possessed a potent and long-lasting inhibitory capability on ERK1/2 signaling pathway in HepG2 cells. The results
suggested that the inhibition of ERK1/2 signaling pathway, instead of PI3K/Akt and p38 MAPK pathways, plays a role in the pro-apoptotic effects of 8-MOP.

**Discussion**

In the present study, it is demonstrated that 8-MOP, independent of the exposure to UVA, inhibits viability and induces apoptosis in human hepatocarcinoma cell line HepG2 cells, exerting an antitumor activity. Its pro-apoptotic effect is accompanied by the loss of mitochondrial membrane potential, translocation of mitochondrial apoptotic proteins and activation of caspases proteins, indicating the involvement of intrinsic apoptotic pathway. In addition, endogenous ROS generation and ERK1/2 pathway inhibition contribute to 8-MOP-induced apoptosis.

8-MOP has been considered as a photosensitizing agent and also been used in combination with UVA in the treatment of some hyperproliferative skin disorders [32, 33]. It is reported for the first time by our group that 8-MOP, independent on the activation of UV, inhibits the proliferation of HepG2 cells by inducing apoptosis [17]. In the present study, we explore the effects and mechanisms of 8-MOP-induced apoptosis in HepG2 cells by investigating the activation of mitochondrial apoptotic pathway, the accumulation of intracellular ROS generation and the involvement of apoptosis-associated intracellular signaling pathways.

In this study, when we determine the sub-G1 hypodiploid cell population using propidium iodide staining and flow cytometric analysis, it is found that sub-G1 hypodiploid cell population is increased by 8-MOP (Fig. 2A). The obvious sub-G1 peak in the DNA histogram after the treatment implies that 8-MOP induces significant loss of DNA content, which is a typical characteristic of cell apoptosis. In the meanwhile, cell cycle arrest is also induced by 8-MOP. The treatment of 100 μM 8-MOP for 48 h significantly increased
the number of S-phase cells, whereas markedly decreased the cell populations in G2/M-phase (Data not shown). It suggests that cell cycle arrest also plays a role in the inhibition of cell proliferation apart from cell apoptosis. S-phase arrest induced by 8-MOP in HepG2 cells indicates its probable inhibition on DNA replication. It agrees with previous report that 8-MOP, independent of the UV irradiation, binds to DNA as an intercalator at low drug load [16]. It is speculated that 8-MOP binds to DNA, subsequently leads to cell cycle arrest and cell proliferation inhibition. It could be important for the anti-cancer effect of 8-MOP, since cell cycle deregulation is featured by most tumor cells. The effects and mechanisms of 8-MOP on cell cycle arrest still need further elucidation. The up-regulation of P53 by 8-MOP is possibly related, since P53 regulates the expression of p21, which directly binds to some Cdk-cyclin complexes, inhibits their kinase activity and sequentially affects cell cycle progression, including S-phase and G2/M-phase [34, 35].

The mitochondrial pathway is important in the process of apoptosis induction. The Bcl-2 protein family, including anti-apoptotic and pro-apoptotic proteins, regulates cell death by modulating mitochondrial membrane permeability during apoptosis. Bcl-2 is anti-apoptotic, while Bax is an antagonist of Bcl-2, which inserts into the mitochondrial outer membrane, allows for the release of Cyt c and AIF, and subsequently activates caspase-dependent or independent apoptotic pathway [21]. In the present study, the involvement of mitochondrial pathway in 8-MOP-induced apoptosis is confirmed by observing changes in MMP, Bax/Bcl-2 ratio and release of Cyt c and AIF (Fig. 3). The results show that 8-MOP results in significant loss of MMP, increase of Bax/Bcl-2 ratio and translocation of Cyt c and AIF. It supports that mitochondrial pathway mediates 8-MOP-induced apoptosis. In addition, that the increase of Bax/Bcl-2 ratio is obviously abolished by the ROS scavenger NAC (Fig. 4E and 4F) suggests that mitochondrial dysfunction is the downstream effector of 8-MOP caused ROS accumulation in the process of apoptosis induction.

The activation of ERK kinases is an important mediator in the proliferation and cell growth of HCC cells [30]. ERK activation exerts an anti-apoptotic effect by inhibiting caspase cascade activation and Cyt c releasing. It plays an important role in apoptosis induced by many diverse anti-cancer drugs [36, 37]. The inhibition on ERK signaling by 8-MOP results in the initiation of cell apoptosis (Fig. 2C and Fig. 6A), which is consistent with previous findings [29]. In addition, the inhibition of ERK activation by 8-MOP happens very quickly, in which 8-MOP significantly suppresses ERK phosphorylation after the incubation lasting for 1 min (Fig. 6A and 6B). The inhibitory effect of 8-MOP on ERK pathway is very potent and perseverative for a long period as 48 h, which is comparable with classical ERK inhibitor PD98059 (Fig. 6C and 6D). This quick and strong reaction pattern on ERK signaling pathway indicates that it is possibly first event after 8-MOP working on the cells and contributes to its pro-apoptotic effects. The inhibition on ERK signaling could probably lead to the activation of mitochondria-mediated apoptotic pathway, eventually provokes cell apoptosis.

ROS plays a key role in mitochondria-mediated apoptosis and is essential for the cytotoxicity of many anti-cancer agents [38]. In low concentrations, ROS has been considered as a second messenger in signaling pathways. However, ROS, if excessive,
may lead to the free radical attack on mitochondrial membrane phospholipids and loss of MMP, which causes the release of apoptosis-inducing factors (Cyt c, AIF, or Smac/Diablo) that activates caspase-dependent or caspase-independent cytosolic signaling events and causes nuclear condensation [28, 39]. ROS acts in the upstream of mitochondria-mediated apoptosis by promoting Bax translocation to mitochondria [40]. The present study shows that the accumulation of ROS is increased by the treatment of 8-MOP in HepG2 cells (Fig. 4A). NAC, a potent antioxidant, inhibits oxidative stress by directly scavenging ROS (Fig. 4B). The pretreatment of NAC rescues the cells from 8-MOP-triggered apoptosis (Fig. 4C and 4D) and abolishes 8-MOP-induced Bax/Bcl-2 ratio increase as well (Fig. 4E and 4F). The results confirm that 8-MOP-promoted ROS accumulation is a key mediator in HepG2 cell apoptosis.

Conclusion

The present study points to three important findings. Firstly, 8-MOP inhibits cell viability and mitochondria-mediated apoptosis in a concentration-dependent manner. Secondly, the accumulation of intracellular ROS is a key mediator in 8-MOP-triggered cell apoptosis by facilitating the loss of mitochondrial integrity and activating mitochondrial apoptotic pathway. Finally, the inhibition of ERK signaling pathway contributes to 8-MOP induced pro-apoptotic effect. Schematic diagram of the present study is shown in Fig. 7. The findings are of great importance in illuminating the mechanisms of 8-MOP as a component in PUVA treatment and suggest its potential as an anti-cancer agent in the HCC chemotherapy.

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Disclosure Statement

The authors declare that there is no conflict of interests.

References


