Synergistic Induction of Apoptosis by Methylseleninic Acid and Cisplatin, The Role of ROS-ERK/AKT-p53 Pathway

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ABSTRACT: Cisplatin-based therapy is one of the most important chemotherapy treatments for cancers. However, its efficacy is greatly limited by drug resistance and undesirable side effects. Therefore, it is of great importance to develop chemosensitizing agents to cisplatin. In the present study, we demonstrated the strategy to use methylseleninic acid (MeSe) as a synergistic agent of cisplatin and elucidated their action mechanisms. The combination of MeSe and cisplatin exhibited synergistic anticancer efficacy and achieved greater selectivity between cancer cell and normal cell. By inducing intracellular oxidative stress, MeSe potentiated cisplatin-induced DNA damage and led to enhanced p53 phosphorylation, followed by increased activation of both mitochondrial and death receptor pathway. Down-regulation of phosphorylated AKT and ERK also played important roles in the synergistic effects of MeSe and cisplatin. Our results suggested that the strategy to apply MeSe as a synergistic agent to cisplatin could be a highly efficient way to achieve anticancer synergism by targeting the intracellular redox system. MeSe might be a candidate for clinical application as a chemosensitizer to cisplatin-based therapy, especially for hepatocellular carcinoma.

KEYWORDS: methylseleninic acid, cisplatin, apoptosis, synergetic effects, ROS

INTRODUCTION

Cancer is a major challenge in public health. Many therapeutic drugs have been explored and developed for clinical treatments. Cisplatin is one of the most well-known anticancer agents. It exerts clinical activities against a wide spectrum of solid neoplasms and has already been largely employed for the treatment of a wide array of solid malignancies. Its prominent cytotoxic mode of action involves the interaction with DNA and the subsequent formation of DNA adducts, especially intrastrand cross-link adducts, which then activate several apoptosis signal transduction pathways. However, many kinds of cancer cells are insensitive to cisplatin-induced apoptosis due to various mechanisms such as Bcl-2 family antiapoptosis proteins overexpression, p53 and MAPKs inactivation, and AKT constituted phosphorylation. As a result, patients have to accept higher doses of cisplatin and this strategy will expose patients to higher risks of side effects such as nephrotoxicity, neurotoxicity, and ototoxicity. Furthermore, insensitiveness of cancer cells to cisplatin will also lead to development of chemoresistance, resulting in therapeutic failure. So far, the high incidence of chemoresistance is still the main limitation to the clinical usefulness of cisplatin as an anticancer drug. Therefore, the development of chemosensitization strategies and chemosensitizing agents are of important clinical implications.

Selenium (Se) is an essential micronutrient of fundamental importance to humans and animals. In the past decades, Se has been extensively studied as a cancer chemopreventive agent. Several cancer chemoprevention trials have shown that supplementation of Se at supranutritional levels might be a safe and effective way to prevent cancers. However, Se exhibited a narrow margin between beneficial and toxic effects. A major limitation in clinical application is that its effective doses are often close to the toxic range. Recent studies have explored the possibility of using selenium to overcome cancer through combination with well-established chemotherapeutic agents. It was reported that Se compounds could enhance the anticancer efficacy of irinotecan in tumor-bearing mice.

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inoculated with irinotecan-resistant cancer cells. This result indicated that Se was a potential effective modulator of therapeutic efficacy of anticancer drugs. Considering the potential toxic effects when applying Se alone, we would like to explore the strategy to employ Se as a sensitizer or a synergistic agent to anticancer drugs. We hope such a strategy can lower the effective dose of Se while at the same time increase the cancer cell sensitivity to anticancer drugs so that we can achieve more efficient and less toxic anticancer therapies.

Different chemical forms of Se exhibited different characteristics and activities. In recent years, organic selenium species, especially naturally existed organic selenocompounds, have attracted more and more attentions and interests. Compared with inorganic selenium species, many naturally existed organic selenocompounds exhibited higher antiproliferative activities and lower toxicity. Methylenedicysteine (MeSe) is a metabolite from animal cells and has been reported to possess therapeutic effects of MeSe and cisplatin on hepatocellular carcinoma, but its effects when applying Se alone, we would like to explore the strategy to employ Se as a sensitizer or a synergistic agent to anticancer drugs. We hope such a strategy can lower the effective dose of Se while at the same time increase the cancer cell sensitivity to anticancer drugs so that we can achieve more efficient and less toxic anticancer therapies.

To date, the management of liver cancer still represented one of the most challenging problems in clinical oncology. Platinum-based chemotherapy was the main treatment for the therapy of hepatocellular carcinoma, but its efficacy was limited by insensitiveness. Studies have shown that half of the Se uptake was accumulated in the liver during metabolism. In the present study, we used a hepatocellular carcinoma cell line HepG2 as an in vitro model. The key mechanisms were also elucidated. Our results showed that MeSe effectively potentiated cisplatin-induced apoptosis and exhibited synergistic effects on inhibition of HepG2 proliferation. MeSe-induced inhibition of AKT and ERK phosphorylation contributed to the synergistic effect by strengthening the downstream apoptosis signal cascade which involved both the mitochondrial (intrinsic) and the death receptor (extrinsic) pathways. Our data also suggested that ROS accumulation might be the earliest and the essential event for the enhancing effect of MeSe. Our study highlighted the strategy of combining application of Se and cisplatin. Our finding also demonstrated that MeSe was an effective synergistic agent to cisplatin and might be a candidate of chemosensitizer for cisplatin-based therapy in clinical application.

**METHODS**

**Cell Culture and Cell Treatments.** HepG2 human hepatocellular carcinoma cell line, MCF-7 human breast cancer cell line, and HK-2 human kidney cell line were obtained from American Type Culture Collection (ATCC) and were maintained in DMEM medium supplemented with fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (50 units/mL) at 37 °C in a humidified incubator with 5% CO₂ atmosphere.

Cells seeded at a density of 8 × 10⁴ cells/mL were pretreated with 10 μM MeSe for 6 h and coincubated with 40 μM cisplatin for another 24 h. For the MeSe treatment alone, the cells were incubated with different concentrations of MeSe for 30 h. For the cisplatin treatment, the cells were incubated with cisplatin for 6 h. In the experiment for ROS was examination, MeSe and cisplatin were given to the cells at the same time. For experiments of p53, AKT and ERK inhibition, the cells were pretreated with 10 μM pifithrin-α (PTF-α), LY294002 for 1 h prior to the addition of drugs. For the experiments of ROS blocking, the cells were pretreated with 5 mM NAC/GSH/Troxol for 2 h prior to the addition of agents.

**Determination of Cell Viability and Flow Cytometric Analysis.** After treatment, cell viability was detected by evaluating the ability of cells to transform MTT to a purple formazan dye. The cell cycle distribution was analyzed by flow cytometric analysis as previously described.

**Evaluation of Mitochondrial Membrane Potential (Δψm).** The Δψm was determined using two fluorescent probes in separate assays, JC-1 and TMRE. Treated cells were loaded with JC-1 probe as described and then analyzed by flow cytometry. The depletion of Δψm was also monitored qualitatively by fluorescence microscopy. Single-cell gel electrophoresis (Comet Assay). Single-cell gel electrophoresis for detection of DNA damage was performed using the Comet assay reagent kit as described. DNA was stained with SYBR Green I (Trevigen) and visualized under a fluorescence microscope (Nikon Eclipse 80i).

**Caspase Activity Assay.** The treated HepG2 cells were harvested by centrifugation and incubated with lysis buffer on ice for 1 h. Total protein (100 μg/well) were placed in 96-well plates and incubated with specific caspase substrates (Ac-DEVD-AMC for caspase-3, Ac-IETD-AMC for caspase-8, and Ac-LEHD-AMC for caspase-9) at 37 °C for 2 h in darkness. Caspase activity was determined by fluorescence intensity with the excitation and emission wavelengths at 380 and 440 nm, respectively.

**Western Blot Analysis.** The total cellular proteins were extracted by incubating the cells in the lysis buffer. The protein concentrations in the cell lysates were determined by BCA kit (Sigma) according to the manufacturer’s protocols. SDS-PAGE was performed in 12% tricine gels with equal amounts of protein loaded per lane. After electrophoresis, the proteins were transferred from the gel to a PVDF membrane, and the membrane was blocked with 5% nonfat milk in TBST buffer for 1 h. The membranes were then incubated with primary antibodies at 1:1000 dilution in 5% nonfat milk overnight at 4 °C. A secondary antibody conjugated with horseradish peroxidase at 1:3000 dilution for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system (Kodak). The expression ratio was calculated according to the reference band of β-actin.

**Measurement of ROS Generation.** The effects of MeSe and cisplatin on intracellular ROS generation were evaluated by
DCF fluorescence assay as described. The ROS level was determined by measuring the fluorescence intensity on a Tecan SAFIRE fluorescence reader, with the excitation wavelength at 488 nm and emission wavelength at 525 nm, respectively. Unlabeled cells in PBS were used as background control. Relative DCF fluorescence intensity of the treated cells was expressed as percentage of control (as 100%).

Statistical Analysis. Experiments were carried out at least in triplicate and repeated three times. All data were expressed as mean ± SD. Statistical analysis was performed using SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc. Chicago, IL). The difference between two groups was analyzed by two-tailed Student’s t test. The difference between three or more groups was analyzed by one-way analysis of variance multiple comparisons. Differences with $P < 0.05$ or $P < 0.01$ was considered statistically significant. Bars with different characters are statistically different at $P < 0.05$ level.

\section*{RESULTS}

Combination of MeSe and Cisplatin Exhibited Synergistic Anticancer Effects and Achieved Greater Selectivity between Cancer Cell and Normal Cell. To evaluate the efficacy of combination, we first examined the minimal cell death-inducing doses of MeSe and cisplatin, respectively. As shown in Supporting Information Figure S1, 5–10 μM of MeSe and 40–80 μM of cisplatin were chosen as the concentrations for combined treatment. To maintain a magnified response to the combination, a time-dependent experiment was conducted. As shown in Figure 1A,B, 6 h and 12 h MeSe pretreatment showed better strengthened effects than other time points. No significant difference between 6 h and 12 h pretreatment was observed. Thus, 6 h MeSe pretreatment was chosen for our further experiments.

Combining MeSe with cisplatin significantly increased cell death of HepG2 human hepatoma carcinoma cells and MCF-7 human breast cancer cells (Figure 1C, Supporting Information Figure S2A). Increased cell death was also observed in HK-2 human normal kidney cells, but the enhanced efficacy was much lower than that of HepG2 and MCF-7 cells, which indicated a potential increase in cell selectivity between cancer and normal cells.

To further evaluate the combination therapy, we wanted to determine whether the anticancer action of MeSe and cisplatin was synergistic, additive, or antagonistic in HepG2 cells, MCF-7 cells, and HK-2 cells. Our data were analyzed by the isobologram analysis. The effects under different ratios of MeSe and cisplatin (1:4, 1:8, and 1:16 to HepG2 cells) were analyzed by isobologram analysis. As shown in Figure 1D, the antiproliferative effects of MeSe and cisplatin were synergistic in all combinations.

Figure 1. MeSe potentiates cisplatin-induced cytotoxicity in HepG2 cells. Cells were pretreated with or without 1.25–80 μM MeSe for different period of time and then cultured in the presence or absence of 10–640 μM cisplatin for 24 h (A–C). Cell viability was examined by MTT assay. (D) Isobologram analysis of the antiproliferative effects of MeSe and cisplatin on HepG2 cells. (E) Morphology of HepG2 cells under different treatments. All data were obtained from three independent experiments and presented as the means ± SD. Statistical difference between each bar was expressed as letters.
2 cells, 1:1, 1:2, and 1:4 to MCF-7) were investigated. In isobologram analysis, if MeSe and cisplatin only have additive effects, the theoretical values of the combination IC\textsubscript{50} value for HepG2 were 181.6 μM for 1:4, 218.5 μM for 1:8, and 248.2 μM for 1:16. The actual detected IC\textsubscript{50} values were 45, 32.4, and 57.8 μM, respectively, which were much lower than the theoretical ones. As shown in isobologram, the locations of the data spots for HepG2 and MCF-7 were far below the line defining an additive effect, indicating strong synergistic effects of MeSe and cisplatin. On the contrary, the spots for HK-2 were near the additive line, showing an obvious difference from that of cancer cells. The combination index (CI) of the IC\textsubscript{50} value of the MeSe and cisplatin for HepG2 were 0.248 (1:4), 0.148 (1:8), and 0.233 (1:16); for MCF-7 they were 0.301 (1:1), 0.233 (1:2), and 0.274 (1:4), further confirming the synergism between MeSe and cisplatin. The CI values for HK-2 were 0.965 (1:4), 0.879 (1:8), and 0.888 (1:16), suggesting a much lower synergistic effect. Taken together, our results clearly demonstrated that the combination of MeSe and cisplatin could effectively enhance anticancer efficacy and achieved greater selectivity between cancer cells and normal cell. The strategy of applying MeSe as chemosensitizer to cisplatin might indicate a more efficient and less toxic anticancer therapy. MeSe in combination with cisplatin at the ratio of 1:8 showed the lowest CI value and therefore was selected as an optimized condition for our further study.

**Methylseleninic Acid Potentiates Apoptosis Induced by Cisplatin in HepG2 Cells.** In the following study, the mechanisms of synergistic effect of MeSe and cisplatin were examined on HepG2 cells, a cell line with a high potential of metastasis and resistance toward the contemporary therapeutic strategies. First, we performed DNA flow cytometric analysis to examine the change in cell cycle distribution. No significant apoptosis (no more than 10% Sub-G1 population) (Figure 2A) was observed in cells exposed to either MeSe or cisplatin alone. However, significant increase in Sub-G1 cell populations was observed in cells coincubated with MeSe and cisplatin. For instance, the cells exposed to MeSe (10 μM) and cisplatin (80 μM) displayed 41.6% of apoptosis. The results of microscopic examination (Figure 1E) also revealed the occurrence of cell

Figure 2. Synergistic induction of apoptosis by MeSe and cisplatin in HepG2 cells. Cells were pretreated with or without 5–20 μM MeSe for 6 h and then cultured in the presence or absence of 40–80 μM cisplatin for 24 h. (A) MeSe increased sub-G1 population. Quantitative analysis of apoptotic cell death induced by MeSe and/or cisplatin were analyzed by flow cytometric analysis. (B) Representative photomicrographs of DNA fragmentation and nuclear condensation in response to MeSe and/or cisplatin treatment, detected by TUNEL assay and DAPI staining. The images shown here are representative of three independent experiments with similar results. (C) Western blot analysis of PARP and caspases cleaved in the apoptosis induced by MeSe and/or cisplatin. Equal protein loading was confirmed by analysis of β-actin in the protein extracts. Similar results were obtained from three independent experiments.
shrinkage, cell rounding, and the appearance of apoptotic bodies after treatments. To confirm the apoptosis induced by MeSe and cisplatin, TUNEL enzymatic labeling and DAPI costaining (Figure 2B) assay was employed. As shown in Figure 2B, compared with cisplatin alone, cells treated with MeSe combination showed significant enhancement in DNA fragmentation and nuclear condensation. These results indicated that apoptosis was the major mode of cell death induced by combined treatment of MeSe and cisplatin and that MeSe could potentiate the apoptosis induced by cisplatin. This finding was further confirmed by cleavage of caspases and PARP. Caspases are a family of cysteine acid proteases, acting as important mediators of apoptosis and contributing to the overall apoptotic morphology by cleavage of various cellular substrates. PARP is an important substrate of caspase and acts as a DNA repair enzyme. The cleavage of PARP is considered as a biochemical hallmark of apoptosis and is mainly mediated by effector caspases such as caspase-3 and caspase-7. As shown in Supporting Information Figure S2A and Figure 2C, combination of MeSe and cisplatin resulted in increased activation of caspase-3 and caspase-7, together with increased cleavage of PARP in HepG2 cells, but not in HK-2 human normal cells (Supporting Information Figure S5).

**Methylseleninic Acid Potentiates Cisplatin-Induced Apoptosis by Activating Both Mitochondria and Death Receptor Signal Pathway.** Apoptosis can be initiated by two central signaling pathways: the extrinsic and intrinsic pathways. To delineate the molecular events initiated by MeSe and cisplatin, activation of three initiator caspases, caspase-8/caspase-10 (Fas/TNF-mediated), and caspase-9 (mitochon-
drial-mediated) were evaluated. The results showed that both caspase-8/caspase-10 and caspase-9 could be activated by MeSe or cisplatin alone, while MeSe mainly activated caspase-9 and cisplatin mainly activated caspase-8/caspase-10 (Supporting Information Figure S2B and Figure 3A). Significant increases in activations of these three caspases were observed in the combined treatment of MeSe and cisplatin, indicating stronger signals of both mitochondria pathway and death receptor pathway. Slight increase of DR5 was observed (Figure 3D) under combined treatment, again suggesting the involvement and enhancement of death receptor pathway. Mitochondria act as a point of integration for apoptotic signals originating from both the extrinsic and intrinsic pathways. Depletion of mitochondrial membrane potential ($\Delta \Psi_m$) is a crucial step in the apoptotic process and is lethal to the cells, which leads to the release of diverse apoptogenic factors from mitochondria into cytoplasm. Therefore, we examined the change of $\Delta \Psi_m$ in HepG2 cells by both TMRE staining assay (Figure 3B) and JC-1 flow cytometric analysis (Figure 3C). The results showed that both MeSe and cisplatin treated alone can induce mitochondrial membrane potential depress (8.4–15.3%), but the event was significantly enhanced under combined treatment (34.5–48.3%).

Bcl-2 family members have been known as key regulators of mitochondrial permeability. Hence, the effects of MeSe and cisplatin on the expression levels of pro-survival and pro-apoptotic Bcl-2 family proteins in HepG2 cells were examined by Western blotting. As shown in Figure 3D, no obvious change of Bcl-XL and Bax expression was observed in cells treated with MeSe or cisplatin alone or combination. No significant difference of Bcl-2, Bad, and Bim expression level were observed in cells treated with cisplatin alone, while cells treated with MeSe alone had up-regulated Bim levels. MeSe and cisplatin cotreatment significantly suppressed the expression level of Bcl-2 and increased the expression levels of Bad and Bim. These results indicated that MeSe and cisplatin cotreatment suppressed expression of Bcl-2 and increased expression of Bad and Bim. These changes of expressions were responsible for mitochondria-mediated apoptosis.

Methylseleninic Acid Potentiates Cisplatin-Induced p53 Dependent Apoptosis. Induction of DNA damage is the major mechanism of cisplatin-induced apoptosis. Our results have shown that MeSe and cisplatin cotreatment could
up-regulate the expression of some p53-inducible genes, such as Bax, Bad, and DR5 (Figure 3D), suggesting the potential involvement of DNA damage mediated p53 pathway. Comet assay was employed to evaluate the DNA damage caused by cotreatment of MeSe and cisplatin. As shown in Figure 3E, a small amount of DNA fragments could be observed in cells treated with cisplatin alone but could hardly be detected in cells treated with MeSe alone. However, significantly increased DNA fragments were observed in cells under cotreatments, suggesting MeSe markedly potentiated DNA damage induced by cisplatin. To further confirm the enhanced effect of DNA damage caused by combined treatment, several DNA damage relative signal molecules, including phospho-Histone H2AX, phospho-p53, total p53, and phospho-MDM2 were evaluated by Western blot. Results (Figure 3F) revealed that both MeSe and cisplatin alone could increase phosphorylation level of p53 and histone H2AX and suppress phosphorylation of MDM2, but strengthened effects were observed in the combination. In contrast, cisplatin-induced phosphorylation of p53 and histone H2AX was not enhanced by MeSe in HK-2 normal cells (Supporting Information Figure S5). To further determine the importance of p53 activation in the synergism, PTF-α, a p53 inhibitor, was introduced and the results showed that PTF-α significantly reversed the apoptosis induced by MeSe and cisplatin (Figure 3. G-H). These results indicate that MeSe potentiate cisplatin-induced apoptosis in a p53-dependent manner.

Methylseleninic Acid Potentiates Cisplatin-Induced Apoptosis by Inhibiting AKT Phosphorylation. Several protein kinase pathways have been known to regulate cell proliferation and survival. AKT is the major signal molecule closely related to the activation of p53 in most cell types.27 The
PI3K/AKT pathway is frequently activated in liver cancer cells, resulting in enhanced resistance to apoptosis through multiple mechanisms. In the present study, the activation of p53 pathway (Figure 3E,F) also suggested the possible involvement of AKT in cell apoptosis. To investigate the possible role of AKT pathway in the potentiated effect of MeSe, we evaluated the phosphorylated and total protein of AKT. Suppression of phospho-AKT at the site of Ser 308 was detected in cells exposed to MeSe alone. Co-treatment of MeSe and cisplatin almost interdicted the phosphorylation of AKT. No significant changes of total AKT protein was detected (Figure 4A), and no significant combination effect of MeSe and cisplatin in the induction of AKT dephosphorylation was detected in HK-2 cells (Supporting Information Figure S5). These results indicate that inhibition of AKT phosphorylation is a very important event in the synergistic anticancer effects.
On the basis of the previous results (Figure 1A), we detected the expression of phospho-AKT in HepG2 cells exposed to MeSe for different periods of times in order to explain why a 6 h MeSe pretreatment is an important condition for the combined treatment. Results in Figure 4B showed that the phosphorylation level of AKT gradually decreased during the first 6 h after the exposure to MeSe. To confirm the importance of the inhibition of phospho-AKT, LY294002, an inhibitor of PI3K, the upstream signal of phospho-AKT, was introduced. The results showed that, similar to MeSe, LY294002 can significantly enhanced apoptosis and DNA damage induced by cisplatin, LY294002 also enhanced apoptosis and DNA damage induced by cotreatment of MeSe and cisplatin (Figure 4C,D). Taken together, these results indicated that inhibition of phosphorylation of AKT contributed to the synergistic apoptosis induced by combination of MeSe and cisplatin.

**Methylseleninic Acid Potentiates Cisplatin-Induced Apoptosis by Inhibiting ERK phosphorylation.** Studies have shown that MAPK signaling pathways played important roles in the resistance to cisplatin in many cancer cells.29 In this study, experiments were conducted to investigate the roles of MAPKs in the potentiated apoptosis induced by MeSe and cisplatin. As shown in Figure 5A, no significant difference was observed in cells exposed to cisplatin alone, while suppression of phospho-ERK was detected in cells treated with MeSe alone. MeSe and cisplatin cotreatment almost interdicted the phosphorylation of ERK, while no significant combination effect of MeSe and cisplatin in the induction of ERK dephosphorylation was detected in HK-2 cells (Supporting Information Figure S5). Phosphorylation of JNK and p38MAPK were undetectable, and total ERK protein stayed stable. These results indicate that inhibition of ERK phosphorylation is another crucial event in combined treatment.

We further detected the inhibition of phospho-ERK in cells exposed to MeSe for different periods of time. Figure 5B showed that the phosphorylation level started to decrease in half an hour after the presence of MeSe and was almost completely inhibited after 6 h. These results suggested that inhibition of phospho-ERK might be an earlier event compared to inhibition of phospho-AKT. To confirm the importance of inhibition of ERK phosphorylation, U0126, an inhibitor of MEK, the upstream signal of phospho-ERK, was introduced. The results showed that, just like MeSe, U0126 can significantly enhanced DNA damage and apoptosis induced by cisplatin. U0126 also enhanced apoptosis and DNA damage induced by MeSe and cisplatin cotreatment (Figure 5C,D). These results revealed that inhibition of ERK phosphorylation also played important roles in synergistic effect of MeSe and cisplatin. MeSe potentiated cisplatin-induced apoptosis and DNA damage as a phospho-ERK inhibitor.

**Methylseleninic Acid Potentiates Cisplatin-Induced Apoptosis by ROS Accumulation.** ROS plays an important role in cancer cell apoptosis induced by DNA damage. The status of the cellular redox system are closely relate to many kinds of kinase, including AKT, MAPKs, and the relative downstream effectors.30,31 Hence, the generation of ROS was evaluated in the current study using DCF fluorescent probe. As shown in Figure 6A, cells exposed to MeSe alone and MeSe–cisplatin combination had similar ROS expression trends. Total ROS generation increased to the maximum after 30 min and decreased after 1 h. No significant increase of ROS was observed in cells treated with cisplatin alone. These results indicated that ROS generation in combined treatment was mainly contributed by MeSe. MeSe potentiate cisplatin-induced apoptosis as a proxidant.

To confirm whether ROS accumulation is a necessary event in the potentiated apoptosis, several ROS scavenger, GSH, NAC, and trolox were induced in our experiment. The results revealed that scavenging of ROS nearly reversed all the detected effects induced by the combined treatment, including the cleaving of PARP and phosphorylation of p53 and histone H2AX. Inhibition of AKT and ERK phosphorylation was also reversed (Figure 6B,C and Supporting Information Figure S4). These results revealed the vital role of ROS in the synergism. The redox system might be the upstream target for MeSe to enhance the apoptosis induced by cisplatin in HepG2 cells.

**DISCUSSION**

This study demonstrates, for the first time, the novel strategy to potentiate cisplatin induced apoptosis in cancer cells by MeSe. Key mechanisms were also studied and elucidated the present study. There are two well characterized caspase activation pathways mediating apoptosis: the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway. The mitochondrial pathway generally involves mitochondrial membrane permeability transition and release of cytochrome c from mitochondria to cytoplasm. Cytosolic cytochrome c then forms apoptosome with Apaf-1 and procaspase-9 and subsequently activates caspase-9. The activated caspase-9 then cleaves and activates the effector caspases like caspase-3 and caspase-7, which play roles in mediating PARP cleavage and DNA digestion.32 The death receptor pathway involves death receptors, adapter protein FADD, procaspase-8, and procaspase-10. These proteins construct a death-inducing signaling complex and trigger the activation of caspase-8 and caspase-10. Caspase-8/caspase-10 can directly activate caspase-3 and caspase-7. The apoptotic signals can be amplified by crosstalk between the two pathways when caspase-8 cleaves Bid, a pro-apoptotic member of the Bcl-2 family, and the truncated Bid translocates to the mitochondria and facilitates release of cytochrome c. In general, activation of both two signal pathways triggers stronger apoptosis effects. In the current work, combination of MeSe with cisplatin resulted in much enhanced activation of DR-5, caspase-8, caspase-10, caspase-9, caspase-7, and caspase-3 and PARP cleavage in HepG2 cells, indicating the enhanced induction and stronger signals of both the extrinsic and intrinsic pathways.

Alterations in any of the factors that regulate and execute apoptosis, be it triggered by DNA damage or oxidative stress via the mitochondrial pathway or be it mediated by the extrinsic route, have the potential to influence cisplatin sensitivity. Several dozens of proteins (including death receptors, pro- and antiapoptotic members of the Bcl-2 protein family, mitochondrial intermembrane proteins, and many others) participate in these lethal cascades, and most of them have been shown to modulate the response to cisplatin in vitro.33 For instance, conclusive clinical data reported that the antiapoptotic members of Bcl-2 family like Bcl-2, Bcl-XL are correlated with cisplatin resistance and tumor recurrence in multiple clinical scenarios.34 Ongoing clinical trials are evaluating the combination of cisplatin with small molecules that inhibit Bcl-2-like proteins for the treatment of ovarian carcinomas.35 In our current work, depression of mitochondrial membrane potential, down-regulation of antiapoptosis Bcl-2 families, and up-regulation of proapoptosis Bcl-2 families were detected in the
cotreatment of MeSe and cisplatin. The alteration of Bcl-2 family pro-apoptotic protein expression ratio potentiated mitochondria-mediated apoptosis, resulting in synergistic apoptosis-inducing effects.

Accumulative evidence suggested that p53 is a transcription factor which can directly or indirectly induce cell apoptosis through both the extrinsic and intrinsic apoptosis pathways. The function and activity of p53 is regulated through transcription, translation, protein–protein interactions with cooperating factors, and extensive post-translational modifications, such as phosphorylation or acetylation on specific amino acids. Growing evidence suggested that p53-mediated apoptosis was activated by DNA strand breaks (DSBs), DNA intrastrand adducts, and DNA–protein cross-links. Moreover, resistance to cisplatin can result from defects in the DNA damage-induced apoptotic signal transduction pathways. The inactivation of p53 is one of the most predominant mechanisms of resistance. This has been documented in vitro by comparing the sensitivity to cisplatin of a wide panel of p53-proficient and -deficient tumor cell lines. In the current work, MeSe potentiated cisplatin-induced apoptosis in a p53 dependent manner. The combination of MeSe and cisplatin significantly increased p53 phosphorylation in HepG2 cells, but not in HK-2 cells, without any changes in total p53 protein. Results of Comet assay clearly showed tails from cells under cotreatment of MeSe and cisplatin, indicating more severe DNA damages and fragmentations. A significant increase of phospho-histone H2AX, a marker of DNA damage, was also detected, suggesting the joint action of MeSe and cisplatin was probably due to enhanced DNA damage and p53 phosphorylation. Another important regulator phospho-MDM2, which can specifically bind to p53 and cause p53 degradation via ubiquitination, was also detected. Significant down-regulation of phospho-MDM2 was observed under combined treatment. These results indicated that the activation of p53 pathway played a vital role in the synergistic effects of MeSe and cisplatin. The presence of MeSe effectively activated p53 pathways and led to amplification of the downstream apoptosis signals, thereby increasing the sensitivity of cancer cells to cisplatin.

Several protein kinase pathways have been known to regulate cell proliferation and survival. MAPKs and AKT are the major signal molecules closely associated with the activation of p53 pathway in most cell types. In addition to AKT, another family of serine–threonine protein kinase, MAPKs have been implicated to play important roles in connection with p53 pathway in diverse cell models. The MAPK family includes three kinase members: c-Jun NH2-terminal protein kinase/ stress activated protein kinases (JNK/SAPKs), p38 MAPK, and extracellular signal-regulated kinase (ERK). In general, JNK and p38 MAPK are activated by diverse stimuli such as oxidative stress, UV irradiation, and osmotic shock and are usually required in the induction of apoptosis. In contrast, ERK plays vital roles in cell growth and division and is generally considered to be a survival mediator and is highly expressed in many cancer cells. Moreover, recent works also found that MEK/ERK and PI3K/AKT pathways have influence on chemotherapeutic drug resistance, such as inducing resistance to trastuzumab, tamoxifen, doxorubicin, and paclitaxel in breast, gastric, and myeloma cancer cells. On the other hand, some studies have reported that the anticancer action of selenocompounds involved the regulation of the MAPK and PI3K/AKT pathways. The activation of p53 pathway (Figure 3) shown in our result also suggested the possibility of the involvement of AKT and/or MAPKs. Hence, we then investigated the role of AKT and MAPK pathways in synergistic effects of MeSe to cisplatin. Our result showed that MeSe enhanced cisplatin-induced apoptosis in HepG2 cells with the involvement of inhibition of ERK and AKT phosphorylation. Introduction of ERK inhibitor U0126 significantly enhanced the apoptosis in HepG2 cells, confirming the important role of ERK and AKT in synergism of MeSe and cisplatin. Moreover, MeSe and MeSe did not show synergistic effect on the dephosphorylation of ERK and AKT in HK-2 cells and MeSe hardly triggered any changes in these two signaling pathways. Therefore, our results suggest that ERK and AKT are key targets for the joint therapeutic effects of MeSe and cisplatin. As ERK and AKT are generally high expressed and phosphorylated in cancer cells, the selective efficacy between cancer cells and normal cells of the combined treatment was very likely due to the targeting of ERK and AKT.

Accumulative evidence suggested that the toxicity of Se was closely related to the induction of oxidative stress and the disruption of redox homeostasis. ROS, including the superoxide anion, hydrogen peroxide, and hydroxyl radical, are produced under normal aerobic growth conditions within cells, but they are elevated under the influence of external stimuli. Superabundant intracellular ROS may attack cellular membrane lipids, proteins, and DNA and then cause oxidative injury. Previous studies have shown that free radicals could cause extensive chemical modifications and alterations in DNA and nucleoproteins, including modified bases and sugars and even breaking strands, which then activates p53 pathway. On the other hand, protein kinase pathways, such as MAPK and PI3K/AKT pathways, are also major oxidative stress sensitive pathways in most cell types. Se was suggested to exhibit anticancer activities as a pro-oxidant by triggering cellular oxidative stress. Our results have shown the participation of p53, MAPK, and AKT in synergistic effects of MeSe and cisplatin, so further investigation of oxidative stress involvement was conducted. Our results showed that MeSe induced accumulation of ROS in HepG2 hepatocellular carcinoma cells in a time dependent manner. To further evaluate the importance of ROS, three antioxidants, NAC, GSH, and trolox, were employed to ascertain the role of oxidative stress signal in induction of apoptosis. Our results showed that blocking of oxidative stress signal by antioxidants effectively prevented cell apoptosis (Figure 6A–C and Supporting Information Figure S4). In addition, owing to the blocking of ROS signal, DNA strand damage, p53 activation, down-regulation of p-MDM2, and inhibition of AKT, ERK phosphorylation induced by MeSe and cisplatin were almost completely reversed. These results suggested that ROS acted as an essential upstream molecular messenger in the synergistic effect of MeSe to cisplatin.

In summary, our results suggest that MeSe potentiates apoptosis induced by cisplatin in HepG2 by the following mechanisms: MeSe acts as a pro-oxidant and induces oxidative stress, followed by AKT and ERK dephosphorylation and DNA damage, which triggers p53 signaling pathway and then enhances the activation of both mitochondrial and death receptor (Figure 6D). Collectively, our results demonstrate that MeSe is an effective synergistic agent to cisplatin, and MeSe might be a candidate of chemosensitizer for cisplatin-based therapy in clinical application.
Molecular Pharmaceutics

ASSOCIATED CONTENT

+$\text{Supporting Information}$

MeSe and cisplatin induce cytotoxicity in HepG2, MCF-7, and HK-2 cells. MeSe potentiates cisplatin-induced cytotoxicity in MCF-7 cells but not HK-2 cells. Synergistic activation of caspases by MeSe and cisplatin in HepG2 cells. Signaling molecules involved in apoptosis induced by MeSe and cisplatin in HK-2 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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