Disulfiram Chelated with Copper Promotes Apoptosis in Osteosarcoma via ROS/Mitochondria Pathway

Yelong Ren, a Yutian Lin, b Jinghao Chen, b and Yonglong Jin a,b

a Department of Anesthesiology, The First Affiliated Hospital of Wenzhou Medical University; Wenzhou, Zhejiang 325000, China; and b Department of Hand Surgery, The First Affiliated Hospital of Wenzhou Medical University; Wenzhou, Zhejiang 325000, China.

Received May 29, 2021; accepted July 26, 2021

INTRODUCTION

Osteosarcoma (OS), a primary malignant bone tumor, is most likely to occur in adolescents with strong aggressiveness, high degree of malignancy and high metastasis rate.1) With the advancement of surgical technology and the development of the new methods of adjuvant chemotherapy, the therapeutic outcome of patients with osteosarcoma and the 5-year survival rate have been obviously improved, but the recurrence rate of patients with postoperative is still high and the outcome is not very satisfactory. The removal of the cancer cells and avoidance of recurrence and metastasis are still difficult, which could bring patients heavy physical and mental pain and economic burden.2) Therefore, there is an urgent need for new therapies to improve the patients’ survival rate and inhibit postoperative recurrence and metastasis and to reduce patient sufferings and economic burden.

Mitochondria are the main sites for ATP metabolism in eukaryotic cells. In addition to providing energy for biological behaviors such as cell’s growth, proliferation and differentiation, they are also widely involved in the pathogenesis and development of apoptosis and tumor drug resistance.3) Once the mitochondrial function appeared disordered, it can rapidly lead to apoptosis. As a regulatory factor, reactive oxygen species (ROS) plays an important role in the progress of apoptosis of mitochondrial pathway.4) Under normal circumstances, the level of ROS in the body is in a dynamic equilibrium state, normal cellular physiological activities can produce a certain amount of ROS. When the level of ROS is slightly higher in the cell, it will be eliminated by various reactive oxygen scavengers in the cell. When various factors induce cell to produce excessive ROS, in the process of ROS treatment, the non-specific permeability transition pore in the mitochondrial inner membrane is opened, and the ions in the mitochondrial inner membrane gap enter the cytoplasmic matrix through the permeability transition pore, resulting in the disappearance of ion concentration gradient on both sides of the mitochondrial membrane, and the decrease of mitochondrial membrane potential (MMP).5) The release of apoptosis factors in the mitochondrial membrane triggers cell dysfunction, and ultimately leads to apoptosis and necrosis. Thus, ROS accumulation and MMP reduction play an important role in mitochondrial apoptotic pathway.

Disulfiram (DSF) is a member of the dithiocarbamates family. It can inhibit acetaldehyde dehydrogenase in liver, make ethanol oxidized into acetaldehyde in body and then can’t continue to decompose and oxidize, resulting in alcoholics’ aversion to alcohol, so it is widely used in clinical anti-alcoholism.6) Therefore, DSF is widely used to resist to alcoholism in clinical practice. At the same time, it has a strong force to chelate metal ions and new potential therapeutic uses for human cancers.7) Some studies have shown that the anti-tumor effect of DSF–Cu is related to the nuclear factor-kappaB (NF-κB) pathway and c-Jun N-terminal kinase (JNK) pathway.8,9) Moreover, because of its cheapness and high safety it has been attached more importance to its researches in the fields of anti-tumor activities in recent years. However, the anti-tumor mechanism of DSF–Cu is still not completely clear, especially in the effect of osteosarcoma, it has rarely been reported.

MATERIALS AND METHODS

Cell Culture and Reagents The human OS cell lines HOS, U2-OS (U2), I43B, SAOS-2and MG-63, and the murine spontaneous OS cell line K7M2 were obtained from the Cell Storage Centre of Wuhan University (Wuhan, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Invitrogen, Grand Island, NY, U.S.A.) supplemented with fetal bovine serum (FBS) (10%, Invitrogen), penicillin (100U/mL) and streptomycin (0.1mg/mL) maintained in a humidified atmosphere at 37°C containing 5% (v/v) CO₂. DSF

© 2021 The Pharmaceutical Society of Japan
and CuCl₂ were purchased from Sigma Chemical (Sigma-Aldrich, St. Louis, MO, U.S.A.). N-Acetyl-L-cysteine (NAC) and cyclosporine A (CsA) were purchased from Aladdin (Shanghai, China).

**Cell Proliferation and Cytotoxicity Assays** Cells were seeded into 96-well plates and treated with different drugs for 24 h. Then the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was added to plate for 2 h, the optical density (OD) values were measured using the SpectraMax microplate reader to the manufacturer’s instructions, the optical density (OD) assay kits (Beyotime Biotech Inc., Jiangsu, China). According to this result (0.2 µM DSF–CuCl₂ (100, 5 mg/kg per os (p.o.), qod) and PBS (equal volume). The tibial tumors were harvested 4 weeks after treatment and each tumor was weighed. Tumor sizes were calculated as volume (cm³) = [width² (cm²) × length (cm)]/2, and the sample was blocked by bovine serum albumin (BSA) then incubated with anti-Active-Caspase-3 (1:300, CST) and anti-mitochondrial fission factor (Mff) (1:200, Proteintech Group, Inc.) specific primary detection antibodies for overnight at 4 °C. The slides were then incubated with the secondary antibody (1:1000) for 2 h at room temperature. After washed with phosphate buffered saline (PBS), slides were re-stained with 4′,6-diamidino-2-phenylindole (DAPI) for 7 min. Fluorescence was observed by a Nikon confocal laser microscope (Nikon, AIPLUS, Tokyo, Japan), blue fluorescence, green fluorescence and red fluorescence. The excitation/emission wavelength and filter are 345/455 nm, UV-2A, 494/518 nm, B-2A and 550/570 nm, G-2A, respectively.

**Animals and Model** The athymic nude BALB/c male mice were procured from the Shanghai Laboratory Animal Center, of Chinese Academy of Science and maintained under specific pathogen-free conditions. The mice were inoculated with K7M2 cells (2 × 10⁶/mouse) via marrow cavity of the right tibia to establish tumor xenograft. After 7 d, those mice were randomly divided into two groups. The mice received y DSF–CuCl₂ (100, 5 mg/kg per os (p.o.), qod) and PBS (equal volume). The tibial tumors were harvested 4 weeks after treatment and each tumor was weighed. Tumor sizes were calculated as volume (cm³) = [width² (cm²) × length (cm)]/2, and the sample was blocked by bovine serum albumin (BSA) then incubated with anti-Active-Caspase-3 (1:300, CST) and anti-mitochondrial fission factor (Mff) (1:200, Proteintech Group, Inc.) specific primary detection antibodies for overnight at 4 °C. The slides were then incubated with the secondary antibody (1:1000) for 2 h at room temperature. After washed with phosphate buffered saline (PBS), slides were re-stained with 4′,6-diamidino-2-phenylindole (DAPI) for 7 min. Fluorescence was observed by a Nikon confocal laser microscope (Nikon, AIPLUS, Tokyo, Japan), blue fluorescence, green fluorescence and red fluorescence. The excitation/emission wavelength and filter are 345/455 nm, UV-2A, 494/518 nm, B-2A and 550/570 nm, G-2A, respectively.

**Statistical Analysis** All data are presented as mean ± standard error of the mean (S.E.M.). One-way ANOVA was used to test the significance of multiple groups and Student’s t-test was used to test differences between the two groups, and p-values <0.05 were defined as significance. All experiments were performed for at least three times.

**RESULTS**

**DSF–Cu Inhibit the Activity of Osteosarcoma Cell** As shown in Figs. 1A–E, the survival rate of OS cells was gradually decreased after DSF–Cu treatment and was dose-dependent. According to this result (0.2 µM DSF), 1 µM Cu was selected for the subsequent cell experiments. The morphology of the DSF–Cu-treated cells shrink in shape and in number (Figs. 1F, G). The invasion and migration of the OS cells was restricted by DSF–Cu (Figs. 1H–K) and the tumorigenesis
Fig. 1. DSF–Cu Inhibits the Activity of OS Cells

(A–E) Cell survival of osteosarcoma cells after different concentrations of DSF–Cu treatment. (F, G) Cellular morphology. (H, I) The migration and invasion of OS cells in different groups. (J, K) The wound-healing assay. (L) The Colony formation analysis. (M–O) The expression of c-caspase-3 protein in OS cells. (*p < 0.05 vs. control group. **p < 0.01 vs. control group. Values represent the mean ± S.E.M., each experiment repeated at least three times.)
Fig. 2. DSF–Cu Promotes Mitochondrial Dysfunction in OS Cells

(A–C) The JC-1 staining analysis of OS cell lines. (D) Mitochondrial fluorescent probe staining (LySOhtraker). (E, F) Immunofluorescence staining of Mff. (G) Represents TEM images of DSF–Cu-treated OS cells. (H–K) Western blot analysis. GAPDH was as the loading control for band density normalization. (*p < 0.05 vs. Control group. **p < 0.005 vs. Control group values represent the mean ± S.E.M., each experiment repeated at least three times.)
ability of OS cells was restrained by DSF–Cu (Fig. 1L). The Western blotting analysis of cleaved-caspase3 showed a significant higher expression of cleaved-caspase3 proteins in the DSF–Cu treatment group compared with the untreated controls group (Figs. 1M–O).

**DSF–Cu Induced Mitochondrial Dysfunction in Osteosarcoma Cell** When the mitochondrial membrane potential is high, JC-1 aggregates in the matrix of the mitochondria to

---

Fig. 3. DSF–Cu Induced Mitochondria-Dependent Apoptosis of OS Cells

(A–D) Detecting the expression levels of GSH and GPx in the OS cells in different groups by ELISA. (E–G) ROS fluorescence probe analysis. (H) The Colony formation analysis. (I–K) Immunofluorescence staining of c-caspase3. (L–P) Cell survival of osteosarcoma cells. (*p < 0.05 vs. Control group. **p < 0.005 vs. Control group. #p < 0.05 vs. DSF–Cu group. ##p < 0.005 vs. DSF–Cu group) values represent the mean ± S.E.M., each experiment repeated at least three times.)
form J-aggregates, which can produce red fluorescence; when the mitochondrial membrane potential is low, JC-1 cannot aggregate in the matrix of mitochondria. At this time, JC-1 is the monomer and can produce green fluorescence. The results of JC-1 staining of OS cells showed that in comparison with the untreated group, the green/red fluorescence ratio increased in the DSF–Cu group, (Figs. 2A–C). The mitochondrial probe (MitoTracker Red) showed that mitochondria becomes rounder after DSF–Cu treatment when compared with control group (Fig. 2D). Furthermore, immunostaining of Mff showed that the expression of Mff were higher in the DSF–Cu group compared with the control group (Figs. 2E, F). The expression of Bax protein showed a significant higher level than that in control group and the expression of BCL-2 and TOM20 proteins showed the adverse trend. Furthermore, we also found that DSF–Cu treatment promotes the release of Cytc from mitochondria into the cytoplasm of OS cells (Figs. 2H–K). To further investigate the effect of DSF on mitochondria in OS, we performed TEM analysis on OS cells, the TEM results showed that the mitochondria ridges of OS cells became blurred and edema after DSF–Cu treatment when compared with control group (Fig. 2G).

**DSF–Cu Induced Mitochondria-Dependent Apoptosis in Osteosarcoma Cell** For the reduction–oxidation (REDOX) system of OS cells, we have investigated the intracellular GSH and the activity of GPx of OS cells. The results revealed that DSF–Cu restrain the amount of intracellular GSH and the activity of GPx in OS cells, and NAC (GSH precursor antioxidants) reversed the trend (Figs. 3A–D). The DCFH-DA staining showed that The level of ROS in OS cells increased when
be exposed to DSF–Cu (Figs. 3E–G). The effects of DSF–Cu on tumorigenesis ability of OS cells was alleviated by NAC (Fig. 3H). The immunofluorescence analysis of cleaved-caspase3 showed a higher expression of cleaved-caspase3 proteins in the DSF–Cu treatment group compared with the DSF–Cu + CsA group (Figs. 3I–K). In order to further investigate the correlation between DSF–Cu-induced apoptosis and mitochondria in OS cells. We added Cyclosporine A or NAC to DSF–Cu treated OS cells and found that Cyclosporine A or NAC mitigate cell death caused by DSF–Cu (Figs. 3L–P).

The Effect of DSF–Cu on OS in Vivo As shown in Fig. 4A, we exerted the treatment process of tumor model in mice. The volume and weight charts of tumor (Figs. 4B–D) illustrated that the sizes of the tumor were increased in the untreated control group, compared with the DSF–Cu group. Hematoxylin and eosin (H&E) staining demonstrated that more tumor cell death in DSF–Cu group compared to untreated group (Fig. 4E) and no significant injury in multiple organs of mice after DSF–Cu treatment (Fig. 4G).

DISCUSSION

At present, there are some problems in the development of new molecular targeted drugs for osteosarcoma, such as high development cost and high price. It is undoubtedly a promising research direction to develop high-efficiency and low-toxic anti-tumor drugs from low-cost and low toxic and side effects of traditional drugs. For example, thalidamide, which was originally used for the treatment of sleep disorders, was later found to have good efficacy in the treatment of multiple myeloma and leprosy. And sildenafil, which is used for sexual dysfunction, is now used to treat pulmonary hypertension. And some studies have shown that intracellular GSH levels are associated with drug resistance in a variety of tumors, such as leukemia, colon cancer, lung cancer and so on. However, reducing the GSH levels in tumor cells by taking some measures can significantly increase the sensitivity of tumor cells to chemotherapeutic drugs and improve the effect of tumor chemotherapies. Our results suggested that DSF–Cu induces apoptosis of osteosarcoma cells accompanied by the decreasing the concentration of intracellular GSH and facilitating the opening of mitochondrial permeability transition pore (PT). These anti-tumor activities can be reversed by Cyclosporine A and NAC.

CONCLUSION

In Conclusion, this study mainly involves the specific molecular mechanism of oxygen free radicals and mitochondrial pathway in apoptosis of osteosarcoma cells which induced by DSF–Cu. DSF–Cu consumes the GSH in cells, promotes the generation of ROS, the openness of PT pores and releases cytochrome c into the cytoplasm, by inhibiting the activity of GPxs and finally activates the mitochondrial pathways. Caspase activation can cause the apoptosis of osteosarcoma cells.

Acknowledgments Funding from the program of Wenzhou Municipal Science and Technology Bureau (No. Y20160375).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Ritter J, Bielack SS. Osteosarcoma. Ann Oncol, 21 (Suppl. 7), vii320–vii325 (2010).
2) Isakoff MS, Bielack SS, Meltzer P, Gorlick R. Osteosarcoma: current treatment and a collaborative pathway to success. J Clin Oncol, 33, 3029–3035 (2015).
3) Amesley SJ, Fisher PR. Mitochondria in health and disease. Cells, 8, 680 (2019).
4) Yang Y, Karakhanova S, Hartwig W, D’Haese JG, Philippov PP, Werner J, Bazhin AV. Mitochondria and mitochondrial ROS in cancer: novel targets for anticancer therapy. J Cell Physiol, 231, 2570–2581 (2016).
5) Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev, 94, 909–950 (2014).
6) Mutschler J, Grosshans M, Soyka M, Rösner S. Current findings and mechanisms of action of disulfiram in the treatment of alcohol dependence. Pharmacopsychiatry, 49, 137–141 (2016).
7) Su ZF, Shukla S, Ambudkar SV. Disulfiram, an old drug with toxic ROS,16,17 Therefore, we infer that the anti-osteosarcoma activity of DSF–Cu may be achieved by increasing the level of ROS. As we expected, our studies show that DSF–Cu can promote the increase of the level of ROS, induce the decline of MMP, lead to changes in mitochondrial function, and then induce the apoptosis of osteosarcoma cells. GSH also plays a key role in the balance between apoptosis and survival of tumor cells. As an early event in the process of apoptosis, GSH depletion will promote the occurrence of apoptosis.18 A study has shown that the apoptosis of gastric cancer accompanied by glutathione decrement via activating mitochondrial dependent pathway.19 There are some studies which have also shown that intracellular GSH levels are associated with drug resistance in a variety of tumors, such as leukemia, colon cancer, lung cancer and so on. However, reducing the GSH levels in tumor cells by taking some measures can significantly increase the sensitivity of tumor cells to chemotherapeutic drugs and improve the effect of tumor chemotherapies. Our results suggested that DSF–Cu induces apoptosis of osteosarcoma cells accompanied by the decreasing the concentration of intracellular GSH and facilitating the opening of mitochondrial permeability transition pore (PT). These anti-tumor activities can be reversed by Cyclosporine A and NAC.
new potential therapeutic uses for human cancers and fungal infections. Mol. Biosyst., 1, 127–134 (2005).

8) Viola-Rhenals M, Patel KR, Jaimies-Santamaria L, Wu G, Liu J, Dou QP. Recent advances in antabuse (disulfiram): the importance of its metal-binding ability to its anticancer activity. Curr. Med. Chem., 25, 506–524 (2018).

9) Zha J, Chen F, Dong H, Shi P, Yao Y, Zhang Y, Li R, Wang S, Li P, Wang W, Xu B. Disulfiram targeting lymphoid malignant cell lines via ROS-JNK activation as well as Nrf2 and NF-kB pathway inhibition. J. Transl. Med., 12, 163 (2014).

10) Ekinci E, Rohondia S, Khan R, Dou QP. Repurposing disulfiram as an anti-cancer agent: updated review on literature and patents. Recent Pat. Anticancer Drug Discov., 14, 113–132 (2019).

11) Pohanka M. Copper and copper nanoparticles toxicity and their impact on basic functions in the body. Bratisl. Lek Listy, 120, 397–409 (2019).

12) Dalecki AG, Crawford CL, Wolschendorf F. Copper and antibiotics: discovery, modes of action, and opportunities for medicinal applications. Adv. Microb. Physiol., 70, 193–260 (2017).

13) Li H, Wang J, Wu C, Wang L, Chen ZS, Cui W. The combination of disulfiram and copper for cancer treatment. Drug Discov. Today, 25, 1099–1108 (2020).

14) McMahon A, Chen F, Li F. Old wine in new bottles: advanced drug delivery systems for disulfiram-based cancer therapy. J. Control. Release, 319, 352–359 (2020).

15) Moloney JN, Cotter TG. ROS signalling in the biology of cancer. Semin. Cell Dev. Biol., 80, 50–64 (2018).

16) Wadhwa S, Mumper RJ. D-penicillamine and other low molecular weight thiols: review of anticancer effects and related mechanisms. Cancer Lett., 337, 8–21 (2013).

17) Li Y, Chen F, Chen J, Chan S, Zhang G. Disulfiram/copper induces antitumor activity against both nasopharyngeal cancer cells and cancer-associated fibroblasts through ROS/MAPK and ferroptosis pathways. Cancers (Basel), 12, 138 (2020).

18) Bansal A, Simon MC. Glutathione metabolism in cancer progression and treatment resistance. J. Cell Biol., 217, 2291–2298 (2018).

19) Wang J, Deng H, Zhang J, Wu D, Li J, Ma J, Dong W. alpha-Hederin induces the apoptosis of gastric cancer cells accompanied by glutathione decrement and reactive oxygen species generation via activating mitochondrial dependent pathway. Phytother. Res., 34, 601–611 (2020).

20) Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronato MA, Marinari UM, Domenicotti C. Role of glutathione in cancer progression and chemoresistance. Oxid. Med. Cell. Longev., 2013, 972913 (2013).

21) Horiyama S, Takahashi Y, Hatai M, Honda C, Suwa K, Ichikawa A, Yoshikawa N, Nakamura K, Komitomo M, Date S, Masujima T, Takayama M. Methyl vinyl ketone, a toxic ingredient in cigarette smoke extract, modifies glutathione in mouse melanoma cells. Chem. Pharm. Bull., 62, 722–728 (2014).

22) Yu Y, Fan SM, Song JK, Tashiro S, Onodera S, Itojima T. Hydroxyl radical (OH) played a pivotal role in oridonin-induced apoptosis and autophagy in human epidermoid carcinoma A431 cells. Biol. Pharm. Bull., 35, 2148–2159 (2012).