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A binuclear complex constituted by diethyldithiocarbamate and copper(I) functions as a proteasome activity inhibitor in pancreatic cancer cultures and xenografts ☆

Jinbin Han [a,b,c,*,1], Luming Liu [a,b,**,1], Xiaoqiang Yue [d], Jinjia Chang [b,e], Weidong Shi [a,b], Yongqiang Hua [a,b]

[a] Department of Integrative Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China
[b] Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China
[c] Shanghai Clinical Center, Chinese Academy of Sciences/Xuhui Central Hospital, Shanghai 200031, China
[d] Department of Traditional Chinese Medicine, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
[e] Department of Medical Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China

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ABSTRACT

It is a therapeutic strategy for cancers including pancreatic to inhibit proteasome activity. Disulfiram (DSF) may bind copper (Cu) to form a DSF–Cu complex. DSF–Cu is capable of inducing apoptosis in cancer cells by inhibiting proteasome activity. DSF is rapidly converted to diethyldithiocarbamate (DDTC) within bodies. Copper(II) absorbed by bodies is reduced to copper(I) when it enters cells. We found that DDTC and copper(I) could form a binuclear complex which might be entitled DDTC–Cu(I), and it had been synthesized by us in the laboratory. This study is to investigate the anticancer potential of this complex on pancreatic cancer and the possible mechanism. Pancreatic cancer cell lines, SW1990, PANC-1 and BXPC-3 were used for in vitro assays. Female athymic nude mice grown SW1990 xenografts were used as animal models. Cell counting kit-8 (cck-8) assay and flow cytometry were used for analyzing apoptosis in cells. A 20S proteasome assay kit was used in proteasome activity analysis. Western blot (WB) and immunohistochemistry (IHC) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used in tumor sample analysis. The results suggest that DDTC–Cu(I) inhibit pancreatic cancer cell proliferation and proteasome activity in vitro and in vivo. Accumulation of ubiquitinated proteins, and increased p27 as well as decreased NF-κB expression were detected in tumor tissues of DDTC–Cu(I)-treated group. Our data indicates that DDTC–Cu(I) is an effective proteasome activity inhibitor with the potential to be explored as a drug for pancreatic cancer.

Introduction

Cancer cells are more sensitive than normal cells to proteasome inhibition, and the proteasome-mediated degradation pathway is considered an important target for cancer treatment (Chen et al., 2006). The proteasome inhibitor bortezomib has been reported to inhibit tumor activity in a variety of cancer models, which was approved by the US Food and Drug Administration (FDA) for clinical treatment in multiple myeloma (MM) (Kane et al., 2006), bringing investigator confidence on inhibition of this pathway. Though copper (Cu) is an essential cofactor for tumor angiogenesis processes, some copper complexes demonstrate the capacity to inhibit proteasome activity, inducing apoptosis in various types of human cancer cells. Disulfiram (DSF) used clinically as an alcohol deterrent in the USA, is capable of binding copper to form a complex named DSF–Cu which acts as a proteasome inhibitor preferable to cancer cells (Chen et al., 2006).

Copper is largely absorbed from the stomach and the small intestine. Absorbed copper is transported to the liver in portal blood bound to albumin and is transmitted to peripheral tissues mainly bound to ceruloplasmin and, to a lesser extent, albumin. During the process of cellular copper uptake, copper enters the cell through various transmembrane transporters and copper(II) is reduced to copper(I) (Knopfel and Solioz, 2002; McKie et al., 2001). In vivo, DSF is rapidly converted to its reduced metabolite, DDTC (Escarabajal and Aragon, 2003; Pike et al., 2001), and if copper ions are available, DDTC–Cu(I) or –Cu(II) complex will be formed (Fig. 1). As other investigators described (Chen et al., 2006; Iljin et al., 2009), the activity of DSF to inhibit the cellular proteasome in vivo might be attributed to DDTC–Cu complexes.

* Correspondence to: J. Han, 966 Middle Huaihai Road, Shanghai 200031, China. Fax: +86 2154033969.
** Correspondence to: L. Liu, Department of Integrative Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China. Fax: +86 2164049440.
E-mail addresses: llm1010@163.com (L. Liu), hanjinbin@gmail.com (J. Han).

[1] The first two authors contribute equally to this work.

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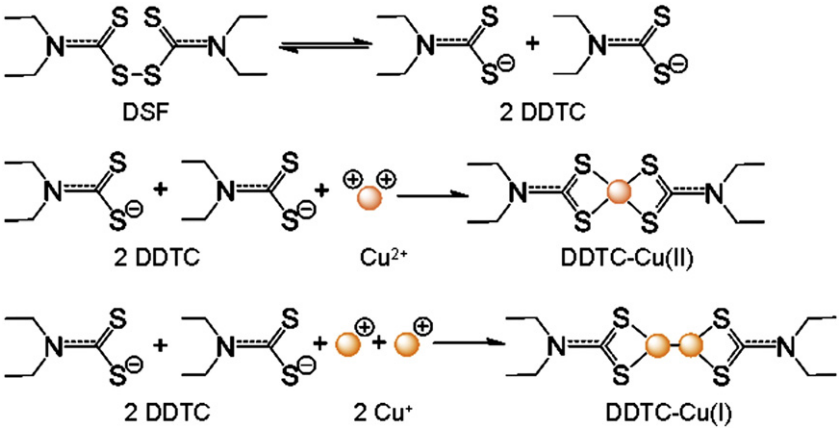

Fig. 1. Disulfiram (DSF) molecule can undergo de-oxidation and transform into diethyldithiocarbamate (DDTC) which can react with metals to form coordination complexes.

We hypothesized that DDTC–Cu(I) play a critical role as a proteasome activity inhibitor in cancer cells The present study is focused on whether DDTC–Cu(I) has the potential to induce apoptosis in pancreatic cancer cultures and xenografts by inhibiting proteasome activity. Though DDTC–Cu complex has been reported by other investigators before (Pang et al., 2007), their studies described the copper(II) complex mainly and this article is the first to describe the copper(I) complex especially to the best of our knowledge.

Materials and methods

Cell lines and reagents. The human pancreatic cancer cell lines, SW1990, PANC-1 and BXPC-3, were originally obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37 °C and 5% CO_2, and used at early passage numbers (passage 3–6). Sodium diethyldithiocarbamate trihydrate ($C_5H_{10}NS_2 \cdot Na \cdot 3H_2O$), copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), and sodium sulfite (Na_2SO_3) were purchased from Sigma-Aldrich Corp. (MO, USA). The 20S proteasome assay kit was purchased from Enzo Life Sciences, Inc. (NY, USA). The rabbit polyclonal antibodies against human ubiquitin or NF-κB p50, and a mouse monoclonal antibody against human p27 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). RPMI 1640 and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA).

Chemical synthesis. The DDTC–Cu(I) complex for cell or animal treatment was synthesized at a concentration of 1 mmol/L as per the quantity equivalent to the molar quantity of DDTC or copper (all the concentrations to describe DDTC–Cu(I) were equivalent to the molar quantity of DDTC or copper in this article). First, sodium diethyldithiocarbamate trihydrate or copper chloride dihydrate as well as sodium sulfite was dissolved in sterilized water to produce a solution of 200 mmol/L concentration respectively. Second, a total of 50 μL of a sodium diethyldithiocarbamate solution, 25 μL of a sodium sulfite and 50 μL of a copper chloride which were prepared in the first step were added to 9 mL of sterilized water orderly and mixed gently. The third, sterilized water was then added to the prepared solution to ensure that the solution had a total volume of 10 mL, and the solution was gently mixed again. Finally, the complex solution was packed and stored at 4 °C.

Cell proliferation assay. Cellular proliferation was assessed by quadruplicate plating at a density of 5000 cells per well in a 96-well plate. The designated concentrations of fresh media containing the drugs or the vehicle were added to cell cultures containing the standard growth media for that cell line on the morning after plating for 12 h once the cells had attached. Viable cells were quantified after the 24-hour treatments using cell counting kit-8 (cck-8) reagent (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. The cck-8 solution (10 μL) was added to each well, followed by incubation for 2 h at 37 °C. The absorbance at 450 nm was determined using an ELx800 Absorbance Microplate Reader (Bio-TEK Instruments Inc., Winooski, VT, USA). Cell viability was expressed as a percentage of that of the control (untreated) cells. For each concentration of the complex, the mean value of the mean absorbance from four wells was calculated. IC_{50} value was calculated from a sigmoidal dose–response curve fit using Prism GraphPad 5 Demo (GraphPad Software, CA, USA).

Flow cytometric apoptosis assay. An Alexa Fluor® Annexin V/Dead Cell Apoptosis kit (Invitrogen, CA, USA) was used, according to the manufacturer's instructions, to distinguish and quantitatively determine the percentage of apoptotic cells after treatment by the drugs. Cells were seeded at a density of 4×10^5 cells/mL in 6-well plates. After treatment, the cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in 100 μL of binding buffer. A total of 5 μL of Annexin V and 10 μL of propidium iodide (PI) were added, and the mixture was incubated for 30 min in the dark. Finally, 400 μL of binding buffer was added to the cells. The labeled cells (10,000 per sample at least) were analyzed by measuring the fluorescence intensity using a FC500 MPL cytometer (Beckman Coulter Inc., Brea, CA, USA) in conjunction with CXP analysis software (Beckman Coulter Inc.).

20S proteasome activity assay. A total of 17.5 ng of 20S proteasome (human) was incubated in 100 μL of assay buffer with or without different concentrations of the DDTC–Cu(I) complex, copper chloride, sodium sulfite, DDTC, or their combinations, and 10 μmol/L of the fluorogenic peptide substrate Suc-LLVY-AMC for 2 h at 37 °C. After incubation, the production of hydrolyzed AMC groups was measured with a Synergy H4 Hybrid Multi-Mode Microplate Reader (Bio-TEK Instruments Inc., Winooski, VT, USA) with an excitation filter of 365 nm and an emission filter of 460 nm. Whole-cell extracts (10 μg) of cells treated as indicated were incubated for 1 h at 37 °C in 100 μL of assay buffer with 20 μmol/L fluorogenic substrate (Chen et al., 2006).

Xenograft experiment. Five-week-old female athymic nude mice were purchased from the Shanghai Laboratory Animal Center (Chinese Academy Science) and housed under specific pathogen-free conditions according to Fudan University animal care guidelines. The experimental animal protocols were reviewed and approved by the animal care committee of the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences and the Fudan University Shanghai Cancer Center. Mice were maintained in laminar flow rooms under constant temperature and humidity.