

Dual Action of Acidic Microenvironment on the Enrichment of the Active Metabolite of Disulfiram in Tumor Tissues[§]

Chongzhuang Tang, Xiaoyan Pang, Zitao Guo, Runcong Guo, Lu Liu, and Xiaoyan Chen

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, P.R. China (C.T., X.P., Z.G., R.G., L.L., X.C.) and University of Chinese Academy of Sciences, Beijing, China (C.T., R.G., L.L., X.C.)

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ABSTRACT

Disulfiram, an antialcoholism drug, could potentially be repurposed as an anticancer drug because of the formation of copper(II) diethyldithiocarbamate (CuET) from dithiocarb (DTC, a reduced metabolite of disulfiram) and Cu²⁺. CuET exhibited preferential distribution to tumor tissues. This study investigated the mechanism of CuET accumulation in tumor tissues by employing MDA-MB-231 human breast cancer cells. The concentration of CuET in cells treated with DTC and Cu²⁺ in acidic culture medium (pH 6.8) was significantly higher than that of the control group (pH 7.4). Subsequently, the effects of pH on the uptake of DTC, Cu²⁺, and CuET were investigated separately. The acidic environment significantly increased the uptake rate of DTC and Cu²⁺ but had no effect on CuET. MDA-MB-231 cells overexpressing copper transporter hCTR1 were constructed to evaluate its intermediate role in CuET accumulation. After treatment with CuCl₂ followed by DTC for 15 minutes, the levels of CuET and Cu²⁺ in hCTR1-overexpressed cells were 2.5 times as much as those of vector group. In the tumors of cancer xenograft models constructed by hCTR1-MDA-MB-231

cells, the concentrations of CuET and Cu were also significantly higher than those of control group. In conclusion, the acidic microenvironment of tumors can promote the enrichment of CuET in tumors through dual action. On the one hand, it can promote transmembrane transport of DTC by converting ionic DTC into molecular state. On the other hand, it enhances Cu²⁺ uptake by activating hCTR1, which ultimately leads to the enrichment of CuET.

SIGNIFICANCE STATEMENT

Increasing evidence suggests that the antitumor activity of disulfiram is related to the formation of a copper(II) diethyldithiocarbamate (CuET) of its reducing metabolite dithiocarb with copper(II) ion, which is preferentially distributed in tumor tissues. We showed that the acidic microenvironment, a common feature of many solid tumor tissues, could promote intracellular CuET accumulation through dual action without changing CuET uptake. This result is helpful for the formulation of clinical dosage regimens of disulfiram in cancer treatment.

Introduction

Disulfiram has been clinically used as an alcohol-deterrent agent for decades (Kranzler and Soyka, 2018). It alters the intermediary metabolism of alcohol (Petersen, 1992) by inhibiting aldehyde dehydrogenase (Veverka et al., 1997). Disulfiram is a relatively nontoxic substance when administered alone. However, when alcohol is consumed after administration of disulfiram, blood acetaldehyde concentrations are markedly increased, resulting in flushing, systemic vasodilation, respiratory difficulties, nausea, hypotension, and other symptoms (Fuller et al., 1986).

In recent years, accumulating evidence indicated that disulfiram showed high selectivity against different cancers with low cost and few side effects (Chen and Dou, 2008). More than 15 clinical trials (data obtained from *Clinicaltrials.gov*) have been or are being prepared to

evaluate the inhibitory effects of disulfiram alone or in combination with other drugs, such as copper gluconate, on different tumors, including breast cancer (NCT03323346\NCT04265274), melanoma (NCT00571116\NCT00256230), glioblastoma (NCT02678975), and prostate cancer (NCT02963051), etc. Disulfiram exerts antitumor effects by suppressing different cancer-associated pathways, including reactive oxygen species, phosphatidylinositol 3-kinase, mitogen-activated protein kinase, nuclear factor- κ B, aldehyde dehydrogenase, and epidermal growth factor receptor/Src/vascular endothelial growth factor (Liu et al., 2012; Skrott et al., 2017; Viola-Rhenals et al., 2018). The currently accepted mechanism is that dithiocarb (DTC), the reductive metabolite of disulfiram, can chelate with copper(II) ions in vivo to form a DTC-copper complex [copper(II) diethyldithiocarbamate (CuET)] (Fig. 1). CuET shows suppressing effects as nuclear protein localization protein 4, an adaptor of p97 segregase, which is essential for the turnover of proteins involved in multiple regulatory and stress-response pathways in cells. Interestingly, after oral administration of disulfiram and copper gluconate, CuET exhibits preferential tissue accumulation in tumors of the xenografted mice constructed with MDA-MB-231 cells (Skrott et al., 2017). To date, the accumulation mechanism of CuET in tumors is not fully elucidated.

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ABBREVIATIONS: CCK-8, Cell Counting Kit-8; CuET, copper(II) diethyldithiocarbamate; DMEM, Dulbecco's modified Eagle's medium; DSF, disulfiram; DTC, dithiocarb; HBSS, Hanks' balanced salt solution; hCTR1, human copper transporter; hGAPDH, halophilic glyceraldehyde 3-phosphate dehydrogenase; ICP-MS, inductively coupled plasma mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; m/z, mass-to-charge ratio; NCT, National Clinical Trial; OATP, organic anion transporting polypeptide; PCR, polymerase chain reaction.

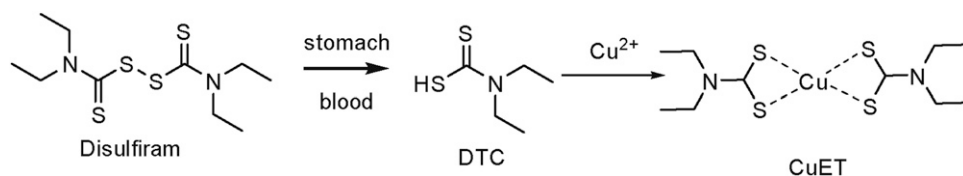


Fig. 1. Scheme of the production of CuET, a copper-chelated metabolite of disulfiram.

High glycolytic activity and hypoxia of solid tumors lead to increased production and secretion of H^+ and lactate to the extracellular space. Noninvasive measurements have shown that extracellular pH ranges from 6.5 to 6.9, whereas intracellular pH remains neutral to alkaline, producing an acid-outside pH gradient typically not observed in normal tissues (Wojtkowiak et al., 2011). The tumor acidic microenvironment may be related to the uptake and cytotoxicity of antitumor drugs, especially acidic or basic drugs, by altering their charged state and lipophilicity. Although CuET is a neutral compound, one of its components, DTC, has an acidic chemical group, and the transmembrane transporter activity of the other component Cu^{2+} can also be affected by the acidic environment. Therefore, we speculated that the acidic microenvironment of the tumor may cause CuET accumulation in tumor tissues.

In this study, the mechanism of CuET accumulation was investigated by employing MDA-MB-231 human breast cancer cells, which could provide new ideas for drug design and a basis for exploring the mechanism of chelator antitumor drugs, such as disulfiram.

Materials and Methods

Materials. Human breast cancer cell line MDA-MB-231 was supplied by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Corning, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, Sacramento, CA) and antibiotics [penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$)] (Gibco, New York) at 37°C in a 5% CO_2 humidified atmosphere. CuET, DTC, disulfiram, clopidogrel, and formic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Hanks' balanced salt solution (HBSS) and lactic acid were supplied by Dalian Meilun Biotechnology Co., Ltd. (Dalian, China).

Effect of pH on the Uptake of Disulfiram, DTC, DTC/ Cu^{2+} , and CuET in MDA-MB-231 Cells. MDA-MB-231 cells were seeded into the wells of 24-well BD BioCoat poly-D-lysine-coated plates (BD Biosciences, Bedford, MA) at a density of 1×10^5 cells per well to evaluate the effect of pH on the uptake of disulfiram, DTC, DTC/ Cu^{2+} , and CuET in these cells. Uptake studies were conducted 2 days after seeding when the cells had grown to confluence.

Before the in vitro uptake experiment, the monolayers of MDA-MB-231 cells in 24-well plates were washed three times with 0.6 ml of prewarmed HBSS (pH 7.4) without serum. After preincubation with 300 μl of HBSS for 15 minutes at 37°C , the equilibration buffer was removed. Afterward, the cell monolayers were incubated separately with HBSS buffer of different pH (pH 7.4 or 6.8) containing 3 μM disulfiram, 3 μM DTC, 6 μM DTC/3 μM CuCl_2 , or 3 μM CuET for 15 minutes, respectively. The pH of the medium was adjusted by dropwise addition of 0.25 M lactic acid. After 15 minutes of incubation, the uptake experiments were terminated by aspirating the incubation medium and washing the cells three times with ice-cold HBSS.

Because of the instability of the free sulfhydryl group in the structure of DTC, chemical derivatization method using iodoacetamide as derivatization reagent was conducted to block the oxidation of sulfhydryl group to determine DTC. An aliquot of 200 μl of acetonitrile containing iodoacetamide (1 mM) was directly added to the wells treated with DTC, and the intracellular DTC was derivatized into 2-amino-2-oxoethyl DTC by a 30-minute reaction with gentle shaking. $[\text{M} + \text{Na}]^+$ ion at mass-to-charge ratio (m/z) 229.045 of the final derivatized product was detected by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. Considering that disulfiram could be rapidly reduced to DTC in the physiologic environment and in cells, the intracellular level of disulfiram was measured in the same manner as DTC.

Cells treated with DTC/ CuCl_2 or CuET were directly disrupted using precooled acetonitrile at -20°C for the determination of CuET. After 3 minutes of shaking, cell lysates were centrifuged at 4°C for 5 minutes, and the supernatant was directly analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Given that DTC and Cu^{2+} could easily form CuET, the concentrations of CuET in the dosing solutions of DTC and CuCl_2 were analyzed directly in two pH media to exclude the possibility that the acidic environment in the solution enhances the formation of CuET.

For cell counting, duplicate samples were performed in parallel following the same procedure, except that 300 μl of deionized water was used as the lysate in the multigelation method. The protein content of solubilized cells was measured using a bicinchoninic acid protein assay kit (Dalian Meilun Biotechnology Co., Ltd.).

Cytotoxicity Assays. Cytotoxicity assays were performed by seeding MDA-MB-231 cells (1×10^4 cell/ cm^2) on 96-well culture plates. Twenty-four hours after plating, the growth medium was replaced with 100 μl of fresh culture medium containing various concentrations of DTC and CuCl_2 (0, 0.625, 1.25, 2.50, 5.00, 10.0, and 20.0 μM DTC with 3.00 or 0 μM CuCl_2 and 0.625, 1.25, 2.50, 5.00, and 10.0 μM CuCl_2 with 3.00 or 0 μM DTC at pH 7.4 and 6.8). Cell viability was assessed after 24 hours treatment using Cell Counting Kit-8 (CCK-8) (Dalian Meilun Biotechnology Co., Ltd.). After removing the medium, CCK-8 solution was added to each well at a final concentration of 10% (v/v) and incubated for 60 minutes at 37°C . Absorbance at 450 nm was determined and used for the measurement of the proportion of surviving cells. The experiments were performed in triplicate, and data were fitted to a dose-response curve using GraphPad Prism 6.01 software.

Effects of Conditions Affecting hCTR1 Activity on CuET Uptake. Based on Lee et al.'s (2002) work, extracellular acidic pH could stimulate the hCTR1-mediated active transport of copper, which is one of the main materials to form CuET. Therefore, the effects of factors affecting hCTR1 activity on CuET accumulation in tumors were evaluated. Several cell uptake media including acidic medium (pH 6.8), high- K^+ medium (145 mM), and ascorbic acid (1 mM) were prepared by adding HCl (1 M), KCl, and ascorbic acid to HBSS, respectively. MDA-MB-231 cells plated in 24-well plate were treated with a mixture of 3 μM DTC and 3 μM CuCl_2 that was prepared in the above medium for 15 minutes. To further prove that the acidic environment promotes the accumulation of CuET by increasing the uptake of Cu^{2+} , 3 μM CuCl_2 instead of the mixture of DTC and CuCl_2 was formulated in the above-mentioned cell uptake medium and incubated with the cells for 15 minutes. Then, the medium was removed and replaced with HBSS containing 3 μM DTC. Finally, the samples were prepared by sample pretreatment method mentioned above to obtain the supernatant of the cell lysate, and the intracellular CuET was measured by LC-MS/MS.

Establishment of Stable hCTR1-Expressing Cell Lines Using Lentiviral Vector. To prove that hCTR1 stimulation in an acidic environment made a contribution to the accumulation of CuET in cancer cells by promoting the uptake of copper ions, MDA-MB-231 cells overexpressing hCTR1 were constructed to simulate the activation of hCTR1. The hCTR1 lentivirus was custom-made by Cyagen Biosciences (Suzhou, China). Briefly, the hCTR1 (GenBank sequence U83460.1) coding sequence was subcloned into GenTarget's lentiviral expression vector. The subcloned insert was constitutively expressed under an elongation factor 1 α promoter. The vector contains a GFP-puromycin resistance fusion gene under the control of a CMV promoter as a dual marker. The sequence-verified lentiviral expression vector was cotransfected with third-generation lentiviral helper plasmids into 293T cells. One day before infection with lentiviral vector, cells were seeded on six-well plates at a density of 5×10^5 cells/well. The cells were infected by exposing the cell monolayer to the lentivirus for 6 hours in the presence of Polybrene (5 $\mu\text{g}/\text{ml}$; Sigma) with gentle shaking every 30 minutes. After 6 hours of incubation, lentivirus-infected cells were added to the complete medium and grown overnight. Two days postinfection, GFP

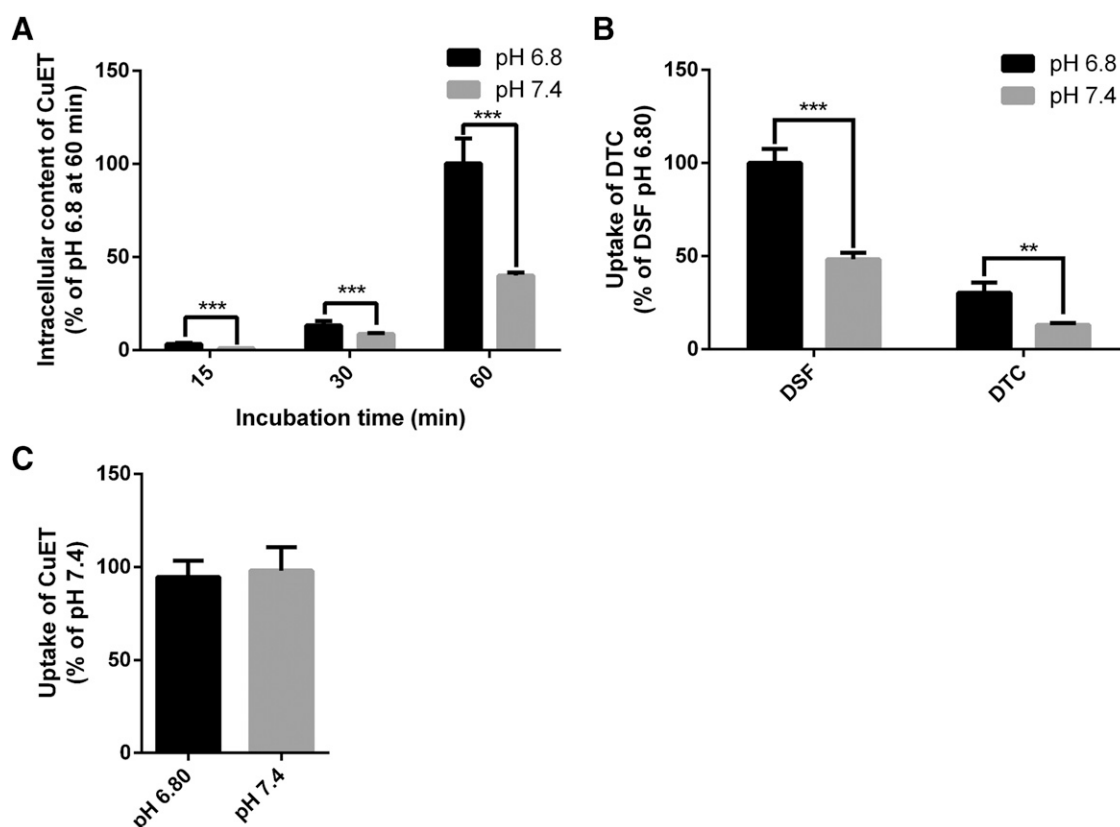


Fig. 2. Uptake of test compounds in MDA-MB-231 cells after incubation with DTC and CuCl₂ (A), DSF or DTC (B), and CuET (C) at pH 7.4 and pH 6.8. Data are expressed as mean \pm S.D. ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-tailed Student's t test).

expression was observed by fluorescence microscopy, and positive clones (hCTR1-MDA-MB-231) were selected using puromycin (1 μ g/ml).

Successive Treatment of hCTR1- and Vector-MDA-MB-231 Cells with CuCl₂ and DTC. hCTR1- and vector-MDA-MB-231 cells were seeded in 24-well plates at the same cell density and cultured for about 48 hours to reach about 90% cell confluence. The original medium was aspirated and replaced with DMEM containing 3 μ M CuCl₂. Three batches of cells were incubated for different times (0, 0.25, 0.50, 2.0, 4.0, and 8.0 hours). Then, the medium was aspirated, and the first batch of cells was washed three times with 0.6 ml of PBS buffer. Adherent cells were scraped with a rubber policeman after adding 0.2 ml of deionized water, and then the copper concentrations in the cells were quantitated by inductively coupled plasma mass spectrometry (ICP-MS). The second batch of cells was washed three times with 0.6 ml DMEM and incubated with 3 μ M DTC for 15 minutes. After incubation, the cells were washed three times with 0.6 ml of PBS buffer, vortexed with precooled acetonitrile for 3 minutes, and centrifuged for LC-MS/MS analysis. The third batch of cells was added with 300 μ l deionized water. After three freeze-thaw cycles, the bichinchonic acid method was used to determine the total protein concentration to be used as a correction for the above two experiments.

Relative Contribution of Extracellular and Intracellular Formation of CuET. To elucidate whether intracellular CuET was generated from CuET formed in the extracellular fluid and then transported into the intracellular compartment or formed intracellularly after the separate uptake of DTC and Cu²⁺, the following experiments were carried out. In acidic (pH 6.8) or neutral (pH 7.4) HBSS uptake media, a batch of hCTR1- and vector-MDA-MB-231 cells was treated with a mixture of 3 μ M DTC and 3 μ M CuCl₂ for 15 minutes. Another batch of cells was treated with 3 μ M CuCl₂ for 15 minutes, washed with HBSS, treated with DTC at the same concentration for 15 minutes, and then processed according to the above sample preparation method. The obtained supernatant was used for intracellular CuET determination.

Mouse Tumor Model and Treatment Regimens. All animal studies were approved by the Institutional Animal Care and Use Committee of Shanghai Institute of Materia Medica. Female BALB/c nude mice (9 weeks old, 18–20 g)

were purchased from Sipper-BK Laboratory Animal Co., Ltd. (Shanghai, China). hCTR1-MDA-MB-231 and vector-MDA-MB-231 tumor cells (5×10^6) suspended in a mixture of serum-free cell culture and Matrigel (BD Biosciences) at a ratio of 50/50 (v/v) were injected subcutaneously (0.2 ml) on the right and left flanks of female BALB/c nude mice. When tumor volumes were greater than 200 mm³, mice were orally administered with 6 mg/kg copper gluconate in the morning and 50 mg/kg disulfiram in the evening, which was consistent with the clinical dosing regimen. After consecutive dosing for 15 days, the mice were sacrificed, and 200 μ l of whole blood was collected from the inferior vena cava. The blood sample was immediately centrifuged at 14,000g to obtain plasma at 4°C. In addition, the tumor tissues on the left and right sides of mice were removed separately and cut into small pieces (50 ± 5 mg) by scissors in the same position. The tumor tissue mass was placed into a homogenizer tube containing ceramic beads and five times the volume of prechilled acetonitrile. Then it was homogenized with a Precellys Evolution homogenizer (Bertin Instruments, Montigny-le Bretonneux, France) at 4°C. The plasma samples were mixed with prechilled acetonitrile and vortexed for 3 minutes. The mixture and tumor homogenate samples were centrifuged at 4°C for 5 minutes, and then the supernatant was transferred to LC-MS/MS directly to determine CuET.

Determination of CuET in Cell and Tissues by LC-MS/MS. CuET was determined using a high performance liquid chromatography (Agilent 1260 Infinity; Agilent Technologies Inc.) coupled with a tandem mass spectrometer (MS/MS, Agilent 6495, Agilent Technologies Inc., Santa Clara, CA, USA). Chromatographic separation was achieved on an inert chromatographic column made from polyetheretherketone instead of stainless steel for the hardware and filled with C₁₈ by Dr. Maisch GmbH (Beim Brückle, Germany). The mobile phase was a mixture of 0.1% formic acid in water (A) and acetonitrile (B) at a ratio of 35:65 (v/v). The column temperature and flow rate were set at 40°C and 0.4 ml/min, respectively. Multiple reaction monitoring (m/z 360.9 \rightarrow 116.0 for CuET and m/z 322.2 \rightarrow 212.1 for clopidogrel, internal standard) was used in the positive electrospray ionization mode. The mass spectrometry parameters employed for the detection of the analyte and clopidogrel were as follows: drying gas temperature, 200°C; drying gas flow, 14 L/min; nebulizer pressure, 30 psi;

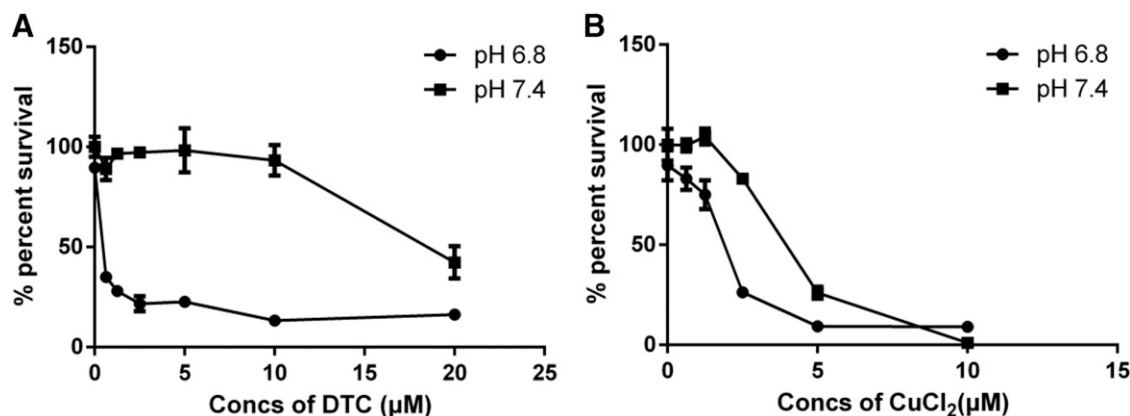


Fig. 3. DTC induces Cu^{2+} -dependent apoptosis at pH 6.8 and pH 7.4. (A) 0, 0.625, 1.25, 2.50, 5.00, 10.0, 20.0 μM DTC with 3.00 μM CuCl_2 (B) 0, 0.625, 1.25, 2.50, 5.00, 10.0 μM CuCl_2 with 3.00 μM DTC.

sheath gas temperature, 325°C; sheath gas flow, 11 l/min; and capillary voltage, 4000 V. The standard curve range was 0.50–100 ng/ml for CuET in tissues and cell lysates.

mRNA Analysis. The mRNA levels of hCTR1 were measured using real-time quantitative PCR. Total cell RNAs were extracted using RNeasy Pure Tissue Kit (TIANGEN, China). cDNA was synthesized using Primescript RT Reagent Kit with gDNA Eraser (Takara, Japan) and oligos from Genewiz (China). The reaction was performed in a volume of 25 μl containing double distilled H_2O , cDNA, corresponding primer, dye, and SYBR Premix Ex Taq (Takara, Japan) on an ABI7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The PCR amplification conditions were 94°C for 5 minutes, 94°C for 15 seconds, and 60°C for 45 seconds for 40 cycles. The sequences of primers were as follows: hCTR1 (forward) 5'- GAGAGCCTGCTGCGTAAGTC -3', (reverse) 5'- CTGTTTGACAGGAGGTGAGGA -3'; and hGAPDH (forward) 5'- GGTGTGAACCATGAGAAGTATGA -3', and (reverse) 5'- GAGTCCTTCCACGATACC AAAG -3'. PCR products were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method with hGAPDH as internal standard.

Measurement of Intracellular Copper Content in MDA-MB-231 Cells and Tumors by ICP-MS. The total copper content of hCTR1- and vector-MDA-MB-231 cells were measured after being incubated for different times (0, 0.25, 0.50, 2.0, 4.0, and 8.0 hours) with medium containing 3 μM CuCl_2 . The determination of copper ions was performed with PerkinElmer's NexION ICP-MS using the Syngistix software for data collection and processing. Total cells (approximately 8×10^6) were collected in 5 ml PBS buffer for total copper uptake experiments and acidified with 200 μl 70% nitric acid incubated 12 hours at 80°C, diluted to 5 ml by H_2O , and used for analysis. A certain amount of liver or tumor tissues (5 mg) was homogenized and then acidified with nitric acid for 12 hours and diluted with deionized water to 10 ml for analysis.

Data Analysis. Data are presented as the mean \pm S.D. ($n \geq 3$). Two-tailed unpaired Student's *t* test or ANOVA analysis, which was followed by a Dunnett multiple comparison *t* test, was conducted to determine the difference using GraphPad Prism software (version 6.01; GraphPad software Inc., San Diego, CA). The level of statistical significance was set at $P < 0.05$.

Results

Increased CuET, DTC, and Cu^{2+} Accumulation in MDA-MB-231 Cells after Treatment under Acidic Microenvironment. Weakly acidic or weakly basic drugs generally exist in the tissue fluid as either nonionized or ionized forms depending on the pH of the environment and their pKa. The unionized components are more permeable to biologic membranes than the ionized fraction. Therefore, the extracellular pH of tissues is an important factor affecting drug distribution (Raghu et al., 2003; Gerweck et al., 2006). The pH of extracellular fluid in healthy tissues is between 7.35 and 7.45, whereas the extracellular pH of solid tumor tissues is usually acidic (pH 6.5–7.2) (Wojtkowiak et al., 2011). To evaluate the effects of acidic environment

on the uptake of DTC, CuET, and Cu^{2+} into tumor cells, MDA-MB-231 cells were treated with DSF, DTC, CuET, and a mixture of DTC and CuCl_2 in normal (pH 7.4) and acidic (pH 6.8) culture media, respectively.

After the treatment of DTC and CuCl_2 mixture in both culture media, CuET was detected in the cell lysates, and its concentrations increased with the incubation time. The intracellular level of CuET in pH-6.8 medium was 2.5 times as much as that in pH-7.4 medium (Fig. 2A), indicating that acidic environment may facilitate the accumulation of CuET in cells.

Next, when MDA-MB-231 cells were treated with disulfiram or DTC alone, the transmembrane transport rate of DTC, one of the components forming CuET, was enhanced by the acidic environment (Fig. 2B). This result could be explained theoretically because DTC is a weakly acidic compound, and more charged DTC was converted to noncharged DTC in a low-pH environment, which easily penetrated the cell membrane.

As shown in Fig. 2C, when treated with CuET directly, the uptake rates of CuET in both media were almost equivalent. Moreover, no significant difference was observed in the amount of CuET produced in the mixture of DTC and CuCl_2 prepared with both media (unpublished data). These two experiments indicated that the acidic microenvironment could not alter the production of CuET and the transmembrane transport of the produced CuET.

Effect of Acid Environment on the Toxicity of DTC/ Cu^{2+} to MDA-MB-231 Cells. In view of the facts that CuET was the main drug-related substance of disulfiram with antitumor activity and the acidic environment could increase the CuET content in MDA-MB-231 cells treated with DTC/ CuCl_2 mixture, the effect of acidic environment on the cytotoxicity of DTC/ CuCl_2 mixture was investigated. The DTC/ CuCl_2 mixture was prepared in two media with different pH (pH 6.8 and 7.4) and used to culture the cells for a period of time. The CCK-8 reagent was used to measure the viability of the cells.

The results are shown in Fig. 3. Neither DTC nor CuCl_2 alone had a toxic effect on MDA-MB-231 cells unless they acted on the cell together, which was consistent with the conclusion in the literature (Allensworth et al., 2015). DTC and CuCl_2 reduced the viability of MDA-MB-231 cells in a concentration-dependent manner. Interestingly, under acidic conditions, the toxic effect was significantly enhanced with higher levels of CuET. After incubation with 3 μM CuCl_2 and different concentrations of DTC, the IC_{50} values in pH 6.8 and pH 7.4 media were 0.0836 μM and 18.6 μM , respectively. Similarly, after incubation with 3 μM DTC and different concentrations of CuCl_2 , the IC_{50} value in pH 6.8 (1.75 μM) was also lower than that of pH 7.4 (3.81 μM).

Effects of Conditions Affecting hCTR1 Activity on CuET Accumulation. As mentioned before, because the acidic microenvironment cannot directly promote the uptake of CuET, the uptake of DTC and copper ions may be enhanced to indirectly lead to an increase in intracellular CuET content. The uptake of DTC has been confirmed to be enhanced in an acidic environment. According to Lee's report, acid microenvironment, ascorbic acid, and high K^+ concentrations could stimulate the uptake of Cu^{2+} . In this experiment, we investigated the effects of several conditions that could promote the uptake of copper ions on the level of CuET in cells.

The intracellular CuET levels are shown in Fig. 4. The intracellular CuET content was significantly higher when treating cells with a mixture of DTC and $CuCl_2$ in acidic or ascorbic acid-containing environments than that in normal HBSS (Fig. 4A). Although not statistically significant, the amount of intracellular CuET in the substrate treated with high concentration of KCl was also higher than that in the control group. Similar to the results of incubation with the DTC/ $CuCl_2$ mixture, when treating cells with $CuCl_2$ and DTC successively, the intracellular CuET content in acidic or ascorbic acid-containing medium was also higher compared with that in normal medium (Fig. 4B). The results indicated that increasing the uptake of Cu^{2+} could increase the intracellular CuET content. However, the result of the high-potassium-chloride group was similar to that of the control group.

Characterization of hCTR1-Overexpressed MDA-MB-231 Cells. After transfection and selection by puromycin treatment, GFP expression was observed in hCTR1- and vector-MDA-MB-231 cells by fluorescence microscopy. Moreover, a real-time quantitative PCR study demonstrated that the mRNA expression of hCTR1 in hCTR1-MDA-MB-231 cells was approximately 25 times as much as that in the vector-MDA-MB-231 cells, which indicated that hCTR1-overexpressed MDA-MB-231 cells were successfully constructed.

In Vitro Cu^{2+} Uptake Assay and the Intermediate Role of hCTR1 in CuET Enrichment in Tumor Cells. Cu^{2+} accumulation was examined in hCTR1- and vector-MDA-MB-231 cells as a function of time. On the basis of the intracellular content of copper, the uptake of Cu^{2+} was significantly increased in hCTR1-MDA-MB-231 cells compared with vector cells (Fig. 5A). Cu^{2+} uptake in cells transfected with the hCTR1 expression plasmid was significantly stimulated in a time-dependent manner, with a greater than 7-fold stimulation over

vector-transfected cells at 8 hours. Similarly, after further treatment with DTC, CuET levels in hCTR1-overexpressed cells were also markedly higher than those in vector cells (about 20-fold, Fig. 5B), which proves that the stimulation or overexpression of hCTR1 can indirectly promote the accumulation of CuET in the tumor by enhancing the uptake of Cu^{2+} .

Acidic Environment Promotes the Accumulation of CuET in Tumors Mainly by Increasing Its Formation in Cells. In this experiment, two methods of treatment were investigated to distinguish the contribution of CuET formation inside or outside the cell. One method was to treat the cells with a mixture of DTC and $CuCl_2$, and the other was to treat the cells with $CuCl_2$ first and then DTC after washing. In both methods, vector- and hCTR1-MDA-MB-231 cells were treated in neutral and acidic uptake media.

Under neutral pH conditions, after vector-MDA-MB-231 cells were treated with a mixture of DTC and $CuCl_2$, the concentration of CuET in the cells was much higher than that of the separate treatment with $CuCl_2$ and DTC (Fig. 6), with the latter being almost undetectable. However, with the decrease of pH, the ratio of CuET in separate treatment groups to the mixture group increased significantly. In hCTR1-MDA-MB-231 cells, under neutral conditions the intracellular CuET concentration of the separate treatment groups was approximately 8% of the treatment with DTC and $CuCl_2$ mixture, which was higher than that of vector-MDA-MB-231 cells. When the pH was decreased to pH 6.8, the levels of CuET in the two groups were comparable.

This result showed that under neutral conditions, CuET in cells was mainly formed extracellularly and transported into the cell through passive diffusion. However, under acidic conditions, a part of CuET in the cell was formed inside the cell by DTC and copper ions entering the cells. In cells overexpressing hCTR1, the formation of CuET inside the cell also accounted for a certain proportion due to the increase in the uptake rate of copper ions, even under neutral conditions. Under acidic conditions, the formation of CuET inside the cell and the transport from outside the cell were comparable.

Accumulation of CuET in hCTR1- and Vector-MDA-MB-231-Cell-Constructed Tumors in BALB/c Nude Mice. Results acquired from in vitro uptake studies revealed that membrane protein hCTR1 played an intermediate role in the CuET accumulation in tumor cells. Therefore, we evaluated whether the overexpression of hCTR1 in tumors in vivo could promote the uptake of CuET.

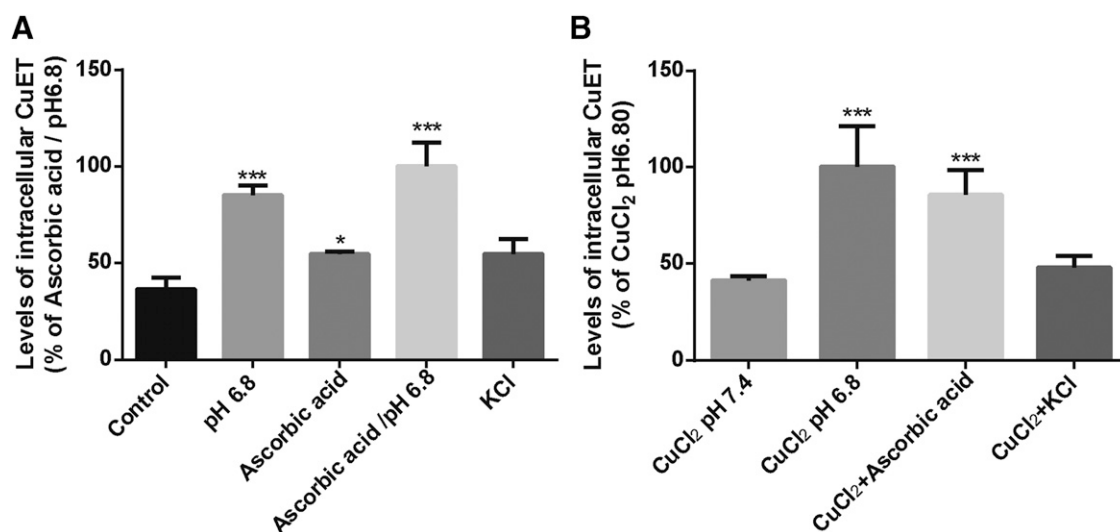


Fig. 4. Intracellular level of CuET after treatments with the mixture of DTC (3 μ M) and $CuCl_2$ (3 μ M) (A) and $CuCl_2$ (3 μ M) followed by DTC (3 μ M) (B) under different conditions. Data are expressed as mean \pm S.D. ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (ANOVA analysis, followed by a Dunnett's multiple comparison t test).

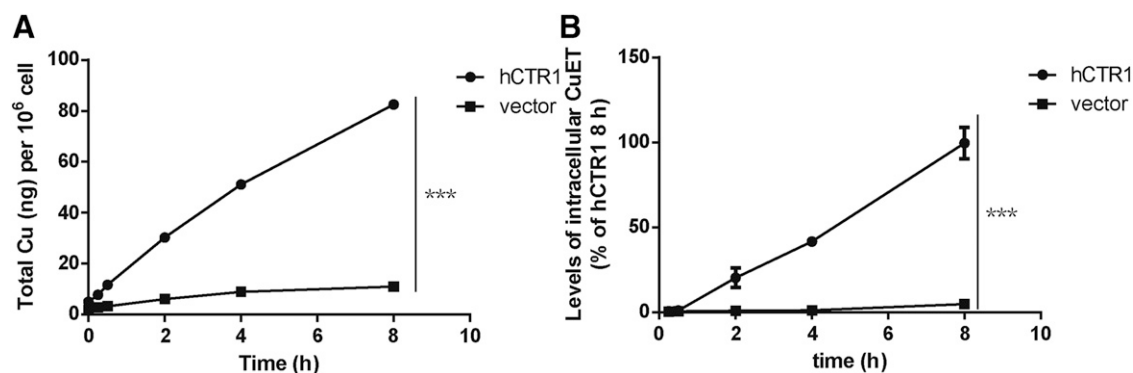


Fig. 5. Intracellular level of Cu²⁺ after treatment with CuCl₂ (A) and intracellular level of CuET (B) after treatment with CuCl₂ followed by DTC in hCTR1-MDA-MB-231 cells and vector-MDA-MB-231 cells. Data are expressed as mean \pm S.D. ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-tailed Student's t test).

After 15-day dosing regimen of copper gluconate in the morning and disulfiram in the evening, the mice were sacrificed, and the CuET concentrations in the tumors were detected using LC-MS/MS. The results showed that the concentrations of CuET and Cu²⁺ in hCTR1-overexpressing tumors were significantly higher than those in the vector group, which was consistent with the results of in vitro experiments. However, the CuET and Cu²⁺ concentrations in the liver were not significantly different between the two groups (Table 1).

Discussion

Disulfiram, an antialcoholism drug, serves as a great example of repurposing an old drug as a promising anticancer drug (Chen and Dou,

2008). Many pharmacological experiments revealed the unique antitumor effect and excellent selectivity of disulfiram on the basis of its mechanism of action (Lin et al., 2011, 2012; Yip et al., 2011; Skrott et al., 2017). In this study, we first described the enrichment mechanism of disulfiram's anticancer active substance CuET in tumors, which explained the selectivity of disulfiram to tumor and normal tissues from a brand-new dimension.

The tissue distribution of drugs is influenced by many factors, including the physiochemical properties of drugs and physiologic factors (Wallace et al., 2012). Uptake and efflux transporters also play an important role in drug tissue distribution. Because of the special metabolic pathways and physiologic environment of tumor tissues, certain transporters are overexpressed in tumor cells (Seker et al., 2000; Calvaresi and Hergenrother, 2013). A typical case is that glucose conjugates exhibit enrichment in tumor cells owing to the active uptake by glucose transporter (Patra et al., 2016). Initially, we suspected that certain transporters that are overexpressed in tumors may mediate the active uptake of CuET and lead to its accumulation in tumor cells. In vitro transporter studies show that the CuET uptake into tumor cells at 37°C was not significantly greater than that at 4°C (unpublished data), which suggests that CuET is less likely to be a substrate for transporters, as the activity of most transporters is temperature-dependent. Moreover, CuET was not the substrate of several common transporters, at least including OATP1B1, OATP1B3, OATP2B1, organic anion transporter 1, organic anion transporter 3, and organic cation transporter 2 (unpublished data). On the basis of these results, combined with some objective information, including the lipophilicity and uncharged nature of CuET (Johansson, 1986), it is more likely to enter cells through passive diffusion rather than active uptake. Therefore, we no longer stuck to the initial assumption.

Tumor tissues may have better uptake for the two raw materials of CuET: DTC and Cu²⁺. In clinical dosing regimens (NCT numbers: NCT03323346 and NCT03323346), copper gluconate and disulfiram were not administered at the same time. One was taken before or with breakfast, and the other was taken after dinner. DTC was the predominant drug-related component of disulfiram in circulation; however, only trace amount of CuET was observed in plasma (Johansson, 1986; Zhang et al., 2013). CuET was easily formed when DTC was mixed with Cu²⁺ (Lewis et al., 2014). Therefore, the distribution of DTC and Cu²⁺ would affect the concentration of CuET in tissues.

The pH of tissues is an important factor affecting drug distribution (Gerweck et al., 2006). Unlike most tissues, solid tumor tissues are usually acidic. In principle, the extracellular acidity of tumors may enhance the uptake and cytotoxicity of weak acid drugs that are membrane permeable in their uncharged state (such as chlorambucil) and inhibit the efficacy of weak bases (such as doxorubicin and

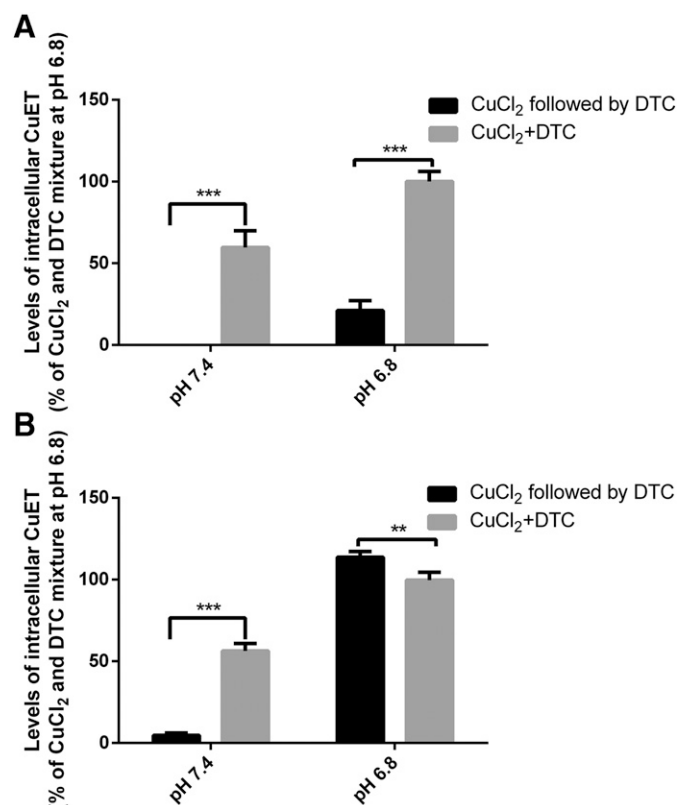


Fig. 6. Intracellular level of CuET after treatments with the mixture of DTC (3 μ M) and CuCl₂ (3 μ M) (A) and CuCl₂ (3 μ M) followed by DTC (3 μ M) (B) in hCTR1-MDA-MB-231 cells and vector-MDA-MB-231 cells. Data are expressed as mean \pm S.D. ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-tailed Student's t test).

TABLE 1
CuET and Cu²⁺ conc. in tumors and liver of mouse xenograft models constructed with hCTR1– and vector–MDA-MB-231 cells

	CuET Conc. (ng/g)		Cu ²⁺ Conc. (μg/g)	
	Tumor	Liver	Tumor	Liver
Vector–MDA-MB-231	10.8 ± 4.2 (n = 9)	1.36 ± 2.33 (n = 11)	3.76 ± 0.79 (n = 9)	4.03 ± 1.62 (n = 11)
hCTR1–MDA-MB-231	21.5 ± 10.4 (n = 11)	2.04 ± 1.62 (n = 11)	6.97 ± 2.14 (n = 11)	3.55 ± 0.99 (n = 11)

mitoxantrone) (Raghunand et al., 2003; Gerweck et al., 2006). However, the common medium in which cells are cultured in vitro is indeed weakly alkaline. To simulate the real environment of tumor tissues, we investigated the effects of acidic environment on the uptake of DTC, CuET, and the mixture of CuCl₂ and DTC. Although transmembrane transport and the formation of CuET could not be enhanced by the acidic microenvironment, the intracellular level of CuET after treatment with DTC and CuCl₂ at pH 6.8 was 2.5 times as much as that at pH 7.4. These results imply the separate transport of DTC and copper in acidic condition. Except as a metal chelator, DTC appears as an acidic compound containing a dithiocarboxylic acid moiety. The theoretical pK_a of DTC was 5.4, as predicted by ACD Laboratories software. With the reduction of initial pH to 6.8, the uncharged DTC would become approximately three times higher than that at pH 7.4, as calculated by the Henderson-Hasselbalch equation. In this study, the uptake rate of DTC in the medium at pH 6.8 was 2.3 times as much as that at pH 7.4 (Tallarida and Murray, 1987), which was consistent with the theoretical situation.

Coincidentally, the uptake rate of copper ions, another component of CuET, is also affected by pH (Lee et al., 2002). The main copper transporter, hCTR1, at the plasma membrane was stimulated by

extracellular acidic pH, ascorbic acid, and high K⁺ concentrations. In this experiment, the cells were treated with copper chloride in a medium containing ascorbic acid or in an acidic medium, and then they were treated with DTC. The results showed that the intracellular content of CuET under the two treatment conditions was higher than that in the control group. This finding also indicated that the stimulation of hCTR1 could indirectly affect CuET content in tumor cells by increasing the uptake of copper ions.

hCTR1-overexpressing MDA-MB-231 cells were constructed to further prove the mediator role of hCTR1. After treatment with CuCl₂, the concentration of copper ions in hCTR1-overexpressing cells was significantly higher than that in vector cells. Further incubation with DTC demonstrated that more CuET was observed in hCTR1-overexpressing cells. In vivo experiment showed a similar result: Higher concentrations of CuET were found in hCTR1-overexpressing tumor constructed by hCTR1–MDA-MB-231 cells compared with the control group.

In general, the dysregulated pH in the tumor microenvironment would lead to the failure of antitumor treatment (Barar and Omidi, 2013). However, according to our in vitro experiments, the acidic environment of tumor cells could promote intracellular CuET accumulation through

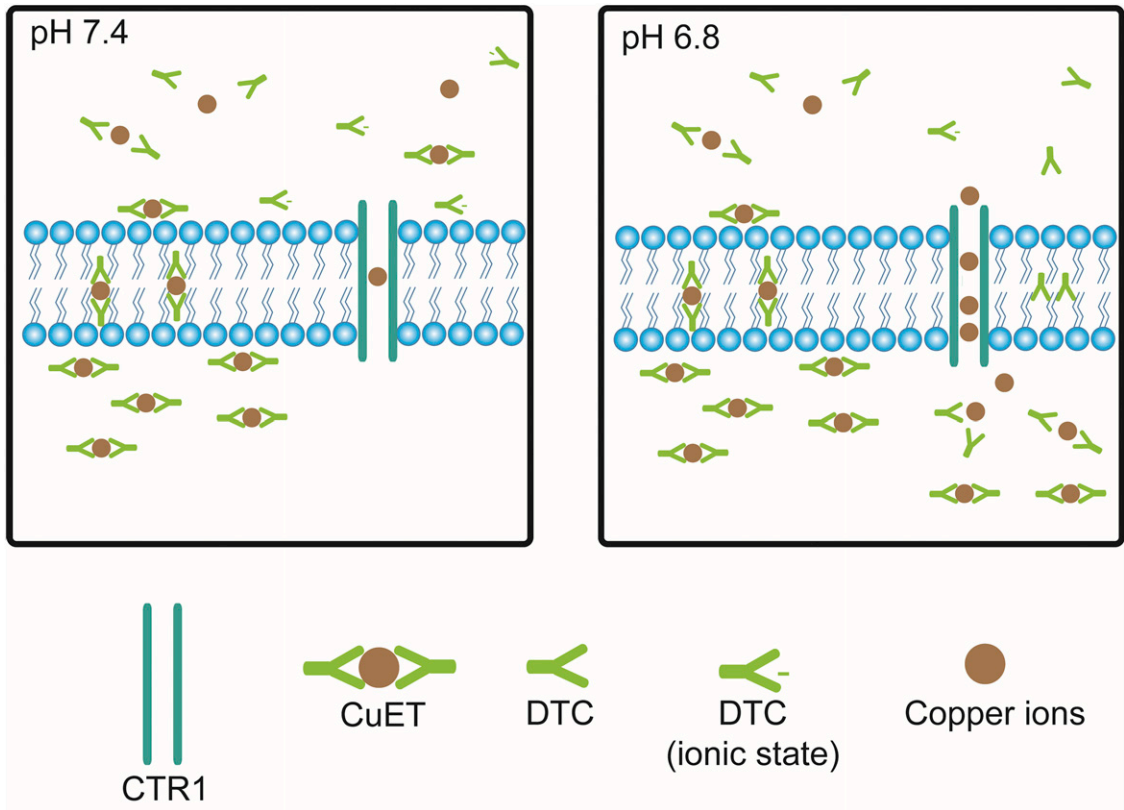


Fig. 7. Schematic diagram of the mechanism of acidic microenvironment promoting CuET accumulation in tumor cells through dual actions. CTR1, copper transporter 1.

the dual action without changing the uptake of CuET. The proposed mechanism is shown in Fig. 7. This mechanism is similar to the formation mechanism of tetracycline teeth. It was not that tetracycline-calcium chelate was formed and entered the teeth, but after taking tetracycline, the two raw materials formed colored metal chelate in the teeth. For CuET, on the one hand, the transmembrane transport of DTC under acidic microenvironment was enhanced by converting the charged DTC to uncharged state to facilitate its passive diffusion. On the other hand, the active uptake of copper ions was promoted by activating hCTR1 on the cell membrane, which increased the two raw materials that made up CuET in the cell and eventually formed more CuET in the cell.

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Authorship Contributions

Participated in research design: Tang, Pang, Chen.

Conducted experiments: Tang, Z. Guo, R. Guo, Liu.

Contributed new reagents or analytic tools: Chen.

Performed data analysis: Tang, Chen.

Wrote or contributed to the writing of the manuscript: Tang, Chen.

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Address correspondence to: Dr. Xiaoyan Chen, Shanghai Institute of Material Medica, Chinese Academy of Sciences, 501 Haik Rd., Shanghai 201203, China.
E-mail: xychen@simm.ac.cn