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Inhibition of Invasion and Angiogenesis by Zinc-Chelating Agent Disulfiram

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ABSTRACT

Cell invasion and angiogenesis are crucial processes in cancer metastasis that require extracellular matrix (ECM) degradation. Proteolytic degradation of the ECM components is a central event of invasion and angiogenesis processes. During these processes, matrix metalloproteinases (MMPs) seem to be primarily responsible for much of the ECM degradation. Disulfiram is frequently used in the treatment of alcoholism and has been reported to possess antiretroviral activity and can eject intrinsic zinc out of human immunodeficiency virus (HIV) nucleocapsid protein. In this report, we show that disulfiram inhibited invasion and angiogenesis in both tumor and endothelial cells at nontoxic concentrations. The ^{3}H-labeled type IV collagen degradation assay suggested that disulfiram has type IV collagenase inhibitory activity, and this inhibition was responsible for blocking invasion and angiogenesis through cell-mediated and non–cell-mediated pathways. However, the mechanisms underlying cell-mediated signal pathways are not fully characterized. Our data demonstrate that the non–cell-mediated pathway is dominant. Thus, disulfiram could directly interact with MMP-2 and MMP-9 and inhibit their proteolytic activity through a zinc-chelating mechanism. Addition of zinc could reverse the inhibition of invasiveness and collagenase inhibition through disulfiram treatment. This finding implies that MMP-2 and MMP-9 may be the inhibitory targets for a potential disulfiram treatment. These observations raise the possibility clinical therapeutic applications for disulfiram used as a potential inhibitor of metastatic cell invasion and angiogenesis.

Tumor invasion and metastasis represent a multistage process that involves detachment of tumor cells from the primary site, controlled degradation of structural barriers, such as basement membrane and extracellular matrix (ECM), and migration of cells through the degraded matrix (Stetler-Stevenson et al., 1993). Among these processes, proteolytic degradation of the ECM components is a central event. Several classes of proteinases, including serine proteinases, cysteine proteinases, and matrix metalloproteinases (MMPs), have been implicated in tumor cell invasion (Duffy, 1992). Among these different proteinases, MMPs seem to be primarily responsible for the ECM degradation in tumor cell invasion and tumor-induced angiogenesis (Johnsen et al., 1998; Kahari and Saarialho-Kere, 1999). MMPs are a family of secreted and transmembrane Zn^{2+}-endopeptidases that function at neutral pH, require Ca^{2+} for activity (Kohn et al., 1994), and can degrade all the components of the ECM, such as fibrillar and nonfibrillar collagens, fibronectin, laminin, elastin, and basement membrane glycoproteins (Woessner, 1994). At least 18 human members of the gene family have been characterized at present, and according to their structure and substrate specificity, they can be divided into subgroups of collagenases, gelatinases, and stromelysins and membrane-type MMPs (Kahari and Saarialho-Kere, 1999).

There is evidence of an association between MMPs and tumorigenicity, invasive behavior, and angiogenesis (Liotta et al., 1991). For example, MMPs, particularly MMP-2 (also known as 72-kDa type IV collagenase) and MMP-9 (also known as 92-kDa type IV collagenase), have been implicated in the progression of colorectal carcinomas, breast cancer (Zucker et al., 1993), and non–small-cell lung cancer (Brown et al., 1993). Moreover, the formation of new capillaries by endothelial cells also requires gelatinases and other proteinases for endothelial cell migration and tissue remodeling (Hanahan and Folkman, 1996). General MMP inhibitors prevent tumor dissemination and formation of metastasis and

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This article is dedicated to the memory of Felicia Y.-H. Wu.

ABBREVIATIONS: ECM, extracellular matrix; MMPs, matrix metalloproteinases; TIMP, tissue inhibitors of metalloproteinase; HIV, human immunodeficiency virus; CAM, chick chorioallantoic membrane; HUVEC, human umbilical vein endothelial cells; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; MICS, membrane invasion culture system; VEGF, vascular endothelial growth factor; NF-κB, nuclear factor-κB; AP-1, activator protein-1.

angiogenesis in animal models, indicating that MMPs are potential targets for therapeutic intervention in cancer.

Recent studies have shown that imbalance between MMPs and their corresponding inhibitors [tissue inhibitors of metalloproteinases (TIMPs)] seems to be an important early sign of metastasis that leads to pathological breakdown of ECM with subsequent tumor cell migration (Khokha and Waterhouse, 1994). TIMPs are naturally occurring proteins that specifically inhibit MMPs, thus maintaining balance between matrix disruption and formation. An imbalance between MMPs and their associated TIMPs may play an important role in the phenotype of malignant tumors. TIMP-1 has been shown to inhibit tumor-induced angiogenesis in experimental systems (Johnson et al., 1994). Nonetheless, TIMPs are probably not suitable for current clinical application because of their short half-life in vivo. Batimastat, also known as BB-94, is a synthetic low molecular weight inhibitor of MMPs. Batimastat has a collagen-like backbone to facilitate binding to the active site of the MMPs and a hydroxamate structure that chelates the zinc in the active site. Batimastat was the first synthetic MMP inhibitor studied in human phase I clinical trials. However, its usefulness has been limited by extremely poor water solubility, which required intraperitoneal administration of the drug as a detergent emulsion (Wojtowicz-Praga et al., 1996). Thus, finding efficient inhibitors to keep the metastatic- or angiogenic-related proteases under control has generated great interest in recent years.

Disulfiram is an inhibitor of aldehyde dehydrogenase and is used clinically in the treatment of alcoholism (Johansson, 1992). It has been reported as a chelating agent and possesses antiretroviral activity and can eject intrinsic zinc out of human immunodeficiency virus (HIV) nucleocapsid protein (McDonnell et al., 1997). In this report, we present data that disulfiram effectively inhibits invasion of tumor and endothelial cells at nontoxic concentrations. In addition, disulfiram displayed antiangiogenic effects in vivo in a 10-day-old chick embryo chorioallantoic membrane (CAM) model. Furthermore, we demonstrated that disulfiram directly interacted with type IV collagenases, especially MMP-2 and MMP-9, and inhibited their proteolytic activity through a zinc-chelating mechanism. Importantly, addition of zinc could reverse the inhibition of invasiveness and collagenase inhibition by disulfiram treatment. These results provide evidence that the phenotype of invasion and angiogenesis is correlated with MMP activity, suggesting that inhibition of MMP activity via the chelating agent disulfiram may serve as a target to inhibit tumor cells invasion and tumor-induced angiogenesis.

Materials and Methods

Cell Culture and Chemicals. Cell lines used in this study were maintained in humidity in a 5% CO_2 incubator. CL1–5 (high metastatic of human lung adenocarcinoma) (Chu et al., 1997) and NTUB1 (human bladder adenocarcinoma derived from that provided by Dr. Pan-Chyr Yang, Department of Internal Medicine, National Taiwan Medical School, Taipei, Taiwan) were cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Human umbilical vein endothelial cells (HUVECs) were cultured in M199 medium containing 10% fetal bovine serum, 2.5 mg/ml endothelial cell growth factor, 5,000 units/ml heparin sulfate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. HUVECs were used between third and fourth passages. Disulfiram, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) dye, dimethyl sulfoxide and cortisone acetate were purchased from Sigma Chemical Co. (St. Louis, MO).

Cytotoxicity Assay. The cytotoxic effects of applied reagents were determined using the MTT assay. In brief, cells were harvested in the exponential phase and seeded into 96-well plates (2×10^4 cells/well) in 100 μl of medium. After overnight incubation at 37°C, 100 μl of culture medium containing the test compound was dispensed into each well. Culture plates were then incubated for 48 h before the addition of a tetrazolium dye. After 4 h of incubation, the medium and MTT dye were removed by slow aspiration and 100 μl of dimethyl sulfoxide was added to dissolve the remaining MTT-formazan crystals. The absorbance at 550 nm was measured using a microtiter plate reader (EL340; Bio-Tek Instruments, Winooski, VT).

In Vitro Invasion Assay. The membrane invasion culture system (MICS) was used to measure a cell line's invasion activity. This assay was performed as described previously (Hendrix et al., 1987). Matrigel, a basement membrane matrix extracted from Engelbreth-Holm-Swarm mouse sarcoma (Becton Dickinson, San Jose, CA) was diluted to 5 mg/ml and coated on a polycarbonate membrane containing 10-μm pores (Nucleopore Corp., Pleasanton, CA). The membrane was placed between the upper and lower chambers of the modified Boyden chamber assay system. Subsequently, cells (2.5×10^4) were seeded into the upper-chamber in RPMI medium containing 10% NuSerum (BD Biosciences Discovery Labware, Bedford, MA). For zinc reversion assay, cells mixed with 10 μM disulfiram and 10 μM zinc were seeded into the upper chamber. After incubation for 48 h at 37°C, cells were removed from the lower chamber with 1 mM EDTA in phosphate-buffered saline and dot-blotted on a polycarbonate membrane with 3-μm pores. After fixing with methanol, blotted cells were stained with Liu stain (Handsel Technologies, Inc., Taipei, Taiwan), and the cell numbers in each blot were counted under a microscope (original magnification, 200×).

Chick CAM Angiogenesis Assay. Angiogenesis assays were performed on the CAMs of 10-day-old chick embryos with minor modifications (Brooks et al., 1994). First, a small hole was made through the shell at the air sac end of the chick egg using a small craft drill. A second hole was drilled on the broad side of the egg directly over embryonic blood vessels. Negative pressure was applied to the original hole, which resulted in the CAM pulling away from the shell membrane, creating a false air sac. A window was cut through the shell over the dropped CAM and filter discs (6-mm diameter) saturated with 25 ng/ml vascular endothelial growth factor (VEGF; R&D Systems Inc., Minneapolis, MN) or disulfiram were placed on the CAM. To avoid inflammation associated with placement of the filter discs, we placed these discs in cortisone acetate solution (3 mg/ml in absolute ethanol) and allowed the disc to air dry in a sterile laminar flow chamber. The discs coated with the cortisone acetate were then ready to be used for growth factor and disulfiram treatment. After 48 h, the filter discs and associated CAM tissue were harvested and examined for angiogenesis under a Stemi SV6 stereomicroscope (Carl Zeiss GmbH, Jena, Germany). The angiogenic index was defined as the mean number of visible blood vessel branch points within the defined area of the filter discs. Photographs were taken at 10× magnification.

^{3}H-Labeled Collagen Type IV Degradation Assay. Collagen type IV degradation assays were performed as described previously with modification (Brooks et al., 1996). Briefly, 96-well plates were coated with 50 μl of ^{3}H-labeled type IV collagen (PerkinElmer Life Sciences, Boston, MA; 500~1,000 cpm/μl) and allowed to dry overnight in a laminar flow hood at room temperature. Plates were then washed with phosphate-buffered saline until free cpm reached the basal level. For cell-mediated collagen degradation assays, cells (1×10^5) in a total volume of 200 μl of medium were incubated for 48 h at 37°C. Disulfiram was included in the incubation at 1, 5, and 10 μM. For cell free collagen degradation assays, 200 μl of various cell

culture media was incubated for 48 h or 0.1 μg of purified recombinant MMP-9 and MMP-2 (Chemicon International Inc., Temecula, CA) was incubated for 48 h in a total volume of 200 μl of reaction buffer containing 2.5% bovine serum albumin, 0.2 mM $MnCl_2$, and 0.1 mM $CaCl_2$ in the presence or absence of various concentrations of disulfiram. For zinc reaction, 0.1 μg of purified recombinant MMP-9 and MMP-2 was incubated for 48 h in the presence or absence of 10 μM disulfiram or 10 μM $ZnCl_2$. Collagen degradation was assessed by measuring the cpm released in 50 μl of medium. Net collagen type IV degradation was determined by subtracting the cpm released from buffer only wells.

Gelatin Zymography. Gelatin zymography was performed on 10% polyacrylamide gel containing 0.1% gelatin as described previously with minor modification (Heussen and Dowdle, 1980). In brief, 0.1 μg of purified human MMP-2 or MMP-9 protein (Chemicon) was incubated for 2 h in the presence or absence of disulfiram, EDTA, or $ZnCl_2$ in a total volume of 20 μl of reaction buffer containing 2.5% bovine serum albumin, 0.2 mM $MnCl_2$, and 0.1 mM $CaCl_2$. After drug treatment, the reaction mixtures were collected and mixed with Laemmli's SDS sample buffer (without β-mercaptoethanol) for electrophoresis. The gel was then soaked in 50 mM Tris-HCl containing 2.5% Triton X-100 at room temperature with gentle shaking for 30 min, followed by an incubation in 50 mM Tris-HCl containing 150 mM NaCl and 10 mM $CaCl_2$ at 37°C overnight. After the gel was stained with Coomassie blue, the gel was destained until the transparent bands were shown on the blue background.

Results

Effect of Disulfiram on Invasion in Vitro. To explore whether the invasion activity was inhibited within highly invasive tumor cells (CL1–5 and NTUB1) and normal endothelial cells (HUVECs) in response to disulfiram, we examined invasion activity using membrane invasion culture system. Figure 1A indicates that after disulfiram treatment, the invasion activity of each cell line was obviously decreased at 48 h; this inhibition occurred in a dose-dependent pattern and was observed at concentrations as high as 10 μM. The inhibition percentage of 10 μM disulfiram at 48 h was about 81% in CL1–5, 79% in NTUB1, and 79% in HUVECs. This inhibition was not caused by cytotoxicity of disulfiram, because we observed no toxic effect of growth inhibition or cell death at treatment with less than 10 μM disulfiram in the 48-h MTT assay (Fig. 1B). Similar results were also obtained with longer treatment (Fig. 1C), suggesting that lower concentrations of disulfiram (< 10 μM) were not toxic to tumor cells and normal endothelial cells. Notably, the median lethal dose (LD_{50}) of disulfiram in HUVECs was about 130 μM, and the LD_{50} of disulfiram in CL1–5 and NTUB1 was about 35 μM (Fig. 1B). This means that the normal endothelial cells were more resistant to disulfiram toxicity than the tumor cells. For example, using 35 μM disulfiram caused 50% cell death of tumor cells but had no effect on endothelial cells. When the disulfiram concentration was raised to 50 μM, there was no significant effect on endothelial cells but 70% cell death of tumor cells (Fig. 1B). These observations revealed that the disulfiram possessed the ability to inhibit cell invasion activity, and the differential toxicity of disulfiram on normal endothelial cells and tumor cells may provide its potential role in clinical application.

Effect of Disulfiram on Angiogenesis in Vivo. To address the question of whether disulfiram played a role in angiogenesis inhibition, we used the CAMs of 10-day-old chick embryos to examine this response. As shown in Fig. 2A, angiogenesis was induced below the 6-mm filter discs saturated with 25 ng/ml of VEGF. Note that the pre-existing vessels (Fig. 2A, Control) were large and less branched than the newly sprouting vessels induced by VEGF (Fig. 2A, VEGF) on stereomicroscopic observation. This angiogenic response was effectively disrupted by the addition of disulfiram (Fig. 2A). Addition of 10 μM disulfiram resulted in a decrease

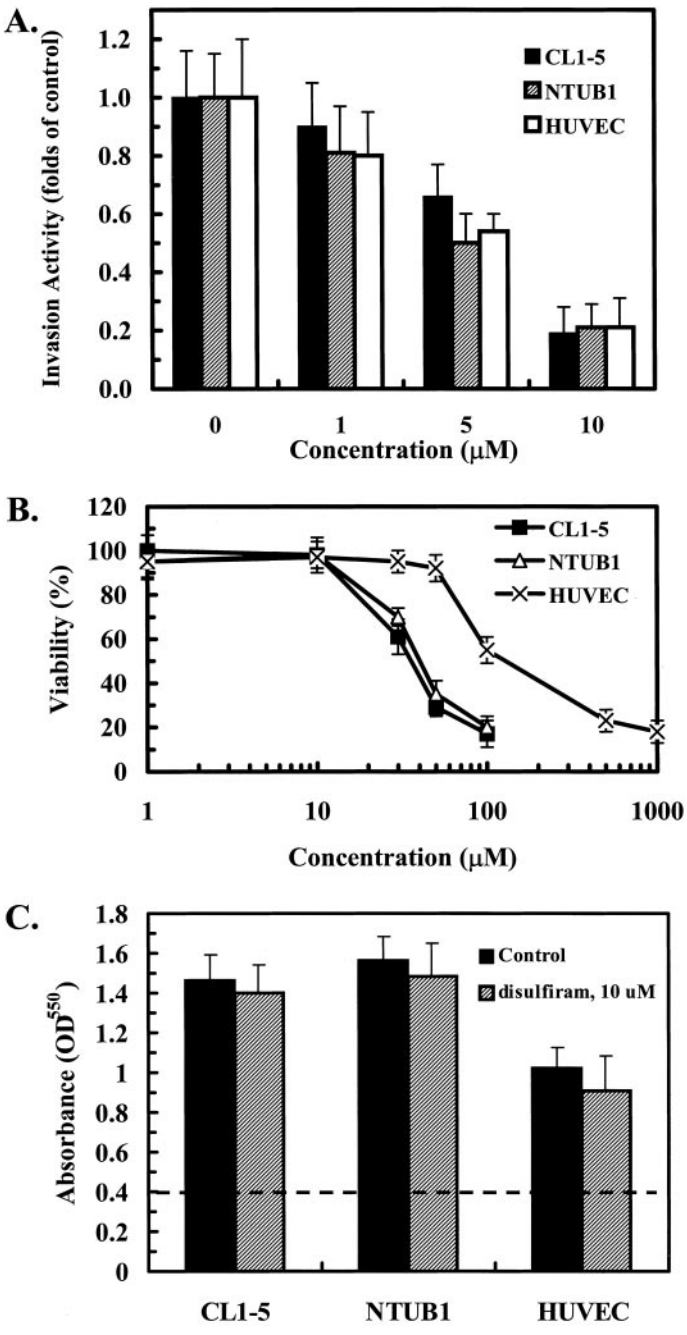

Fig. 1. Effects of disulfiram on cultured cells. A, disulfiram blocked invasion activity in vitro. The invasion activity of each cell line was measured using a MICS chamber. B, sensitivity of tumor cells (CL1–5 and NTUB1) and normal cells (HUVEC) to disulfiram treatment. The percentage of surviving cells was measured using the MTT dye assay. C, cytotoxicity of CL1–5, NTUB1, and HUVEC cells treated long-term with 10 μM disulfiram. The growth rates of each cell line were measured spectrophotometrically at wavelength of 550 nm on day 4 for CL1–5 and NTUB1 cultures and on day 7 for HUVEC cultures. The hatched line represents the absorbance of the three cell lines at day 0. Data are means ± S.E. from triplicate wells.

of more than 80%in the angiogenic index as determined by counting the number of blood vessel branch points directly within the area of the filter discs (Fig. 2B). Furthermore, this antiangiogenic effect of disulfiram was dose-dependent (Fig. 2C). These results provide evidence that disulfiram has the ability to inhibit angiogenic effect in vivo.

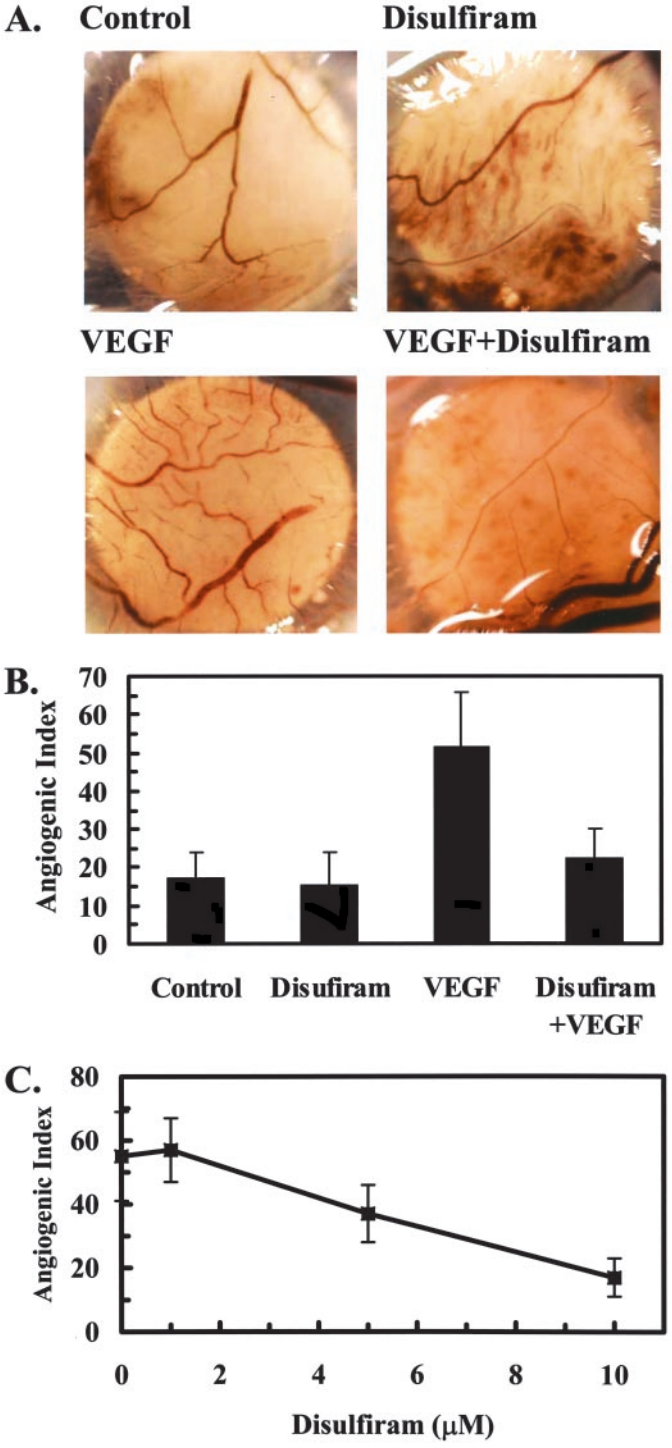

Fig. 2. Disulfiram blocked angiogenesis in vivo. Angiogenesis was induced on the chorioallantoic membranes (CAMs) of 10-day-old chicken embryos using 6-mm filter discs saturated with 10 μM disulfiram or 25 ng/ml of VEGF placed on an the CAM. After 48 h, the CAM tissue associated with the filter disc was removed and photographed on a stereomicroscope. A, representative examples of filter disc-associated CAM tissues taken from typical experiments. B, angiogenesis was quantified by counting the number of blood vessel branch points directly within the area of the filter discs. C, dose-response of disulfiram on angiogenesis as determining by counting the blood vessel branch points. Data are means ± S.E. from six embryos per treatment.

Disulfiram Inhibits MMP-2 and MMP-9 Activity. Disulfiram inhibited the invasion activity of tumor cells and angiogenic ability of endothelial cells, suggesting that the drug may interfere with the common targets of the invasion and angiogenesis pathways. Proteolytic degradation of the extracellular matrix components is a central event of invasion and angiogenic processes. During these processes, MMPs seem to be primarily responsible for much of the ECM degradation. To verify the possible mechanism by which disulfiram affects the invasion and angiogenesis behavior through MMP inhibition, we examined the effects of disulfiram on collagenolytic activity. Figure 3A shows that addition of disulfiram to CL1–5, NTUB1 and HUVEC cells resulted in a dose-dependent decrease in collagen type IV degradation activity. The inhibition percentage of 10 μM disulfiram was about 73% in these cell lines. To clarify whether this inhibition was through a cell-mediated pathway or through direct drug-protein interaction, we used a cell free collagen degradation assay system to assess collagen type IV degradation activity. Briefly, 200 μl of 48-h culture medium from CL1–5, NTUB1, and HUVEC cells was incubated in the 96-well plates coated with ^{3}H-labeled type IV collagen for another 48 h in the presence or absence of disulfiram. As shown in Fig. 3B, the inhibition pattern was also dose-dependent and similar to the results of Fig. 3A. Interestingly, the inhibition intensity of collagenolytic activity in the cell free system (about 40%) was weaker than in cell-mediated system at 10 μM disulfiram, suggesting that these two mechanisms together contribute to collagenase inhibition of disulfiram treatment. Nevertheless, the pathway via drug-protein interaction was dominant, suggesting that disulfiram might directly inhibit some type IV collagenases, alternatively some types of MMPs, and subsequently reduce the collagen type IV degradation activity. MMP-2 and MMP-9 have been frequently associated with invasive and angiogenic potential of tumor cells and endothelial cells, respectively, and they are type IV collagenases. Therefore, we used the purified human MMP-2 and MMP-9 proteins to test the possible drug-protein interaction mechanism. Indeed, both MMP-2 and MMP-9 showed significantly decreased collagenolytic activity after incubation with 10 μM disulfiram, as shown in Fig. 4A. Furthermore, we analyzed the gelatinases activity of the medium below the Matrigel well; as shown in Fig. 4B, the activities of MMP-2 and MMP-9 were drastically decreased by disulfiram treatment. These findings demonstrate that disulfiram inhibits type IV collagenase activity, especially for MMP-2 and MMP-9. Furthermore, this inhibition is mediated largely through direct drug-protein interaction rather than by the cell-mediated pathway.

Zymographic Analysis of MMPs Activity. It has been reported that disulfiram has the ability to eject zinc from the nucleocapsid protein of HIV in the NMR assay, and this ejection activity was more effective than high-affinity zinc-chelating agents such as EDTA (McDonnell et al., 1997). To compare MMP inhibition activity of disulfiram and EDTA, we used gelatin zymography to analyze the inhibition patterns of each. Four major gelatinolytic bands of 180 kDa

(dimer), 88 kDa (proform), 84 kDa (intermediate form), and 82 kDa (active form) were detected from purified human MMP-9 protein (Fig. 5A, lane 1), and two major bands of 68 (proform) and 60 kDa (active form) were detected from purified human MMP-2 protein (Fig. 5B, lane 1). Addition of increasing concentrations of disulfiram resulted in a dose-dependent reduction of the all forms of MMP-2 and MMP-9 proteins (Fig. 5, A and B, lanes 1–4). The zinc-chelating agent EDTA showed a similar inhibition pattern, but the inhibition strength was less than that of disulfiram treatment (Fig. 5A, B, lane 5–8). Moreover, addition of 10 μM zinc could reverse the collagen type IV degradation activity of MMP-2 and MMP-9 under 10 μM disulfiram treatment (Fig. 6A). Importantly, 10 μM zinc also reversed the invasion ability of CL1–0, NTUB1, and HUVEC cells inhibited by disulfiram (Fig. 6B). However, when the concentration of zinc was more than 25 μM, apoptotic cell death was observed (data not shown).

Discussion

In this study, we attempted to elucidate the mechanisms of the anti-invasion or antiangiogenic effects induced by disulfiram. The results suggest that disulfiram is an MMP inhibitor, which chelates the zinc ion from the active site of MMPs and subsequently inhibits the proteolytic activity of MMPs.

Initially, to investigate the influence the inhibition of disulfiram on cell invasiveness, the in vitro invasion assay through Matrigel-coated membranes was performed (Fig. 1). Our results demonstrated that disulfiram efficiently inhibited the in vitro invasion of both human tumor cell lines (CL1–5 and NTUB1) and one endothelial cell line (HUVEC). Almost 80% of the invasive ability of these cells was repressed by treatment with 10 μM disulfiram. Furthermore, at this concentration, disulfiram produced no apparent toxicity in this study. In fact, disulfiram did not influence the growth phenotype or proliferation rate of these cells, even with long-term treatment, supporting the notion that the anticancer activity of disulfiram is caused by its anti-invasion effects not its toxicity.

Disulfiram has been given to humans to eradicate a number of protozoan parasites, including *Giardia lamblia* (Nash and Rice, 1998), *Plasmodium falciparum* (Scheibel et al., 1979), and *Trypanosoma cruzi* (Lane et al., 1996) for decades. Its toxicity to humans is relatively low, with few significant

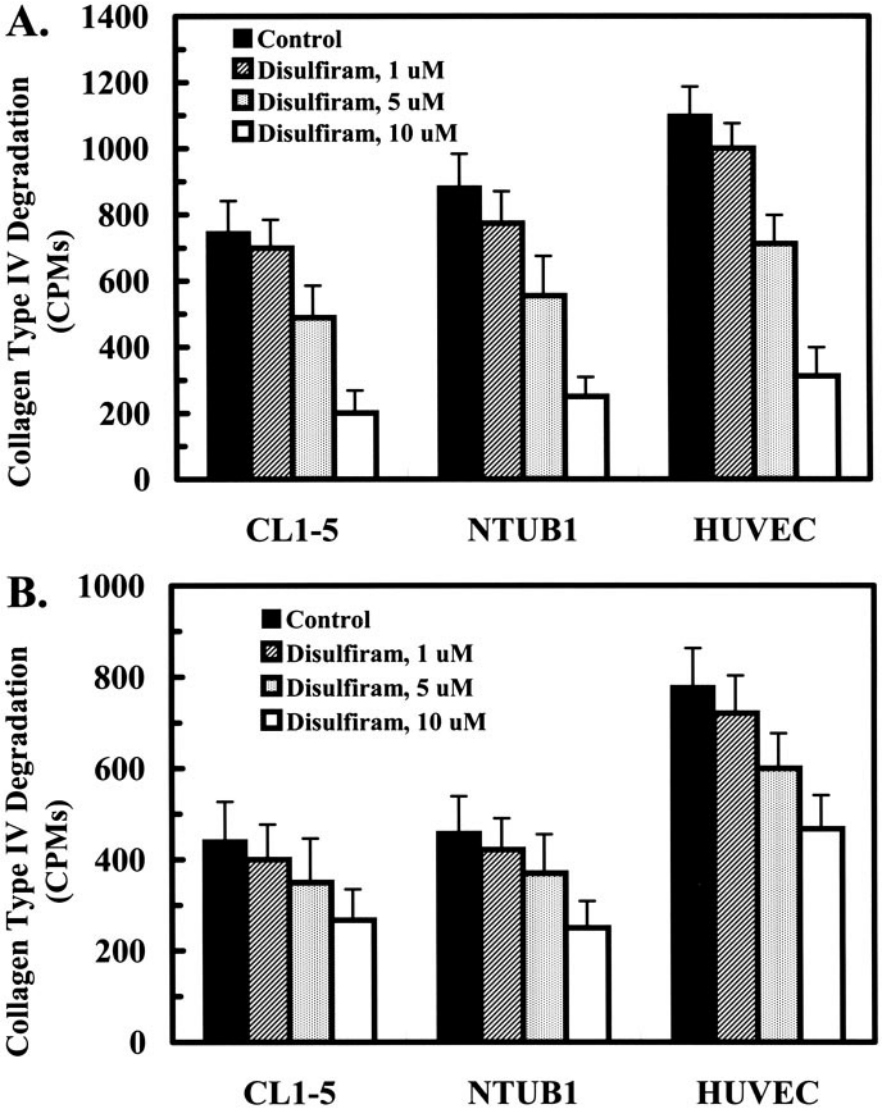

Fig. 3. Dose-response relationship for disulfiram blocking collagen type IV degradation. A, in the cell-mediated system, CL1–5, NTUB1, and HUVEC cells were cultured with ^{3}H-labeled type IV collagen and treated with 0 to 10 μM disulfiram. B, in the cell free system, 200 μl of various cell culture media was treated with disulfiram described as above. Collagen degradation was assessed as the radiolabeled material released per 50 μl of culture medium after 48 h, corrected for the cpm released from buffer only wells. Data are means ± S.E. from triplicate wells.

side effects. Nonetheless, in anticancer clinical trials of some MMP inhibitors such as marimastat and batimastat, there were considerable side effects during therapy (Wojtowicz-Praga et al., 1998) caused by disruption of numerous physiological processes (Goetzl et al., 1996). On the other hand, disulfiram has been used for treatment of alcohol abuse for many years (Garbutt et al., 1999) because of its ability to inhibit the liver mitochondrial form of aldehyde dehydrogenase (Ploemen et al., 1996). Disulfiram alone is a relatively nontoxic substance (Chick, 1999) but the combined intake of disulfiram and ethanol provokes an unpleasant reaction (including nausea, hypotension, and flushing), which is the basis of its therapeutic use (Ploemen et al., 1996). Taken together, these findings suggest that disulfiram is a relatively low risk drug for the pharmacological treatment of several human diseases.

In addition to its effects on cell invasion, the anticancer activity of disulfiram is also likely to depend on its ability to inhibit angiogenesis, as shown in the chick CAM assay (Fig. 2). In animal studies, disulfiram has proven to be a potential angiogenesis inhibitor (Marikovsky et al., 2002). Angiogenesis is an important process in tumor development; tumors must recruit blood vessels to support their progressive growth and metastasis (Folkman, 1992). Disulfiram totally inhibited chick CAM angiogenesis, suggesting that disulfiram may possibly disrupt an essential step in new blood vessel formation, as well as endothelial cell invasion.

The next question we wanted to resolve was how disulfiram inhibits invasion and angiogenesis during tumor progression. Our data suggest that the mechanism of action of disulfiram in suppressing invasion and angiogenesis seems to be related to its ability to block type IV collagenase activities, especially for MMP-2 and MMP-9. Disulfiram could directly interact with MMP-2 and MMP-9, and then inhibit their proteolytic activity, as in the gelatin zymography and collagen type IV degradation assay (Figs. 3, 4 and 5). MMP-2 and MMP-9 are commonly overexpressed in highly aggressive human tumors and are thought to be responsible for extracellular proteolysis (Seftor et al., 1998). Proteolytic processes seem to be important for tumor invasion and angiogenesis (Liotta et al., 1991). Our experimental cell lines also expressed high levels of MMP-2 and MMP-9 in culture, indi-

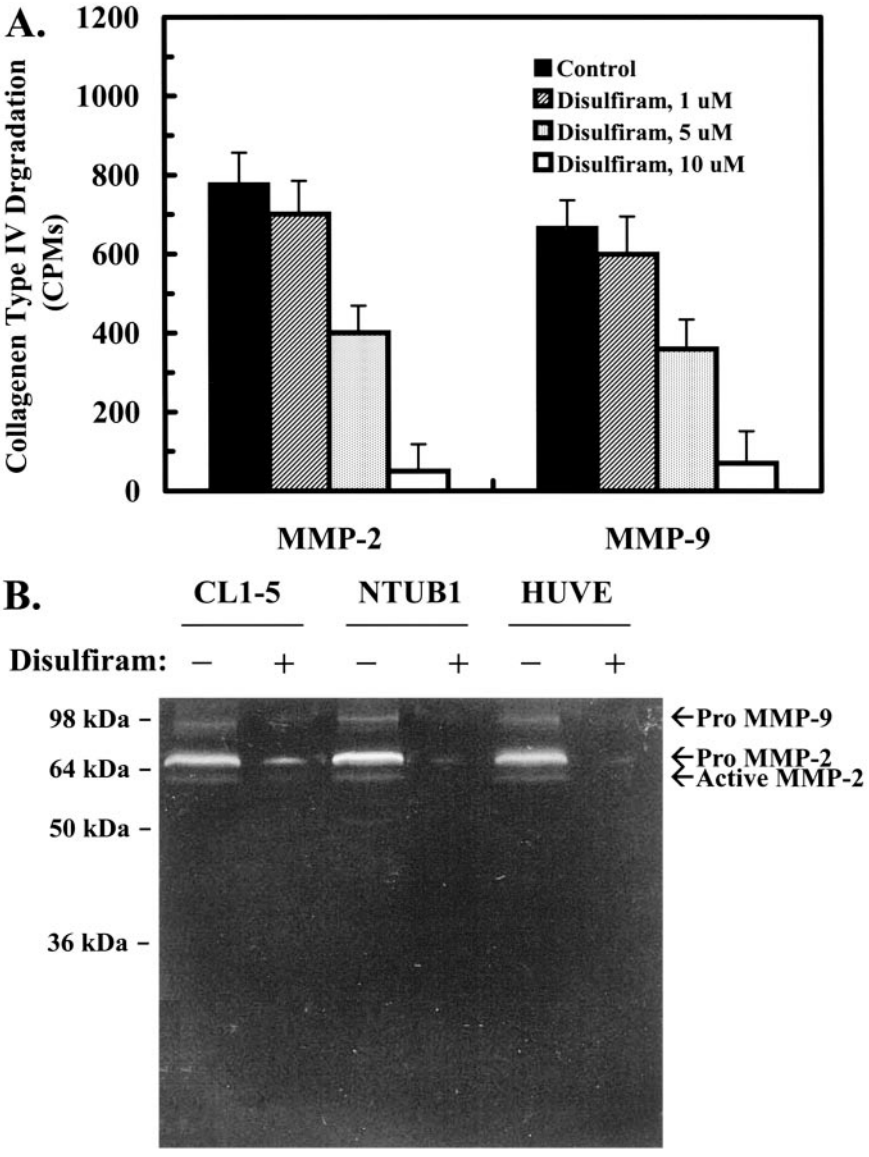

Fig. 4. Disulfiram inhibited MMP-2 and MMP-9 activities. A, 0.1 μg of purified recombinant MMP-2 and MMP-9 proteins were treated with disulfiram in 200 μl of reaction buffer. Data are means ± S.E. from triplicate wells. B, gelatinase activity of the medium below the Matrigel chamber. Briefly, 25 μl of culture medium in the lower well of the invasion chamber was subjected to electrophoresis on 10% polyacrylamide gel containing 0.1% gelatin. Two major gelatinolytic bands of 88 (pro MMP-9) and 68 kDa (pro MMP-2) were detected from culture medium.

cating the importance of extracellular proteolysis during invasion and angiogenesis. It is worth noting that disulfiram inhibited type IV collagen degradation, including cell-mediated and non–cell-mediated pathways, suggesting that there are at least two possible mechanisms involved in this inhibition process. Disulfiram has been reported to react with sulfhydryl groups of various proteins by forming intermolecular mixed disulfides (Neims et al., 1966). The formation of intermolecular disulfide bonds between the active site thiol and the thiol of another cysteine residue potentially causes the loss of invasion and angiogenesis-related enzyme activities (Hogg, 2002). On the other hand, some authors showed that disulfiram could down-regulate c-Jun/c-Fos and NF-κB expression levels and then decrease the DNA binding activity of AP-1 and NF-κB (Liu et al., 1998). These may also influence gene expression responsible for regulating invasion and angiogenesis. For example, the mitogen-activated protein kinase pathway up-regulates MMP-1, MMP-3 and MMP-9 expression through AP-1–dependent transcriptional activation in human skin fibroblasts and glioma cells; this activation plays a crucial role in stimulation of the proteolytic capacity of normal fibroblast as well as glioma cells during wound repair and tumor invasion (Lakka et al., 2002; Reunanen et al., 2002). Moreover, the activation of transcription factor NF-κB was important for breast cancer invasion by induction of the urokinase-type plasminogen activator secretion (Sliva et al., 2002). In addition, disulfiram could attenuate superoxide dismutase activity and subsequently inhibit angiogenesis (Marikovsky et al., 2002). Taken together, these phenomena may provide possible cell-mediated signal pathways as a result of disulfiram interference.

Our data strongly suggested that disulfiram could directly interact with MMP-2 and MMP-9 proteins through a non–cell-mediated pathway (Figs. 3, 4, and 5). Disulfiram has

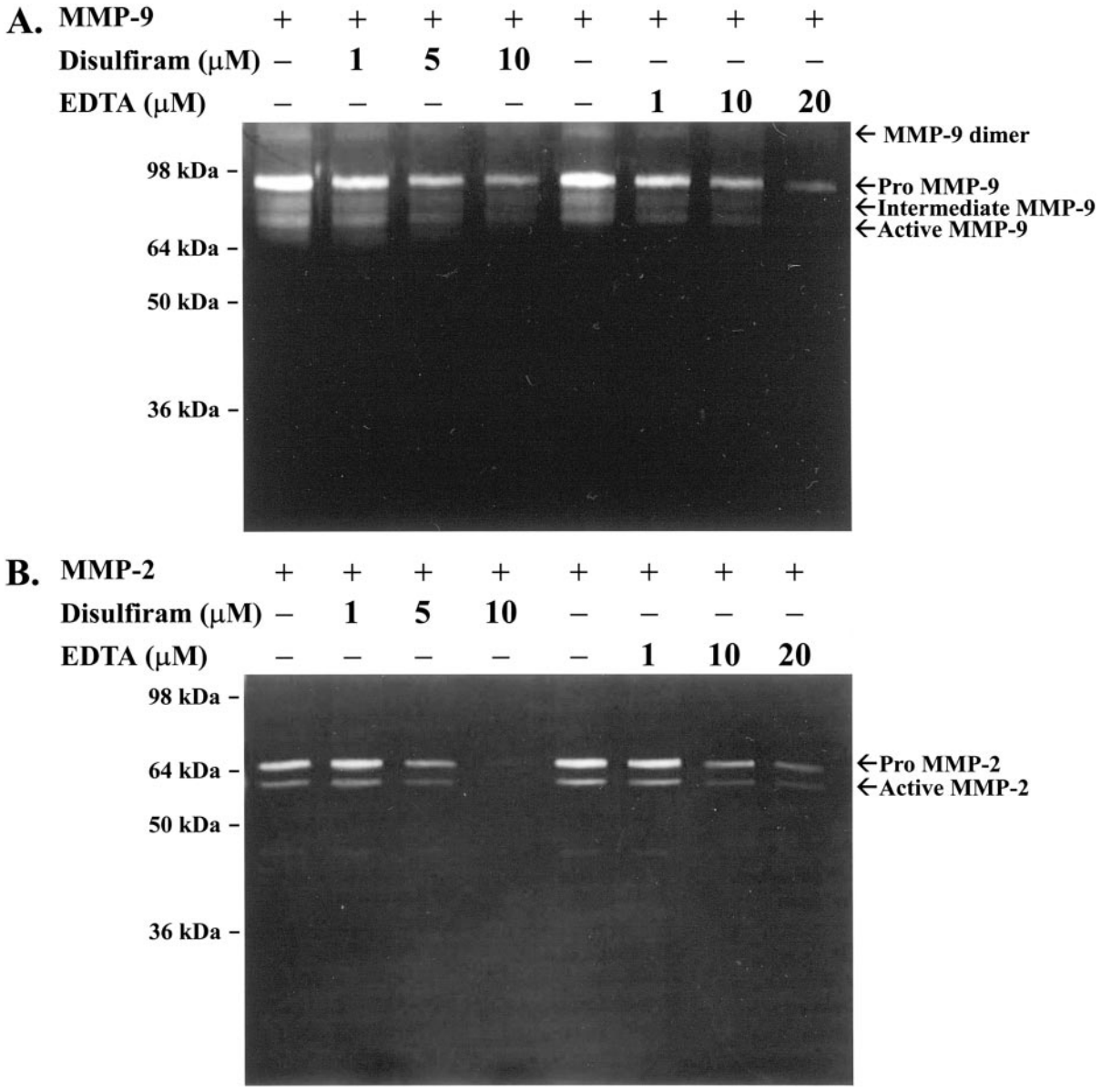

Fig. 5. Zymographic analysis of purified MMP-2 and MMP-9 proteins treated with increasing concentrations of chelating agents. Purified MMP-9 (A) or MMP-2 (B) protein (0.1 μg) was mixed with various concentrations of disulfiram or EDTA in reaction buffer. The mixture was then electrophoresed on 10% polyacrylamide gel containing 0.1% gelatin. Supershift bands in A are the MMP-9 dimer (~180 kDa).

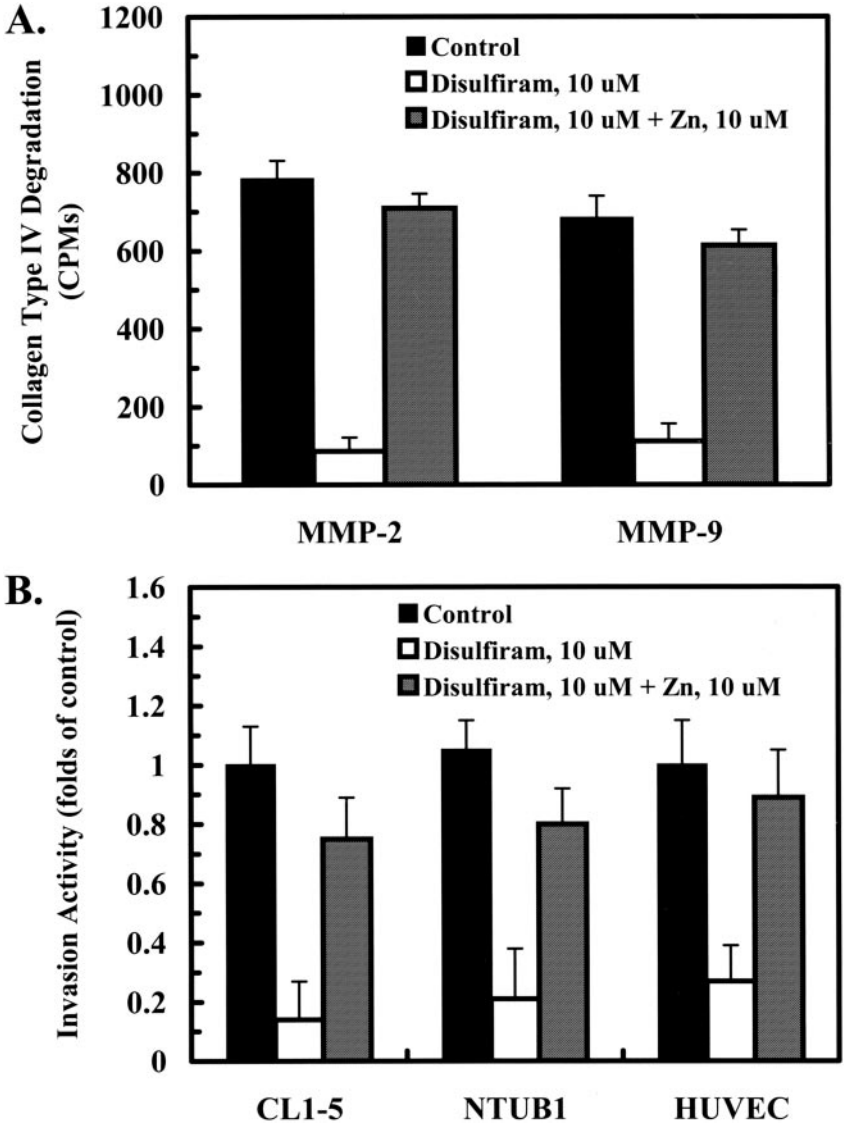

Fig. 6. Effects of zinc on disulfiram inhibition. A, 0.1 μg of purified recombinant MMP-2 and MMP-9 proteins were treated with disulfiram for 48 h in the presence or absence of 10 μM zinc. B, the invasion activity was measured using a MICS chamber under 10 μM disulfiram treatment for 48 h in the presence or absence of 10 μM zinc. Data are means ± S.E. from triplicate wells.

been identified as a potent candidate for an anti-HIV compound for the treatment of acquired immunodeficiency syndrome (Rice et al., 1995). Its inhibition of HIV is proposed to be mediated by direct interaction with the HIV nucleocapsid protein, acting as a protease inhibitor (Condra et al., 1995). The NMR assay revealed that disulfiram could readily eject zinc from synthetic peptides with sequences corresponding to the HIV nucleocapsid protein zinc finger, as well as from the intact HIV nucleocapsid protein (McDonnell et al., 1997). These facts suggest that disulfiram has zinc-ejecting capability and is a zinc ejector. MMPs belong to the zinc binding proteinase family, and zinc is essential for their proteolytic capacity of ECM degradation. MMPs are generally inhibited by compounds containing reactive zinc-chelating groups, such as thiol or hydroxamate (Talbot and Brown, 1996). However, zinc could reverse the collagenase activity inhibited by 10 μM disulfiram treatment. Importantly, the invasion ability inhibited by disulfiram among the CL1–5, NTUB1, and HUVEC cells was also reversed by addition of zinc (Fig. 6). Thus, disulfiram may, through its zinc chelating ability, inhibit MMP-2 and MMP-9 activity and subsequently inhibit invasion ability. Although our experiments showed that disulfiram possesses type IV collagenase inhibitory activity, suggesting that both MMP-2 and MMP-9 play a key role in tumor and endothelial cell invasion and angiogenesis, we cannot entirely rule out the possibility that targets other than gelatinases might exist in vivo.

In conclusion, our results indicate that a low concentration of disulfiram potently inhibits MMP-2 and MMP-9 activity in vitro, which is responsible for extracellular proteolysis inhibition, which in turn resulted in reduction of cell invasion and angiogenesis (in vivo). These findings suggest that MMPs are potential targets for therapeutic intervention in cancer therapy, and disulfiram may be a good MMP inhibitor for clinical application.

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