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TITLE: Examination of Potential Anti-Tumor Activity of N-Thiolated b-Lactam Antibiotics  
in Nude Mice Bearing Human Breast Tumors

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| 14. ABSTRACT<br><br>Activation of the cellular apoptotic program is a current strategy for the prevention and treatment of human cancer including breast cancer. Because of the ease of synthesis and structural manipulation, small molecules with apoptosis-inducing ability have great potential to be developed into chemotherapeutic drugs. The beta-lactam antibiotics have for the past 60 years played an essential role in treating bacterial infections without causing toxic side effects in the host. We hypothesized that active N-thiolated b-lactams can damage DNA and induce apoptosis in human breast cancer cells in nude mice. In this final report, we have first evaluated potencies of many novel synthetic beta-lactams to inhibit proliferation and induce apoptosis in human cancer cells. We then determined whether several of these b-lactams, L1, HY2, HY14, HY15 and SC4, could damage breast tumor cell DNA and inhibit breast tumor growth in vivo. We have found that these b-lactams inhibited growth of implanted MDA-MB-231 breast tumors in a concentration-dependent manner, associated with their DNA-damaging activities. Our studies have provided strong support for proof-of-concept of the potential use of these N-thiolated beta-lactams in breast cancer prevention and treatment. |                  |                         |                            |                                                           |                                           |
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## INTRODUCTION

Activation of the cellular apoptotic program is a current strategy for the prevention and treatment of human cancer including breast cancer (1). Because of the ease of synthesis and structural manipulation, small molecules with apoptosis-inducing ability have great potential to be developed into chemotherapeutic drugs (2). One particularly important class of small molecule drugs, the beta-lactam antibiotics, have for the past 60 years played an essential role in treating bacterial infections without causing toxic side effects in the host (3, 4). Recently we uncovered new members of this family of drugs, termed N-thiolated beta-lactams, which are highly effective at inhibiting bacterial growth in drug-resistant strains of *Staphylococcus aureus* (5, 6). Their mode of action appears to differ from that of traditional beta-lactam antibiotics. Most innovatively, we have discovered and characterized for the first time the anti-proliferative and apoptosis-inducing properties of N-thiolated beta-lactam antibiotics against human tumor cells (7). However, whether N-thiolated beta-lactam antibiotics have actual anti-breast cancer effects *in vivo* remains unknown.

## BODY

For details, please see the included APPENDICES. Please also see below KEY RESEARCH ACCOMPLISHMENTS.

**Structure-activity relationships of N-methylthiolated beta-lactam antibiotics with C<sub>3</sub> substitutions and their selective induction of apoptosis in human cancer cells (8 and see Appendices)**. The development of novel anti-cancer drugs that induce apoptosis has long been a focus of drug discovery. Beta-lactam antibiotics have been used for over 60 years to fight bacterial infectious diseases with little or no side effects observed. Recently a new class of N-methylthiolated beta-lactams has been discovered that have potent activity against methicillin resistant *Staphylococcus aureus*. Most recently, we determined the potential effects of these N-thiolated beta-lactams on tumorigenic cell growth and found that they are apoptosis-inducers in human cancer cell lines. In the current study, we further determined the effects of the substitution of the O-methyl moiety on C<sub>3</sub> and stereochemistry of the beta-lactams on the anti-proliferative and apoptosis-inducing abilities. We have found that Lactam 18, in which C<sub>3</sub> is substituted with an acrylate ester group, is a very effective proliferation inhibitor against human premalignant and malignant **breast**, leukemic, and simian virus 40-transformed fibroblast cells. Generally speaking, increasing the size of the moiety on C<sub>3</sub> decreases its anti-proliferation potency, possibly indicating steric hindrance with the cellular target or decreased permeability through the cell membrane. We also found that the stereochemistry of the beta-lactams plays an important role in their potency. The 3*S*,4*R* isomers are more effective than their enantiomers (3*R*,4*S*), suggesting that 3*S*,4*R* configuration is more favorable for target interaction.

**Beta-lactams and their potential use as novel anticancer chemotherapeutics drugs (9 and see Appendices)**. The discovery of natural and synthetic antibiotics is one of the most important medical breakthroughs in human history. Many diseases, such as bacterial meningitis, pneumonia, and septicemia, are now curable with the use of antibiotics. Antibiotics are efficacious, generally well tolerated in patients, and have a low toxicity level. It is for these reasons antibiotics remain an attractive target for drug discovery. Traditional beta-

lactam antibiotics (e.g. penicillins, penems, cephalosporins) have a bicyclic ring structure that is conformationally rigid and functions to inhibit bacterial cell wall synthesis. In addition to the bactericidal action of antibiotics, it has been discovered that many antibiotics are capable of inhibiting tumor cell growth. There are currently many antitumor antibiotics approved for cancer therapy, which work to inhibit tumor cell growth by DNA intercalation. The use of beta-lactams as prodrugs has also met with success by aiding delivery of the chemotherapeutic directly to tumor sites. Recently, a novel class of N-thiolated monobactams, so termed because they possess a monocyclic ring instead of the bicyclic ring, has been found to induce apoptosis potently and specifically in many tumor cell lines but not in normal, non-transformed cell lines. Other beta-lactams, such as the polyaromatics, have been found to slow or inhibit tumor cell growth, and the 4-alkylidene beta-lactams are capable of inhibiting matrix metalloproteinases and leukocyte elastase activity. These data indicate that synthesis and evaluation of beta-lactams are a promising area for further development in anticancer research.

#### **Unpublished Data: Effects of Novel beta-lactams on growth of human breast cancer cells *in vitro* and *in vivo*.**

**Cell death-inducing activities of novel beta-lactams.** In order to discover more potent  $\beta$ -lactams for *in vivo* studies as proposed in this *Concept Award*, we have first examined the growth-inhibiting and cell death-inducing activities of numerous of  $\beta$ -lactams that were synthesized by our chemistry collaborators. There are 10 compounds in HY group (HY2 to HY21), 24 compounds in JG group (JG1 to JG 24) and several SC compounds (such as SC4). Chemical structures of some of the compounds are shown in Fig. 1. Our results indicate that in JG group, JG19 and JG5 were most potent cell death inducers when tested in human leukemia HL60 and Raji cell lines (Fig. 2A, B) and that in HY group, the order of potency to induce HL60 cell death was: HY20 > HY18 > HY16 = HY 15 > HY14 > HY19 > HY17 (Fig. 2C). Beta-lactam L-1 was used as a comparison (Fig. 2). The results from Western blot analysis also showed that JG19 and JG20 could induce PARP cleavage, a cellular apoptotic marker (Fig. 3).

**Beta-lactams effectively inhibit proliferation and induce apoptosis in human breast cancer cells.** Previously we reported that L-1 has the great potency to induce apoptosis in cancer cells showed by MTT assay and PARP cleavage (7). In the current experiment we screened more beta-lactams (Fig. 4) in order to discover more potent analogs for *in vivo* studies. Human breast cancer MCF-7 cells were treated with each of the indicated beta-lactams at 1, 25 or 50  $\mu$ M or DMSO (as solvent control) for 24 h, followed by performance of an MTT assay, which measures the status of cell viability and, thus, cell proliferation. After treatment with 50  $\mu$ M of L-47, cellular viability of MCF-7 was decreased by 73%. Compared with 63% inhibition by 50  $\mu$ M of L-1, L-47 was the most potent one in the tested beta-lactams (Fig. 5A).

We then treated another human breast cancer cell line MDA-MB-231 with 50  $\mu$ M of L-1, L-30, L-47 or L-53 for different time points, followed by preparation of cell lysates and measurement of PARP cleavage, a cellular apoptotic marker, by Western blotting. The results showed that among the tested lactams, L-47 had the greatest potency to induce PARP cleavage within 8 h of treatment (Fig. 5B).

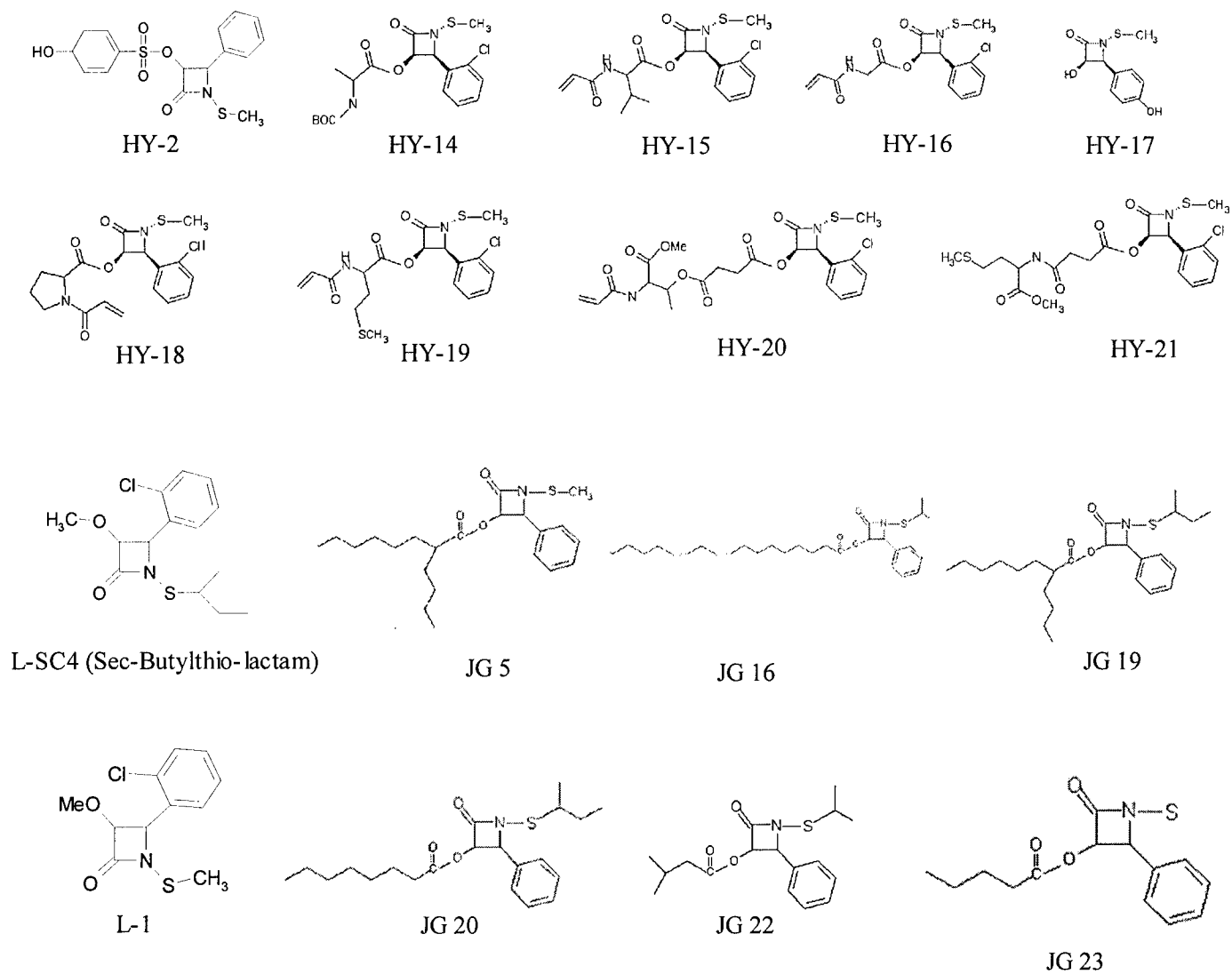
**Beta-lactam HY14, an analog of L-47, significantly inhibits the growth of breast cancer xenografts, associated with its DNA damaging activity *in vivo*.** The data described above clearly demonstrate that  $\beta$ -lactams are apoptosis inducers in cultured leukemia and breast cancer cells. Our experimental results also showed that HY14 was more potent than L-1 in inducing cell death in cancer cells (Fig. 2C). Since we have a large quantity of HY14, we then examined anti-tumor activity of HY14 *in vivo*. We implanted MDA-MB-231 cells s.c. in nude mice. When the tumors became  $\sim 200 \text{ mm}^3$ , the mice were *i.p.* treated with either vehicle control or HY14 at 0.3 or 3.0 mg/kg/day. The inhibition (up to 53%) of tumor growth by 3.0 mg/kg/day treatment of HY14 was observed after 30 days injection but only 13% inhibition of tumor growth was showed by 0.3 mg/kg/day treatment (Fig. 6A), indicating that HY has anti-tumor activity which is dose-dependent. The immunohistochemistry results showed that the apoptosis-specific TUNEL positivity was found mainly in MDA-MB-231 tumors treated with HY14 at 3.0 mg/kg/day, less in those treated with 0.3 mg/kg/day of HY14, but none in vehicle-treated tumor (Fig. 6B).

**The anti-breast tumor activities of Beta-lactams L1, HY-2, HY15 and SC4.** We then examined the antitumor activities of several other beta-lactams of which we have a large quantity. Again, MDA-MB-231 cells were s.c. implanted in nude mice. When the tumors became  $\sim 100 \text{ mm}^3$ , the mice were *i.p.* treated with either vehicle control or each selected beta-lactam at 10 or 20 mg/kg/day. We found that HY-2, HY15 and SC4 at 10 mg/kg/day inhibited tumor growth by 17%, 46% and 64%, respectively (Fig. 7). L1 at 10 and 20 mg/kg/day caused 30% and 48% inhibition, respectively (Fig. 7), indicating a dose-dependence. The tumor tissue samples are currently analyzed by immunohistochemistry assays.

**Beta-lactam 1 is able to damage tumor DNA *in vivo* and inhibits the growth of breast cancer MDA-MB-231 xenografts.** Since we have a large quantity of L1, we then examined its anti-tumor activity at higher concentrations. We implanted MDA-MB-231 cells s.c. in nude mice. When the tumors became  $\sim 100 \text{ mm}^3$ , the mice were *i.p.* treated with either vehicle control or L1 at 20 or 30 mg/kg/day. Up to 72% inhibition of tumor growth by 30 mg/kg/day treatment of L1 was observed after 30 days injection (Fig. 8), but only  $\sim 40\%$  inhibition was found by 20 mg/kg/day treatment with L1, again indicating that L1's anti-tumor activity is dose-dependent (Fig. 8). The immunohistochemistry results showed that the apoptosis-specific condensed nuclei and TUNEL positivity were found mainly in MDA-MB-231 tumors treated with L1 at 30 mg/kg/day, less in those treated with 20 mg L1/kg/day, but none in vehicle-treated tumor (Fig. 9). **Therefore, these beta-lactam antibiotics are able to selectively damage tumor cell DNA *in vivo*, resulting in cell death and growth inhibition.**

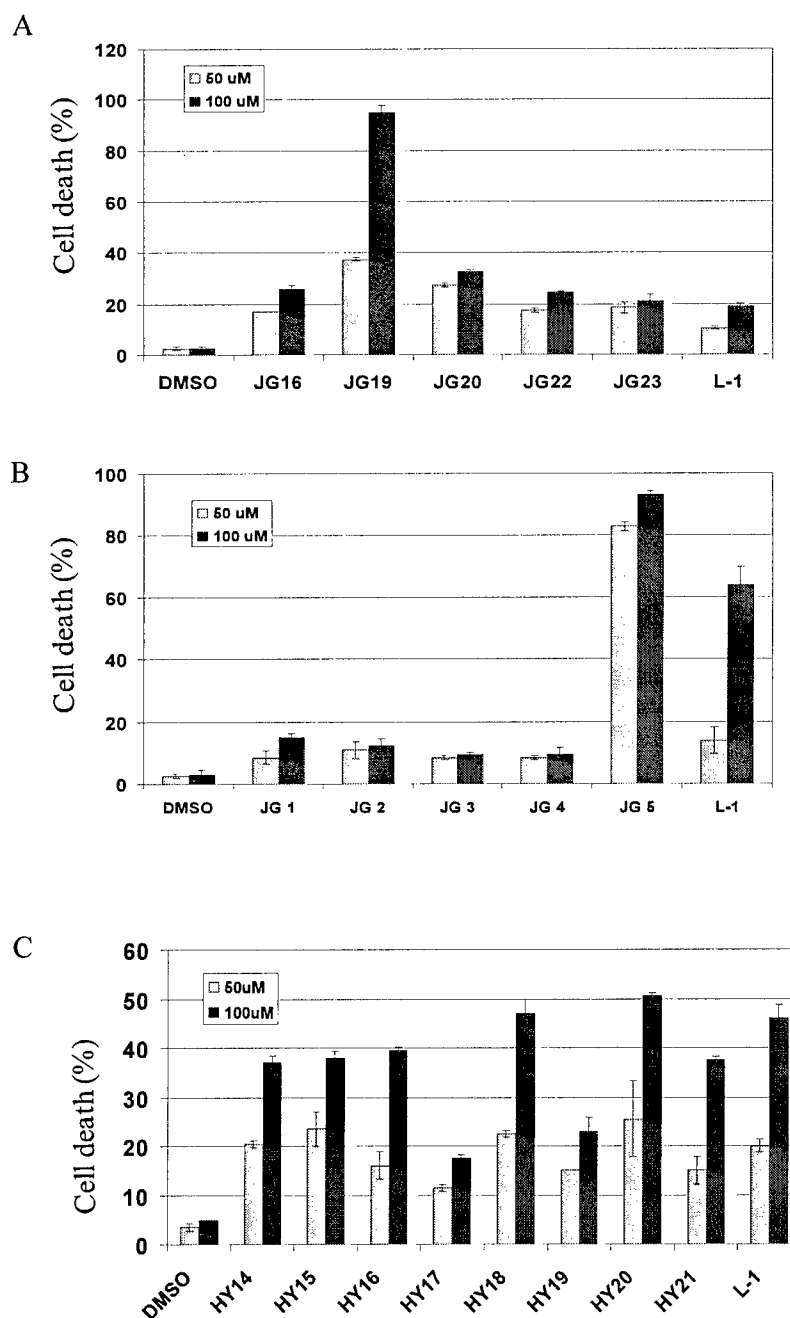
We are repeating some of the animal experiments and are processing all the collected tissue samples. These results will be summerized in one or more manuscripts for publicaition in the near future. **These studies should provide strong support for proof-of-concept of the potential use of these N-thiolated beta-lactams in breast cancer prevention and treatment.**

**Figure 1. Chemical structure of related beta-lactams**



# **Figure 2. Beta-lactams could induce cell death in human leukemia cells.**

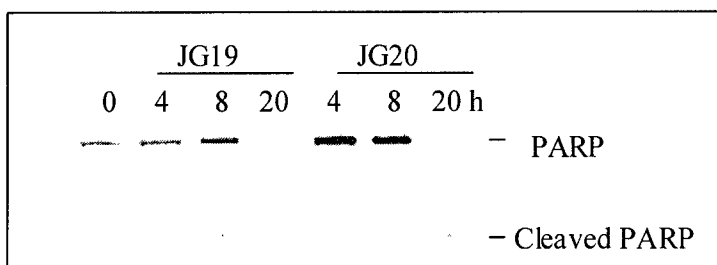
Trypan blue assays: human leukemia HL60 (A, B) and Jurkat cell line (C) were treated with 50 or 100  $\mu$ M of each beta-lactam for 24 hrs, and then non-viable cells were determined by Trypan Blue. L1 was used as a positive control.



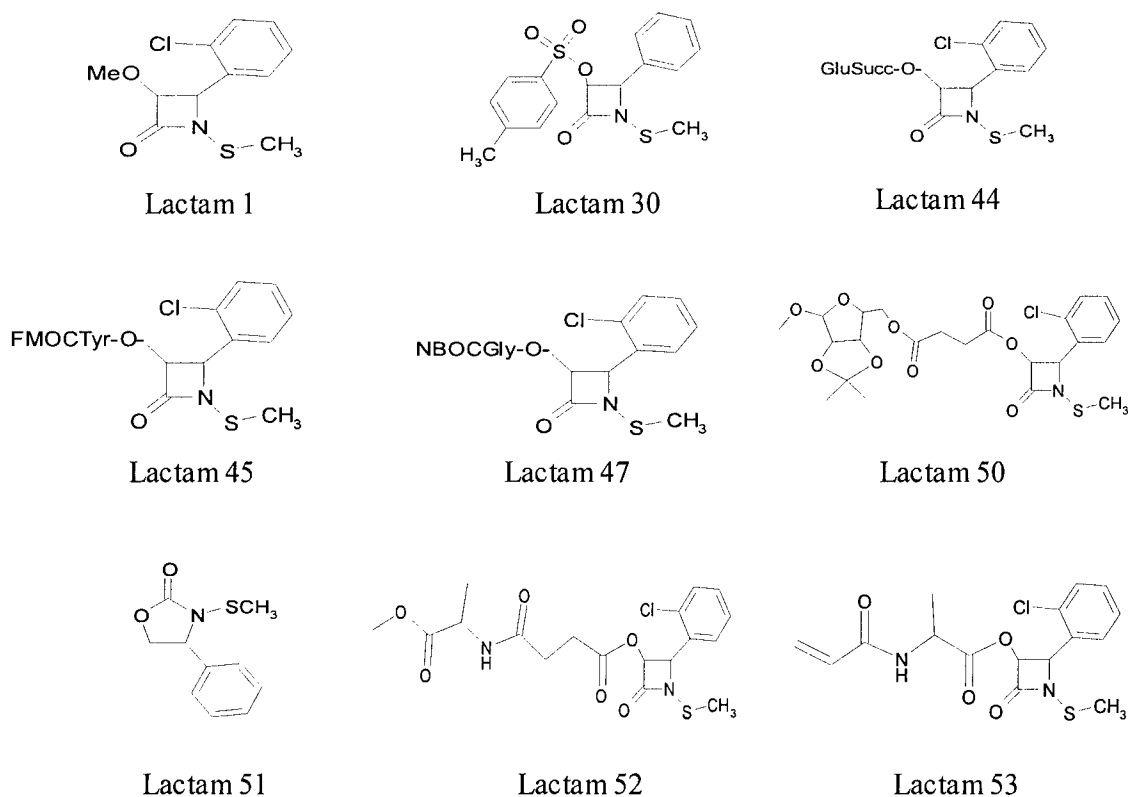
**Figure 3. JG19 and JG20 could induce apoptosis-associated PARP cleavage.**

Raji cells were treated with 50  $\mu$ M JG19 or JG20 for 4, 8, or 20 hours before being harvested.

The results show that both JG19 and JG20 induce PARP cleavage, indicative of apoptosis.

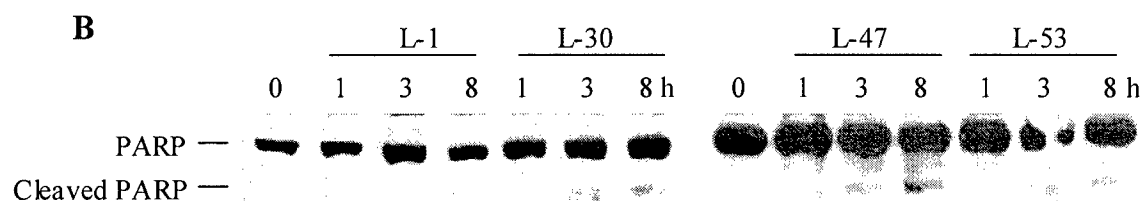
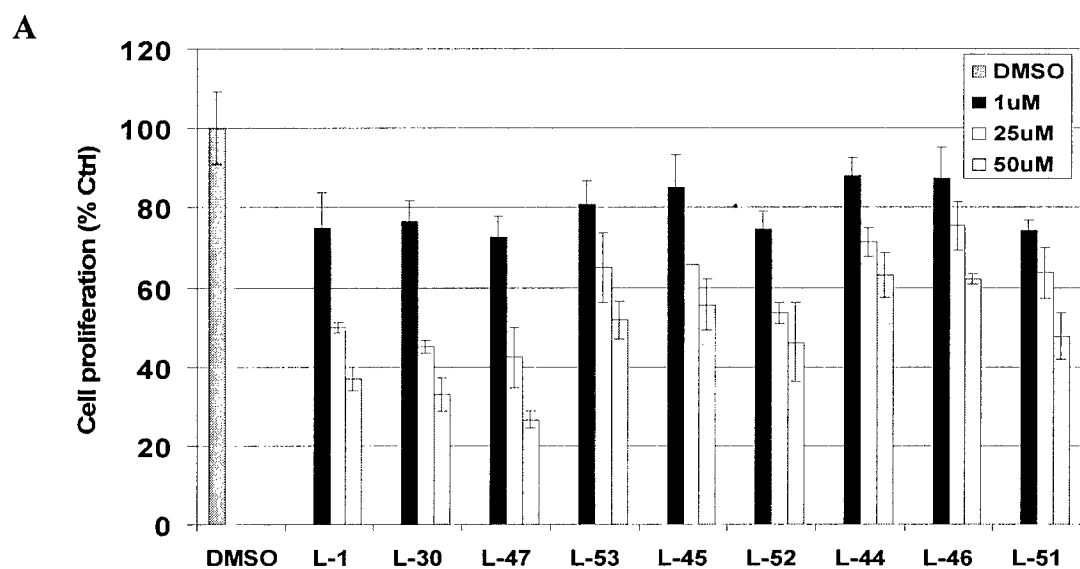


**Figure 4. Chemical structures of more beta-lactams**



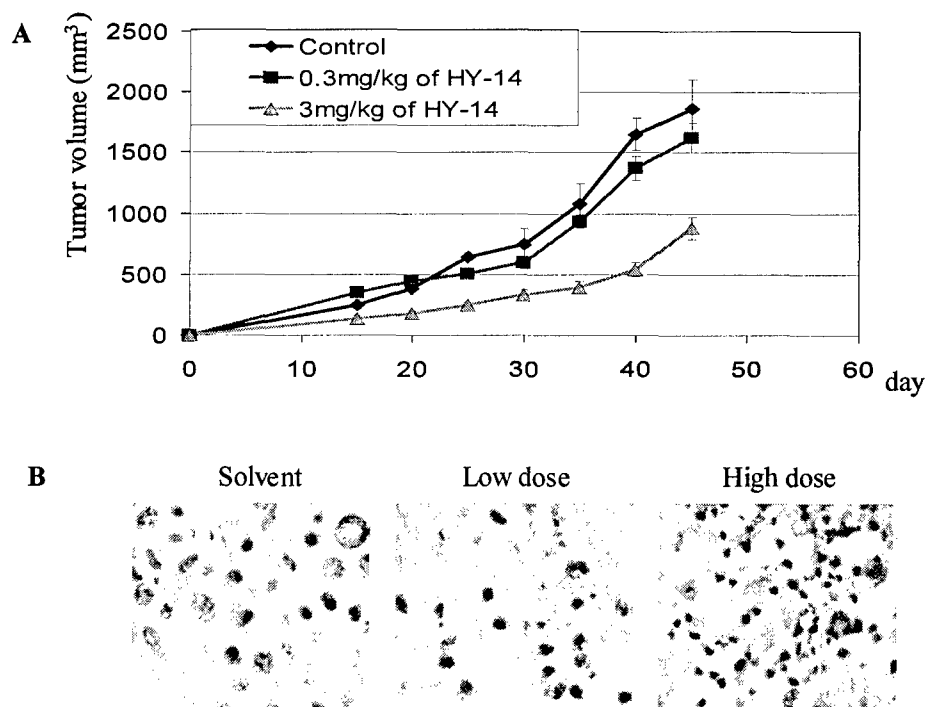
**Figure 5.  $\beta$ -lactams could effectively inhibit proliferation and induce apoptosis in human breast cancer cells.**

- A.** MCF-7 cells were treated with either 1, 25 or 50  $\mu$ M of indicated lactams or DMSO as solvent control (Ctrl) for 24 h, followed by performance of an MTT assay
- B.** MDA-MB-231 cells were treated with 50  $\mu$ M of indicated  $\beta$ -lactams for different time points, followed by preparation of cell lysates and measurement of PARP cleavage by Western blot.

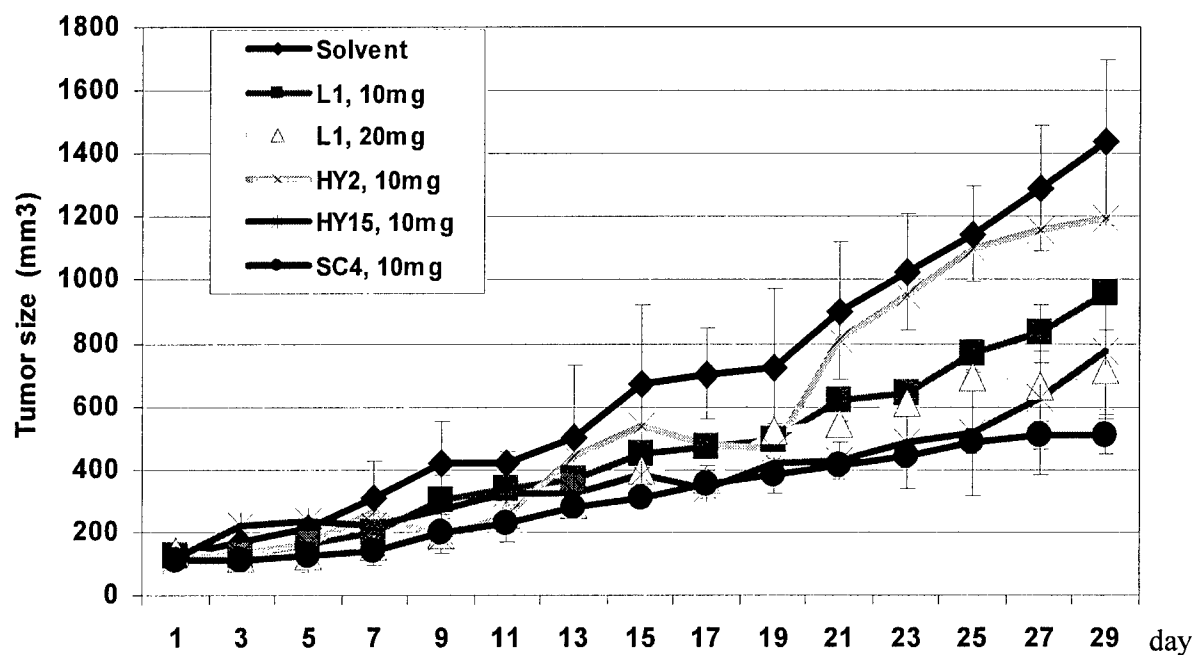


**Figure 6.  $\beta$ -lactam HY-14 could inhibit the tumor growth in nude mice implanted by human breast cancer MDA-MB-231 cells.**

Female athymic nude mice (NCRNU-M) were xenografted by injection of  $6 \times 10^6$  MDA-MB-231 cells. 15 days after the injection, the mice were divided into three groups: solvent control, low dose (0.3 mg/kg) and high dose (3 mg/kg) treatment with  $\beta$ -lactam HY-14 by subcutaneous injection daily. Tumor size was measured every 5 days and tumor volume (V) was determined by the equation:  $V = (L \times W^2) \times 0.5$ , where L is the length and W is the width of a tumor. Tumor volume was calculated and expressed as cubic millimeters (A). TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) analysis of tumor tissues from three different groups was performed. Nuclei stained in dark brown indicate TUNEL positive (B).

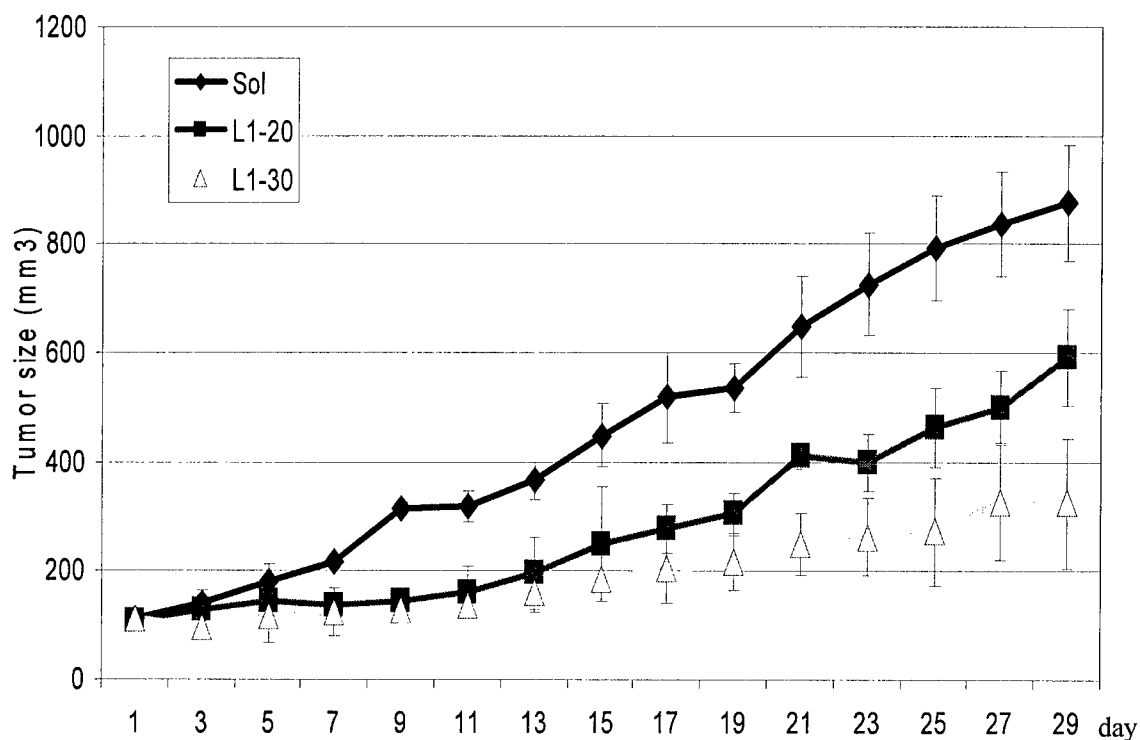


## Effect of Beta-lactams on MDA-MB-231 xenografts



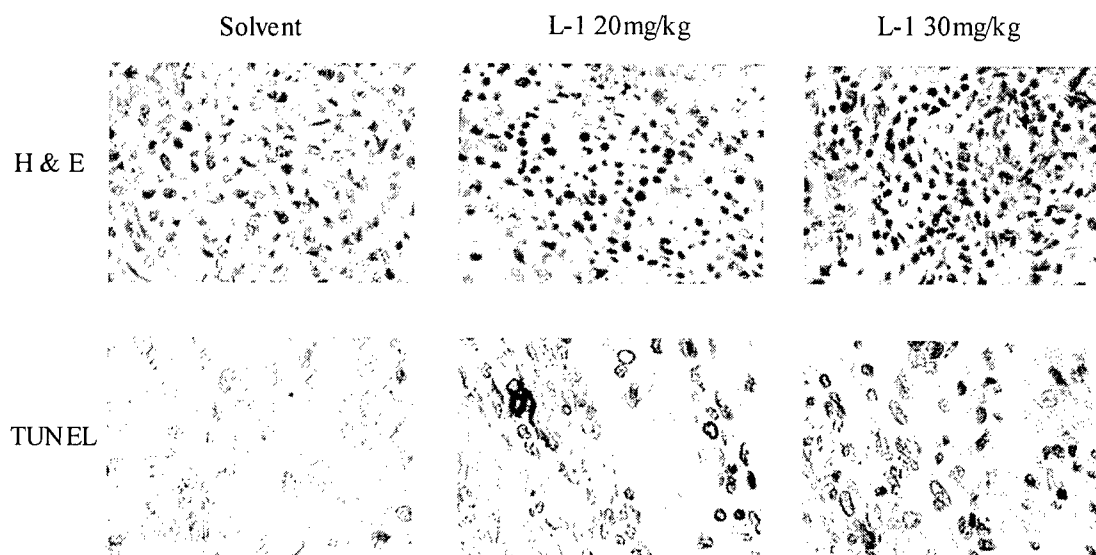
**Fig. 7. Effects of other four  $\beta$ -lactams L-1, HY-2, HY-15 and SC4 on tumor growth in nude mice implanted by human breast cancer MDA-MB-231 cells.**

Female athymic nude mice (NCRNU-M) were xenografted by injection of  $6 \times 10^6$  MDA-MB-231 cells. When the tumor size reached  $\sim 100 \text{ mm}^3$ , the mice were divided into six groups: solvent control, 10 mg/kg or 20 mg/kg of L-1, HY-2, HY-15 or SC4 (all at 10 mg/kg). Treatment was performed by daily subcutaneous injection. Tumor size was measured every other day.



**Fig. 8. Re-test of  $\beta$ -lactams L-1 with higher concentration in mice bearing xenograft breast cancer**

Female athymic nude mice (NCRNU-M) were xenografted by injection of  $6 \times 10^6$  MDA-MB-231 cells. When the tumor size reached  $\sim 100$  mm<sup>3</sup>, the mice were treated with solvent, 20 mg/kg or 30 mg/kg of L-1 by daily subcutaneous injection. Tumor size was measured every other day.



**Fig. 9. Analysis of the tumor tissues by immunohistochemistry**

**Other related publications** (For details, please see the included **APPENDICES**. Please also see below **KEY RESEARCH ACCOMPLISHMENTS**).

**Apoptotic-Inducing Activity of Novel Polycyclic Aromatic Compounds in Human Leukemic Cells (10)**. Persistent, but relatively limited research has been devoted to the use of compounds related to polycyclic aromatic hydrocarbons (PAH) as anticancer agents. In previous reports we have described the cytotoxicity of a number of new and novel PAH against human cancer cell lines. However, the involved molecular mechanisms of inducing cell death were not elucidated. In the current study, we describe the apoptotic pathway as apparently playing a crucial role in cell death induced in human leukemia Jurkat T cells by several diamide and diamine PAH that contain chrysene as their core aromatic ring system. Structure-activity relationships were analyzed. Importantly, no effect was demonstrated in a normal, non-transformed line of human natural killer cells. These results provide additional evidence for the potential chemotherapeutic use of PAH.

**Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells (11 and see Appendices)**. **Introduction** A physiological feature of many tumor tissues and cells is the tendency to accumulate high concentrations of copper. While the precise role of copper in tumors is cryptic, copper, but not other trace metals, is required for angiogenesis. We have recently reported that organic copper-containing compounds, including 8-hydroxyquinoline-copper(II) and 5,7-dichloro-8-hydroxyquinoline-copper(II), are a novel class of proteasome inhibitors and tumor cell apoptosis inducers. In the current study, we investigate whether clioquinol (CQ), an analog of 8-hydroxyquinoline and an Alzheimer's Disease drug, and pyrrolidine dithiocarbamate (PDTC), a known copper-binding compound and antioxidant, can interact with copper to form cancer-specific proteasome inhibitors and apoptosis inducers in human breast cancer cells. Tetrathiomolybdate (TM), a strong copper chelator currently being tested in clinical trials, is used as a comparison. **Methods** Breast cell lines, normal, immortalized MCF-10A, premalignant MCF10AT1K.cl2, and malignant MCF10DCIS.com and MDA-MB-231, were treated with CQ or PDTC with or without prior complexation with copper, followed by measurement of proteasome inhibition and cell death. Inhibition of the proteasome was determined by levels of the proteasomal chymotrypsin-like activity and ubiquitinated proteins in protein extracts of the treated cells. Apoptotic cell death was measured by morphological changes, Hoechst staining, and Poly(ADP-Ribose) Polymerase cleavage. **Results** When complexed with copper, both CQ and PDTC, but not TM, can inhibit the proteasome chymotrypsin-like activity, block proliferation, and induce apoptotic cell death preferentially in breast cancer cells, less in premalignant breast cells, but are nontoxic to normal/non-transformed breast cells at the concentrations tested. In contrast, CQ, PDTC, TM or copper alone had no effects on any of the cells. Breast premalignant or cancer cells, that contain copper at concentrations similar to those found in patients, when treated with just CQ or PDTC alone, but not TM, undergo proteasome inhibition and apoptosis. **Conclusions** The feature of breast cancer cells and tissues to accumulate copper can be used as a targeting method for anticancer therapy through treatment with novel compounds such as CQ and PDTC that become active proteasome inhibitors and breast cancer cell killers in the presence of copper.

**INHIBITION OF PROSTATE CANCER CELLULAR PROTEASOME ACTIVITY BY A PYRROLIDINE DITHIOCARBAMATE-COPPER COMPLEX IS ASSOCIATED WITH SUPPRESSION OF PROLIFERATION AND INDUCTION OF APOPTOSIS (12 and see**

**Appendices).** Recent research suggests that copper could be used as a novel selective target for cancer therapies. Copper is a co-factor essential for tumor angiogenesis processes and high levels of copper have been found in many types of human cancers, including prostate, breast and brain. We have reported that organic copper-containing compounds, such as 8-hydroxyquinoline-copper(II), are a novel class of proteasome inhibitors and tumor cell apoptosis inducers (Daniel et al., Biochem Pharmacol. 2004;67:1139-51). Most recently, we have found that when complexed with copper, the known antioxidant pyrrolidine dithiocarbamate (PDTC) forms a potent proteasome inhibitor in human breast cancer, but not normal cells (11). In the current study, we investigate whether the PDTC-copper complex can play similar roles in inhibiting the proteasomal activity and consequently inducing apoptosis in human prostate cancer cells. We used tetrathiomolybdate (TM), a strong copper chelator currently being tested in clinical trials, as a control. We report here that after binding to copper, PDTC, but not TM, can inhibit the proteasomal chymotrypsin-like activity, suppress proliferation, induce apoptotic cell death, and inhibit uptake of radiopharmaceutical 2-[<sup>18</sup>F]Fluoro-2-deoxy-D-glucose in cultured human prostate cancer cells. In contrast, PDTC, TM or copper alone or a TM-copper mixture had no such effects. Our study suggests that high copper levels in human prostate cancer *in vivo* can be targeted by a ligand such as PDTC, resulting in formation of an active proteasome inhibitor and apoptosis inducer specifically in prostate tumor, but not normal cells.

**Disulfiram, A Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts via Inhibition of the Proteasome Activity (13 and see Appendices).** Disulfiram (DSF), a member of the dithiocarbamate family capable of binding copper and an inhibitor of aldehyde dehydrogenase, is currently being used clinically for the treatment of alcoholism. Recent studies have suggested that DSF may have antitumor and chemosensitizing activities although the detailed molecular mechanisms remain unclear. Copper has been shown essential for tumor angiogenesis processes. Consistently, high serum and tissue levels of copper have been found in many types of human cancers, including breast, prostate and brain, supporting the idea that copper could be used as a potential tumor-specific target. Here we report that the DSF-copper complex potentially inhibits the proteasomal activity in cultured breast cancer MDA-MB-231 and MCF10DCIS.com, but not normal, immortalized MCF-10A, cells, prior to induction of apoptotic cancer cell death. Furthermore, MDA-MB-231 cells that contain copper at concentrations similar to those found in patients, when treated with just DSF, undergo proteasome inhibition and apoptosis. In addition, when administered to mice bearing MDA-MB-231 tumor xenografts, DSF significantly inhibited the tumor growth (by 74%), associated with *in vivo* proteasome inhibition (as measured by decreased levels of tumor tissue proteasome activity and accumulation of ubiquitinated proteins and natural proteasome substrates p27 and Bax) and apoptosis induction (as shown by caspase activation and apoptotic nuclei formation). Our study demonstrates that inhibition of the proteasomal activity can be achieved by targeting tumor cellular copper with the non-toxic compound DSF, resulting in selective apoptosis induction within tumor cells.

**A Novel Anticancer Gold(III) Dithiocarbamate Compound Inhibits the Activity of a Purified 20S Proteasome and 26S Proteasome in Human Breast Cancer Cell Cultures and Xenografts (14).** Although cisplatin has been used for decades to treat human cancer, some toxic side effects and resistance are observed. It has been suggested that gold(III) complexes, containing metal centers isoelectronic and isostructural to cisplatin, are promising anticancer drugs. Gold(III) dithiocarbamate complexes were shown to exhibit *in vitro*

cytotoxicity, comparable to and even greater than cisplatin, however, the involved mechanism of action remained unknown. Because we previously reported that copper(II) dithiocarbamates are potent proteasome inhibitors, we hypothesized that gold(III) dithiocarbamate complexes could suppress tumor growth *via* direct inhibition of the proteasome activity. Here, for the first time, we report that a synthetic gold(III) dithiocarbamate, Compound 2, potently inhibits the activity of a purified rabbit 20S proteasome and 26S proteasome in intact, highly metastatic, MDA-MB-231 breast cancer cells, resulting in the accumulation of ubiquitinated proteins and the proteasome target protein p27 and induction of apoptosis. The Compound 2-mediated proteasome inhibition and apoptosis induction were completely blocked by addition of a reducing agent, 1,4-Dithio-DL-threitol or N-Acetyl-L-Cysteine, demonstrating that process of oxidation is required for proteasome inhibition by Compound 2. Treatment of MDA-MB-231 breast tumor-bearing nude mice with Compound 2 resulted in significant inhibition of tumor growth, associated with proteasome inhibition and massive apoptosis induction *in vivo*. Our findings reveal the proteasome as a primary target for gold(III) dithiocarbamates, and support the idea for their potential use as anticancer therapeutics.

### **KEY RESEARCH ACCOMPLISHMENTS**

- Published 18 articles and 7 abstract
- At least two more manuscript in preparation
- Gave 16 scientific presentations
- Trained and graduated two Ph.D. students
- Partially supported several personnel (Deborah Kuhn, Ph.D., Kenyon Daniel, Ph.D., Di Chen, Ph.D., Lihua Li, M.D., Guoqing Shi, Ph.D., Shirley Orlu, B.S., Cindy Cui, B.S.)

### **REPORTABLE OUTCOMES**

Provide a list of reportable outcomes that have resulted from this research to include:

#### **Manuscript Publication (see Appendices):**

Kuhn D, Coates C, Daniel K, Chen D, Bhuiyan M, Kazi A, Turos E, Dou QP. Beta-lactams and their potential use as novel anticancer chemotherapeutics drugs. *Front Biosci*. 2004 Sep 1;9:2605-17.

Kazi A, Hill R, Long TE, Kuhn DJ, Turos E, Dou QP. Novel N-thiolated beta-lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells. *Biochem Pharmacol*. 2004;67:365-74.

Kuhn DJ, Wang Y, Minic V, Coates C, Reddy GS, Daniel KG, Shim JY, Chen D, Landis-Piowar KR, Miller FR, Turos E, Dou QP. Structure-activity relationships of N-methylthiolated beta-lactam antibiotics with c3 substitutions and their selective induction of apoptosis in human cancer cells. *Front Biosci*. 2005 May 1;10:1183-90.

Chen D, Peng F, Cui QC, Daniel KG, Orlu S, Liu J, and Dou QP. Inhibition of prostate cancer cellular proteasome activity by a pyrrolidine dithiocarbamate-copper complex is associated

with suppression of proliferation and induction of apoptosis. *Frontiers in Bioscience*, 2005; 10: 2932-2939.

Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, and Dou QP. Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells. *Breast Cancer Res*, 2005; 7:R897-R908

Landis-Piwowar KR, Chen D, Cui QC, Minic V, Becker FF, Banik BK, and Dou QP. Apoptotic-Inducing Activity of Novel Polycyclic Aromatic Compounds in Human Leukemic Cells. *Intl. J Mol Med.*, 2006;17(5):931-5.

Yang HJ, Chen D, Cui QC, Yuan X, and Dou QP. Celastrol, a triterpene extracted from the Chinese Thunder of God Vine, is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. (A figure was selected as the cover of the issue) *Cancer Research* 66, 4758-4765, May 1, 2006

Chen D, Cui QC, Yang HJ, and Dou QP. Disulfiram, A Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts *via* Inhibition of the Proteasome Activity. *Cancer Research*, (in press)

Milacic V, Chen D, Ronconi L, Landis-Piwowar KR, Fregona D, and Dou QP. A Novel Anticancer Gold(III) Dithiocarbamate Compound Inhibits the Activity of a Purified 20S Proteasome and 26S Proteasome in Human Breast Cancer Cell Cultures and Xenografts. *Cancer Research*, (in press)

Pelley RP, Chinnakannu K, Murthy S, Strickland FM, Menon M, Barrack ER, Dou QP and Reddy GPV. Calmodulin-androgen receptor interaction: calcium-controlled, calpain-mediated breakdown of androgen receptor in LNCaP prostate cancer cells. *Cancer Research*, (in press)

Adsule S, Barve V, Chen D, Ahmed F, Dou QP, Padhye S, Sarkar FH. Novel Schiff base copper complexes of quinoline-2 carboxaldehyde as proteasome inhibitors in human prostate cancer cells. *J Med Chem*, (in press)

Kuhn D, Coates C, Daniel KG, Chen D, Bhuiyan M, Kazi A, Turos E, and Dou QP. Beta-Lactams and Their Potential Use As Novel Anti-Cancer Chemotherapies. *Frontiers in Bioscience*, 2004; 9: 2605-2617

Daniel KG, Harbach RH, Guida WC, and Dou QP. Copper Storage Diseases: Wilson's, Menkes and Cancer. *Frontiers in Bioscience*, 2004; 9: 2652-2662

Smith DM, Daniel KG and Dou QP. Exploiting the Ubiquitin-Proteasome Pathway for Anticancer Drug Discovery: Unanswered Questions and Future Directions (invited review). *Letters in Drug Design & Discovery*, 2005; 2: 77-84

Kuhn DJ and Dou QP. The Role of Interleukin-2 Receptor Alpha in Cancer. *Frontiers in Bioscience*, 2005; 10: 1462-1474

Daniel KG, Kazi A, Kuhn DJ and Dou QP. Anti-angiogenic and anti-tumor properties of proteasome inhibitors (invited review). *Curr Cancer Drug Targets*, 2005; 5: 529-541

Reddy GPV, Barrack ER, Dou QP, Menon M, Pelley R, Sarkar FH, and Sheng S. Regulatory processes affecting androgen receptor expression, stability and function: potential targets to treat hormone-refractory prostate cancer. *J. Cell. Biochem.*, 2006; 98: 1408-1423.

Daniel KG, Chen D, Yan B and Dou QP. Copper-binding compounds as proteasome inhibitors and apoptosis inducers in human cancer. *Frontiers in Bioscience*, 2007; 12: 135-144

### **Abstracts (see Appendices):**

Deborah Kuhn, Yang Wang, Vesna Minic, Cristina Coates, G. Suresh Kumar Reddy, Kenyon, G. Daniel, Jeung-Yeop Shim, Di Chen, Kristin R. Landis-Piwowar, Edward Turos, And Q. Ping Dou, Ph.D. Relationships of structures of N-methylthiolated beta-lactam antibiotics to their apoptosis-inducing activity in human breast cancer cells. Poster presentation. ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting, Pennsylvania Convention Center, Philadelphia, Pennsylvania, June 8-11, 2005 (ref. 15)

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of  $\beta$ -Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005

Di Chen, Marina Chen, Kenyon G. Daniel, Deborah J. Kuhn, Kristin Landis, and Q. Ping Dou. Dietary Flavonoids as Proteasome Inhibitors and Apoptosis Inducers in Human Leukemia Cells and Their Structure-activity Relationships. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005

Di Chen, Qiuzhi Cindy Cui, Huanjie Yang, Fazlul H. Sarkar, G. Prem Veer Reddy, Shijie Sheng, Raul A Barrea and Q. Ping Dou. Clioquinol, A Therapeutic Agent For Alzheimer's Disease, Has Proteasome-Inhibitory, Apoptosis-Inducing And Anti-Tumor Activities In Prostate Cancer Cells And Xenografts. 6<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006

Huanjie Yang, Di Chen, Qiuzhi Cindy Cui, Xiao Yuan, and Q. Ping Dou. Celastrol, A Triterpene Extracted From The Chinese Thunder Of God Vine, Is A Potent Proteasome Inhibitor And Suppresses Human Prostate Cancer Growth In Nude Mice. 6<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006 (The First Place Poster Award)

Xiaohua Li, Di Chen, Shuping Yin, Yiwei Li, Huanjie Yang, Kristin R. Landis-Piwowar, Fazlul Sarkar, Prem Veer G. Reddy, Q. Ping Dou, Shijie Sheng. Proteasome Inhibition Up-regulates Apoptosis-sensitizing Maspin. 6<sup>th</sup> Annual Symposium, Michigan Prostate Research

Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006

Di Chen, Qiuzhi Cindy Cui, Huanjie Yang and Q. Ping Dou. Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts *via* inhibition of the proteasome activity. The AACR International Conference on Frontiers in Cancer Prevention Research, Boston, MA, November 12-15, 2006 (ref. 16).

**Presentations:**

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of  $\beta$ -Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, Detroit, MI, September 8, 2004

Dou QP. Invited Speaker. Synthetic beta-lactam antibiotics and a selective breast cancer cell apoptosis inducer: Significance in breast cancer prevention and treatment. The Breast Cancer Group, Karmanos Cancer Institute, Detroit, MI, May 6, 2004

Dou QP. Invited Speaker. Discovery of Novel Small Molecules: Rational Design, Structure-Activity Relationships, Cellular Targets, and Potential Uses for Cancer Treatment and Prevention. The Developmental Therapeutics Group, Karmanos Cancer Institute, Detroit, MI, September 8, 2004

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of  $\beta$ -Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005

Deborah Kuhn, Yang Wang, Vesna Minic, Cristina Coates, G. Suresh Kumar Reddy, Kenyon, G. Daniel, Jeung-Yeop Shim, Di Chen, Kristin R. Landis-Piwowar, Edward Turos, And Q. Ping Dou, Ph.D. Relationships of structures of N-methylthiolated beta-lactam antibiotics to their apoptosis-inducing activity in human breast cancer cells. Poster presentation. ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting, Pennsylvania Convention Center, Philadelphia, Pennsylvania, June 8-11, 2005

Dou QP. Invited Speaker. Copper as a novel target for determining fate of AR and prostate cancer cells. Karmanos Cancer Institute Research Retreat, Detroit, MI, October 7, 2005

Dou QP. Invited Speaker. Roles of Diet, Biometals, and Environmental Factors in Cancer Prevention. Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China, April 19-20, 2006

Dou QP. Invited Speaker. Nutrient-Gene-Environment Interactions and Molecular Prevention of Cancer. The Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, April 24, 2006

Dou QP. Invited Speaker. Discovery of Novel Small Molecules for Cancer Therapies: - *From Nature to Laboratories and ... back*. The Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, April 25, 2006

Dou QP. Invited Speaker. A Common Target of Dietary Factors, Traditional Medicine and Chemopreventive Agents in Human Prostate Cancer: the Significance in Molecular Prevention. 6<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006

Dou QP. Invited Speaker. Molecular Prevention of Human Cancer: An Example of Diet-Gene-Environment Interaction. Institution of Environmental Health Sciences, Wayne State University, Detroit, MI, June 15, 2006.

Dou QP. Invited Speaker. A Lesson Learned from Thymidine Kinase Transcription at G1/S and later Stories. Symposium Honoring Dr. Pardee on the occasion of his 85th birthday. Boston, MA, June 24, 2006.

Dou QP. Invited Speaker. Discovery of Novel Natural and Synthetic Compounds for Molecular Prevention of Human Cancer. Henry Ford Health Systems, Detroit, MI, August 3, 2006.

Dou QP. Invited Speaker. Molecular Cancer Prevention and Therapies. Shandong Institute of Cancer Prevention and Treatment, Jinan, Shandong, China, October 18, 2006

Dou QP. Invited Speaker. Discovery of Novel Small Molecules for Cancer Prevention and Therapies. Wuhan Botanical Garden/ Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, China, October 20, 2006

Dou QP. Invited Speaker. The Proteasome as a Potential Cellular Target of Organic Toxic Metals. The 3<sup>rd</sup> International Symposium on Persistent Toxic Substances, Beijing, China, October 22-25, 2006

**Patents and licenses applied for and/or issued:**

None

**Degrees obtained that are supported by this award:**

Kenyon Daniel, Ph.D., graduated from Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine on April 5, 2004. Dissertation Title: "Strategies for Cancer Therapy Through Regulation of Apoptotic Proteases" (Advisor: Q. Ping Dou). Currently working as a postdoctoral fellow in my laboratory at Karmanos Cancer Institute, Wayne State University

Deborah Kuhn, Ph.D., graduated from Cancer Biology Program, University of South Florida College of Medicine on November 7, 2004. Dissertation Title: "Novel Approaches to Targeting

Tumor Cell Apoptotic Signaling Pathways" (Advisor: Q. Ping Dou). Currently working as a postdoctoral fellow in University of North Carolina.

**Development of cell lines, tissue or serum repositories; infomatics such as databases and animal models, etc:**

None.

**Funding applied for based on work supported by this award:**

Alliance For Cancer Gene Therapy. Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/08.

The Michigan Technology Tri-Corridor Fund, Fiscal Year 2005 Competition. DUAL-AGENT NANOPARTICLES TO OVERCOME DRUG RESISTANCE. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/07.

NIH R21. Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/07.

Wayne State University Stimuli\_responsive Nanosystems Proposal. Stimulus Controlled Nanosystems for Cancer Imaging and Treatment. (Co-I: Q. Ping Dou; PI: Stephanie L. Brock).

Susan Komen Foundation. Nanoparticle-mediated combination photodynamic and chemotherapy to overcome refractory tumors. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 05/01/06-04/30/08.

VA Merit. Ubiquitin Protease System in Malignant Pleural Mesothelioma. 10% Effort. (Co-I: Q. Ping Dou; PI: Anil Wali).

NIH R01. Copper as a novel target for determining fate of AR and prostate cancer cells. 20% Effort (PI: Q. Ping Dou). 07/01/06-06/30/11.

NIH R21 (resubmission). Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/06-06/30/08.

NIH R01. The Chinese Thunder of God Vine: Active Components & Biological Targets in Cancer. 20% Effort (PI: Q. Ping Dou). 12/01/06-11/30/11.

NIH P01. AR-mediated cell cycle-dependent anti-apoptotic events in prostate cancer. (PI: Reddy GPV; Co-Directors: Dou QP and Menon M). Project #3: Targeting 26S proteasome for determining fate of AR and prostate cancer cells. 40% Effort (PI: Q. Ping Dou). 12/01/06-

11/30/11.

DOD Breast Cancer Research Program-Concept Award. Chemosensitization of human breast cancer cells by an active compound purified from the Chinese medicine Thunder of God vine. 5% Effort (PI: Q. Ping Dou). 07/01/06-06/30/07.

NIH R01. Maspin in Hormone Refractory Prostate Cancer Intervention (Co-I: Q. Ping Dou, 5%; PI: Shijie Sheng). 12/01/06-11/30/11.

NIH R01 (resubmission). Elucidation of molecular mechanisms for the ubiquitin-proteasome pathway in malignant pleural mesothelioma (Co-I: Q. Ping Dou; PI: Anil Wali). 12/01/06-11/30/11.

MICHIGAN ECONOMIC DEVELOPMENT CORPORATION (MEDC). Development of natural pharmaceuticals to protect against low-intensity radiation exposure. 5% Effort (Co-PI: Q. Ping Dou; PI: Michael C Joiner). 10/01/06-09/30/09.

National Natural Science Foundation of China (NSFC). Synthesis and Mechanistic Study of Catechin Glycosides as Proteasome Inhibitors. Co-PI: Q. Ping Dou (PI: Sheng Biao Wan). 10/01/06-09/30/08.

DOD Prostate Cancer Research Program-Idea Development Award. Targeting the proteasome/ NF $\kappa$ B/ Androgen receptor-mediated survival pathway to chemosensitize human prostate cancer cells. 20% Effort (PI: Q. Ping Dou). 12/01/06-11/30/09.

DOD Breast Cancer Research Program/IDEA Award. Overcoming breast cancer drug resistance by a medicinal compound isolated from Indian Winter Cherry. 15% Effort (PI: Q. Ping Dou). 12/01/06-11/30/09.

NIH R21 (resubmission). Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/07-06/30/09.

**Employment or research opportunities applied for and/or received based on experience/training supported by this award:**

Deborah Kuhn, Ph.D.  
Kenyon Daniel, Ph.D.  
Huanjie Yang, Ph.D.  
Haiyan Pang, Ph.D.  
Lihua Li, M.D.  
Guoqing Shi, Ph.D.  
Di Chen, Ph.D.  
Shirley Orlu, B.S.  
Cindy (Qiuzhi) Cui, Technician

**CONCLUSIONS**

We have determined whether several novel beta-lactams, L1, SC4, HY2, HY14 and HY15, can inhibit tumor growth *in vivo*. We have found that these beta-lactams inhibited growth of implanted MDA-MB-231 breast tumors in a concentration-dependent manner, associated with their DNA-damaging and apoptosis-inducing activities. Our studies have provided strong support for proof-of-concept of the potential use of these N-thiolated beta-lactams in breast cancer prevention and treatment.

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9. Kuhn D, Coates C, Daniel K, Chen D, Bhuiyan M, Kazi A, Turos E, Dou QP. Beta-lactams and their potential use as novel anticancer chemotherapeutics drugs. *Front Biosci.* 2004 Sep 1;9:2605-17.
10. Landis-Piowar KR, Chen D, Cui QC, Minic V, Becker FF, Banik BK, and Dou QP. Apoptotic-Inducing Activity of Novel Polycyclic Aromatic Compounds in Human Leukemic Cells. *Intl. J Mol Med.*, 2006;17(5):931-5.
11. Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, and Dou QP. Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells. *Breast Cancer Res*, 2005; 7:R897-R908
12. Chen D, Peng F, Cui QC, Daniel KG, Orlu S, Liu J, and Dou QP. Inhibition of prostate cancer cellular proteasome activity by a pyrrolidine dithiocarbamate-copper complex is associated with suppression of proliferation and induction of apoptosis. *Frontiers in Bioscience*, 2005; 10: 2932-2939.
13. Chen D, Cui QC, Yang HJ, and Dou QP. Disulfiram, A Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts *via* Inhibition of the Proteasome Activity. *Cancer Research*, (in press)
14. Milacic V, Chen D, Ronconi L, Landis-Piowar KR, Fregona D, and Dou QP. A Novel Anticancer Gold(III) Dithiocarbamate Compound Inhibits the Activity of a Purified 20S Proteasome and 26S Proteasome in Human Breast Cancer Cell Cultures and Xenografts. *Cancer Research*, (in press)
15. Deborah Kuhn, Yang Wang, Vesna Minic, Cristina Coates, G. Suresh Kumar Reddy, Kenyon, G. Daniel, Jeung-Yeop Shim, Di Chen, Kristin R. Landis-Piowar, Edward Turos, And Q. Ping Dou, Ph.D. RELATIONSHIPS OF STRUCTURES OF N-METHYLTHIOLATED BETA-LACTAM ANTIBIOTICS TO THEIR APOPTOSIS-INDUCING ACTIVITY IN HUMAN BREAST CANCER CELLS. ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting, Pennsylvania Convention Center, Philadelphia, Pennsylvania—June 8-11, 2005

16. Di Chen, Qiuzhi Cindy Cui, Huanjie Yang and Q. Ping Dou. Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts *via* inhibition of the proteasome activity. The AACR International Conference on Frontiers in Cancer Prevention Research, Boston, MA, November 12 - 15, 2006.

## APPENDICES

Kazi A, Hill R, Long TE, Kuhn DJ, Turos E, Dou QP. Novel N-thiolated beta-lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells. *Biochem Pharmacol*. 2004 Jan 15;67(2):365-74.

Kuhn DJ, Wang Y, Minic V, Coates C, Reddy GS, Daniel KG, Shim JY, Chen D, Landis-Piwowar KR, Miller FR, Turos E, Dou QP. Structure-activity relationships OF N-methylthiolated beta-lactam antibiotics with c3 substitutions and their selective induction of apoptosis in human cancer cells. *Front Biosci*. 2005 May 1;10:1183-90.

Kuhn D, Coates C, Daniel K, Chen D, Bhuiyan M, Kazi A, Turos E, Dou QP. Beta-lactams and their potential use as novel anticancer chemotherapeutics drugs. *Front Biosci*. 2004 Sep 1;9:2605-17.

Chen D, Peng F, Cui QC, Daniel KG, Orlu S, Liu J, and Dou QP. Inhibition of prostate cancer cellular proteasome activity by a pyrrolidine dithiocarbamate-copper complex is associated with suppression of proliferation and induction of apoptosis. *Frontiers in Bioscience*, 2005; 10: 2932-2939.

Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, and Dou QP. Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells. *Breast Cancer Res*, 2005; 7:R897-R908

Chen D, Cui QC, Yang HJ, and Dou QP. Disulfiram, A Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts *via* Inhibition of the Proteasome Activity. *Cancer Research*, (in press)

Deborah Kuhn, Yang Wang, Vesna Minic, Cristina Coates, G. Suresh Kumar Reddy, Kenyon, G. Daniel, Jeung-Yeop Shim, Di Chen, Kristin R. Landis-Piwowar, Edward Turos, And Q. Ping Dou, Ph.D. RELATIONSHIPS OF STRUCTURES OF N-METHYLTHIOLATED BETA-LACTAM ANTIBIOTICS TO THEIR APOPTOSIS-INDUCING ACTIVITY IN HUMAN BREAST CANCER CELLS. ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting, Pennsylvania Convention Center, Philadelphia, Pennsylvania—June 8-11, 2005.

Di Chen, Qiuzhi Cindy Cui, Huanjie Yang and Q. Ping Dou. Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts *via* inhibition of the proteasome activity. The AACR International Conference on Frontiers in Cancer Prevention Research, Boston, MA, November 12-15, 2006.

Curriculum vitae.



## Novel *N*-thiolated $\beta$ -lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells

Aslamuzzaman Kazi<sup>a</sup>, Randy Hill<sup>a</sup>, Timothy E. Long<sup>b</sup>, Deborah J. Kuhn<sup>a</sup>,  
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### Abstract

Historically, it has been shown that the  $\beta$ -lactam antibiotics play an essential role in treating bacterial infections while demonstrating selectivity for prokaryotic cells. We recently reported that certain *N*-methylthio-substituted  $\beta$ -lactam antibiotics had DNA-damaging and apoptosis-inducing activities in various tumor cells. However, whether these compounds affect human normal or nontransformed cells was unknown. In the current study, we first show that a lead compound (lactam **1**) selectively induces apoptosis in human leukemic Jurkat T, but not in the nontransformed, immortalized human natural killer (NK) cells. Additionally, we screened a library of other *N*-methylthiolated  $\beta$ -lactams to determine their structure–activity relationships (SARs), and found lactam **12** to have the highest apoptosis-inducing activity against human leukemic Jurkat T cells, associated with increased DNA-damaging potency. Furthermore, we demonstrate that lactam **12**, as well as lactam **1**, potently inhibits colony formation of human prostate cancer cells. We also show that lactam **12** induces apoptosis in human breast, prostate, and head-and-neck cancer cells. Finally, lactam **12** induces apoptosis selectively in Jurkat T and simian virus 40-transformed, but not in nontransformed NK and parental normal fibroblast, cells. Our results suggest that there is potential for developing this class of  $\beta$ -lactams into novel anticancer agents.

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**Keywords:** *N*-thiolated  $\beta$ -lactam; Antibiotics; DNA damage; Apoptosis; Anticancer drugs

### 1. Introduction

Apoptosis, or programmed cell death, is a highly regulated process important in embryonic and immune system development and tissue homeostasis [1,2]. Perturbation of this pathway can lead to autoimmunity, acquired immune deficiency syndrome, neurodegenerative disorders, and cancer [3,4]. Initiation, commitment, and execution are

the three fundamental steps of apoptosis [5]. Several apoptotic stimuli, such as death receptor-binding ligands, signal to activate the initiator caspases (caspases-2, -8, -9, -10), which in turn activates downstream effector caspases (caspases-3, -6, -7). The effector caspases can also be activated through the release of key mitochondrial proteins, such as cytochrome *c*, cell death inducer second mitochondria-derived activator of caspases (Smac), and apoptosis initiating factor [6]. It is generally believed that proteolytic cleavage of a variety of intracellular substrates, including poly(ADP-ribose) polymerase (PARP) [7,8] and the retinoblastoma protein (RB) [9–11], by effector caspases leads to apoptosis.

For nearly 60 years  $\beta$ -lactam compounds have been used in the treatment of bacterial infections [12]. Following the initial introduction of penicillin, a variety of other classes of  $\beta$ -lactam antibiotics were subsequently identified and used clinically, including cephalosporins, penems,

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**Abbreviations:** SAR, structure–activity relationship; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling; NK cells, natural killer cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

carbapenems, nocardicins, and monobactams [13]. The bacterial targets of these antibiotics are membrane-bound transpeptidases referred to as the penicillin-binding proteins, which are responsible for creating crosslinks within the bacterial cell wall [13]. By disrupting these cross-linking proteins, the  $\beta$ -lactams induce structural deformities within the cell wall, which cause the bacteria to lyse. Recently, a novel class of *N*-thiolated  $\beta$ -lactams has been shown to inhibit *Staphylococcus aureus* and methicillin-resistant *S. aureus* growth [14–16].

Previously we showed that *N*-thiolated  $\beta$ -lactams, such as  $\beta$ -lactam **1**, induced DNA damage, inhibited DNA replication, and induced tumor cell apoptosis in a time- and concentration-dependent manner [17]. Our current study shows, for the first time, that the *N*-thiolated  $\beta$ -lactam **1** can preferentially induce apoptosis in leukemic Jurkat T cells, but not nontransformed, immortalized human NK cells. Additionally, we also show that lactam **12**, an analog of lactam **1**, has enhanced apoptosis-inducing activity in Jurkat T cells compared to lactam **1**. Furthermore, this study reveals that lactam **12** can induce apoptosis in other human solid tumor cell lines such as breast, prostate, and head and neck. Lactam **12** also induces apoptosis selectively in Jurkat T, but not human NK, cells, and in simian virus 40 (SV40)-transformed human fibroblasts (VA-13), but not in their parental counterpart (WI-38). Both lactams **1** and **12** are able to activate caspase-3 in human prostate cancer cells and inhibit colony formation of these cells in soft agar. These data indicate that further study of *N*-thiolated  $\beta$ -lactams in the treatment of cancer is warranted.

## 2. Materials and methods

### 2.1. Materials

Fetal bovine serum (Tissue Culture Biologicals), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and trypan blue were purchased from Sigma-Aldrich. RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), MEM nonessential amino acids solution, MEM sodium pyruvate solution, penicillin, and streptomycin were purchased from Invitrogen. Fluorogenic peptide substrate Ac-DEVD-AMC (the specific caspase-3/-7 substrate) was obtained from Calbiochem. Polyclonal antibody to human PARP was obtained from Roche Molecular Biochemicals. The APO-DIRECT kit for terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) staining was purchased from BD Pharmingen.

### 2.2. Synthesis of $\beta$ -lactams

The  $\beta$ -lactam analogs (Fig. 1A) were prepared as racemates (with *cis* stereochemistry) using a procedure described previously [14,15].

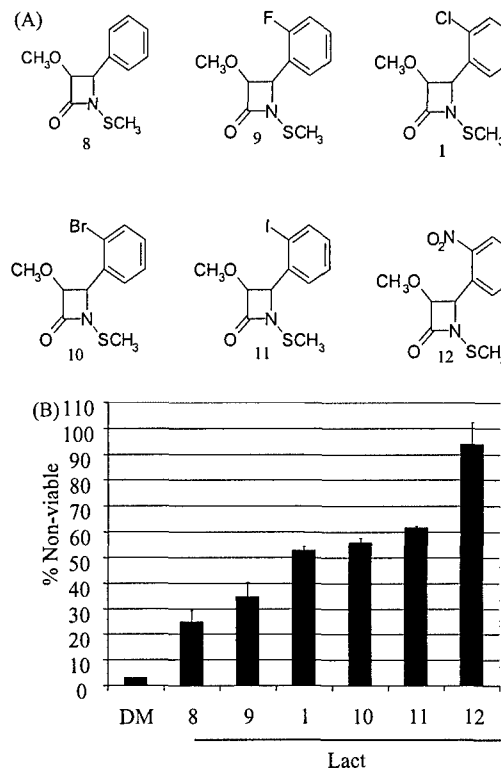


Fig. 1. Screen for more potent analogs of the lactam **1**. (A) Structures of the *N*-thiolated  $\beta$ -lactam compounds studied. Numerical designations were given to each compound. (B) Jurkat T cells were treated with the solvent (DMSO) or 50  $\mu$ M of each indicated analog for 24 hr, followed by trypan blue dye exclusion assay. The numbers given are percentages of nonviable cells to total cells. Standard deviations are shown with error bars from a mean of at least three different experiments.

### 2.3. Cell culture, protein extraction, and Western blot assay

Human Jurkat T cells and human prostate cancer LNCaP cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Human YT cells were cultured in RPMI 1640 medium supplemented with 1 mM MEM sodium pyruvate solution, 0.1 mM MEM nonessential amino acids solution, 10% fetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Human breast cancer MCF-7 cells, head-and-neck cancer PCI-13 cells, prostate cancer DU-145 cells, normal (WI-38) and SV-40 transformed (VA-13) human fibroblasts cells were grown in DMEM containing 10% fetal calf serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. All cell lines were maintained at 37° in a humidified incubator with an atmosphere of 5% CO<sub>2</sub>.

A whole-cell extract was prepared as described previously [18]. Briefly, cells were harvested, washed with PBS and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°. Afterwards, the lysates were centrifuged

at 12,000 g for 15 min at 4° and the supernatants collected as whole-cell extracts. Equal amounts of protein extract (60 µg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell) using a Semi-Dry Transfer System (Bio-Rad). The enhanced chemiluminescence Western blot analysis was then performed using specific antibodies to the proteins of interest.

#### 2.4. Trypan blue assay

The trypan blue dye exclusion assay was performed by mixing 20 µL of cell suspension with 20 µL of 0.4% trypan blue dye before injecting into a hemocytometer and counting. The number of cells that absorbed the dye and those that exclude the dye were counted, from which the percentage of nonviable cell number to total cell number was calculated.

#### 2.5. Morphological assessment of apoptosis

To assess morphological changes of cells, 50 µL of treated or untreated cell suspension were transferred to a glass slide at the indicated time points. The slides were observed under a phase-contrast microscope (Leica) and photographs were taken (100×). Apoptotic cells were identified by their distinct morphological changes.

#### 2.6. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) was used to determine the extent of DNA strand breaks [19]. The assay was performed following manufacturer's instruction using the APO-Direct kit. In brief, the harvested cells were fixed in 1% paraformaldehyde for 15 min on ice, washed with PBS, and then fixed again in 70% ethanol at –20° overnight. The cells were then incubated in DNA labeling solution (containing terminal deoxynucleotidyl transferase (TdT) enzyme, fluorescein-conjugated dUTP and reaction buffer) for 90 min at 37°. After removing the DNA labeling solution by rinsing cells with Rinsing Buffer, the cells were incubated with the propidium iodide/RNase A solution, incubated for 30 min at room temperature in the dark, and then analyzed by flow cytometry within 3 hr of staining.

#### 2.7. Caspase-3/-7 activity assay

To measure cell-free caspase-3/-7 activity, whole-cell extracts (20–30 µg) from untreated or treated LNCaP, MCF-7, PCI-13, DU-145, VA-13, and WI-38 cells were incubated with 20 µM of the fluorogenic substrate caspase-3/-7 (Ac-DEVD-AMC) for 30 min at 37° in 100 µL of assay buffer (50 mM Tris, pH 8.0). Measurement of the hydrolyzed AMC groups was performed on a VersaFluor™ Fluorometer (Bio-Rad) as described previously [20].

#### 2.8. Soft agar assay

The soft agar assay was performed as described previously [21] with a few modifications. In brief, in a 6-well plate, a bottom feeder layer (0.6% agar) was prepared with DMEM media containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. A top layer (0.3% agar) was prepared with DMEM and the same media as described above but containing  $2 \times 10^4$  prostate cancer LNCaP cells and 50 µM of lactam **1** or **12**, or equal volume of solvent (DMSO) as a control. Plates were incubated at 37° in 5% CO<sub>2</sub> in a humidified incubator for 3 weeks. MTT (1 mg/mL) was added to each well and incubated overnight to allow complete formation of purple formazan crystals. The plates were then scanned and photographed, and the number of colonies was quantified by *Quantity one* v. 4.0.3 software (Bio-Rad).

#### 2.9. Nuclear staining

To assay nuclear morphology, the detached or remaining attached cells were washed with PBS, fixed with 70% ethanol for 1 hr, and stained with Hoechst 33342 (1 mM) for 30 min. The nuclear morphology was visualized by fluorescence microscopy (40×; Leitz) [18].

### 3. Results

#### 3.1. Screening for more apoptotically active analogs of lactam **1**

Lactam **1** contains a *chloro* (–Cl) group in the *ortho* position on the benzene ring (Fig. 1A). To examine whether deletion or substitution of the Cl group could affect its cell death-inducing ability, other halogen and nonhalogen analogs of lactam **1** were synthesized (Fig. 1A). These compounds were then tested in the trypan blue dye exclusion assay, using lactam **1** as a comparison (Fig. 1B). Jurkat T cells were treated with each of these compounds at 50 µM for 24 hr, followed by measurement of loss of cell membrane permeability, indicative of a late apoptotic stage (Fig. 1B) [22,23]. As a control, lactam **1** induced ~52% cell death (Fig. 1B). Interestingly, removal of the Cl group from the benzene ring significantly decreased the cell death-inducing activity to ~25% (lactam **8**; Fig. 1B). Furthermore, replacement of the Cl group with a smaller halogen atom (–F; lactam **9**) also decreased the death-inducing activity (to ~35%), while analogs with a larger halogen group (–Br and –I; lactams **10** and **11**, respectively; Fig. 1A) increased the cell death rates to 55 and 60% (Fig. 1B). These data suggest that the size of the group in the *ortho* position on the benzene ring is important for the compound's cell death-inducing activity. Indeed, the analog with –NO<sub>2</sub> substitution, lactam **12** (Fig. 1A), exhibited the strongest effect with a total of

~94% cell death (Fig. 1B). Therefore, the order of potency of the tested compounds was:  $X = H < F < Cl < Br < I < NO_2$ .

### 3.2. Lactam **1** induces apoptosis preferentially in leukemic Jurkat T over nontransformed, immortalized NK cells

Previously, we reported that  $\beta$ -lactam analogs, such as lactam **1** (Fig. 1A) [17], were able to induce tumor cell apoptosis. However, whether lactam **1** affects normal or nontransformed cells was unknown. To determine whether lactam **1** was able to induce apoptosis preferentially in tumor/transformed vs. normal/nontransformed cells, we treated human leukemic Jurkat T cells and immortalized, nontransformed NK cells (YT line) [24] with lactam **1** in both concentration- and time-dependent experiments. Treatment of Jurkat T cells with 10  $\mu$ M of lactam **1** for 24 hr induced apoptosis-specific PARP cleavage fragment p85 (Fig. 2A), whose levels were further increased when 25  $\mu$ M of lactam **1** was used (Fig. 2A). After treatment with 50  $\mu$ M of lactam **1**, PARP degradation was further increased, as evidenced by a significant decrease in expression of intact PARP protein (Fig. 2A). In contrast, no PARP cleavage was detectable in the YT cells after treatment with lactam **1** at even 50  $\mu$ M (Fig. 2A).

In the kinetic experiment, both Jurkat T and YT cells were treated with 30  $\mu$ M of lactam **1** for 3, 6, or 24 hr. PARP cleavage was detected in Jurkat T cells first at 3 hr,

which was then increased at 6 hr (although the levels of PARP/p85 fragments at 24 hr were decreased in this Western blotting; Fig. 2B). Importantly, no PARP cleavage was observed in YT cells in the same kinetics experiment (Fig. 2B). To confirm the tumor cell-selective killing activity of lactam **1**, a trypan blue dye exclusion assay was performed in the same kinetic experiment. After 24 hr, there was 42% cell death in the Jurkat T cells compared to 9% in YT cells (Fig. 2C). Furthermore, by using phase-contrast microscopy, more cell death was observed in Jurkat T cells than YT cells (Fig. 2D). These data support the conclusion that lactam **1** could induce apoptotic cell death selectively in tumor *over* nontransformed cells.

### 3.3. Lactam **12** has enhanced apoptosis-inducing activity specific to Jurkat T, but not normal YT cells

To determine if lactam **12** is capable of inducing apoptosis at lower concentrations than lactam **1**, a dose-response experiment was performed with both compounds. Jurkat T cells were treated with lactam **12** at 2, 10, 25, and 50  $\mu$ M for 24 hr, using 50  $\mu$ M of lactam **1** as a comparison. Again, treatment with lactam **1** caused ~50% cell death, measured by trypan blue exclusion assay (Fig. 3A). Under the same experimental conditions, lactam **12** induced cell death in a concentration-dependent manner: 25% at 10  $\mu$ M, 45% at 25  $\mu$ M, and 80–90% at 50  $\mu$ M (Fig. 3A). Therefore, lactam **12** is ~2-fold more potent than lactam **1**. This conclusion was further supported by PARP cleavage

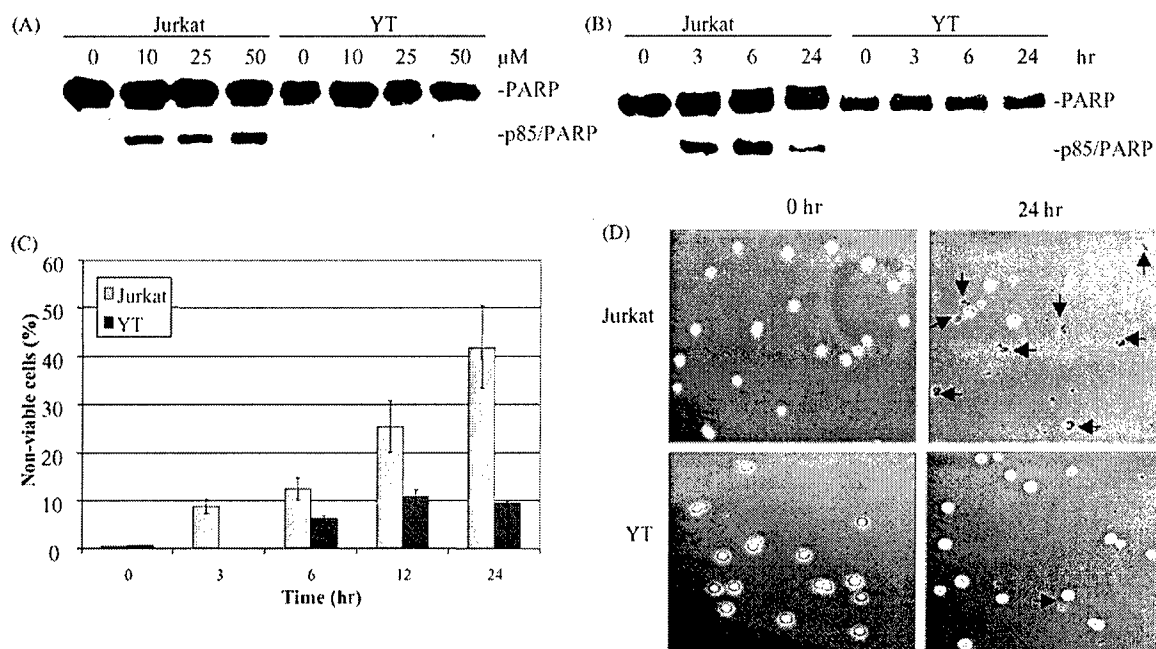


Fig. 2. Selective induction of apoptosis by lactam **1** in leukemic Jurkat T *over* immortalized/nontransformed NK cells. Jurkat T and NK (YT) cells were treated with 10, 25, and 50  $\mu$ M of lactam **1** for 24 hr (A) or with 30  $\mu$ M of lactam **1** for indicated hours (B–D). (A and B) Measurement of PARP cleavage in Western blot assay. The intact PARP (116 kDa) and a PARP cleavage fragment (p85) are shown. (C) Trypan blue dye exclusion assay. The numbers given are percentages of nonviable cells to total cells. Standard deviations are shown with error bars from a mean of at least three different experiments. (D) Morphological changes of Jurkat T and YT cells after treatment. Photographs under a phase-contrast microscope (100 $\times$ ).

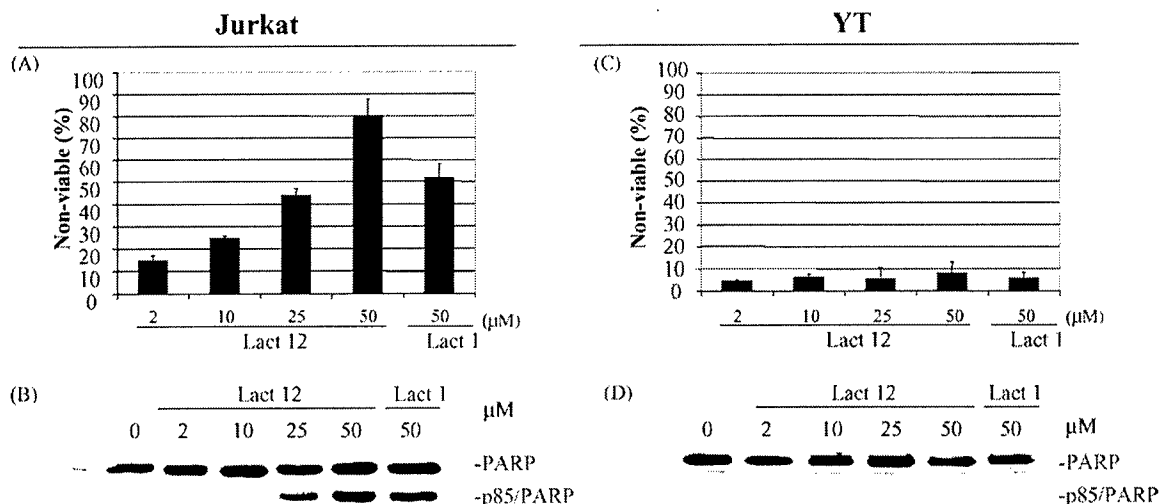


Fig. 3. Dose-response comparison between Jurkat T and YT cells treated with lactams 12 and 1 to induce cell apoptosis. Jurkat T (A and B) and YT cells (C and D) were treated with 2, 10, 25, and 50  $\mu$ M of lactam 12 vs. 50  $\mu$ M of lactam 1 for either 12 (B and D) or 24 hr (A and C), followed by trypan blue exclusion (A and C) or Western blot assay using anti-PARP antibody (B and D). Results are representative of three different experiments. Standard deviations are shown with error bars from a mean of at least three independent experiments (A and C).

assay using lysates prepared after 12-hr treatment (Fig. 3B). Cleavage of PARP occurred in lactam 12-treated cells in a dose-dependent manner with the highest level of PARP cleavage observed at 50  $\mu$ M (Fig. 3B). The levels of PARP cleavage induced by 50  $\mu$ M of lactam 1 were equivalent to ~50% of that by 50  $\mu$ M of lactam 12 (Fig. 3B).

In the same experiment, when immortalized, nontransformed NK cells were treated with lactam 12 (using lactam 1 as a control), neither cell death (Fig. 3C) nor PARP cleavage (Fig. 3D) were observed. Therefore, like lactam 1, lactam 12 also induces apoptotic cell death preferentially in tumor over nontransformed cells.

To further compare the potency of lactams 1 and 12, Jurkat T cells were treated with 25  $\mu$ M of lactam 12 vs. 50  $\mu$ M of lactam 1 for 3, 6, 12, and 24 hr, followed by determination of trypan blue incorporation and PARP cleavage. After 3 hr, lactam 12 at 25  $\mu$ M caused 15% vs. 11% cell death with lactam 1 at 50  $\mu$ M (Fig. 4A). Similarly, at 6 hr, 24% of trypan blue-positive cells were found after 25  $\mu$ M lactam 12 treatment, while only 20% observed in 50  $\mu$ M lactam 1-treated cells (Fig. 4A). Only at later time points (12 and 24 hr), lactam 1 at 50  $\mu$ M was slightly more potent than lactam 12 at 25  $\mu$ M (Fig. 4A). Similar levels of cleaved PARP were observed in Jurkat T cells treated with either 25  $\mu$ M of lactam 12 or 50  $\mu$ M of lactam 1 at each time point (Fig. 4B). Therefore, lactam 12 is able to induce similar amounts of apoptosis in Jurkat T cells at a concentration half of that of lactam 1.

Furthermore, we examined levels of sub- $G_1$  populations, as a measurement of cells with DNA fragmentation [18], in Jurkat T cells treated with lactam 12 or 1. Treatment with 50  $\mu$ M of lactam 12 increased the sub- $G_1$  populations by 34 and 57%, respectively, at 12 and 24 hr (Fig. 5A). In comparison, 50  $\mu$ M of lactam 1 treatment for 12 and

24 hr induced sub- $G_1$  populations by 10 and 16%, respectively [17], confirming the greater potency of lactam 12.

### 3.4. Lactam 12 is able to induce DNA damage in Jurkat T cells

We have previously shown that lactam 1 induces damage to DNA, leading to the inhibition of DNA replication and

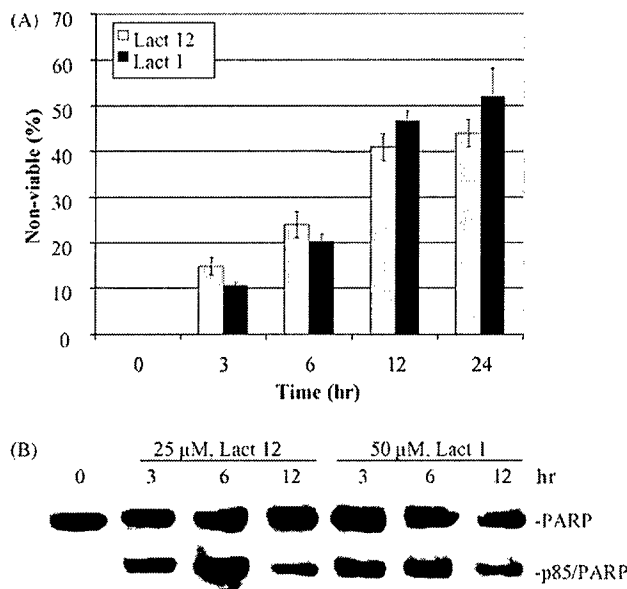


Fig. 4. A kinetic comparison between lactams 12 and 1 to induce apoptosis in Jurkat T cells. Jurkat T cells were treated with 25  $\mu$ M of lactam 12 vs. 50  $\mu$ M of lactam 1 for 3, 6, 12, and 24 hr, followed by trypan blue dye exclusion assay (A), and PARP cleavage in Western blot assay (B). Results are representative of three different experiments. Standard deviations are shown with error bars from a mean of at least three independent experiments.

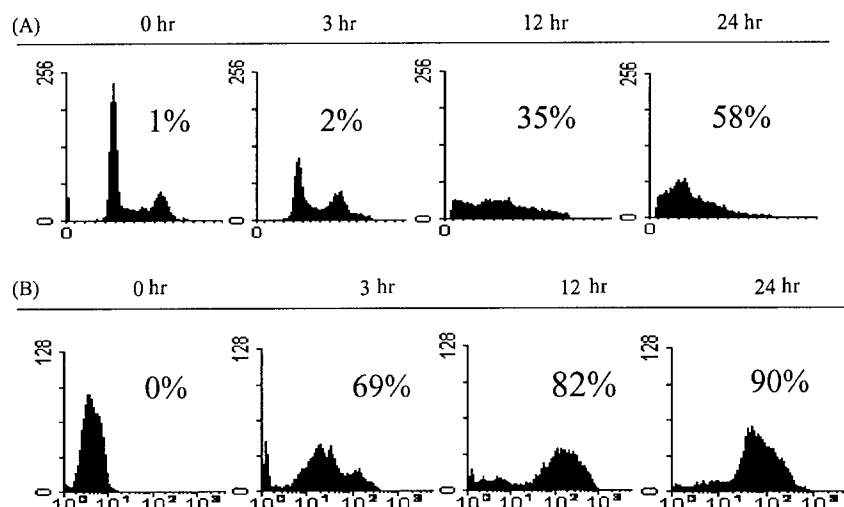


Fig. 5. Lactam 12 induces sub-G<sub>1</sub> cell population and TUNEL-positivity. Jurkat T cells (0 hr) were treated with 50  $\mu$ M of lactam 12 for the indicated hours. (A) Measurement of sub-G<sub>1</sub> DNA content by flow cytometry analysis. The percentage of sub-G<sub>1</sub> cell population represents the cell populations with DNA fragmentation. (B) Measurement of DNA strand breaks by TUNEL assay. The numbers indicate the percentage of TUNEL-positive population. Results of representative of three independent experiments are shown.

subsequent induction of apoptosis [17]. To determine whether lactam 12 is also capable of damaging tumor cell DNA, Jurkat T cells were treated with 50  $\mu$ M of lactam 12, followed by performance of TUNEL assay, which detects DNA strand breaks [17]. A significant population ( $\sim$ 70%) of the cells exhibited DNA strand breaks after 3 hr of incubation with lactam 12 (Fig. 5B). A total of 82–90% of the cells were TUNEL-positive after 12–24-hr treatment with lactam 12 (Fig. 5B). In this experiment, 66% of TUNEL-positive cells were observed after treatment with 50  $\mu$ M of lactam 1 for 24 hr (data not shown). Thus, the increased DNA-damaging capability of lactam 12 is most likely responsible for its enhanced cell death-inducing activity (Figs. 1–4).

### 3.5. Lactams 1 and 12 induce apoptosis and inhibit colony formation in human prostate cancer cells

So far, we demonstrated that lactam 12, like lactam 1, is able to induce DNA damage and subsequently induce apoptosis in human leukemia cells (Figs. 1–5) [17]. To determine if this lactam could also activate death program in solid tumor cells, human prostate cancer LNCaP cells were treated for 48 hr with lactam 12 at 2–25  $\mu$ M or lactam 1 at 50  $\mu$ M (as a control), followed by measurement of cell-free caspase-3/-7 activity. A dose-dependent increase in caspase-3/-7 induction was observed in LNCaP cells treated with lactam 12: by 2-, 2.5-, and 4.2-fold, respectively, at 2, 10, and 25  $\mu$ M (Fig. 6A). Treatment with 50  $\mu$ M of lactam 1 also increased levels of caspase-3/-7 activity by 2.5-fold over the control (Fig. 6A). These data are consistent with the conclusion that lactam 12 has greater apoptosis-inducing potency than lactam 1.

We then investigated the *in vivo* effects of these two lactams in a soft agar assay that measures the transforming

activity of human tumor cells. LNCaP cells were plated in soft agar along with 50  $\mu$ M of lactam 1, 50  $\mu$ M of lactam 12, or solvent (DMSO), followed by a 21-day incubation to allow for colony formation. The solvent (DMSO)-treated plates allowed for the development of  $\sim$ 500 colonies (Fig. 6B and C). Lactam 1 inhibited 91%, and lactam 12 completely blocked ( $\sim$ 100%), colony formation of LNCaP cells (Fig. 6B and C). Therefore, both lactams are able to inhibit the transformation capability of prostate cancer cells.

### 3.6. Lactam 12 induces apoptosis in several solid tumor cell lines and SV-40-transformed, but not normal, human fibroblasts

In a previous study, we showed that lactam 1 induced apoptosis in several solid tumor cell lines [17]. In this study we also investigated the effects of lactam 12 on several solid tumor cell lines including human breast (MCF-7), head-and-neck (PCI-13), and prostate (DU-145) cancer cells. Furthermore, we wanted to investigate whether lactam 12-induced cell death was selective in transformed (VA-13) over the normal (WI-38) human fibroblasts. We treated these cell lines with 50  $\mu$ M lactam 12 or an equal percentage of DMSO, followed by separation of the attached and detached cell populations. Both attached and detached cell populations were then used for detection of apoptotic nuclear change. We found that after a 48-hr treatment with lactam 12,  $\sim$ 60% of MCF-7 and PCI-13 cells and  $\sim$ 50% of DU-145 and VA-13 cells became detached. However, no detachment was observed in WI-38 cells after treatment with lactam 12. Little or no detachment was observed in all the cell lines treated with DMSO. All the detached tumor or transformed cells exhibited typical apoptotic nuclear condensation

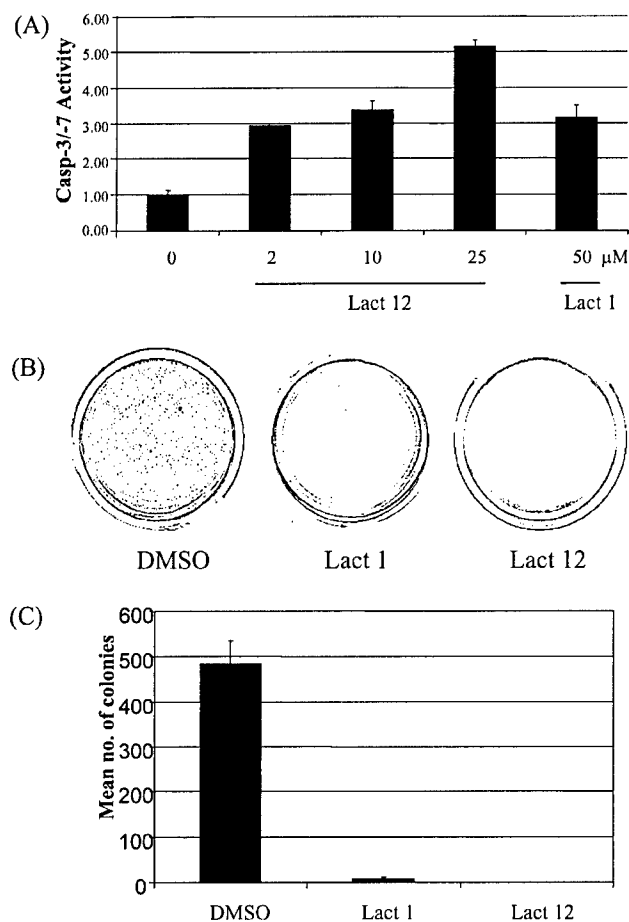


Fig. 6. Effects of  $\beta$ -lactams on caspase activation and colony formation. (A) Prostate cancer LNCaP cells were treated for 48 hr with 2, 10, and 25  $\mu$ M of lactam 12 vs. 50  $\mu$ M of lactam 1. Cell-free caspase-3/-7 activity was then determined by incubating whole-cell extracts with caspase-3/-7 substrate and measuring free AMC. (B and C) LNCaP cells were plated in soft agar with the solvent DMSO or 50  $\mu$ M of the indicated  $\beta$ -lactams. Cells were then cultured for 21 days without addition of new drug. The plates were scanned and a representative well from each treatment was selected for presentation (B). Colonies were quantified with an automated counter and presented as mean values from triplicate independent experiments. Error bars denote standard deviations (C).

and fragmentation (Fig. 7A). In addition, apoptosis-specific nuclear condensation was also observed in the remaining attached solid tumor (MCF-7, PCI-13, and DU-145) and transformed (VA-13), but not the normal (WI-38), cells (Fig. 7A). These results strongly suggest that lactam 12 induces apoptosis that lead to detachment preferentially in the tumor and transformed cells.

To confirm lactam 12-mediated apoptotic cell death, in the same experiment, aliquots of both detached and attached cells of each line were combined and used for whole-cell extract preparation. This was followed by measurement of cell-free caspase-3/-7 activity. Consistent with the apoptotic nuclear changes (Fig. 7A), treatment of MCF-7, PCI-13, DU-145, and VA-13 cells with lactam 12 also increased levels of caspase-3/-7 activity by 11.0-, 10.2-, 5.2-, and 5.3-fold, respectively, over the control

DMSO-treated cells (Fig. 7B). In addition, accompanying the lack of the detachment in normal WI-38 cells treated with lactam 12 (Fig. 7A), there was little or no induction of caspase-3/-7 activity observed in these cells (Fig. 7B). Taken together, these data further support the conclusion that lactam 12 is able to induce apoptotic cell death preferentially in tumor and transformed over the normal cells.

#### 4. Discussion

Developing novel anticancer drugs that induce apoptosis in tumor cells has long been a goal of cancer drug discovery research. Many of the drugs in current use focus on targeting dysregulated cell cycle and apoptosis programs in cancer cells [25]. We previously have shown that *N*-thiolated  $\beta$ -lactams cause DNA damage in tumor cells that leads to induction of apoptosis through p38 activation, cytochrome *c* release, and caspase activation [17]. Here we show that lactam 1 selectively induces apoptosis in human leukemic Jurkat T cells, but not nontransformed, immortalized human NK cells (Fig. 2). Furthermore, lactam 1 is capable of inducing Jurkat cell apoptosis at concentrations as low as 10  $\mu$ M after 24-hr treatment (Fig. 2A).

Often, addition/substitution of groups on a molecule leads to development of more potent drugs. In order to determine whether structural changes to lactam 1 could produce a more potent tumor cell death inducer, analogs of lactam 1 were synthesized (Fig. 1A). Substitutions of the -Cl group with other halogens of higher atomic mass (-Br, -I) did increase the efficacy of the compound. In contrast, substitution with a lower atomic mass halogen (-F) or a hydrogen (H) atom had a concomitant decrease in cell death induction (Fig. 1). Lactam 12, containing an -NO<sub>2</sub> substituent, proved to be a highly active compound and induced 93–100% of cell death at 50  $\mu$ M vs. 52% of cell death by lactam 1 at the same concentration (Fig. 1B). Furthermore, lactam 12 was superior to lactam 1 at inducing apoptosis in human Jurkat T cells because lactam 12 can induce the same amount of PARP cleavage at a lower concentration than lactam 1 (Figs. 3 and 4). Additionally, lactam 12 at 25  $\mu$ M was able to exert its cell death-inducing effect at as early as 3 hr (Fig. 4A and B). We also found that lactam 12 had greater potency than lactam 1 when used in human prostate cancer cells to activating caspase-3/-7 and inhibiting colony formation (Fig. 6). Similar to our previous results with lactam 1 [17], we found that lactam 12 induces apoptosis in several solid tumor cell lines (e.g. MCF-7, PCI-13, DU-145) in a caspase-dependent manner (Fig. 7). Due to lack of caspase-3 in MCF-7 cells, it was believed that lactam 12-mediated MCF-7 cell death was associated with caspase-7 activity (Fig. 7). Additionally, like lactam 1, lactam 12 was also able to selectively induce apoptosis in human leukemic Jurkat T cells over nontransformed,

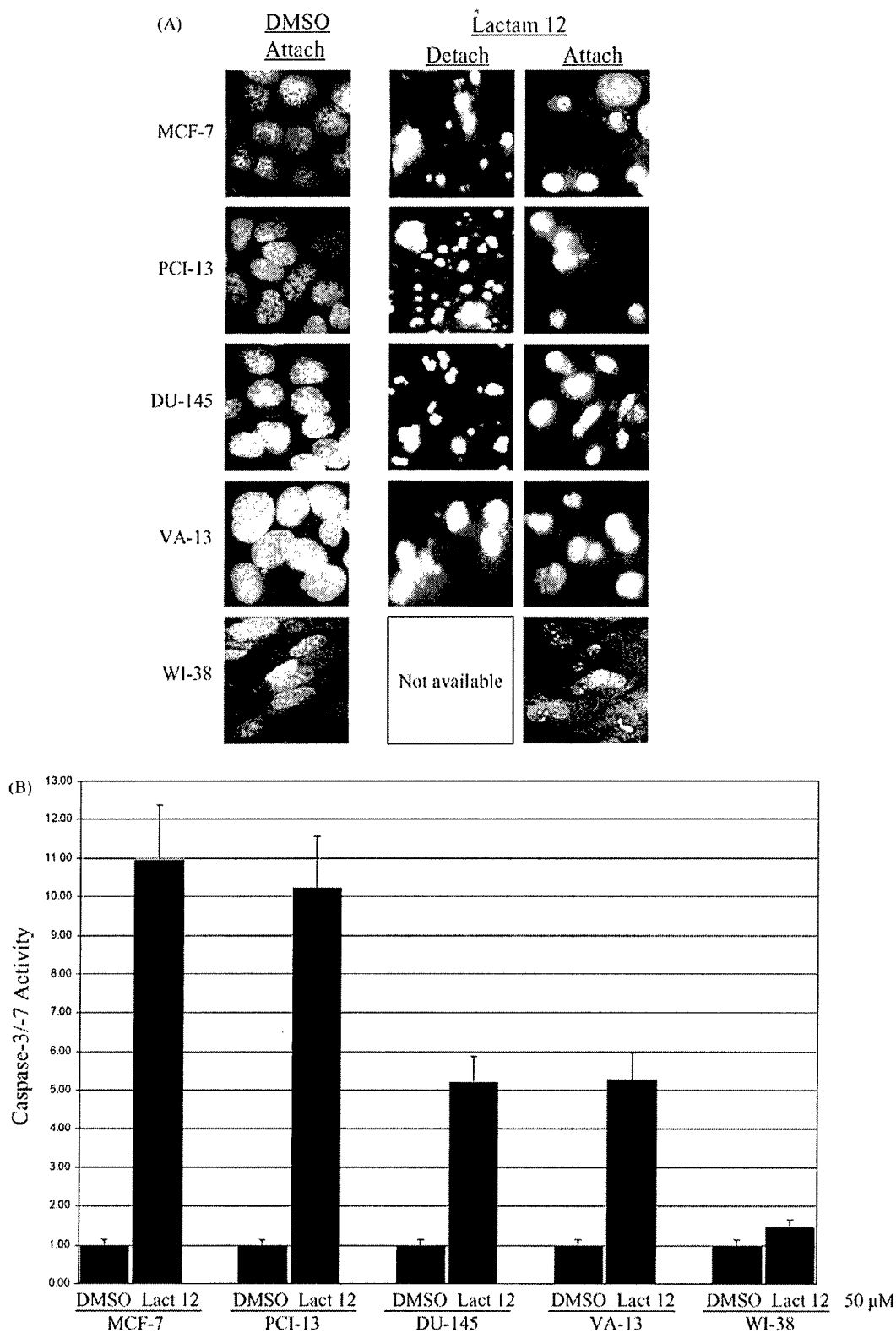


Fig. 7. Lactam 12 induces apoptosis and caspase activation in different solid tumor cell lines and SV40-transformed but not normal cells. (A) Nuclear staining assay. MCF-7, PCI-13, DU-145, VA-13, and WI-38 cells were treated with 50  $\mu$ M lactam 12 or DMSO for 48 hr, followed by collection of both detached and attached cell populations. After lactam 12 treatment, ~60% of MCF-7 and PCI-13 cells and ~50% of DU-145 and VA-13 cells became detached, whereas <5% were detached from each of these cell lines after DMSO treatment. No detachment was found in WI-38 cells after each treatment. Both detached and attached cell populations were stained with the nuclear staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology. (B) Cell-free caspase-3/-7 activity assay. Aliquots of the above detached and attached cells of each line were combined for whole-cell extraction. Cell-free caspase-3/-7 activity was then determined by incubating each whole-cell extract with caspase-3/-7 substrate and measuring free AMCs. Error bars denote standard deviations. Similar results were obtained in three independent experiments.

immortalized human NK cells (Fig. 3). Also, lactam **12** was able to selectively induce apoptotic cell death in simian virus 40-transformed, but not the parental normal, human fibroblasts (Fig. 7). The molecular mechanism for the enhanced activity in lactam **12** remains unknown. One interpretation is that the presence of  $-\text{NO}_2$  group in this drug increases its binding to the cellular target(s). Alternatively, this drug might have increased uptake rates by the cells.

The mechanism of action of many chemotherapeutic drugs is through DNA damage and then subsequent apoptosis induction in tumor cells [26]. As mentioned above, we have recently shown that lactam **1** is capable of inducing apoptosis after DNA damage [17]. In the present study, we show, by TUNEL assay, that lactam **12** also causes DNA damage in ~70% of cells just after 3-hr treatment (Fig. 5B). At this time, there was only 2% cell death (Fig. 5A), suggesting that the DNA damage occurs much earlier than apoptotic cell death. However, apoptotic cells increased at later time points with increased TUNEL-positive cells (Fig. 5A and B). This result is consistent with our previous study [17] and several other studies that have shown that several traditional chemotherapeutic drugs or DNA-damaging agents cause DNA strand breaks that trigger apoptotic cell death [27,28].

Malignant transformation of a cell can lead to tumor formation and metastasis. The desired effect of any anticancer drug is to inhibit tumor growth and formation *in situ*. Soft agar colony forming assay is an assay that has been developed to mimic tumor cellular growth in tissue. We hypothesized that the *N*-thiolated  $\beta$ -lactams that induce cell death should be able to inhibit colony formation in soft agar assay. Indeed, when LNCaP prostate cancer cells were cultured in the presence of lactam **1** or **12**, 91 and 100% inhibition of colony formation was observed, respectively, as compared to the solvent control (Fig. 6).

Based on our previous [17] and current studies, we propose that these *N*-thiolated  $\beta$ -lactams act by inducing DNA damage that leads to apoptosis preferentially in cancer and transformed *over* normal/nontransformed cells. Although it appears that the *N*-methylthio moiety is necessary for the cell death-inducing activity [17], addition of a larger group in the *ortho* position on the phenyl ring can also increase the effectiveness of the compound (Fig. 1). Our results strongly suggest the potential for developing this class of  $\beta$ -lactams into novel anticancer agents. Immediate future studies focusing on determining the molecular targets and chemical action of the *N*-thiolated  $\beta$ -lactams would help rational development of these compounds.

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## STRUCTURE-ACTIVITY RELATIONSHIPS OF *N*-METHYLTHIOLATED BETA-LACTAM ANTIBIOTICS WITH C<sub>3</sub> SUBSTITUTIONS AND THEIR SELECTIVE INDUCTION OF APOPTOSIS IN HUMAN CANCER CELLS

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### 1. ABSTRACT

The development of novel anti-cancer drugs that induce apoptosis has long been a focus of drug discovery. Beta-lactam antibiotics have been used for over 60 years to fight bacterial infectious diseases with little or no side effects observed. Recently a new class of *N*-methylthiolated beta-lactams has been discovered that have potent activity against methicillin resistant *Staphylococcus aureus*. Most recently, we determined the potential effects of these *N*-thiolated beta-lactams on tumorigenic cell growth and found that they are apoptosis-inducers in human cancer cell lines. In the current study, we further determined the effects of the substitution of the *O*-methyl moiety on C<sub>3</sub> and stereochemistry of the beta-lactams on the anti-proliferative and apoptosis-inducing abilities. We have found that lactam 18, in which C<sub>3</sub> is substituted with an acrylate ester group, is a very effective proliferation inhibitor against human premalignant and malignant breast, leukemic, and simian virus 40-transformed fibroblast cells. Generally speaking, increasing the size of the moiety on C<sub>3</sub> decreases its anti-proliferation potency, possibly indicating steric hindrance with the cellular target or decreased permeability through the cell membrane. We also found that the stereochemistry of the beta-lactams plays an important role in their potency. The 3*S*,4*R* isomers are more effective than their enantiomers (3*R*,4*S*), suggesting that 3*S*,4*R* configuration is more favorable for target interaction.

### 2. INTRODUCTION

Selectively targeting tumorigenic cells *versus* normal cells is a primary goal in anti-cancer drug discovery. Small molecules with apoptosis-inducing ability have great potential to be developed into novel chemotherapeutic drugs because of the ease of synthesis and structural manipulation (1-3). Initiation, commitment, and execution are the three fundamental steps of apoptosis (4). Several apoptotic stimuli, such as irreparable DNA damage, signal to activate the initiator caspases (*e.g.* caspases-8/10), which in turn activate downstream effector caspases (*e.g.* caspases-3/7). The effector caspases can also be activated through the release of mitochondrial proteins, such as cytochrome *c* (5). It is generally believed that proteolytic cleavage of a variety of intracellular substrates by effector caspases leads to apoptosis (6-9).

One particularly important class of small molecule drugs, the beta-lactam antibiotics, have played an essential role in treating bacterial infections without causing toxic side effects in the host for the past 60 years. Sir Alexander Fleming first coined

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the name "penicillin" in 1928 after his discovery that molds from the *Penicillium* genus secrete powerful antimicrobial compounds, called beta-lactams (10). X-ray crystallography revealed that penicillin is a thiazolidine ring fused to a four membered beta-lactam ring (11). The beta-lactams are powerful and potent inhibitors of bacterial growth and many different moieties of bicyclic beta-lactams have been isolated or synthesized since the discovery of penicillin (12). There are several classes of bicyclic beta-lactams that possess antibacterial properties, including the penams, penems, carbapenems, cephalosporins, and clavulanic acids (10).

A novel class of beta-lactams ~~were~~ was discovered by the Squibbs and Takeda laboratories in 1981, which have an *N*-sulfonic acid group attached directly to the nitrogen in the lactam ring (13, 14). The term "monobactam" was coined for these lactams, which have a flexible monocyclic ring, and lack the carboxylic acid moiety, yet still retain a high bactericidal potency. Recently, a structurally related family of *N*-thiolated compounds, termed *N*-methylthio beta-lactams, ~~were~~ was found to inhibit growth of *Staphylococcal* and methicillin-resistant *S. aureus* (MRSA) (15-17). Additionally, we have shown that these *N*-methylthio beta-lactams possess potent anti-proliferative properties, and are capable of inducing DNA strand breakage, inhibiting DNA replication, and inducing apoptosis in a time- and concentration-dependent manner when tested in several human cancer, but not normal cell lines (18, 19).

In this study, we screened several additional *N*-thiolated beta-lactams with substitutions made to the *O*-methyl moiety of carbon 3 ( $C_3$ ) for their structure-activity relationships and found that increasing the size of the  $C_3$  substitution results in decreased anti-proliferative activity in human breast cancer cells. Additionally, increasing the size of the  $C_3$  substituent may interfere with cellular uptake. We identified one particularly active lactam (lactam **18**), which possesses an acrylate ester moiety off of  $C_3$ , for further study. Lactam **18** induces caspase-3 activation and apoptosis, associated with increased Hsp70 protein expression and p38 phosphorylation. We have also found that the stereochemistry plays an important role in the activities of *N*-thiolated beta-lactam antibiotics, including anti-proliferation, S/G<sub>2</sub>/M cell cycle arrest, and apoptosis induction. The 3S,4R-configured [(+)] isomers of lactam **18** and another lactam **19** are both more potent than their 3R,4S-configured isomers or the racemic mixtures. Furthermore, these (+)-lactams are more efficacious than racemic lactam **1**, which was identified from our previous studies (18). These effects of beta-lactams were found mainly in cultured human cancer and transformed cells, but not in normal/non-transformed cells. These data indicate that further study of *N*-thiolated beta-lactams in the treatment of cancers is warranted.

## MATERIALS AND METHODS

### 3.1. Reagents

Fetal Bovine Serum was purchased from Tissue Culture Biologicals (Tulare, CA). Mixture of penicillin-streptomycin-L-glutamine, RPMI, Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 (1:1) medium, horse serum, MEM non-essential amino acids solution, MEM sodium pyruvate solution, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), epidermal growth factor (EGF), sodium bicarbonate, hydrocortisone, cholera enterotoxin, bovine insulin, propidium iodide and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies to actin, monoclonal antibodies to HSP70 and p-p38, and anti-goat and anti-mouse IgG-horseradish peroxidase were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). CaspACE FITC-VAD-FMK marker was purchased from Promega (Madison, WI). Fluorogenic peptide substrate Ac-DEVD-AMC (for caspase-3/-7 activities) was obtained from Calbiochem (San Diego, CA).

### 3.2. Synthesis of beta-lactams

The beta-lactam analogs (Figure 1) were prepared as racemates and enantiomers (with *cis* stereochemistry) using a procedure described previously (15, 16).

### 3.3. Cell culture, protein extraction, and Western blot assay

Human leukemic Jurkat T cells and natural killer YT cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Further supplementation with 1 mM MEM sodium pyruvate solution, 0.1 mM MEM non-essential amino acids solution was added to YT cells. Human breast cancer MCF-7 cells, normal (WI-38) and SV-40 transformed (VA-13) human fibroblasts cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin. Premalignant MCF10AT1Kcl.2 transformed human breast cells (20) were cultured in DMEM/F12 (1:1) supplemented with 10 µg/ml bovine insulin, 100ng/ml cholera enterotoxin, 20 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 29 mM sodium bicarbonate, 5% horse serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. All cell lines were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO<sub>2</sub>. A whole-cell extract was prepared and Western blotting was performed as described previously [18].

### 3.4. Cellular proliferation assay

The MTT assay was used to determine the effects of beta-lactams on overall proliferation of tumor cells. Cells were plated in a 96-well plate and grown to 70-80% confluency, followed by addition of each compound at an indicated concentration for 24 h. MTT (1 mg/ml) in PBS was then added to wells and incubated at 37°C for 4 hours to allow for complete cleavage of the tetrazolium salt by metabolically active cells. Next, MTT was removed and 100 µl of DMSO was added, followed by

## SAR of N-methylthiolated beta-lactams

colorimetric analysis using a multilabel plate reader at 560 nm (Victor<sup>3</sup>; Perkin Elmer). Absorbance values plotted are the mean from triplicate experiments.

### 3.5. Trypan blue assay

The trypan blue dye exclusion assay was performed by mixing 20  $\mu$ l of cell suspension with 20  $\mu$ l of 0.4% trypan blue dye before injecting into a hemocytometer and counting. The number of cells that absorbed the dye and those that exclude the dye were counted, from which the percentage of nonviable cell number to total cell number was calculated.

### 3.6. Cell cycle assay

Cell cycle analysis based on DNA content was performed as follows. Cells were harvested, counted, and washed twice with PBS. Cells ( $5 \times 10^6$ ) were then suspended in 0.5 ml of PBS, pipetted, and fixed in 5 ml of 70% ethanol for at least 2 h at -20°C. Cells were centrifuged, resuspended in 1 ml of propidium iodide staining solution (50  $\mu$ g propidium iodide, 1 mg RNase A, and 1 mg of glucose per ml of PBS) and incubated at room temperature for 30 min before flow cytometry analysis. The cell cycle distribution is shown as the percentage of cells containing G<sub>1</sub>, S, G<sub>2</sub>, and M DNA judged by propidium iodide staining.

### 3.7. Caspase-3 activity assay

To measure cell-free caspase-3 activity, whole cell extracts (30  $\mu$ g) from untreated or treated cells were incubated with 20  $\mu$ M of the fluorogenic substrate caspase-3/-7 (Ac-DEVD-AMC) for 30 min at 37°C in 100  $\mu$ l of assay buffer (50 mM Tris, pH 8.0). Measurement of the hydrolyzed AMC groups was performed on a Victor<sup>3</sup> Multilabel plate reader<sup>TM</sup> (Perkin Elmer) as described previously [18].

### 3.8. Immunostaining of apoptotic cells

Immunostaining of apoptotic cells was performed by addition of the FITC-VAD-FMK marker and visualized on an Axiovert 25 microscope (Zeiss; Thornwood, NY). Briefly, cells were grown to ~80% confluency in 60 mm dishes, and then treated under conditions described in the figure legends. Detection of caspase activity was determined according to the manufacturer's protocol with a few modifications. Briefly, total cell population was collected and incubated with a 10  $\mu$ M FITC-VAD-FMK for 20 min in the dark. Cells were then centrifuged at 300  $\times$ g for 3 minutes, washed 3X in PBS, and then resuspended in 50  $\mu$ l PBS. Cell suspension was then transferred to glass slides in the presence of Vector Shield mounting medium with DAPI. Images were captured using AxioVision 4.1 and adjusted using Adobe Photoshop 6.0 software. Apoptotic cells were quantified by counting the number of apoptotic cells over the total number of cells in the same field.

### 3.9. Nuclear staining

After each drug treatment, both detached and attached populations of VA-13 and WI-38 lines were stained with Hoechst 33342 to assess apoptosis. Briefly, cells were washed 2X in PBS, fixed for 1 h with 70% ethanol at 4°C, washed 3X in PBS, and stained with 50  $\mu$ M Hoechst for 30 min in the dark at room temperature. Detached cells were plated on a slide and attached cells were visualized on the culture plate with a fluorescent microscope at 10X or 40X resolution (Zeiss, Thornwood, NY). Images were obtained using an AxioVision 4.1 and adjusted using Adobe Photoshop 6.0.

## 4. RESULTS

### 4.1. Structure-activity relationship analysis of N-thiolated beta-lactams

We have previously shown that lactam **1**, which contains an *O*-methyl moiety at carbon-3 (C<sub>3</sub>) of the beta-lactam ring, induces apoptosis in a relatively selective manner in tumor and transformed cells, but not normal or non-transformed cell lines (18). More than 35 *N*-thiolated beta-lactam analogs were then screened using an MTT assay in breast cancer MCF-7 cells to assess their anti-proliferative potency compared to lactam **1**. Several of these analogs with substitutions to the *O*-methyl group at C<sub>3</sub> (Figure 1) were then selected for further structure-activity relationship (SAR) studies (Figure 2). MCF-7 cells were treated with 50  $\mu$ M of selected lactams for 24 h, followed by MTT assay. Lactam **18**, which possesses an ester moiety at C<sub>3</sub>, is twice as potent as lactam **1** (Figure 2). Additionally, it was found that as the C<sub>3</sub> group increased in size, the effectiveness to inhibit proliferation decreased. For instance, increasing the size of the halogen, from -Cl to -I (lactam **13** versus lactam **14**), lead to a 2-fold decrease in potency (Figure 2). A similar observation was made in comparing the bioactivities of C<sub>3</sub>-sulfonated lactams **16** and **17**. Mesyl lactam **16** inhibited 57% of MCF-7 cell growth, while dansyl lactam **17** has much less effect on inhibiting proliferation (Figure 2). A possible explanation may be that lactam **17** with the large dansyl group is incapable of crossing the cell membrane. Although lactam **15** with an N<sub>3</sub> group at C<sub>3</sub> is less potent than lactam **14** with I at C<sub>3</sub>, as predicted, it is yet unclear why lactam **15** is less potent than mesyl lactam **16** (Figure 2). The order of potency is determined as follows: Lactam **18** > **1** > **16**  $\geq$  **13** > **14** > **17** > **15**.

### 4.2. Lactam **18** is more potent than lactam **1** at inducing apoptosis associated with Hsp70 expression and p38 phosphorylation

We decided to focus on lactam **18** due to its increased anti-proliferative potency over lactam **1** (Figure 2). To determine whether lactam **18** is capable of inducing apoptosis, we treated leukemia Jurkat T cells with 20  $\mu$ M lactam **18** for 24 h, using lactam **1** as a control. A fluorescent marker specific for activated caspases was then added to the cells and then visualized by fluorescence microscopy (Figure 3A). Lactam **18** displayed greater apoptosis-inducing activity than lactam **1** (Figure 3A).

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Microarray analysis showed up-regulation of many genes by lactam 1 treatment, including *HSP70* (manuscript in preparation). To confirm this finding, Jurkat T cells were treated with lactam 1 and lactam 18 for 16 h at either 25 or 50  $\mu$ M. It was found that Jurkat T cells had increased Hsp70 protein expression after treatment of both lactams; lactam 18 induced a greater amount of Hsp70 expression at 25  $\mu$ M than lactam 1 at the same concentration (Figure 3B). This is consistent with the idea that the beta-lactams induce a stress response in the cell, most likely due to their disruption of DNA synthesis (18).

We have previously reported that p38 MAP kinase activation is associated with  $\beta$ -lactam-induced apoptosis (18). Activation of p38 MAP kinase can trigger apoptosis following multiple stimuli, such as DNA damage (21, 22). Western blot for phosphorylated (activated) pp38 reveals that treatment with 25  $\mu$ M lactam 18 leads to an increase in pp38 levels (9.0-fold), compared to that of 25  $\mu$ M lactam 1 (2.6-fold) (Figure 3B). Actin was used as a loading control.

### 4.3. (+)-Lactam 19 inhibits cellular proliferation more effectively than (-)-lactam 19

To determine if the stereochemistry has any bearing on the potency of beta-lactams, lactams 18 and 19 were synthesized in enantiomerically pure forms (Figure 1). Premalignant MCF-10AT1Kcl.2 breast cancer cells were treated with (+)-lactam 19 (3S,4R-configuration), (-)-lactam 19 (3R,4S-configuration), or racemic lactam 1 (as a control) for 24 h at indicated doses (Figure 4A). All of these lactams inhibited proliferation in a dose-dependent manner, with (+)-lactam 19 at 50  $\mu$ M inhibiting 85% cell growth, (-)-lactam 19 inhibiting 56% and lactam 1 inhibiting 49% at the same concentration (Figure 4A). These results are similar to that from another experiment using malignant MCF-7 breast cancer cells (data not shown). Trypan blue incorporation also shows increased tumor cell killing with (+)-lactam 19 to that of (-)-lactam 19, 42% versus 26%, respectively, at 25  $\mu$ M (Figure 4B).

### 4.4. beta-Lactams 18 and 19 induce tumor cell-selective apoptosis

We have previously shown that lactam 1 preferentially induces apoptosis in human cancer cells over normal, non-transformed cells lines (19). To determine if lactam 18 possessed a similar tumor cell-specific activity, human leukemic Jurkat T cells and immortalized, non-transformed natural killer cells (YT) were treated with lactam 1 and lactam 18, and the effects were determined. After 16 h treatment, it was found that lactam 18-treated Jurkat cells had a 19-fold increase in caspase-3 activity at 25  $\mu$ M, compared to 9-fold induced by lactam 1 at the same concentration (Figure 5A). Both lactam 18 and lactam 1 had little or no apoptosis-inducing effects on the immortalized, non-transformed YT cells (Figure 5A).

To assess whether the isomers of lactam 19 (Figure 1) also have explicit activity against cancer cells, we treated Jurkat T cells and YT cells with (+)-lactam 19, (-)-lactam 19 and racemic lactam 1 (as a control), and determined the effects on apoptotic cell death. We found that only the Jurkat, but not YT, cells exhibited high levels of caspase-3 activity when treated with these beta-lactams (Figure 5B). Additionally, (+)-lactam 19 at 25  $\mu$ M induced a 7.4-fold increase in caspase-3 activity compared to 5.5-fold increase by (-)-lactam 19 and 3.1-fold increase by lactam 1 at 25  $\mu$ M (Figure 5B). Therefore, the 3S,4R-configured isomer, (+)-lactam 19, is more potent than (-)-Lactam 19 and racemic lactam 1.

To further confirm the effect of stereoselectivity on the apoptosis-inducing effects of beta-lactams, we synthesized an isomeric pair of lactam 18, 3S,4R-isomer [(+)-lactam 18] and 3R,4S-isomer [(-)-lactam 18] (Figure 1). Jurkat and YT cells were then treated with 50  $\mu$ M (+)-lactam 18, (-)-lactam 18, racemic lactam 18 or lactam 1 for 24 h, followed by trypan blue dye exclusion assay (Figure 6A). (+)-lactam 18 induced much higher amount of cell death than its isomer (-)-lactam 18, 98% vs. 58%, respectively (Figure 6A). Interestingly, the racemic lactam 18 was almost equally potent to that of (+)-lactam 18 (Figure 6A). All lactam 18 compounds initiated more cell death than lactam 1 (Figure 6A). Again, it was found that normal, non-transformed YT cells did not undergo cell death after treatment with any of these beta-lactams (Figure 6A).

Another experiment using SV-40 transformed (VA-13) and normal (WI-38) human fibroblasts demonstrates again that (+)-lactam 18 is the more active isomer. A nuclear stain of VA-13 and WI-38 cell lines treated with 50  $\mu$ M of each beta-lactam for 24 h reveals that there is a high degree of detachment and DNA condensation, characteristics indicative of apoptosis, in cells treated with (+)-lactam 18, racemic lactam 18 and lactam 1 (Figure 6B). (-)-lactam 18, on the other hand, showed decreased activity when compared with (+)-lactam 18 and racemic lactam 18. There was a very minor amount of cellular detachment observed in the normal WI-38 fibroblasts treated with (+)-lactam 18 (data not shown), supporting that these beta-lactams selectively kill transformed VA-13 cells (Figure 6B).

### 4.5. (+)-Lactam 18 induces S/G2/M cell cycle arrest

We have previously shown that beta-lactams decrease G<sub>1</sub> population, associated with DNA damage (18). To further investigate the cause of apoptosis after N-methylthio beta-lactam treatment, analysis of cell cycle changes were performed on an exponentially growing cell population (Table 1). As a control, lactam 1 was found to decrease G<sub>1</sub> phase DNA content by 6% after 6 h incubation (Table 1). Racemic lactam 18 had a very similar effect on cell cycle as racemic lactam 1 (Table 1). However, when cells were treated with (+)-lactam 18, there was a 15% decrease in G<sub>1</sub>, demonstration that the 3S,4R configuration has increased growth-inhibitory activity (Table 1).

## 5. DISCUSSION

Currently, many anticancer therapies, from radiation treatment to chemotherapeutic agents, are very toxic. Therefore, drug discovery for anticancer therapy is as concerned with selectivity of normal *versus* cancer tissues, as it is the potency of the therapy itself. Antibiotic therapies have typically used the unique molecular targets of microbes in order to avoid toxicity to the patient during treatment. Recently we have shown that some of these compounds possess anti-proliferation activity in human tumor cells (18). Thus, these compounds that are already known to be essentially non-toxic to humans may be anticancer agents as well. Of particular interest are the *N*-thiolated beta-lactams, which we have previously found to be potent against MRSA (17). Additionally, we found these compounds act as potential anticancer agents through S-phase arrest, DNA damage, and apoptosis induction (18). These compounds are also able to selectively induce apoptosis in cancerous *over* normal cells (19).

This novel class of *N*-thiolated beta-lactams possesses potent anti-cancer activity, which is directly related to the nature of the substituents on each of the four ring sites. We have previously reported on the effects of additions/substitutions to the *N*-thio group and aryl ring (18, 19). The work reported here is a further characterization of the SAR between the various substitution groups on beta-lactam ring. At the core of the beta-lactam molecule is a four-membered ring that is substituted at each position (Figure 1). Each of the positions, we have previously published, plays a role in the potency of the compound. Position 1 is the *N*-methylthio position and changes at this position that either eliminate the methylthio moiety or lengthen the chain result in decreased potency (18). Position 2 is substituted with a benzene ring and changes here also effect potency (19). Physical position on the ring with regards to ortho-, para-, or meta substitution as well as the nature of the substituent affected the potency dramatically [for details see (19)]. Position 4, which is substituted with a double-bonded oxygen, is the "backbone" of the beta-lactam and therefore cannot be changed without losing the general beta-lactam framework (10, 23). The work presented here examines the SAR at position 3 and completes the survey of each position of the four-membered ring.

We determined that the size and polarity of the group at C<sub>3</sub> is important for their lactam's activity. As these C<sub>3</sub> substituents increase in size or in polarity, the efficacy of the compound seems to drop. For example, replacing the chloro (Cl) moiety of lactam 13 for azido (N<sub>3</sub>), lactam 15, decreases the anti-proliferative activity from 75 to 5% respectively (Figure 2). However, a simple single atom change in the same period (Cl to I; lactam 13 vs. lactam 14) seems to result in only a partial increase in potency. Similarly, the potencies of C<sub>3</sub>-sulfonated compounds 16 and 17 can be directly attributed to their C<sub>3</sub> substitutions: lactam 17 with its large, polar dansyl moiety has significantly diminished activity compared to its smaller, less polar mesyl analog, lactam 16 (Figure 2). Likewise, lactam 16 seems to be similar in or slightly less potent than lactam 14, indicating that the size of the substituent may be slightly more significant than the overall charge (Figure 2). Lactam 18 displays very potent activity with its acrylate ester off C<sub>3</sub>. This may indicate that these substitutions may affect the capability of these compounds to cross the cell membrane.

Of primary importance in anticancer drug research is that the compound being investigated demonstrate selectivity between normal cells and tumor cells. Cytotoxic agents are less desirable than those compounds that can differentially activate apoptosis in cancer cells *vs.* tumor cells. Previously we have reported that lactam 1-induced apoptosis is caspase-dependent and associated with cytochrome c release (18). Here we show that lactam 18-induced apoptosis is also caspase-dependent (Figure 5A). However, the efficacy to induce apoptosis by lactam 18 is much improved over lactam 1 (Figure 3) and that the apoptosis induced by lactam 18 is tumor cell-specific (Figure 5A). beta-Lactams cause DNA strand breakage and subsequent cell cycle arrest (18). Our microarray studies show a 3.5-fold increase in *HSP70* expression in Jurkat T cells treated with lactam 1 (data not shown). Increased expression of Hsp70 protein (Figure 3B) indicates that treatment with lactam 18 induces a stress response in leukemic Jurkat T cells. Another important molecular event in lactam-induced apoptosis is the increase in p38 phosphorylation. Abrogation of pp38 activity with a specific inhibitor (PD169316) leads to tumor cell survival (18). Not only is lactam 18 capable of inducing p-38 activation, it is capable of inducing a greater amount of pp38 levels at 25  $\mu$ M compared to 50  $\mu$ M of lactam 1 (Figure 3B).

Stereochemistry can play an important role in the efficacy of a particular compound. Often only one of the isomers displays a significant selectivity for the molecular target while the other can cause adverse side effects (24-26). Here we find that two 3S,4R-configured beta-lactam compounds, (+)-lactam 18 and (+)-lactam 19, do have a higher potency than their 3R,4S enantiomers or a racemic mixture. Specifically, (+)-lactam 19 has greater anti-proliferation and cell death-inducing activities than both (-)-lactam 19 and the racemic lactam 1 (Figure 4). (+)-Lactam 19 triggers an equivalent amount of caspase-3 activation at half the concentration of lactam 1 and this activity again is tumor cell-selective (Figure 5). Additionally, another isomer, (+)-lactam 18, displays a similar potency to (+)-lactam 19 while still retaining the tumor cell-selectivity (Figures 5B and 6).

A vast amount of anti-cancer research is ongoing to develop apoptosis-inducing drugs. While the molecular targets and chemical actions of *N*-thiolated beta-lactams are not fully characterized, we believe that the compounds possess great potential for chemotherapeutic drug development. These antibiotics compounds are predicted to have little to no effect on normal cells, supported by our results. Thus, the anti-tumor potential and expected lack of toxicity of these beta-lactams makes them excellent candidates for anticancer drug development. Our ongoing studies focus on identifying the molecular interactions of beta-lactams in human cancer cells and their anti-tumor activities *in vivo*.

## 6. ACKNOWLEDGMENTS

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**Key Words:** beta-lactams, cancer, apoptosis, antibiotics, structure-activity relationship

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**Figure 1.** Chemical structures of N-methylthiolated beta-lactams.

**Figure 2.** Structure-activity relationship (SAR) analysis of N-thiolated beta-lactams. MCF-7 cells were plated in a 96-well plate and grown to 70-80% confluency followed by addition of 50  $\mu$ M beta-lactam for 24 h. Cells were then incubated with 1 mg/ml MTT for 3 h and proliferation rates were determined using a multi-label plate reader (Victor<sup>3</sup>, Perkin Elmer;  $\pm$ SD).

**Figure 3.** Lactam 18 induces caspase activity associated with Hsp70 expression and p38 phosphorylation. **A.** Jurkat T cells were treated with 20  $\mu$ M lactam 1 or lactam 18 for 24 h. Following the treatment, the cells were then incubated with a FITC-conjugated marker that binds to activated caspases. Cell suspension was then transferred to glass slides in the presence of Vector Shield mounting medium with DAPI. Images were captured using AxioVision 4.1 and adjusted using Adobe Photoshop 6.0 software. **B.** Jurkat cells treated with 25 or 50  $\mu$ M of lactam 1 or lactam 18 for 16 h, followed by Western blot analysis using specific antibodies to HSP70, p-p38, and Actin. Data shown are representative from three independent experiments.

**Figure 4.** (+)-Lactam 19 effects proliferation and cell death in a dose-dependent manner. **A.** MCF10AT1Kcl.2 cells were plated in a 96-well plate and grown to 70-80% confluency followed by addition of 50  $\mu$ M of indicated beta-lactams for 24 h. Cells were then incubated with 1 mg/ml MTT for 4 h and proliferation rates were determined using a multi-label plate reader (Victor<sup>3</sup>, Perkin Elmer;  $\pm$ SD). **B.** Jurkats T cells treated with lactam 1, (+)-lactam 19, (-)-lactam 19 at indicated doses and assayed for cell death by trypan blue incorporation ( $\pm$ SD).

**Figure 5.** beta-Lactams induce apoptosis in a tumor cell-specific manner. **A.** Jurkat T and YT cells were treated with lactam 1 and lactam 18 at indicated concentration for 16 h, followed by measurement of cell-free caspase-3 activity by incubating whole cell extracts with caspase-3 substrate and measuring free AMCs. **B.** (+)-lactam 19 is 2-fold more potent than lactam 1 at inducing apoptosis in a tumor cell specific manner. Jurkat T and YT cells were treated for 24 h with 25 and 50  $\mu$ M of lactam 1 versus 25

## SAR of N-methylthiolated beta-lactams

$\mu\text{M}$  of (+)-lactam **19** and (-)-lactam **19**. Cell-free caspase-3 activity was then determined by incubating whole cell extracts with caspase-3 substrate and measuring free AMCs.

**Figure 6.** (+)-Lactam **18** induce apoptosis selectively in tumorigenic cells. **A**, Leukemic Jurkat T and non-transformed YT cells were treated with lactam **1** or isomers of lactam **18** at 50  $\mu\text{M}$  for 24 h. Cell death is given as a percent of dead cells over total cell population ( $\pm\text{SD}$ ). **B**, Both detached and attached VA-13 and WI-38 fibroblast cell populations were collected and stained with the nuclear staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology.

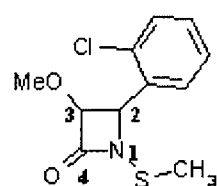
**Table 1.** Cell cycle analysis of asynchronous Jurkat T cells treated with beta-lactams at 50  $\mu\text{M}$  for 6 h.<sup>1</sup>

|                                                        | No treat | Lactam <b>1</b> | Lactam <b>18</b> | (+)-Lactam <b>18</b> |
|--------------------------------------------------------|----------|-----------------|------------------|----------------------|
| % G <sub>0</sub> /G <sup>1</sup>                       | 42       | 36              | 36               | 27                   |
| % S                                                    | 42       | 38              | 41               | 46                   |
| % G <sub>2</sub> /M                                    | 16       | 26              | 23               | 27                   |
| % G <sub>0</sub> /G <sub>1</sub> $\Delta$ <sup>2</sup> | --       | -6              | -6               | -15                  |

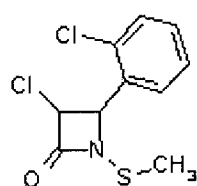
<sup>1</sup>The cell cycle distribution was measured as the percentage of cells that contain G<sub>1</sub>, S, G<sub>2</sub> and M DNA (G<sub>1</sub>/S/G<sub>2</sub>/M = 100%).

<sup>2</sup>The percent change from control cells (no treat) is shown as %  $\Delta$ .

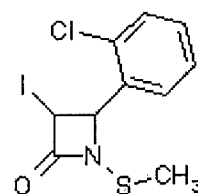
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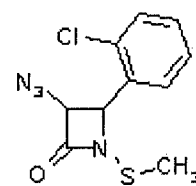
Lactam 1  
*racemic*



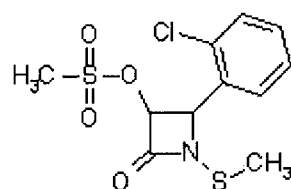
Lactam 13  
*racemic*



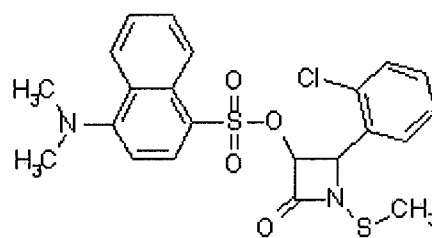
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*racemic*



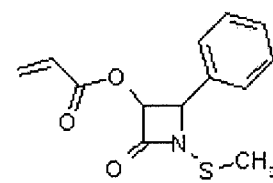
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*racemic*



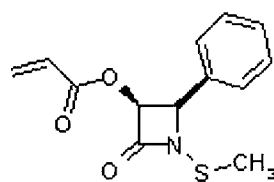
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*racemic*



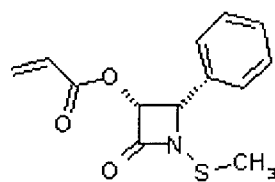
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*racemic*



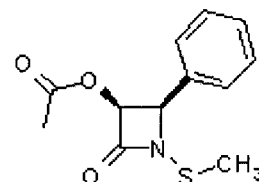
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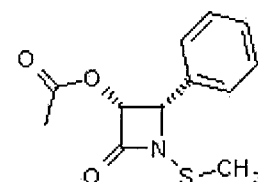
Lactam (+)-18  
*3S, 4R-configuration*



Lactam (-)-18  
*3R, 4S-configuration*



Lactam (+)-19  
*3S, 4R-configuration*



Lactam (-)-19  
*3R, 4S-configuration*

Figure 1

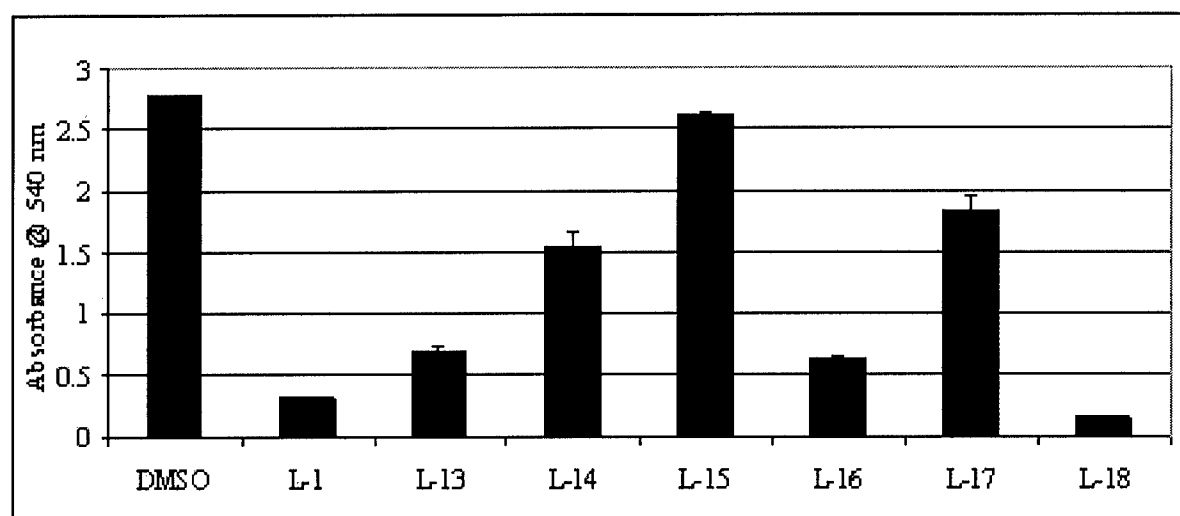
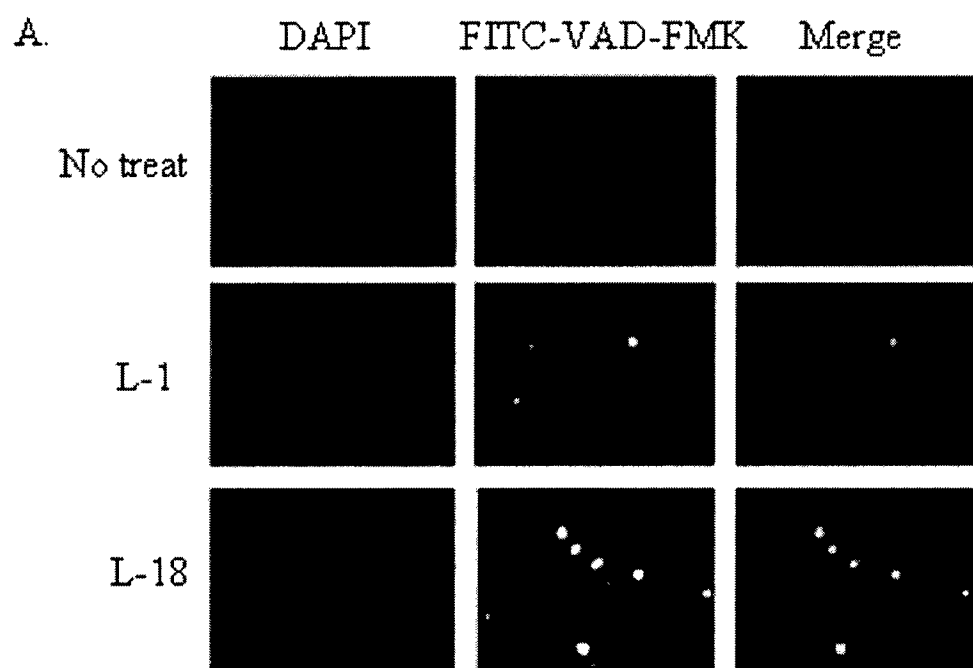


Figure 2

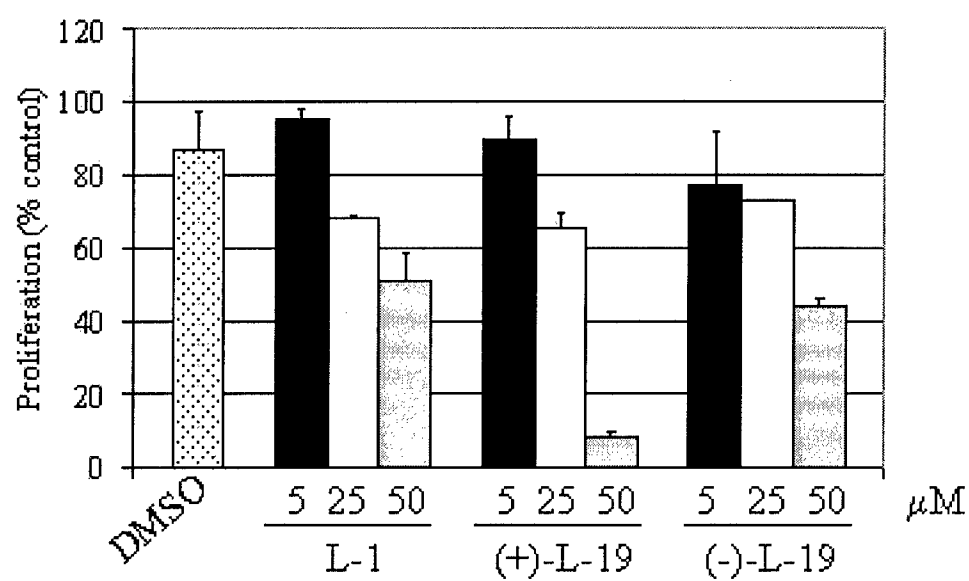


B.



Figure 3

A.



B.

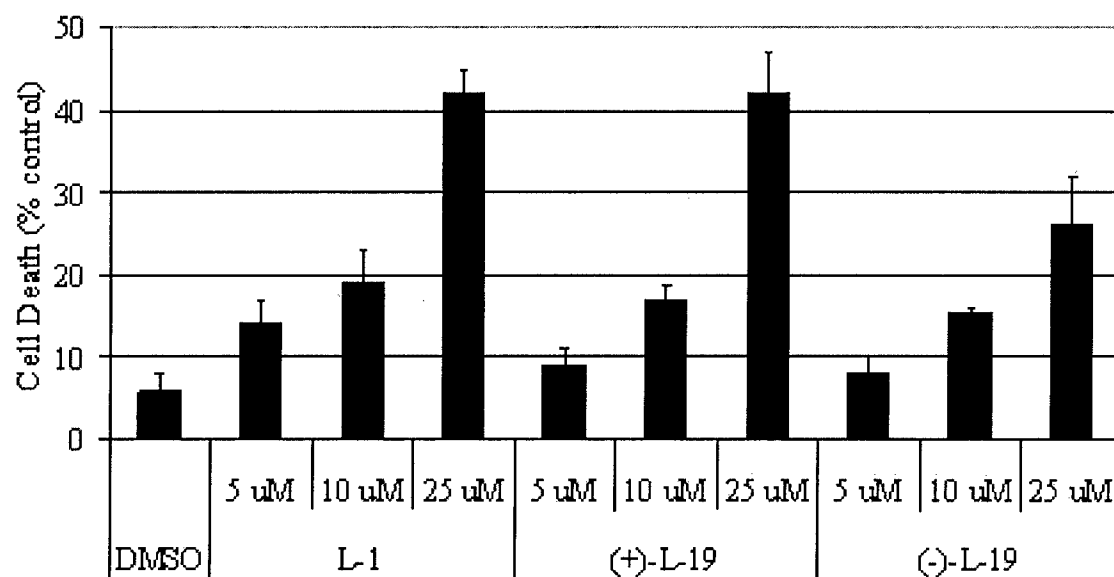
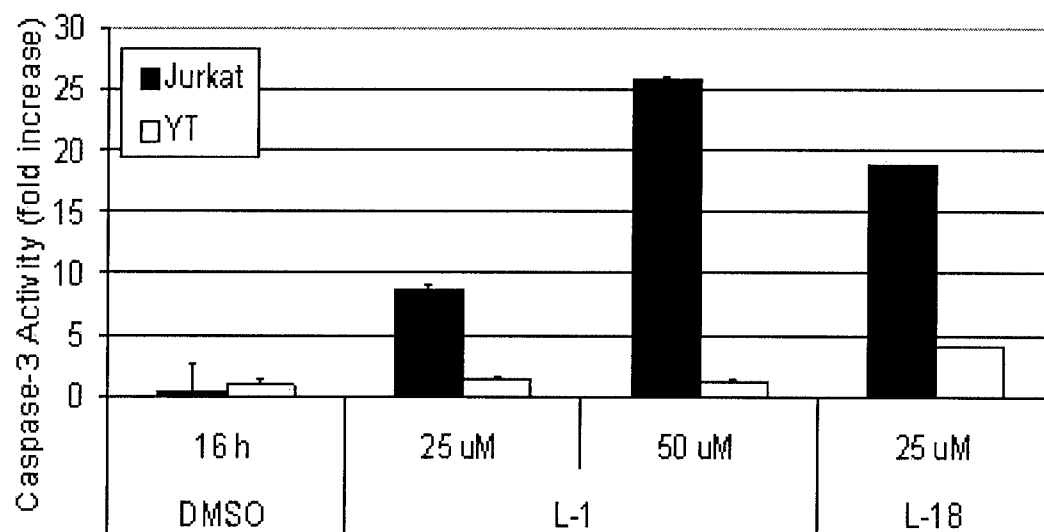


Figure 4

A.



B.

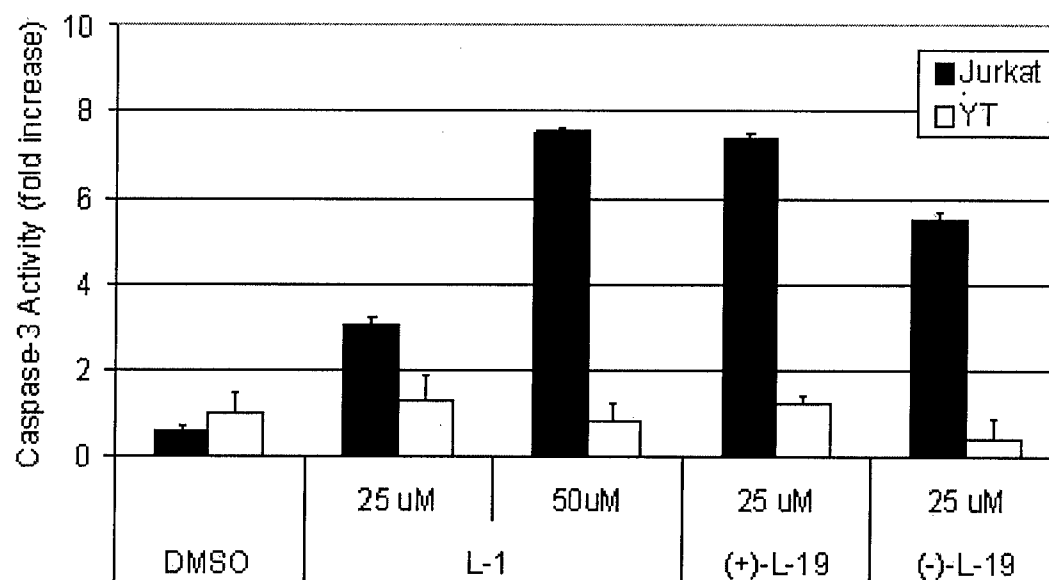
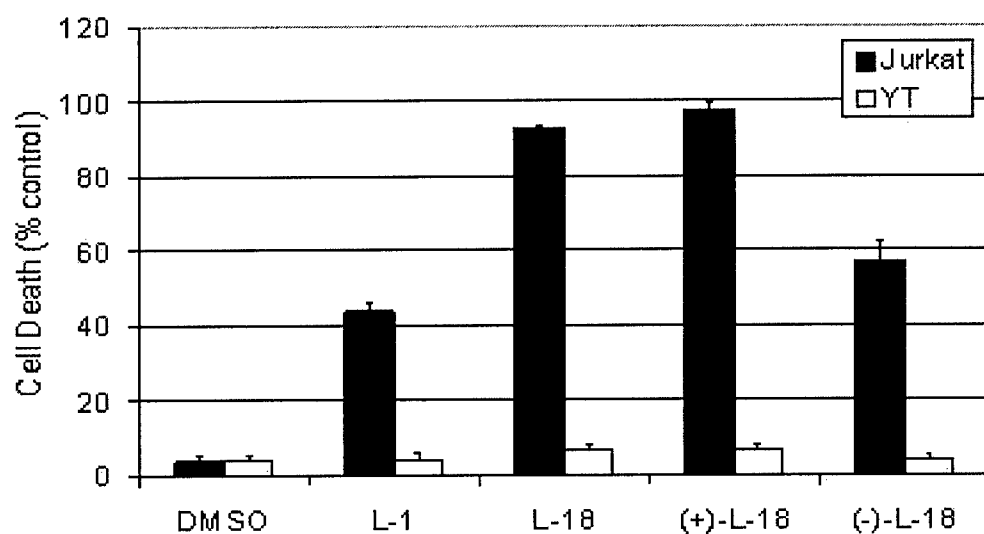


Figure 5

A.



B.

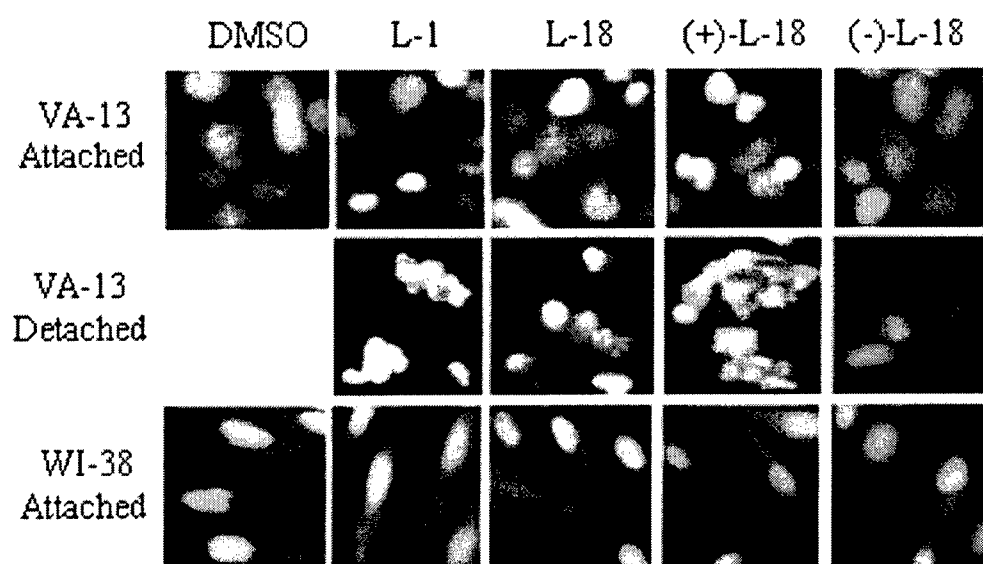


Figure 6

## BETA-LACTAMS AND THEIR POTENTIAL USE AS NOVEL ANTICANCER CHEMOTHERAPEUTIC DRUGS

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### 1. ABSTRACT

The discovery of natural and synthetic antibiotics is one of the most important medical breakthroughs in human history. Many diseases, such as bacterial meningitis, pneumonia, and septicemia, are now curable with the use of antibiotics. Antibiotics are efficacious, generally well tolerated in patients, and have a low toxicity level. It is for these reasons antibiotics remain an attractive target for drug discovery. Traditional beta-lactam antibiotics (e.g. penicillins, penems, cephalosporins) have a bicyclic ring structure that is conformationally rigid and functions to inhibit bacterial cell wall synthesis. In addition to the bactericidal action of antibiotics, it has been discovered that many antibiotics are capable of inhibiting tumor cell growth. There are currently many antitumor antibiotics approved for cancer therapy, which work to inhibit tumor cell growth by DNA intercalation. The use of beta-lactams as prodrugs has also met with success by aiding delivery of the chemotherapeutic directly to tumor sites. Recently, a novel class of N-thiolated monobactams, so termed because they possess a monocyclic ring instead of the bicyclic ring, has been found to induce apoptosis potently and specifically in many tumor cell lines but not in normal, non-transformed cell lines. Other beta-lactams, such as the polyaromatics, have been found to slow or inhibit tumor cell growth, and the 4-alkylidene beta-lactams are capable of inhibiting matrix metalloproteinases and leukocyte elastase activity. These data indicate that synthesis and evaluation of beta-lactams are a promising area for further development in anticancer research.

### 2. INTRODUCTION

Cancer is a heterogeneous disease and can be characterized as the growth of a malignant cell population that eventually leads to the interference of normal physiological functions. Anticancer drug research focuses on inhibition of tumor cell growth and induction of apoptosis in the malignant cell population. Apoptosis, or programmed cellular death, first described by Kerr *et al.* in 1972, is characterized by the ability of a cell to undergo a step-by-step self suicide program without affecting neighboring or adjoining cells (1). Activation of the apoptotic program in tumorigenic cells is essential for cancer prevention and treatment. A significant focus in anticancer drug discovery is to selectively induce tumor cell apoptosis with limited toxicity to normal cells. Tumor cells often have multiple alterations in their apoptotic machinery and/or signaling pathways that lead to increased levels of

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growth and proliferation. The absence of a tumor suppressor protein (such as p53) or the activation of an oncogenic protein (such as Bcl-2) can inhibit tumor cell apoptosis (2,3). Therefore, overriding these mutations can lead to stimulation of the apoptotic signaling pathway and cell death in tumor cells.

Currently, the beta-lactams are the most exploited family of antibiotics used for the treatment of bacterial infections (figure 1A). Beta-lactams are secreted by molds from the *Penicillium* genus and Sir Alexander Fleming first coined the name "penicillin" in 1928. Fleming observed bacteriolysis in a broth contaminated with *Penicillium* at St. Mary's Hospital in London, England (4). It would be many years until the import of this discovery was fully appreciated. Later studies at Oxford by Abraham, Florey and Chain resulted in the isolation of penicillin and subsequent drug trials (4). X-ray crystallography performed by Dorothy Hodgkin revealed that penicillin is a thiazolidine ring fused to a four membered beta-lactam ring (5; figure 1A). Later research focused on identifying several other antibiotics isolated from natural sources. Bacteria from the genus *Cephalosporium* also excrete beta-lactam containing compounds. Today the cephalosporin antibiotics, and their derivatives, comprise a large portion of the antibiotic therapies used (6,7). Several other classes of bicyclic beta-lactams were found to also possess antibacterial properties, such as penams, carbapenems and clavulanic acids (figure 1A).

The beta-lactams are powerful and potent inhibitors of bacterial growth and many different moieties of bicyclic beta-lactams have been isolated or synthesized since the discovery of penicillin (8). In 1981, two independent groups from the Squibbs and Takeda laboratories isolated the first *N*-thiolated beta-lactams from natural sources (9,10). These beta-lactams were the first to have a *N*-sulfonic acid group attached directly to the nitrogen in the lactam ring. The term "monobactam" was coined for these lactams, which have a flexible monocyclic ring, lack the carboxylic acid moiety, yet still retain a high bactericidal property (figure 1B).

Of late, a new class of *N*-thiolated beta-lactams was found to inhibit *Staphylococcal* and methicillin-resistant *S. Aureus* (MRSA) growth (11-13). The novel lactams most active against MRSA have an *N*-methylthio-substitution. These compounds are unaffected by penicillinases, such as beta-lactamase, an enzyme produced by some bacteria that degrades beta-lactams (14).

## **3. CURRENT ANTIBIOTIC MODALITIES IN CANCER TREATMENT**

The tetracyclines are antibiotics that have been used for the treatment of infection for decades (15). This family of compounds includes: tetracycline, doxycycline, and minocycline. Although these compounds are known for their effects on mitochondria, their ability to inhibit matrix metalloproteinases, the enzymes required for angiogenesis, may prove more beneficial (15). Col-3, a modified tetracycline, is now in clinical trials. Doxycycline has been shown to reduce tumor burden in mouse models and osteolytic bone metastasis as a result from breast cancer (15). These results have enabled doxycycline to enter clinical trials.

Rapamycin (RAPA) is a microbial macrolide from *Streptomyces hygroscopicus* and is used as an immunosuppressive to prevent organ rejection (16). It can also induce anti-proliferative effects by inhibiting cyclin-dependent kinases and inhibit retinoblastoma protein phosphorylation leading to cell cycle arrest (17-19). RAPA has been shown to have growth-suppressive effects in a broad range of cancers (20). For instance, Nepomuceno *et al.* have shown that RAPA is capable of preventing the growth of Epstein Barr virus positive (EBV+) B-cell lymphomas. Severe Combined Immune Deficient (SCID) mice treated with 1.5 mg/kg/day RAPA remained tumor free for up to six weeks after injection with peripheral blood from liver transplant patients, while mice without RAPA treatment developed tumors within three weeks (21). Their results suggest that rapamycin may be able to control other EBV-related cancers and those associated with organ transplant.

The novel histone deacetylase inhibitor, FK228, has been recently isolated from *Chromobacterium violaceum* (22). This antibiotic is a bicyclic peptide with a non-cysteine disulfide bridge that has been found to reverse H-ras transformed NIH-3T3 cells (22). The activity of FK228 against tumor cells is in the ng/mL range while effects against normal cells are not seen below concentrations of 1 µg/mL (23). The action of FK228 indicates that it is more effective against large tumors with an established blood supply over small tumors that do not yet require a capillary network (23). Specifically, FK228 down-regulates mRNA levels of vasoendothelial growth factor, a principle component of the angiogenesis pathway. This evidence strongly supports FK228 as a potential candidate for cancer chemotherapy.

Lavendamycin, which possesses a quinoline-5,8-dione core, is an antibiotic derived from *Streptomyces lavendulae* and isolated in 1981 (24). While the native compound did not pass clinical trials due to poor solubility and general toxicity, newly designed analogs show promise as potential chemotherapeutic drugs (25). The analogs of this compound possess a modified ring structure of the native compound to increase solubility and selectivity for p53-deficient cells (25). When lung carcinoma A549 cells were treated with the analog MB-97, the cells accumulated and activated p53 (25). These results suggest that this compound acts as a strong DNA damaging agent, a property it shares with other antibiotic/antitumor compounds like streptonigrin and the anthracyclines (25). Parental compounds of the MB-97 lavendamycin analog showed *in vivo* toxicity in the range of 0.4 mg/kg, whereas the new analog did not display toxicity until a treatment of 400 mg/kg was reached. Administration of another lavendamycin analog, MB-51, at doses of 300 mg/kg to mice bearing tumors resulted in an 80% reduction of tumor mass (25). These results strongly suggest that further examination of lavendamycin analogs as chemotherapeutic agents is necessary.

## **N-thiolated beta-lactams as anticancer drugs**

Discovered in 1966, bleomycin (derived from *Streptomyces verticillus*) is a well-studied antibiotic/antitumor agent (26). This compound is a principle treatment for testicular cancer and demonstrates reduced myelotoxicity (27). The most well known mode of action for this compound is its oxygen-dependent degradation of DNA (27). Unfortunately, bleomycin possesses potential fatal pulmonary toxicity (27). A recent 2003 clinical trial examined the effects of bleomycin treatment with mitomycin C as a follow up treatment to postoperative irradiation for patients with advanced head and neck cancer (28). This combined therapy improved survivability and the toxic effects (primarily mucositis) were considered within acceptable limits (28).

The anthracycline class of antibiotics include doxorubicin, daunorubicin, idarubicin, and epirubicin. Doxorubicin (DOX) and daunorubicin (DNR) have been used for over 30 years to treat a variety of solid and hematological tumors. DOX and DNR work by intercalation into DNA and inhibition of Topoisomerase II via binding to the Topo II/DNA ternary complex to promote its stabilization (29,30). Unfortunately, DOX and DNR also have high toxicity due to their mechanism of action, production of reactive oxygen species (ROS), which leads to toxicity of the cardiomyocytes and subsequent chronic and acute cardiomyopathies (31-33). Improvements in DOX and DNR structures lead to the development of idarubicin and epirubicin. Although these two analogs do have decreased toxicity and improved activity, there still is a significant risk to patients using these chemotherapeutic drugs (34,35).

Antibiotics are an intriguing class of compounds, not only for their ability to control bacterial infection but also for their capability to function as chemotherapeutic agents in cancer. There are an unlimited number of compounds bacteria can create. Based on the properties of existing antibiotics, studies into active analogs or novel synthetic compounds continue. Typically, antibiotics have reduced or no toxicity though there are exceptions, e.g. high doses can result in toxicity. Furthermore, these compounds can serve the dual roles of treating cancer or fighting potential infection during chemotherapy as an adjunct treatment. One of the more recent antibiotics to enter into the class of antibiotic/antitumor compounds is the class of beta-lactams.

## **4. TRADITIONAL ROLES OF BETA-LACTAMS**

The family of beta-lactams, so named because they all contain a beta-lactam ring, have been used for many years to treat bacterial infections. Traditional beta-lactam antibiotics, such as the penicillins and cephalosporins, contain, in addition to the beta-lactam ring, a carboxyl group in close proximity to the lactam nitrogen, which is required for antimicrobial activity. These antibiotics act as bactericidal agents by serving as a substrate for peptidoglycan transpeptidase, the enzyme responsible for crosslinking the *N*-acetylglucuronic acid (NAG) and *N*-acetylmuramic acid (NAM) moieties in the peptidoglycan layer surrounding the periplasmic space and membrane of bacteria. The transpeptidase enzyme is acylated by the beta-lactam, which results in a weakened cell wall and osmotic lysis of the bacteria. Since the commercial availability of penicillin in 1940, many other beta-lactam antibiotics with medicinal effects have been isolated and synthesized. Many thought that the war on infectious diseases was over after the discovery of penicillin as we have used antibiotics to treat and cure many diseases that were once fatal, namely tuberculosis, bacterial meningitis, and pneumonia. Unfortunately, bacteria developed resistance by producing enzymes to hydrolyze the beta-lactam moiety rendering the antibiotic inactive (36). This resistance has spawned a renewed interest in identifying and synthesizing new active antibiotics to treat resistant strains, derived mainly through resistant genes located in plasmids. Bacterial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are currently susceptible to only one current antibiotic (vancomycin).

Antibiotics are substances created by microorganisms or synthetically designed to kill or inhibit the growth of bacteria. These compounds are the staple of treatment for bacterial infection. In addition, there are numerous antitumor antibiotics that are currently used to treat cancer, such as the anthracyclines, bleomycin, mitomycin C, dactinomycin, and mithramycin. The major mechanism of action for these antitumor antibiotics is DNA intercalation or inhibition of DNA synthesis. Beta-lactam antibiotics are traditionally used only for bacterial infections, however, several novel classes of beta-lactams have been shown to possess anticancer properties as well. We have found that a class of beta-lactams, the *N*-thiolated beta-lactams, induce tumor cell apoptosis by introducing DNA damage in a potent, and more importantly, a tumor cell-specific manner with little or no effect on normal cells (37,38). Cainelli *et al.*, describe that 4-alkylidene-beta-lactams inhibit matrix metalloproteinases-2, and -9 (MMP), essential for the tumor induced neovascularization (39). Banik *et al.*, also show that beta-lactams with polyaromatic substituents induce tumor cell death in a variety of cancer cell lines, such as ovarian, prostate, breast, colon, and leukemic *in vitro* and demonstrated inhibition of tumor cell growth in mice (40) (see Section 6 for details).

## **5. BETA-LACTAMS AS PRODRUGS FOR ANTICANCER CHEMOTHERAPIES**

### **5.1. Background**

The most widely applied beta-lactams for prodrug based cancer chemotherapy have been the cephalosporins. The cephalosporins were chosen as prodrug candidates because of their inherent reactivity when hydrolyzed by beta-lactamase enzymes. Hydrolytic cleavage of the beta-lactam ring causes a secondary reaction that triggers the expulsion of the 3'-substituent (figure 2). The cytotoxic component can be attached to this position and then released when the cephalosporin beta-lactam ring is hydrolyzed by the enzyme.

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Cephalosporins have also been used in the selective targeting of anticancer compounds to tumor cells using Antibody Directed Enzyme Prodrug Therapy (ADEPT) and this method has received much attention in recent years (41,42). ADEPT is a drug delivery strategy that employs an enzyme covalently attached to a monoclonal antibody that is specific for a tumor cell antigen. This strategy allows for the delivery of the cytotoxic agent masked as a prodrug to specifically target tumor cells. To achieve site-specific generation of the cytotoxic agent, the monoclonal antibody-enzyme immunoconjugate (mAb-enz) is given to the patient first. This allows for prelocalization of the mAb-enz on the targeted tumor cell surface. The prodrug, which is the substrate of the enzyme, is then administered leading to targeted release of the drug.

The benefits of this method are many compared to administering the prodrug or parent drug alone (43-46). The catalytic nature of the enzyme will allow for the conversion of a stoichiometric excess of prodrug substrate. In addition, lower doses of the antibody-enzyme conjugate and higher doses of the prodrug can be administered, which reduces the toxicity. The surrounding tumor cells not bearing the target epitope can also be effectively dosed with the cytotoxic drug. This can lead to lower side effects due to tumor-specific localization of the parent drug. Furthermore, beta-lactamase enzymes are not endogenous to humans, making them highly exploitable for ADEPT and therefore eliminating premature cleavage of the prodrug by other naturally occurring enzymes. In order for the monoclonal antibody-enzyme immunoconjugate system to be successful, there are several criteria required for therapeutic efficacy. First, the mAb-enzyme conjugate should not elicit an immune response and have a high tumor/blood ratio. Secondly, the antibody portion of the immunoconjugate must selectively target and have a high binding affinity for the tumor cell antigen. Lastly, the unbound conjugate must be readily cleared from the blood-stream before the prodrug is administered. The following section will discuss antibody-enzyme-prodrug systems that have undergone evaluation to treat various types of cancers.

### **5.2. Nitrogen Mustards**

The alkylating agents or nitrogen mustards include cyclophosphamides, chloroambucil, and melphalan, and function by replacing an alkyl group for a hydrogen atom, leading to the formation of DNA adducts, or abnormal base pairing and cross linking of DNA. Cyclophosphamide and ifosfamide exist as prodrugs and are activated by hepatic enzymes into active species (47). While the nitrogen mustards are efficacious, they are extremely cytotoxic which results in unwanted side effects. For example, 7-(phenylacetamido) cephalosporin mustard (CM) prodrug was at least 50 times less toxic than phenylenediamine mustard (PDM) toward H2981 human adenocarcinoma cell line *in vitro* (48). The monoclonal antibody-enzyme conjugate L6-BC $\beta$ L was shown to activate CM in an immunologically specific manner, which resulted in a level of cytotoxicity comparable to PDM (48). *In vivo* studies indicated that CM was less toxic to nude mice than PDM, however treatment was hampered due to severe tail necrosis following intravenous injection (49).

A modified analog of CM, 7-(4-carboxybutamido) cephalosporin mustard (CCM), showed higher activity *in vitro* when administered with L6-ECI $\beta$ L. CCM was found to be less cytotoxic than PDM on H2981 cells, with IC<sub>50</sub> values of 25-45  $\mu$ g and 1.5  $\mu$ g, respectively. When the monoclonal antibody-enzyme conjugate L6-ECI $\beta$ L was administered 96 h prior to CCM, there was observed significant antitumor activity *in vivo* (49). The *in vivo* experiments concluded that administration of CCM in nude mice was less toxic than CM, and both prodrugs (CM and CCM) were significantly less toxic than PDM.

The prodrug C-Mel, a cephalosporin carbamate derivative of melphalan, was also shown to have antitumor activity (50). C-Mel was activated in an immunologically specific manner by the L49-sFv- $\beta$ L conjugate. *In vitro* cytotoxicity assays using 3677 human melanoma cells treated with the C-Mel prodrug in conjunction with L49-sFv- $\beta$ L showed that c-Mel was 40 times less toxic than melphalan alone, IC<sub>50</sub> = 53 $\mu$ g and 1.3  $\mu$ g, respectively. *In vivo* studies demonstrated that nude mice with growing tumors treated with L49-sFv- $\beta$ L and C-Mel at 150 mg/kg/injection underwent complete regressions, and 3 out of 5 mice were eventually cured (50).

### **5.3. Methoxytrexate**

Methotrexate (MTX) is a folic acid analog, or antimetabolite, which was first developed and used clinically in the 1940's (51). MTX interacts with dihydrofolate reductase (DHFR), an enzyme critical in folate metabolism (52). Unfortunately, drug resistance can often occur due to increased endocytosis of the antifolate by multidrug resistance protein (MDRPs) pumps and folate transporters (53-56). The prodrug of MTX is a potent cytotoxic agent and antimetabolite developed for ADEPT therapy. MTX was found to be a good substrate of beta-lactamase but showed identical cytotoxicity to that of the parent drug alone (45). No further evaluations have been conducted.

### **5.4. 5-Fluorouracil**

Another group of antimetabolites include the pyrimidine analogs, such as 5-fluorouracil and gemcitabine, and the purine analogs, such as 6-mercaptapurine and 6-thioguanine. These analogs substitute for nucleic acid bases in both DNA and RNA synthesis, but drug resistance to these antimetabolites has been implicated due to the nucleoside transporters (NT), which mediate uptake of nucleic acids into dividing cells (57). 5-Fluorouracil is an anticancer drug often used in the treatment of colon cancer. The prodrug of 5-fluorouracil was shown to be cleaved by beta-lactamase, however, the *in vitro* cytotoxicity was found to be the same as that of the parent drug alone (58).

## **N-thiolated beta-lactams as anticancer drugs**

### **5.5. Vinca Alkaloids**

The vinca alkaloids are potent anticancer drugs derived from natural sources that are used to treat the acute leukemias, lymphomas, and some solid tumors. Two cephalosporin-vinca alkaloid prodrugs were found to have a 5-fold less cytotoxicity to LS174T colon adenocarcinoma cells than the parent drug LY233425 and LY266070 (59,60). LY233425-cephalosporin prodrug was found to be equipotent when administered with the F(ab')<sub>2</sub>-beta-lactamase conjugate with the parent drug (59). *In vivo* studies with mouse models of human colorectal carcinoma tumors demonstrated long term regressions when LY266070-cephalosporin prodrug was administered with mAb-beta-lactamase (60).

### **5.6. Doxorubicin**

Cephalosporin-doxorubicin prodrugs have been developed that show promising anticancer properties when used in conjunction with mAb-beta-lactamase conjugates. *In vitro* studies of C-Dox on H2981 lung adenocarcinoma cells revealed that the prodrug was less toxic than doxorubicin alone. The prodrug was also immunospecifically activated by the L6-ECI $\beta$ -lactamase conjugate to release doxorubicin *in vitro* (61). Additionally, another study showed that a different mAb-beta-lactamase/cephalosporin-doxorubicin prodrug system effectively delivered doxorubicin to a series of MCF7 breast carcinoma, OVCAR3 ovarian carcinoma, and T380 and LS174T colon tumor xenografts (62). The maximum tolerated dose of the prodrug was equivalent to that of the free drug when compared to the degree of tumor suppression, however tumors did not regress. A polymer prodrug of cephalosporin-doxorubicin has been developed and was shown to increase the survival rate and decrease the tumor growth rate of mice when treated in conjunction with a polymer bound beta-lactamase enzyme (63). The combination of polymer-prodrug and polymer-enzyme was non-toxic with the doses used in the study.

### **5.7. Mitomycin C**

Two cephalosporin prodrugs of mitomycin C were evaluated against H2987 lung adenocarcinoma and clone 62 melanoma cell lines (64). *In vitro* studies showed that one of the prodrugs (prodrug 1) had comparable cytotoxicity to the parent drug, whereas another prodrug (prodrug 3) was 40- and 10- fold less toxic toward H2987 and clone 62 melanoma cells. Prodrug 3 also was immunospecifically activated by L6-F(ab')<sub>2</sub>-beta-lactamase and 96.5-F(ab')<sub>2</sub>-beta-lactamase conjugates that are selective toward H2987 and clone 62 cells, respectively (64).

### **5.8. Paclitaxel**

Anticancer drugs derived from natural sources comprise a large body of the drugs currently approved for chemotherapies. Treatments with paclitaxol (Taxol), derived from *Taxus brevifolia* (Pacific yew tree) and a semisynthetic analog, docetaxol, target rapidly dividing cancer cells by increasing microtubule polymerization, thereby inhibiting anaphase during cell cycle (65,66).

A prodrug of paclitaxel has been shown to be immunospecifically activated by the fusion protein L49-sFv-beta-lactamase (67). *In vitro* cytotoxicity assays performed on 3677 melanoma cells expressing the melanotransferrin (p97) antigen revealed that the prodrug was 12 to 30 times less cytotoxic than the parent drug (67).

### **5.9. Radioimmunoconjugates**

Radioimmunotherapy is a method to deliver a radioisotope to a specific target area namely tumor cells. One disadvantage of method is that it can lead to dose-limiting toxicities through radiation exposure to non-targeted organs. A recent study has shown that a radioimmunoconjugate containing a cleavable linker can release the radioisotope upon administering an enzyme thus lowering systemic radiation exposure (68). This approach utilized a <sup>131</sup>I-labeled cephalosporin conjugated to Tositumomab, a mAb specific for the CD20 antigen via a synthetic linker. Upon administration of the beta-lactamase enzyme, the radiolabel would be released causing rapid clearance from the blood and normal organs. *In vivo* studies of mouse models with human Ramos B lymphoma tumor xenografts revealed no decrease of the injected dose after 1 h of beta-lactamase treatment (68). However, after 4 h there was a noticeable decrease in the radioactive content from the tumor as well as blood, liver, lung and marrow demonstrating that there was rapid clearance of the radiolabel after injection of the radioimmunoconjugate and beta-lactamase enzyme (68). In addition, there was an enhanced tumor to blood % injected dose ratio at the beginning time points of the study.

The ADEPT system allows for the use of agents that, when given systemically, are too toxic for use in the clinic. The diversity of cancer drugs that are utilized is well demonstrated. Additionally, many studies have shown that the active drug is generated at the tumor site and at concentrations that could not be used in normal systemic administration of the parent drug. Further advancements to improve the efficacy of mAb-enzyme/prodrug therapies have resulted in modifying the mAb-beta-lactamase conjugate. Recombinant formed monoclonal antibody-beta-lactamase conjugates showed improved anticancer therapeutic activities compared to the synthetically formed conjugates (69).

## **6. POTENTIAL USE OF BETA-LACTAMS AS ANTICANCER DRUGS**

### **6.1. N-Thiolated beta-Lactams**

#### **6.1.1. Structure-Activity Relationships**

## ***N*-thiolated beta-lactams as anticancer drugs**

Currently, there are many types of antibiotics (e.g. anthracyclines, bleomycin) that have been used to treat cancer. However, research into the possibility of utilizing beta-lactam antibiotics as potential anticancer medications has been relatively non-existent. Our most recent studies suggest that beta-lactams could play a role as anticancer drugs (37,38). In the following sections, we will discuss the different *N*-methylthio beta-lactams and their effects on cancer cells as well as some insights into possible mechanisms of action. We have demonstrated that *N*-thiolated beta-lactams have tumor cell-killing ability through induction of DNA-damage and subsequent apoptosis (37). Synthesis of other beta-lactams have aided in identifying important structure-activity-relationships (SARs). Additionally, we have also shown that *N*-thiolated beta-lactams have the ability to preferentially induce apoptosis in tumor cells, but not in normal or non-transformed cell (38).

Perhaps one of the most important findings with beta-lactams was that they did not need to possess a bicyclic ring with its rigid conformity to be bactericidal (9,10). This allows for a broader range of synthetic analogs to be made that possess antibacterial or antitumor activity. We have screened a large number of synthetic beta-lactams for their ability to promote tumor cell death (37,38). Of the compounds screened, we chose a lead compound, Lactam 1, to be the basis for additional synthesis of analogs (figure 3). Our findings yielded several important SARs. Lactam 1 was shown to induce apoptosis in a variety of tumor cell lines, namely, breast (MDA-MB-231, MCF-7), prostate (PC-3, DU-145), head-and-neck (PCI-13), SV-40 transformed lung cells (VA-13) and leukemic (Jurkat T) cell lines (37,38).

Other important SARs were observed also when cells were treated with the beta-lactams in cellular toxicity assays. Foremost, is the necessity of the *N*-methyl-thio group that when absent abolishes the apoptosis-inducing activity. Also observed was the inverse relationship between the number of carbon atoms off the *N*-thio group. Increasing the number of carbons from one to two decreased the amount of apoptosis observed by ~50% (Lactam 1 vs. Lactam 3). A four carbon chain off the *N*-thio group further decreased apoptosis-inducing activity by ~65%, and substitution of the *N*-methylthio group with a *N*-benzylthio group lead to ~70% decrease in apoptosis. Observations about the position of the chloro group off the phenyl ring also provided key SAR information. Isomers with the -Cl group in the *meta* or *para* position revealed that Lactams-5 and -6, while still capable of inducing apoptosis, were less potent than Lactam 1 (37). To determine if deletion or substitution of the *ortho* -Cl on the phenyl ring would increase or decrease activity, several analogs of Lactam 1 were synthesized with substituted halogen or non-halogen groups for the -Cl (figure 3). It was found that increasing the size of the group in the *ortho* position correlated with increased cell death (38). In fact, elimination of the *ortho* substituted group resulted in the least amount of activity, while substitution with a nitro group lead to the greatest amount of activity. The -NO<sub>2</sub> substituted analog, Lactam 12, exhibited the strongest effect and consistently induced apoptosis comparable with Lactam 1, but at half the concentration (38).

According to these results, several key features should be retained for future design and synthesis of beta-lactams with antitumor properties: 1) the *N*-methyl-thio group must remain intact without additional carbon chains, 2) although *meta* and *para* substitutions on the phenyl ring still preserve their apoptotic producing abilities, substitutions in the *ortho* position are most potent, and lastly 3) the larger groups in the *ortho* position correlates with enhanced apoptotic-inducing activity (37,38).

### **6.1.2. Apoptosis Induction**

A number of synthetic *N*-methylthio beta-lactam compounds have been found to induce apoptosis in a number of tumor cell lines, such as the human leukemia Jurkat T cells, breast cancer (MCF-7, MDA-MB-231), prostate (PC-3, DU-145), and head-and-neck (PCI-13) cells (37,38). Several of these compounds (figure 3) caused induction of caspase-3/-7 activity, effector caspases whose activation is indicative of the apoptosis (70). The potency of these beta-lactams is as follows, Lactam 12 > 10 > 11 > 1 > 6 > 5 > 3 > 4 > 9 > 8 > 7 > 2 (37,38). A nuclear stain to determine the morphological changes of apoptotic nuclei showed that Lactam 12 induced cellular detachment of 50-60 % of total cell population (38). Additionally, beta-lactam treatment instigated cleavage of poly(ADP-ribose) polymerase (PARP) from its 116 kDa full length form to the 85 kDa fragment (37) which occurred in conjunction with caspase-3 activity, the caspase shown to directly cleave PARP (71). Caspase-8, an initiator caspase capable of mitochondria-dependent and -independent apoptosis initiation (72), was also found to become active after beta-lactam treatment (37). Cytochrome *c* is a mitochondrial protein that is released during apoptosis when the membrane potential of the mitochondria is compromised and combines with several other proteins (dATP, Apaf-1, caspase-9) to form the apoptosome, which is capable of activating caspase-3 (73). The *N*-methylthio beta-lactams are also able to cause cytochrome *c* release from the mitochondria, prior to activation of caspase-3, in time- and concentration-dependent manners (37). These data confirm that beta-lactams can indeed cause apoptosis in tumor cells.

There are other lactam compounds that can also induce apoptosis. Watabe *et al.* found that gamma-lactams, which contain a five-membered ring, are capable of inducing apoptosis in HL-60 cells (74). MT-21, a synthetic gamma-lactam, activates caspase-9 followed by the subsequent activation of caspase-3. Unlike our findings with beta-lactams, caspase-8 was not found to participate in the apoptosis signaling cascade after gamma-lactam treatment (74). Lactacystin, a gamma-lactam possessing a thio ester moiety and originally isolated from actinomycetes (75), has been found to be a potent inhibitor of chymotryptic- and tryptic-like catalytic activities of the proteasome through covalent bonding to the N-terminal threonine of the beta-subunits (76). Proteasome inhibition leads to an accumulation of p27 (77), I $\kappa$ B- $\alpha$  (78), and Bax (79), which can cause G<sub>1</sub> cell cycle arrest and apoptosis (80, 81). It is for these reasons many believe that proteasome inhibitors are good candidates for anticancer chemotherapeutic drugs (82-84).

### **6.1.3. DNA-Damage and Signal Transduction Pathways**

To further investigate the cause of apoptosis after *N*-methylthio beta-lactam treatment, analysis of cell cycle changes were performed. Lactam 1 was found to increase S-phase DNA content and initiate a concomitant decrease in G<sub>1</sub> phase DNA. This S-phase cell cycle arrest was found to be due to an inability for treated cells to undergo DNA replication as was found from a [<sup>3</sup>H] thymidine incorporation assay. DNA replication was inhibited in a time- and concentration-dependent manner with a half-maximal inhibition (IC<sub>50</sub>) of [<sup>3</sup>H] thymidine in Jurkat cells at 32 μM with Lactam 1 treatment (37). A terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay, which detects DNA strand breaks, then provided additional data to support to the hypothesis that the DNA replication inhibition was due to damage of the genomic DNA. After just 1 h of treatment with, over half of the cell population contained DNA strand breaks and after 4 h 98 % of the cells showed DNA strand breakage (37). In a similar experiment it was found that Lactam 12 induced greater DNA damage than Lactam 1 (by 27%) after a 24 h period (37).

It was also determined that p38 MAP kinase activation is required for beta-lactam induced apoptosis (37). Activation of p38 MAP kinase can trigger apoptosis following multiple stimuli, such as DNA damage (85, 86). Protein levels of phosphorylated p38 increased significantly with Lactam treatment and cotreatment with the p38 inhibitor (PD169316) inhibited PARP cleavage and activation of caspase-3, -8, and -9 (37). Additional experiments revealed that p38 activation occurs upstream of caspase activation and that p38 activity was necessary for caspase-mediated cell death in beta-lactam treatment. Conversely, DNA strand breaks were still observed after cotreatment with PD169316 and Lactam 1, indicating that *N*-thiolated beta-lactams induced DNA damage leading to p38 activation, followed by caspase activation and subsequent apoptotic cell death.

### **6.1.4. Preferential Tumor Cell Killing**

Many currently used chemotherapeutic drugs for cancer intercalate with cellular DNA, thus making it impossible for the cell to function which leads to subsequent apoptotic death. Unfortunately, these drugs are not "tumor-specific" and they will intercalate with any rapidly dividing cell, such as the epithelial cells lining the gastrointestinal tract, which can lead to nausea and vomiting. Tumor cell specific therapies are those that solely target tumor cell characteristics exclusively. For instance, Gleevec (STI571) is an ATP inhibitor that targets growth and proliferative signaling pathways stimulated by the Bcr-Abl oncoprotein in chronic myelogenous leukemia (87).

To determine if Lactam 1 possessed a tumor cell-specific activity human leukemic Jurkat T cells and immortalized, non-transformed natural killer cells (YT cells) were treated with Lactam 1 and the effects were determined. It was found that only the Jurkat, but not YT, cells showed apoptosis-specific PARP cleavage and decreased cell viability in both time- and concentration-dependent manner (38). Additionally, treatment with Lactam 12, which substitutes the -Cl moiety for a -NO<sub>2</sub> on the benzene ring, was found to potently and specifically induce apoptosis in only the Jurkat T cells while not affecting the non-transformed YT cells. Both Lactam 1 and Lactam 12 inhibited colony formation, indicative of cellular transformation, of prostate cancer LNCaP cells as observed in a soft agar assay. These lactams were also able to induce TUNEL-positive cells as well as caspase-3/-7 activity and apoptotic nuclei in a number of transformed tumor cell line types, but not in non-transformed cell lines (38). For example, Lactam 12 treatment induced apoptotic morphological changes and caspase-3 activity exclusively in SV-40 transformed human fibroblasts (VA-13) but not in normal non-transformed fibroblasts (WI-38) (38). This is consistent with the idea that beta-lactams could be developed into tumor-specific drugs.

### **6.2. 4-Alkylidene-beta-Lactams**

The matrix metalloproteinases (MMPs) are a class of mammalian proteases that can, among other functions, degrade the extracellular matrix (88). Angiogenesis, the formation of new blood vessels, requires the activity of the MMPs to digest the basement membrane. The MMPs play a pivotal role in cancer progression by allowing neovascularization, which is essential for tumor growth, invasion, and metastasis (89). MMPs can be constitutively activated in cancer cells, but not in normal cells (90). Thus, targeting MMP expression and activity is a unique approach in the field of cancer research.

A class of beta-lactams, the 4-alkylidene-azetidin-2-ones, has been identified that exhibit inhibitory activity to both MMP-2 and MMP-9 as well as leukocyte elastase (LE) (39). LE can activate MMP-2 and MMP-9, and inactivate their tissue inhibitor (91). Compounds with protected hydroxy groups (Compounds 1 and 8) were found to inhibit LE. Compound 8 in particular showed an IC<sub>50</sub> of 9 μM to LE activity. The green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG), a known LE inhibitor, was used as a comparison and was found to be ~22-fold more active (IC<sub>50</sub>=0.4 μM) than Compound 8 (39). When the hydroxyl group was unprotected, or removed all together, the beta-lactam lost its potent activity against LE, but gained considerable activity against MMP-2 and MMP-9. Two compounds in particular (Compounds 2 and 18) showed the greatest inhibitory activity on MMP-2 with IC<sub>50</sub>s of 85 μM and 60 μM, respectively (39). This is a promising area of drug research because inhibition of angiogenesis not only inhibits tumor growth, but also prevents invasion and metastasis *via* the circulatory system.

### **6.3. Polyaromatic Beta-Lactams**

In 2001, Banik *et al.* described polyaromatic imine beta-lactams with biological activity against cancer cells (40). Several synthesized compounds were tested *in vitro* for their cytotoxicity on nine cancer cell lines (40). Compounds with phenanthrene and chrysene substituents had the most activity *in vitro*, as measured by MTT assay. The maximal activity

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concentrations ranged from 2.5 to 40.6  $\mu\text{M}$ , some well within the therapeutic range. Conversely, beta-lactams with naphthalene, anthracene, and pyrene substituents showed virtually no cytotoxicity (40).

A series of *in vivo* assays using athymic nude (nu/nu) mice was performed with the active beta-lactams mentioned above. Mice were injected with K-562 leukemia, HT-29 colon, or SKOV-3 ovarian cancer cells. A variety of treatment times and regimens were tested (40). Mice given polyaromatic beta-lactam treatments (Compound 17a) at 60 mg/kg showed negligible toxicity compared to the control mice that were given cisplatin and adriamycin. The polyaromatic induced only a slight weight loss (3.52 g), which was quickly recovered after discontinuation of treatment (40). It was also found that treatment with the beta-lactam compound delayed the onset of tumor formation by  $7 \pm 2$  days in mice injected with HT-29 cells. Additionally, many of the mice injected with SKOV-3 cells did not form any tumors at all (40).

## **7. CONCLUSIONS AND PERSPECTIVES**

Cancer is lethal to 42% of those diagnosed. With millions of new patients each year, the initiative to develop suitable chemotherapeutic agents is a driving focus of medical research. However, the currently available chemotherapeutic agents are incapable of selectively targeting cancer cells from normal cells leading to treatments that are almost as hazardous as the disease itself. Beta-lactams are compounds that have been used for many years to combat microbial infections. Therefore, it is already known that these compounds possess minimal effects on non-bacterial cells; this trait is desirable of chemotherapeutic agents. Recently, the potential of beta-lactams as anticancer agents has come to light. Beta-lactams can be used as pro-drugs that are capable of specifically targeting tumor cells. Likewise, the *N*-methylthiolated beta-lactams are capable of inducing apoptosis in a wide array of tumor cells types, with little effect on normal cells. Thus making these compounds, and other beta-lactams (eg. 4-alkylidene and polyaromatics), attractive targets for structure-activity relationship studies and analog synthesis. While further study on this class of compounds in animal models should be performed to completely assess their toxicity, selectivity, and efficacy *in vivo*, the profiles reported here show an optimistic future for expanding the role of these compounds from simple antibiotics to anticancer therapeutics.

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**Figure 1.** Structure of bicyclic (A) and monocyclic (B) beta-lactam families.

**Figure 2.** Diagramatic presentation of cephalosporin prodrug reaction. Beta-lactamase induces hydrolytic cleavage of the beta-lactam ring, which causes a secondary reaction leading to the release of the anticancer drug in the 3' position.

**Figure 3.** Structure of *N*-thiolated monobactams.

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1. Title: Change 'CHEMOTHERAPIUTICS' to 'CHEMOTHERAPEUTICS'
2. Section 3, p. 3, paragraph 1: Change last sentence to, "This combined therapy improved survivability and the toxic effects (primarily mucositis) **were considered** within acceptable limits."
3. Section 4, p. 3, paragraph 1: Change last sentence from "methicillin-resistance" to "methicillin-resistant".
4. Section 4, p. 3, paragraph 2, line 8: Sentence should read, "Cainelli et al., describe that 4-alkylidene-beta-lactams..."
5. Section 6.1.3, p. 7, paragraph 1, lines 3-4: Sentence should read, "...cells to undergo DNA replication as was found from a [3H] thymidine..."
6. Section 6.2, p. 7, paragraph 2, line 3: Change "...hydroxy groups (Compound 1 and 8)..." to hydroxy groups (Compounds 1 and 8)..."

INHIBITION OF PROSTATE CANCER CELLULAR PROTEASOME ACTIVITY BY A PYRROLIDINE DITHIOCARBAMATE-COPPER COMPLEX IS ASSOCIATED WITH SUPPRESSION OF PROLIFERATION AND INDUCTION OF APOPTOSIS

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| 1. ABSTRACT                                                                                   |  |

Recent research suggests that copper could be used as a novel selective target for cancer therapies. Copper is a co-factor essential for tumor angiogenesis processes and high levels of copper have been found in many types of human cancers, including prostate, breast and brain. We have reported that organic copper-containing compounds, such as 8-hydroxyquinoline-copper(II), are a novel class of proteasome inhibitors and tumor cell apoptosis inducers (Daniel et al., *Biochem Pharmacol.* 2004;67:1139-51). Most recently, we have found that when complexed with copper, the known antioxidant pyrrolidine dithiocarbamate (PDTC) forms a potent proteasome inhibitor in human breast cancer, but not normal cells (Daniel, Chen, et al., submitted). In the current study, we investigate whether the PDTC-copper complex can play similar roles in inhibiting the proteasomal activity and consequently inducing apoptosis in human prostate cancer cells. We used tetrathiomolybdate (TM), a strong copper chelator currently being tested in clinical trials, as a control. We report here that after binding to copper, PDTC, but not TM, can inhibit the proteasomal chymotrypsin-like activity, suppress proliferation, induce apoptotic cell death, and inhibit uptake of radiopharmaceutical 2-[<sup>18</sup>F]Fluoro-2-deoxy-D-glucose in cultured human prostate cancer cells. In contrast, PDTC, TM or copper alone or a TM-copper mixture had no such effects. Our study suggests that high copper levels in human prostate cancer *in vivo* can be targeted by a ligand such as PDTC, resulting in formation of an active proteasome inhibitor and apoptosis inducer specifically in prostate tumor, but not normal cells.

## 2. INTRODUCTION

Copper is an essential trace metal for animals. The amount of copper in an organism is tightly regulated (1, 2). High levels of copper have been found in many types of human cancers including prostate, breast, colon, lung, and brain (3-11). Consistently, angiogenesis, the formation and differentiation of blood vessels, is essential for tumor growth, invasion, and metastasis (12-15). Indeed, molecular processes of angiogenesis require of copper, but not other trace metals, as an essential cofactor (12-21).

The ubiquitin/proteasome system plays an important role in the degradation of cellular proteins. This proteolytic system involves two distinct steps: ubiquitination and degradation (22, 23). The eukaryotic proteasome contains at least three known

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activities, which are associated with its  $\beta$  subunits. These are the chymotrypsin-like (cleavage after hydrophobic residues,  $\beta 5$  subunit), trypsin-like (cleavage after basic residues,  $\beta 2$  subunit), and caspase-like or peptidyl-glutamyl peptide-hydrolyzing (cleavage after acidic residues,  $\beta 1$  subunit) activities (24, 25). Inhibition of the proteasomal chymotrypsin-like, but not trypsin-like, activity has been found to be associated with induction of apoptosis in tumor cells (26-31).

Dithiocarbamates are a well-defined class of metal-chelating compounds. These compounds have been previously used for treating bacteria, fungi and AIDS (32, 33). Pyrrolidine dithiocarbamate (PDTC; Figure 1A), a member of the dithiocarbamates, is an antioxidant that has been used as a therapeutic agent for treatment of inflammation, atherosclerosis and metal intoxication (34, 35). It has been shown that PDTC is able to inhibit NF $\kappa$ B activation and induce tumor cell apoptosis (36). Although the involved molecular mechanism remains unknown, it has been found that copper uptake is required for PDTC-mediated activities (37) and that PDTC is a copper-binding compound (38). PDTC and other dithiocarbamates have been found to induce apoptosis in conjunction with copper in different types of cancer cells (37, 39).

Tetrathiomolybdate (TM) is a copper chelator that was originally used for patients with Wilson's disease (17, 18). TM has been found to be effective in impairing the growth of mammary tumors in HER2/neu transgenic mice (40) and lung metastatic carcinoma in C57BL/6/J mice (41). In a phase I clinical trial with patients suffering from metastatic cancers, TM therapy achieved stable disease in 5 of 6 patients who became copper-deficient (20). However, the disease advanced in some other patients before copper levels were sufficiently lowered (17, 18, 20). These reports support the idea of copper control as an anticancer strategy.

We have previously demonstrated that certain types of copper-binding compounds are potent proteasome inhibitors (42). Most recently, we have also found that PDTC, after interacting with copper, forms a proteasome inhibitor in human breast cancer cells (Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, Dou QP, submitted). In the current study, we investigated the effects of the PDTC-Cu complex in human prostate cancer cells. We report that PDTC is capable of binding copper, spontaneously forming a new complex that has proteasome-inhibitory and apoptosis-inducing activities to human prostate cancer cells. In contrast, copper, PDTC or TM alone, or TM-copper complex had no effects under the same experimental conditions. We propose that targeting highly elevated copper could be tumor-specific and that formation of an active proteasome inhibitory complex between PDTC and copper is a novel strategy that has great potential for prostate cancer therapies.

### 3. MATERIALS AND METHODS

#### 3.1. Chemicals, reagents, and radiopharmaceuticals

Cupric chloride ( $\text{CuCl}_2$ ), dimethyl sulfoxide (DMSO), pyrrolidine dithiocarbamate (PDTC), and ammonium tetrathiomolybdate (TM), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and bisbenzimidazole Hoechst #33258 stain were all purchased from Sigma-Aldrich. The substrate for the chymotrypsin-like activity of the proteasome, Suc-Leu-Leu-Val-Tyr-AMC, was purchased from Calbiochem. Polyclonal antibody to poly(ADP-ribose) polymerase (PARP) was obtained from Boehringer, Mannheim. Monoclonal antiubiquitin antibody, secondary antibodies, anti-mouse IgG horseradish peroxidase, and anti-rabbit IgG-horseradish peroxidase were from Santa Cruz Biotechnology Inc. Radiopharmaceutical 2-[ $^{18}\text{F}$ ]Fluoro-2-deoxy-D-glucose ([ $^{18}\text{F}$ ]FDG), a tracer widely used for positron emission tomography (PET) imaging of cancers, is prepared by the method of Hamacher et al (43) using 1,3,4,6-tetra-O-acetyl-2-O-triflyl- $\beta$ -D-manno-pyranose as the labeling precursor and  $^{18}\text{F}$  radioisotope produced with a cyclotron housed at the PET Center, Children's Hospital of Michigan.

#### 3.2. Preparation of the PDTC-Cu complex

To prepare PDTC-Cu complex, equal molar amounts of PDTC and  $\text{CuCl}_2$ , both dissolved in DMSO at 50 mM, were mixed by drop-wise addition of the  $\text{CuCl}_2$  solution into the PDTC solution. The mixture was kept at room temperature for 10 minutes to allow formation of the PDTC-Cu complex. The freshly prepared PDTC-Cu complex was used for experiments.

#### 3.3. Cell culture, drug treatment and whole cell extract preparation

LNCaP human prostate cancer cell line was purchased from ATCC. Cell culture medium (RPMI 1640), antibiotics, and trypsin solution were purchased from GIBCO. LNCaP cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/mL of penicillin, 100 mg/mL of streptomycin and 10mM HEPES at 37 °C in a humidified incubator with an atmosphere of 5%  $\text{CO}_2$ . Cells were treated with noted molar amounts of compounds, mixtures, or with an equivalent volume of solvent control as indicated in the figure legends. Whole cell extracts were prepared as described previously (44, 45). Briefly, cells were harvested, washed with cold PBS twice, and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4 °C. Afterwards, the lysates were centrifuged at 12,000 g for 15 min, and the supernatants were collected as whole cell extract.

#### 3.4. Cell proliferation inhibition assay

The effects of each compound and their copper mixtures on LNCaP cells were determined using the MTT dye uptake method. Briefly, LNCaP cells ( $10^4$  /well/ 0.1 ml) were seeded in triplicate in a 96-well plate and incubated at 37 °C in a humidified incubator with an atmosphere of 5%  $\text{CO}_2$  until 70-80% confluence. This was followed by an additional 24-h incubation with fresh medium containing different concentrations of each compound and their copper mixtures. The same

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amount of DMSO was added as the solvent control. Thereafter, 100  $\mu$ L MTT solution (1 mg/ml in serum-free medium) replaced the medium in each well. After 4 h at 37 °C incubation, the MTT solution was replaced by 100  $\mu$ L DMSO. When the blue crystals were dissolved, the optical density (OD) was measured in a Wallac Victor3<sup>TM</sup> multilabel plate reader (Perkin Elmer, USA) at a wavelength of 560 nm. The formula used to evaluate the result is as follows: inhibition of cell proliferation (%) = (OD of the experimental samples/OD of the control)  $\times$  100%.

### 3.5. Cellular and nuclear morphology analysis

LNCaP cells were treated as indicated (see figure legends). Afterwards, the cells were visualized by microscopic imaging with either phase contrast for cellular morphology or Hoescht staining with fluorescence for nuclear morphology. Briefly, the cells were washed with PBS and fixed in 75% ethanol for at least 1 h at 4 °C followed by washing with PBS three times and staining in 50  $\mu$ M of Hoechst 33258 for 30 min at 4 °C in dark. Subsequently the cells were washed and resuspended in 50  $\mu$ L PBS and mounted for viewing. Copper mixture-induced apoptosis was monitored by the extent of nuclear fragmentation. Nuclear fragmentation was visualized by Hoechst 33258 staining of apoptotic nuclei using Zeiss Axiovision fluorescence microscope (Carl Zeiss Microscope Inc., Hallbergmoos, Germany).

### 3.6. *In vitro* proteasome activity assay

The cell-free chymotrypsin-like activity of the proteasome was determined by measuring the release of the AMC groups from a substrate as previously described (26). Briefly, after each treatment, whole cell extract (10  $\mu$ g) of LNCaP cells was incubated in 100  $\mu$ L of assay buffer (50 mM Tris-HCl, pH 7.5) and 40  $\mu$ M fluorogenic peptide substrates, Suc-Leu-Leu-Val-Tyr-AMC (for the chymotrypsin-like activity) for 1 h at 37 °C. After incubation, production of hydrolyzed AMC groups was measured using a Wallac Victor3<sup>TM</sup> multilabel plate reader with an excitation filter of 380 nm and an emission filter of 460 nm. Changes in fluorescence were calculated against non-treated controls and plotted with statistical analysis using Microsoft Excel<sup>TM</sup> software.

### 3.7. Western Blot Assay

To evaluate ubiquitinated protein accumulation and PARP cleavage induced by PDTC-Cu complex treatment, cell lysates (30  $\mu$ g) from treated LNCaP cells were subject to SDS-PAGE analysis and then transferred to a nitrocellulose membrane. The Western blot analysis was performed using specific antibodies to ubiquitin and PARP as described previously (42, 45), followed by visualization *via* enhanced chemiluminescence (ECL) (Amersham Biosciences).

### 3.8. Cellular <sup>18</sup>F-FDG uptake assay

To evaluate the effects of PDTC-Cu complex on cellular uptake of <sup>18</sup>F-FDG, LNCaP cells (5  $\times$  10<sup>5</sup> cells in 1 ml of RPMI1640 /well) were seeded in triplicate on a 12-well cell culture plate 12 hours prior to treatment of cells with PDTC-Cu complex and other control reagents. Following treatment for 12 hours, LNCaP cells were incubated with 5  $\mu$ Ci (185 kBq) of <sup>18</sup>F-FDG added in the cell culture medium for 1 hour at 37 °C, in an atmosphere containing 5% CO<sub>2</sub>. At end of incubation, the culture medium containing <sup>18</sup>F-FDG was removed and the cells were washed with PBS three times. Subsequently, the cells were digested with 5% trypsin solution and collected in a tube for radioactivity count using a Packard RIAStar multiwell gamma counter (Packard Instrument Co, Meriden CT). Radioactivity of the cells was calculated as percentage of inoculation dose (ID%) normalized to cell number (5  $\times$  10<sup>5</sup>/well), in reference to radioactivity of 5  $\mu$ Ci (185 kBq) of <sup>18</sup>F-FDG.

### 3.9. Statistical analysis

All data were expressed as mean  $\pm$ SD. Statistical analysis was performed with the paired, two-tailed T test using Microsoft Excel software and *p* values < 0.05 or < 0.01 were considered to represent statistical significance.

## 4. RESULTS

### 4.1. PDTC spontaneously reacts with copper to form a new complex.

In order to use endogenous elevated copper in prostate tumor tissues as a targeting mechanism for cancer therapy, it is prerequisite for a ligand to react spontaneously with copper to form a new complex. To test the reactivity of PDTC with copper, 50 mM of PDTC was added to a 50 mM of copper (II) chloride (Figure 1). The reaction of PDTC with copper results in a dramatic color change (Figure 1), indicating formation of a new metal complex. This result is consistent with a previous publication showing that PDTC is strong copper chelator (37). Therefore, PDTC may be capable of combining with endogenous copper in tumor tissues and forming an active proteasome-inhibitory complex.

### 4.2. The PDTC-Cu complex inhibits the chymotrypsin-like activity of the proteasome in intact LNCaP cells

Previously we reported that organic copper complexes inhibit the cellular proteasomal activity (42; Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, Dou QP, submitted). We then investigated whether the PDTC-copper could inhibit the proteasome activity in human prostate cancer cell. Prostate cancer LNCaP cells were treated for 24 h with copper, PDTC, or PDTC-copper mixture, using TM and TM-copper mixture as controls. After treatment, the cells were collected and protein extracts were prepared for analysis of proteasome inhibition. Inhibition of the proteasome was measured by two assays: the chymotrypsin-like activity assay (Figure 2A), and the accumulation of ubiquitinated proteins by Western blotting (Figure 2B). We found that the prepared PDTC-copper mixture significantly inhibited the proteasome activity in LNCaP cells, as indicated by low levels of the

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proteasomal chymotrypsin-like activity (Figure 2A) and accumulation of ubiquitinated proteins (Figure 2B, lane 5). Copper or PDTC alone does not inhibit the proteasome activity (Figure 2A, B, lanes 2, 3). We found that neither TM nor the TM-Cu mixture was able to inhibit the proteasome activity in the prostate cancer cells (Figure 2), further supporting that TM acts as a passive copper chelator and eliminator and TM-copper is an inactive complex (42). These data support the hypothesis that a PDTC-Cu complex is capable of proteasome inhibition.

### 4.3. PDTC-Cu mixture effectively inhibits proliferation of human prostate cancer cells

Since PDTC can form a complex with copper, as indicated by color change (Figure 1), and is able to inhibit the proteasomal activity in prostate cancer cells (Figure 2), we then tested whether the complex is able to inhibit proliferation of human prostate cancer cells. Prostate cancer LNCaP cells were treated with copper, PDTC, PDTC-copper mixture, TM or TM-copper mixture for 24 h. We found that the PDTC-copper mixture inhibited LNCaP cell proliferation in a dose-dependent manner (Figure 3). The PDTC-copper mixture showed 50% inhibition at 1  $\mu$ M and greater than 90% inhibition at 5-10  $\mu$ M (Figure 3). The  $IC_{50}$  value of the PDTC-copper mixture was determined as  $1 \pm 0.07$   $\mu$ M. In contrast, copper alone, PDTC alone, or TM and TM mixed with copper had no significant effect (Figure 3).

### 4.4. LNCaP cells treated with the PDTC-Cu complex undergo apoptosis

Having demonstrated that the PDTC-copper complex is capable of inhibiting cellular proliferation in prostate cancer cells (Figure 3), we then determined whether this mixture could induce prostate cancer cell apoptosis. Exponentially growing LNCaP cells were treated with copper, PDTC, TM or their mixtures for 24 h, followed by observing cellular and nuclear morphological changes by phase contrast or fluorescence microscope, respectively (Figure 4). The cells treated with the PDTC-copper mixture became spherical and detached (Figure 4A). By staining with Hoescht 33258, the most of the nuclei in the PDTC-copper-treated cells were brighter and denser, compared to those treated with copper, PDTC, TM alone or TM-copper (Figure 4B). These results suggested that the cells treated with the PDTC-copper mixture were undergoing apoptosis. Apoptosis induction was further verified by cleavage of PARP (Figure 2C). Treatment with the PDTC-copper mixture, but not others, induced loss of the intact PARP and appearance of a PARP cleavage fragment (Figure 2C). Taken together, PDTC, when combined with copper, forms an active complex that has the proteasome-inhibitory, proliferation-inhibitory and apoptosis-inducing activities.

### 4.5. Reduction of F18-FDG uptake by LNCaP cells after treatment with PDTC-Cu complex

To prepare for evaluation of anti-tumor activity of the PDTC-Cu complex *in vivo* with  $^{18}$ F-FDG PET imaging, we tested effects of the PDTC-Cu complex on  $^{18}$ F-FDG uptake by LNCaP cells following treatment with the PDTC-Cu complex *in vitro* (Figure 5). Cellular  $^{18}$ F-FDG uptake was dramatically reduced in LNCaP cells treated with 10  $\mu$ M of the PDTC-Cu complex (ID% 0.05), in comparison to LNCaP cells treated with 10  $\mu$ M PDTC (ID% 0.70), 10  $\mu$ M copper chloride (ID% 0.74), 5% DMSO (ID% 0.94), or no treatment (ID% 1.02) (Figure 5).

## 5. DISCUSSION

In regards to the current anti-cancer chemotherapies, most drugs used in clinics are toxic to some extent. This is mainly due to their inability to distinguish normal cells from tumor cells. In order to eliminate toxicity, it is necessary to identify some specific properties or features of cancer cells *different from* normal cells. An increased understanding of the tumor-specific differences will facilitate the development of novel anti-cancer drugs that selectively kill cancer cells with limited toxicity or no toxicity to the normal cells.

One such difference is the ubiquitin-proteasome pathway. In cancer cells, dysregulation of this system plays a critical role for tumor progression, drug resistance and altered immune surveillance (46). Previously we reported that proteasome inhibitors induce apoptosis selectively in tumor cells but not in normal cells (47). Bortezomib, the first proteasome inhibitor as a potential anticancer drug, is currently being tested in phase III clinical trials (48, 49). Another attractive tumor-specific feature is high levels of copper found in many types of human cancers including prostate, breast, colon, lung, and brain (3-11). Molecular processes of angiogenesis include: the requirement of copper, but not other trace metals, as an essential cofactor, stimulation of endothelial growth by tumor cytokine production (*i.e.*, vasoendothelial growth factor), degradation of extracellular matrix proteins by metalloproteinases, and migration of endothelial cells mediated by integrins (12-21).

It has been shown that cancer cells are more sensitive to proteasome inhibition than normal cells (27, 47, 50-52). We hypothesize that an inactive or nontoxic organic ligand could bind with elevated copper found in tumor tissues and result in a complex capable of proteasome inhibition. After the ligand binds with endogenous tumor cellular copper, the formed complex would inhibit the proteasome activity, leading to inhibition of the processes of angiogenesis in tumor tissues and induction of apoptosis in the tumor cells.

In search for new proteasome inhibitors of high potency and low toxicity, we found that several organic copper complexes possessing potent proteasome-inhibitory and apoptosis-inducing activities in tumor but not in normal cells (42). Since PDTC, an antioxidant used to treat inflammation, atherosclerosis and metal intoxication (34, 35), could form a copper complex (37), we hypothesized that PDTC-Cu complex may also possess proteasome-inhibitory activity. Indeed, this hypothesis was

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supported by the most recent study using human breast cancer cells (Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, Dou QP, submitted). In the current study, we have also demonstrated that PDTC can form a new complex with copper, and the complex can inhibit proteasomal activity and cellular proliferation and induce apoptosis in prostate cancer LNCaP cells.

Once we verified that PDTC could spontaneously bind with copper and form a new complex (Figure 1), we then tested the complex in LNCaP cells to determine whether or not the complex was a proteasome inhibitor. We examined both cellular proteasome activity (Figure 2A) and accumulation of ubiquitinated proteins (Figure 2B). We found that the cells treated with PDTC-copper mixture had significantly reduced chymotrypsin-like activity (Figure 2A) and accumulation of ubiquitinated proteins (Figure 2B), indicating that proteasome inhibition had occurred. In contrast, copper, PDTC alone, TM alone, or TM mixed with copper was incapable of inhibiting the proteasome (Figure 2A and B).

We then tested whether or not the complex was capable of inhibiting proliferation of human prostate cancer cells. The results showed that only PDTC-copper mixture could inhibit proliferation of LNCaP cells in dose-dependent manner, but not copper, PDTC alone, TM alone, or TM-copper mixture (Figure 3).

Afterward we verified the association between PDTC-copper induced inhibition of proteasomal activity and proliferation, we then determined apoptosis-inducing activities of each compound in prostate cancer cells. Our result demonstrated that LNCaP cells treated with PDTC-copper mixture underwent apoptosis, as showed by PARP cleavage (Figure 2C) and cellular (Figure 4A) and nuclear (Figure 4B) morphological changes. However, LNCaP cells treated with copper, PDTC alone, TM alone, or TM-copper mixture failed to undergo apoptosis (Figs. 2C, 4A and B).

Positron-emission tomography (PET) is a sensitive, quantitative, non-invasive molecular imaging technology, which can be used to evaluate response of tumors to anticancer drugs. We tested effects of PDTC-Cu complex on  $^{18}\text{F}$ -FDG uptake by LNCaP cells. As expected,  $^{18}\text{F}$ -FDG uptake was dramatically reduced in LNCaP cells treated with PDTC-Cu complex (Figure 5), which suggests that the therapeutic effects of PDTC-Cu complex against prostate cancer may be monitored *in vivo* with  $^{18}\text{F}$ -FDG-PET imaging. Slight inhibition of  $^{18}\text{F}$ -FDG uptake was observed in the cells treated with PDTC alone, which is probably related to anti-tumor effects of small amount of PDTC-Cu complex formed with trace amount of intracellular copper. Unexpectedly, slight reduction of  $^{18}\text{F}$ -FDG was also observed in LNCaP cells treated with 10  $\mu\text{M}$  copper chloride (Cu), which may be related to inhibition of glucose transporter activity in LNCaP cells or other unknown mechanisms. These results demonstrated that it would be feasible to evaluate anti-tumor activity of PDTC-Cu complex *in vivo* non-invasively with  $^{18}\text{F}$ -FDG-PET imaging.

The data presented here supports the novel concept of using accumulated copper in prostate cancer cells and tissues as a selection method for chemotherapy. Nontoxic organic compounds such as PDTC can spontaneously bind with tumor cellular copper and form a proteasome inhibitor and an apoptosis inducer. PDTC has been previously explored for use in other diseases and we believe our data support further investigation of this and other similar compounds in an anticopper/anticancer strategy.

## 6. ACKNOWLEDGEMENTS

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**Abbreviations:** PDTC, pyrrolidine dithiocarbamate; TM, tetrathiomolybdate; PARP, poly(ADP-ribose) polymerase; AMC, 7-amido-4-methyl-coumarin; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; [<sup>18</sup>F]FDG, radiopharmaceutical 2-[<sup>18</sup>F]fluoro-2-deoxy-D-glucose; PET, positron emission tomography.

**Key Words:** Copper; Anti-copper drugs; Chelator; Proteasome inhibitors; Drug discovery; PET

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**Figure 1.** Copper complex formation as indicated by color change. A. Chemical structure of PDTC. B. 50 mM of PDTC was mixed in a 1:1 molar ratio with CuCl<sub>2</sub>. All solutions were made in DMSO. The appearance of intensified color of mixture indicated formation of copper complex.

**Figure 2.** Inhibition of proteasome activity and induction of apoptosis in LNCaP prostate cancer cells by the PDTC-copper mixture. LNCaP prostate cancer cells were treated with 10 μM copper (Cu), PDTC (P), TM, PDTC-copper (PC), or TM-copper (TC), using DMSO (DM) as a control. Cells were collected after 24 h treatment and analyzed for proteasome inhibition and apoptosis. A. Proteasome activity as measured by release of AMCs from the substrate specific for chymotrypsin-like activity as described in Materials and Methods. Values are mean triplicates and error bars denote standard deviations. B. Western analysis was performed using anti-ubiquitin antibody for accumulation of ubiquitinated proteins as an indicator of proteasome inhibition. C. Western analysis for cleavage of PARP as an indication of apoptosis using anti-PARP antibody.

**Figure 3.** Anti-proliferative effects of the PDTC-copper mixture. LNCaP cells were treated for 24 h with the following: copper (Cu; at 30 μM), PDTC (P; 10 μM), TM (T; 30 μM), PDTC-copper (PDTC-Cu; 1, 5, 10 μM), or TM-copper (TM-Cu; 10, 20, 30 μM). After 24 h the media was removed and cells were treated with MTT solution as described in Materials and Methods. Measurement of MTT conversion by absorbance at 560 nm showed that LNCaP cells responded in a dose-dependent manner to PDTC-copper mixture but not to TM-copper mixture. (\* P<0.05; \*\* P<0.01)

**Figure 4.** Cellular and nuclear morphological changes indicate induction of apoptosis by PDTC-copper mixture in LNCaP cells. LNCaP prostate cancer cells were treated for 24 h with 10 μM copper (CuCl<sub>2</sub>), PDTC, TM, PDTC-copper (PDTC-Cu), or TM-copper (TM-Cu), using DMSO as a control. A. Cellular morphology changes were visualized by phase contrast imaging that cellular spherical and detached changes indicated apoptosis (Magnification 100x). B. Treated cells were stained with Hoescht dye for determination of apoptotic nuclei. Nuclei that were punctate or granular and bright were considered apoptotic (Magnification 100x).

**Figure 5.** Cellular <sup>18</sup>F-FDG uptake assay on LNCaP cells treated with PDTC-Cu complex. Cellular uptake of <sup>18</sup>F-FDG was dramatically reduced in LNCaP cells treated with the PDTC-Cu complex in comparison to controls. PDTC, pyrrolidine

## PDTC-copper complex as novel proteasome inhibitor and potential anti-cancer drug

dithiocarbamate; DMSO, dimethyl sulfoxide; Cu, copper chloride, NT, no treatment, Isotope Uptake ID%, percentage of inoculation dose of  $^{18}\text{F}$ -FDG.

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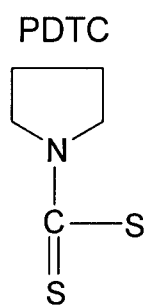
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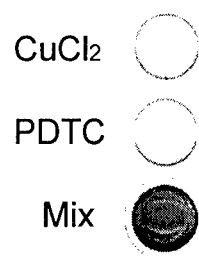


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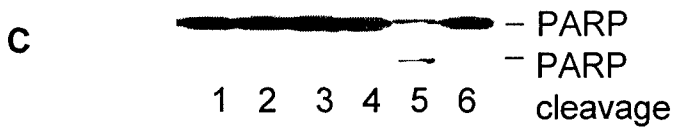
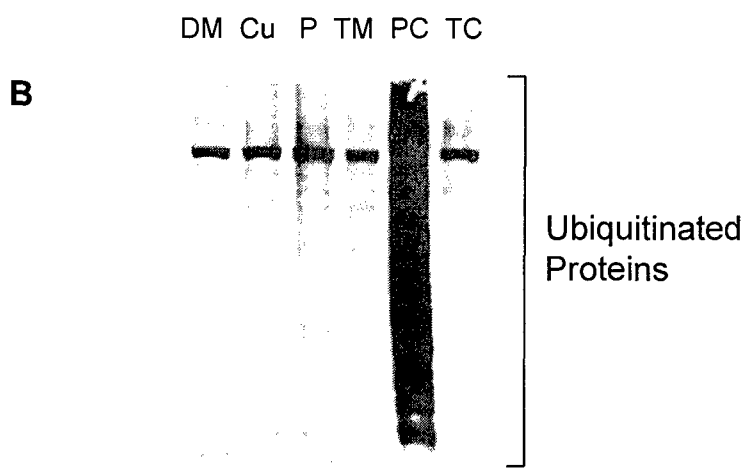
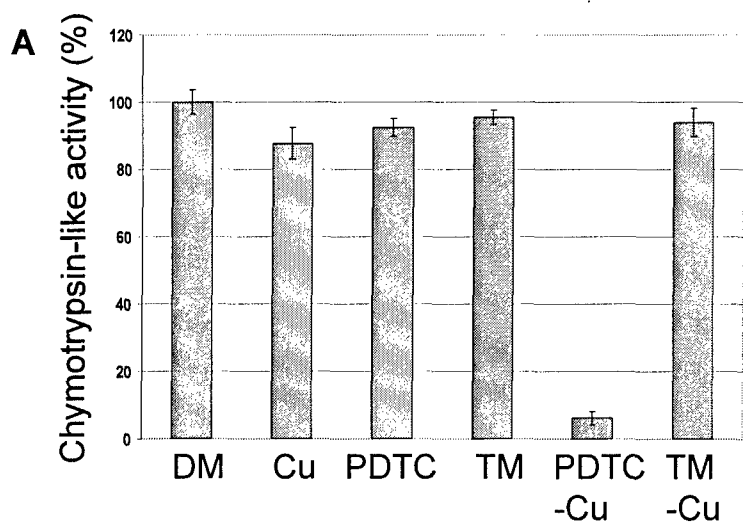


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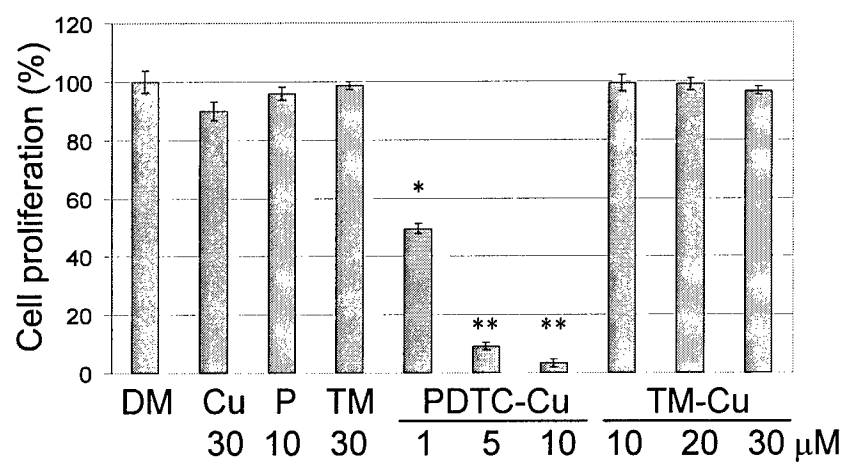


Figure 3

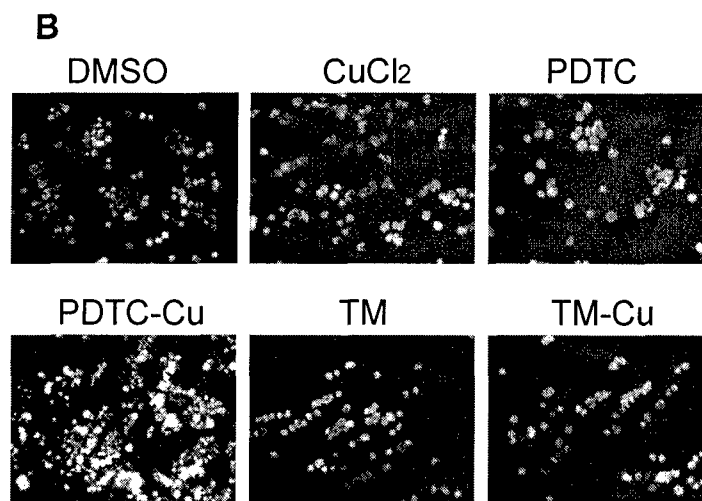
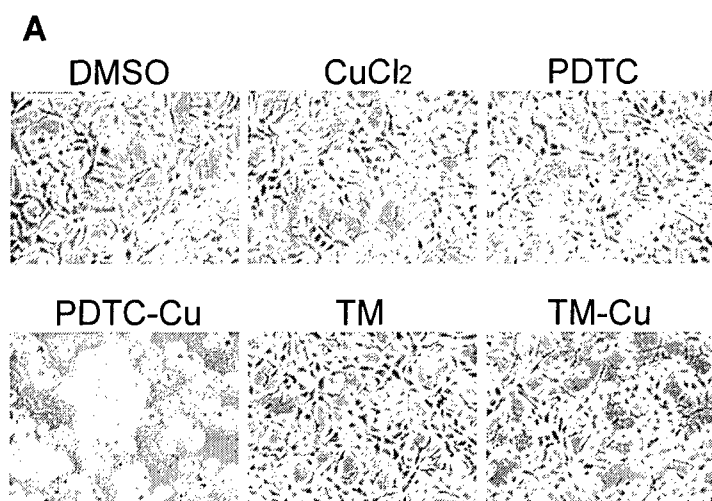


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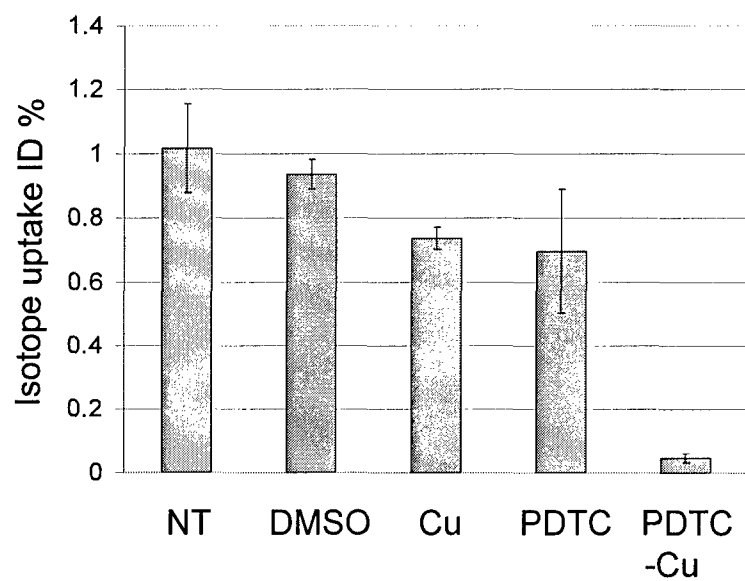


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# Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells

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## Abstract

**Introduction** A physiological feature of many tumor tissues and cells is the tendency to accumulate high concentrations of copper. While the precise role of copper in tumors is cryptic, copper, but not other trace metals, is required for angiogenesis. We have recently reported that organic copper-containing compounds, including 8-hydroxyquinoline-copper(II) and 5,7-dichloro-8-hydroxyquinoline-copper(II), comprise a novel class of proteasome inhibitors and tumor cell apoptosis inducers. In the current study, we investigate whether clioquinol (CQ), an analog of 8-hydroxyquinoline and an Alzheimer's disease drug, and pyrrolidine dithiocarbamate (PDTC), a known copper-binding compound and antioxidant, can interact with copper to form cancer-specific proteasome inhibitors and apoptosis inducers in human breast cancer cells. Tetrathiomolybdate (TM), a strong copper chelator currently being tested in clinical trials, is used as a comparison.

**Methods** Breast cell lines, normal, immortalized MCF-10A, premalignant MCF10AT1K.cl2, and malignant MCF10DCIS.com and MDA-MB-231, were treated with CQ or PDTC with or without prior interaction with copper, followed by measurement of proteasome inhibition and cell death. Inhibition of the proteasome was determined by levels of the proteasomal chymotrypsin-like activity and ubiquitinated proteins in protein extracts of the treated cells. Apoptotic cell death was measured

by morphological changes, Hoechst staining, and poly(ADP-ribose) polymerase cleavage.

**Results** When in complex with copper, both CQ and PDTC, but not TM, can inhibit the proteasome chymotrypsin-like activity, block proliferation, and induce apoptotic cell death preferentially in breast cancer cells, less in premalignant breast cells, but are non-toxic to normal/non-transformed breast cells at the concentrations tested. In contrast, CQ, PDTC, TM or copper alone had no effects on any of the cells. Breast premalignant or cancer cells that contain copper at concentrations similar to those found in patients, when treated with just CQ or PDTC alone, but not TM, undergo proteasome inhibition and apoptosis.

**Conclusion** The feature of breast cancer cells and tissues to accumulate copper can be used as a targeting method for anticancer therapy through treatment with novel compounds such as CQ and PDTC that become active proteasome inhibitors and breast cancer cell killers in the presence of copper.

## Introduction

Copper is an essential trace metal for animals. The amount of copper in an organism is tightly regulated [1,2]. Angiogenesis,

the growth of a tumor blood supply, is essential for tumor growth, invasion, and metastasis [3-6]. It has been shown that tumors, without a blood supply, do not grow larger than 1 to 2

5,7-DiCl-8-OHQ = 5,7-dichloro-8-hydroxyquinoline; 8-OHQ = 8-hydroxyquinoline; APS = advanced photon source; CQ = clioquinol; DMEM = Dulbecco's modified Eagle medium; DMSO = dimethylsulfoxide; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PARP = poly(ADP-ribose) polymerase; PBS = phosphate buffered saline; PDTC = pyrrolidine dithiocarbamate; TM = tetrathiomolybdate.

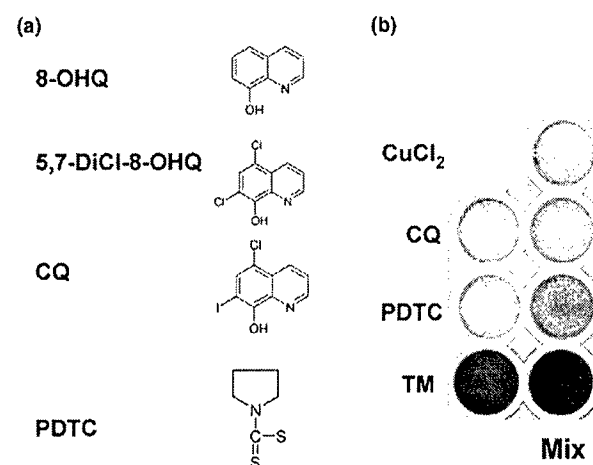
mm<sup>3</sup> [7]. Molecular processes of angiogenesis that require copper as an essential cofactor include stimulation of endothelial growth by tumor cytokine production (i.e., vasoendothelial growth factor), degradation of extracellular matrix proteins by metalloproteinases, and migration of endothelial cells mediated by integrins [3-12]. Consistently high levels of copper have been found in many types of human cancers, including breast, prostate, colon, lung, and brain [13-21]. Three anti-copper drugs have been tested in clinical trials [8,9], particularly tetrathiomolybdate (TM), a copper chelator, which was originally used for patients with Wilson's disease [8,11]. TM has been found to be effective in impairing the growth of mammary tumors in HER2/neu transgenic mice [22] and lung metastatic carcinoma in C557BL6/J mice [23]. In a phase I clinical trial with patients suffering from metastatic cancers, TM therapy achieved stable disease in five of six patients who were copper-deficient [11]. However, the disease advanced in some other patients before copper levels were sufficiently lowered [8,9,11]. These reports support the idea of copper control as an anticancer strategy.

Apoptosis, an evolutionarily conserved form of cell death, is the process by which a cell will actively commit suicide under tightly controlled circumstances [24]. Apoptosis occurs in two physiological stages, commitment and execution [25,26]. Activation of effector caspases leads to apoptotic execution probably through the proteolytic cleavage of important cellular proteins [27], such as poly(ADP-ribose) polymerase (PARP) [28], and the retinoblastoma protein [29-31]. Other hallmarks of apoptosis include cellular shrinkage, membrane blebbing, and DNA fragmentation [25-27].

The ubiquitin/proteasome system plays an important role in the degradation of cellular proteins. This proteolytic system involves two distinct steps, ubiquitination and degradation [32,33]. The eukaryotic proteasome contains at least three known activities, which are associated with its  $\beta$  subunits. These are the chymotrypsin-like (cleavage after hydrophobic residues,  $\beta 5$  subunit), trypsin-like (cleavage after basic residues,  $\beta 2$  subunit), and caspase-like or peptidyl-glutamyl peptide-hydrolyzing (cleavage after acidic residues,  $\beta 1$  subunit) activities [34,35]. Inhibition of the proteasomal chymotrypsin-like activity has been found to be associated with induction of apoptosis in tumor cells [36-41].

Most recently, we discovered that several organic-copper (but not zinc or nickel) compounds, such as bis-8-hydroxyquinoline-copper(II), potently and specifically inhibited the chymotrypsin-like activity of the proteasome *in vitro* and in human tumor cell culture [42]. Inhibition of the proteasome activity by organic copper compounds occurs very rapidly in tumor cells (15 minutes), followed by induction of apoptosis. Neither proteasome inhibition nor apoptosis were found in human normal or non-transformed cells under the same treatment. Most importantly, proteasome inhibition and apoptosis were also

Figure 1



Copper complex formation as indicated by color change. (a) Chemical structures of compounds referenced in the text: 5,7-DiCl-8-OHQ, 5,7-dichloro-8-hydroxyquinoline; 8-OHQ, 8-hydroxyquinoline; CQ, clioquinol; PDTc, pyrrolidine dithiocarbamate. (b) 50 mM of PDTc, CQ, or tetrathiomolybdate (TM) were mixed in a 1:1 molar ratio with CuCl<sub>2</sub>. All solutions were made in dimethylsulfoxide. In each case the appearance of intensified color indicated formation of a copper complex.

detected in copper-containing tumor cells treated with 8-hydroxyquinoline (8-OHQ; Fig. 1a). None of these events occurred in cells treated with either inorganic copper, ligand-treated cells that did not contain copper, or pretreatment with the closely related nickel followed by addition of the ligand [42]. We also found that 5,7-dichloro-8-hydroxyquinoline (5,7-DiCl-8-OHQ; Fig. 1a) synthesized to contain copper was a potent proteasome inhibitor and apoptosis inducer [42].

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline; CQ; Fig. 1a) belongs to the quinoline class of compounds and is structurally similar to 5,7-DiCl-8-OHQ. This class of compounds possesses an established toxicology profile with the US Pharmacopoeia [43]. During the 1950s to the 1970s, CQ was used as an antibiotic [44,45]; however, it was withdrawn due to association with subacute myelo-optic neuropathy possibly due to overdose and/or a reversible vitamin B<sub>12</sub> deficiency [44,46-48]. Recently, interest in CQ has reemerged due to studies involving its use, in combination with B<sub>12</sub>, for treatment of Alzheimer's disease [43,49,50]. Regardless of it being a controversial compound, CQ can still serve as a model compound from which analogs could be developed that exploit its copper binding potential but avoid its negative associations. CQ is a lipophilic compound that is capable of forming stable complexes with copper(II) ions [51]. In a phase II clinical trial, CQ, at a starting concentration of 3.3 mg/kg, the same order of magnitude of treatment used in mice, was found to be well-tolerated and suitable for further study [49]. Examination of CQ in animal studies has continued to further characterize its effects [52].

Dithiocarbamates are a class of metal chelating compounds. These compounds have previously been used in the treatment of bacterial and fungal infections, and have been considered for use in the treatment of AIDS [53,54]. Pyrrolidine dithiocarbamate (PDTC; Fig. 1a) is a synthetic antioxidant and inhibitor of NF- $\kappa$ B that is capable of binding copper [55,56]. PDTC and other dithiocarbamates have been found to induce apoptosis in conjunction with copper in different types of cancer cells [55,57]. Previously we found a synthetic PDTC containing copper was a potent proteasome inhibitor and apoptosis inducer [42].

Here we show that CQ and PDTC are capable of binding copper, spontaneously forming new complexes that have proteasome-inhibitory and apoptosis-inducing activities to cancer but not normal/non-transformed breast cells, and that premalignant or cancer breast cells cultured to contain elevated copper are sensitive to treatment with CQ or PDTC alone. In contrast, TM-copper or TM alone had no effects in the same experiments. We propose that targeting highly elevated copper can be tumor-specific and that formation of an active anticancer proteasome inhibitory complex between CQ or PDTC and tumor cellular copper is a novel strategy that has great potential for breast cancer therapies.

## Materials and methods

### Chemicals and reagents

CQ, PDTC, disulfiram (tetraethyl thiuram disulfide), tetramethyl thiuram disulfide, methyl propyl disulfide, allyl disulfide, isopropyl disulfide, TM, CuCl<sub>2</sub>, dimethylsulfoxide (DMSO), bisbenzimidazole Hoechst No. 33258 stain, cholera toxin, hydrocortisone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), epidermal growth factor, and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). F12 medium, DMEM, horse serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) was obtained from Calbiochem (San Diego, CA, USA). Mouse monoclonal antibody to human PARP was from Roche Applied Science (Indianapolis, IN, USA). Mouse monoclonal antibody to human ubiquitin was from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

### Cell culture and lysates preparation

MCF10A (normal-MCF10), MCF10AT1K.cl2 (pre-malignant-MCF10), and MCF10dcis.com (malignant-MCF10) cells were cultured as described previously [58]. Briefly, normal-MCF10 and pre-malignant-MCF10 cells were cultured in 1:1 F12/DMEM prepared as follows: 500 ml of media was supplemented with 5.26% (v/v) horse serum, 100 units/ml of penicillin, 100  $\mu$ g/ml of streptomycin, 52.55  $\mu$ g of cholera endotoxin, 5 mg insulin, 10 ml of 1 M NaHCO<sub>3</sub>, 10  $\mu$ g of epidermal growth factor, and 250  $\mu$ g hydrocortisone. Malignant-MCF10 cells were cultured in 1:1 F12/DMEM media supplemented with 5.26% (v/v) horse serum, 10 ml of 1 M NaHCO<sub>3</sub>, 100

units/ml of penicillin, and 100  $\mu$ g/ml of streptomycin. MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM media containing 10% (v/v) fetal bovine serum and 100 units/ml of penicillin, 100  $\mu$ g/ml of streptomycin. All cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO<sub>2</sub>. For copper enrichment experiments, pre-malignant-MCF10 or MDA-MB-231 cells were cultured in media further supplemented with 25  $\mu$ M CuCl<sub>2</sub> for 3 days to 2 weeks. Whole cell extracts were prepared as described previously [29]. Briefly, cells were harvested, washed with PBS twice, and homogenized in a lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP40 (v/v), 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°C. Afterwards, the lysates were centrifuged at 12,000 g for 30 minutes, and the supernatants were collected as whole cell extracts.

### Color change and precipitate formation reactions

CQ, PDTC, TM, and CuCl<sub>2</sub> were dissolved in DMSO to a final concentration of 50 mM. Then CuCl<sub>2</sub> was mixed with each in a 1:1 ratio and qualitatively examined for color change and precipitate formation. After mixing, solutions were heated and vortexed repeatedly until clear. For the visual studies, solutions were examined for color change and precipitation as indicators of complex formation. In cellular studies, however, stock concentrations were kept lower (10 and 20 mM) prior to dilution during mixing in order to prevent precipitation.

### Cell proliferation assay

The MTT assay was used to determine the effects of these agents on overall proliferation of cells. Cells were plated in a 96-well plate and grown to 70–80% confluency, followed by addition of each compound at an indicated concentration for 24 h. MTT (1 mg/ml) in PBS was then added to wells and incubated at 37°C for 4 h to allow for complete cleavage of the tetrazolium salt by metabolically active cells. Next, MTT was removed and 100  $\mu$ l of DMSO was added, followed by colorimetric analysis using a multilabel plate reader at 560 nm (Victor<sup>3</sup>; PerkinElmer (Wellesley, MA, USA)). Absorbance values plotted are the mean from triplicate experiments.

### Cellular and nuclear morphology analysis

A Zeiss (Thornwood, NY, USA) Axiovert 25 microscope was used for all microscopic imaging with either phase contrast for cellular morphology or fluorescence for nuclear morphology with Hoechst staining. For fluorescent nuclear morphology analysis, Hoechst stain was used as follows. Cells, either attached in plates or collected as a detached fraction, were washed once with ice cold PBS. Cells were then fixed in ethanol for 1 h and afterwards washed with ice cold PBS. Cells were stained with 50  $\mu$ M Hoechst and kept in the dark at 4°C for 30 minutes and then visualized using fluorescence microscopy. Punctate and bright staining, or granular and bright staining nuclei were considered apoptotic.

### Copper pretreatment and ligand post-treatment

To simulate the *in vivo* copper status of cancer cells, premalignant-MCF10 and MDA-MB-231 cells were cultured in media containing 25  $\mu$ M copper as done previously with prostate PC-3 cells [42]. MDA-MB-231 cells were cultured for a minimum of 48 h and premalignant-MCF10 cells were cultured for a minimum of 2 weeks. After copper enrichment culturing, cells were washed with PBS and then treated for the indicated hours using standard cell media containing TM (25  $\mu$ M), CQ (1 to 100  $\mu$ M), or PDTC (1 or 10  $\mu$ M).

### Cellular copper measurement

Premalignant-MCF10 cells were cultured for 2 weeks in culture media with or without 25  $\mu$ M  $\text{CuCl}_2$ . Cells were collected and counted to determine total cells in the sample. Samples were spun down, washed with PBS, and provided to Quantum Labs (Wixom, MI, USA) for graphite furnace analysis to determine total copper in each sample.

### Western blot analysis

Cells were treated as indicated (see Figure legends). Afterwards, cells were harvested and lysed. Cell lysates (50  $\mu$ g) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, followed by visualization using the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). Western blot analysis was performed using specific antibodies to ubiquitin and PARP as described previously [36]. Proteasome inhibition was measured as accumulation of ubiquitinated proteins and apoptosis by cleavage of PARP [36].

### Analysis of the proteasome chymotrypsin-like activity in whole cell extracts

Whole cell extracts (10  $\mu$ g) of cells treated as indicated were incubated for 60 minutes at 37°C in 100  $\mu$ l of assay buffer (50 mM Tris-HCL, pH 7.5) with 40  $\mu$ M of fluorogenic substrate for the proteasomal chymotrypsin-like activity. After incubation, production of hydrolyzed 7-amino-4-methylcoumarin (AMC) groups was measured using a Victor 3 Multilabel Counter with an excitation filter of 380 nm and an emission filter of 460 nm (PerkinElmer, Boston, MA, USA). Changes in fluorescence were calculated against non-treated controls and plotted with statistical analysis using Microsoft Excel™ software.

## Results

### CQ and PDTC spontaneously react with copper to form a new complex

In order to use endogenous elevated tumor copper as a targeting mechanism for breast cancer therapy (Fig. 2), it is necessary that the ligand under consideration be capable of reacting spontaneously with copper to form a new complex. Complex formation reactions, particularly those involving metal, can result in dramatic color changes and/or precipitate formation. To test the reactivity of CQ and PDTC with copper, 50 mM of each was added to a 50 mM solution of copper (II) chloride

Figure 2

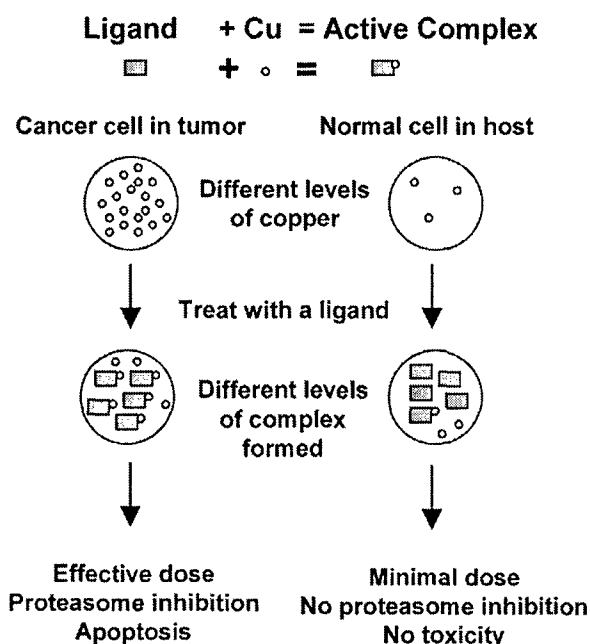


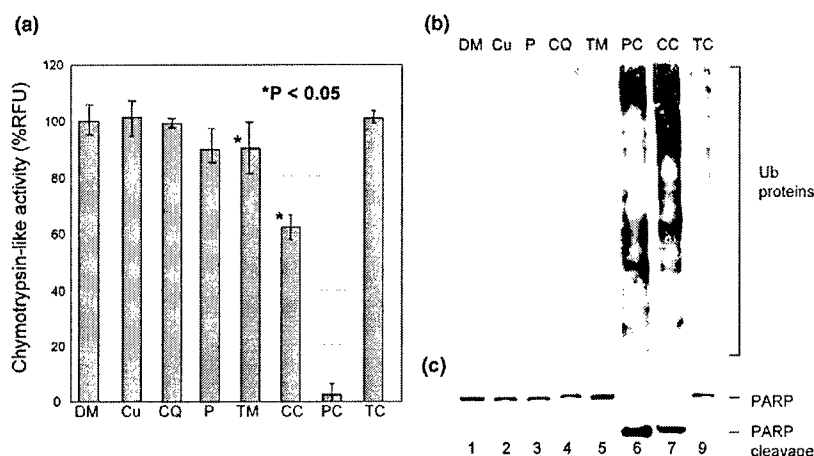
Diagram of a proposed copper-targeting therapeutic strategy. Cancer cells contain high levels of copper compared to normal cells. Upon treatment with a copper-binding ligand, a proteasome inhibiting copper complex will be formed. Only a minimal amount of complex should be formed in normal cells, therefore making them resistant to proteasome inhibition. In contrast, cancer cells may have a high dose of complex formed and are thus sensitive to proteasome inhibition, resulting in apoptosis. Copper forms the basis of the selection criteria between normal and tumor cells.

(Fig. 1). The reaction of CQ and PDTC with copper, in DMSO, results in a dramatic color change (Fig. 1), indicating a chemical reaction has occurred and a metal complex has formed. These results are consistent with previous publications showing that both CQ and PDTC are strong copper chelators [51,55]. Therefore, these ligands may be capable of combining with endogenous tumor copper and forming a reactive complex.

The CQ-copper mixture has been further examined by the advanced photon source (APS) of Argonne National Laboratories (Argonne, IL, USA). The result is consistent with formation of a new complex between CQ and copper in solution (unpublished data). Furthermore, samples of a PDTC-copper mixture will be analyzed by the APS to confirm complex formation and the resulting structure. The details of these studies will be presented in a future manuscript.

### CQ and PDTC combine with copper to form proteasome-inhibitory complexes

As both compounds can form a complex with copper, as indicated by color change (Fig. 1), we then tested whether these

**Figure 3**

Inhibition of proteasome activity and apoptosis induction in MDA-MB-231 breast cancer cells by clioquinol (CQ) + copper and pyrrolidine dithiocarbamate (PDTC) + copper. MDA-MB-231 breast cancer cells were treated with 20  $\mu$ M copper (Cu), CQ, CQ + copper (CC), tetrathiomolybdate (TM), TM + copper (TC), or 10  $\mu$ M PDTC (P), or PDTC + copper (PC), using DMSO (DM) as a control. Cells were collected after 24 h treatment and analyzed for proteasome inhibition. **(a)** Proteasome activity as measured in released fluorescence units (RFUs) by release of 7-amino-4-methylcoumarins (AMCs) from substrate specific for chymotrypsin-like activity. **(b)** Western analysis for accumulation of ubiquitinated proteins as an indicator of proteasome inhibition. Treatment with PDTC + copper (PC; 10  $\mu$ M) or CQ + copper (CC; 20  $\mu$ M) results in reduced release of AMCs and ubiquitinated protein accumulation, suggesting proteasome inhibition. **(c)** Western analysis for cleavage of poly(ADP ribose) polymerase (PARP) as an indication of apoptosis. Treatment with CQ + copper (20  $\mu$ M) or PDTC + copper (10  $\mu$ M) results in cleavage of PARP, indicating that these complexes are capable of inducing apoptosis.

complexes were capable of inhibiting the proteasome activity in intact cells. Breast cancer MDA-MB-231 cells were treated with copper, CQ, CQ-copper mixture, PDTC, or PDTC-copper mixture, using TM and TM-copper mixture as controls. After a 24 h treatment, cells were collected and the cell extracts were prepared for analysis of proteasome inhibition by the chymotrypsin-like activity assay (Fig. 3a) and accumulation of ubiquitinated proteins (Fig. 3b). We found that both CQ-copper and PDTC-copper mixtures significantly inhibited the proteasome activity in MDA-MB-231 cells, as indicated by decreased levels of the proteasomal chymotrypsin-like activity (Fig. 3a) and accumulation of ubiquitinated proteins (Fig. 3b). The PDTC-copper mixture is more potent than that of CQ-copper (Fig. 3a). Copper, CQ, or PDTC alone had no effect. Interestingly, we found that TM and the TM-copper mixture had little to no proteasome-inhibitory activity (Fig. 3), supporting the inactive complex nature of TM-copper [42]. These data support that CQ and PDTC can combine spontaneously with copper to form a proteasome-inhibitory complex.

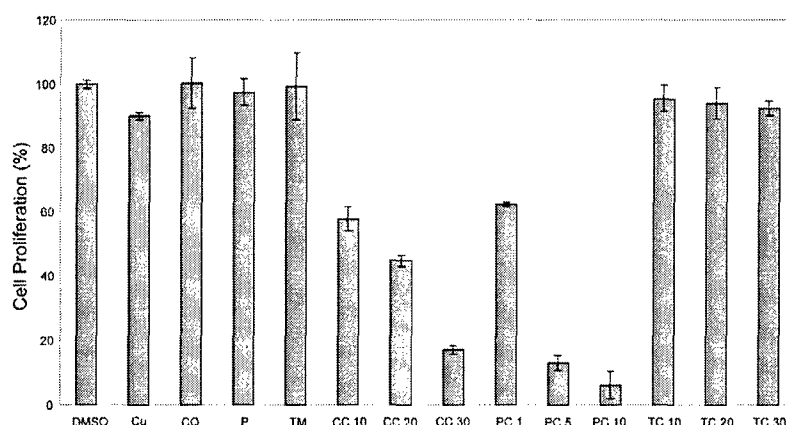
Although we have shown that copper alone can inhibit the activity of a purified proteasome [42], it is still possible that dithiocarbamates could be oxidized by copper to thiuram disulfides [59], which could be responsible for the observed proteasome inhibition. We therefore tested the effects of two thiuram disulfides and three disulfides on the proteasome activity. In the absence of copper, disulfiram (tetraethyl thiuram disulfide) and tetramethyl thiuram disulfide are incapable of inhibiting the proteasomal activity of MDA-MB-231 cell extract

at micro-molar concentrations (data not shown). In addition, none of the tested disulfides, methyl propyl disulfide, allyl disulfide, and isopropyl disulfide, could inhibit the proteasome activity under the cell-free conditions (data not shown). This result suggests that complex formation between PDTC and copper, rather than general oxidation of PDTC to thiuram disulfide, is the likely mechanism of proteasome inhibition. Furthermore, we have found and reported that production of  $H_2O_2$  does not occur in this system and that reductants do not block copper-mediated proteasome-inhibitory activity, supporting the idea that mechanisms other than oxidation are involved in proteasome inhibition [42]. This suggests that general oxidation or oxidation of dithiocarbamates is not sufficient to result in proteasome inhibition at these concentrations in these systems.

#### CQ and PDTC when mixed with copper block proliferation of breast cancer MDA-MB-231 in a dose-dependent manner

After finding that CQ-copper and PDTC-copper mixtures could inhibit proteasome activity (Fig. 3a,b), we measured the effects of each compound on breast cancer cell proliferation (Fig. 4). We found that, associated with proteasome inhibition, the CQ-copper and PDTC-copper complexes inhibited cellular proliferation in a dose-dependent manner. CQ-copper showed 40% inhibition at 10  $\mu$ M and increased to approximately 80% inhibition at 30  $\mu$ M (Fig. 4). The PDTC-copper mixture inhibited proliferation by 40% at 1  $\mu$ M and greater than 90% inhibition at 10  $\mu$ M (Fig. 4). In contrast, copper, CQ,

Figure 4



Anti-proliferative effects of clioquinol (CQ) + copper and pyrrolidine dithiocarbamate (PDTC) + copper. MDA-MB-231 cells were treated for 24 h with the following: copper (Cu; at 30  $\mu$ M); CQ (CQ; at 30  $\mu$ M); PDTC (P; 10  $\mu$ M); tetrathiomolybdate (TM; 30  $\mu$ M); CQ + copper (CC; 10, 20, 30  $\mu$ M); PDTC + copper (PC; 1, 5, 10  $\mu$ M); TM + copper (TC; 10, 20, 30  $\mu$ M); or dimethylsulfoxide (DMSO) as a control. After 24 h the media was removed and cells were treated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) solution. Measurement of MTT conversion by absorbance at 560 nm showed that MDA-MB-231 cells responded in a dose-dependent manner to CQ-copper and PDTC-copper complexes. This suggests that these organic-copper complexes act as anti-proliferative agents.

PDTC, or TM alone or TM mixed with copper had no significant effect (Fig. 4). The ranking of these compounds with respect to their ability to inhibit breast cancer cell proliferation matches well with their ability to inhibit the cellular proteasome activity (Figs. 4 versus 3a). Due to the nature of the MTT assay and the inability to separate apoptosis from growth arrest, both possible outcomes of the proteasome inhibition, IC<sub>50</sub> values of these complexes were not measured. These data suggest that CQ and PDTC can spontaneously combine with copper to form an anti-proliferative complex.

#### **CQ and PDTC combine with copper to form a product toxic to malignant-MCF10 and MDA-MB-231 and premalignant-MCF10 cells, but non-toxic to normal-MCF10 breast cells**

We found that the same CQ-copper and PDTC-copper complexes capable of proteasome inhibition (Fig. 3a, b) also demonstrated apoptosis induction, as shown by cleavage of PARP (Fig. 3c). In the absence of copper, neither CQ nor PDTC was able to induce apoptosis at these concentrations (Fig. 3c, lanes 6 and 7 versus lanes 3 and 4). TM, in the presence or absence of copper, does not induce apoptosis, further supporting TM's action as passive chelation and elimination of copper. These data support the idea that CQ and PDTC, but not TM, can combine spontaneously with copper to form a proteasome-inhibitory and apoptosis-inducing complex.

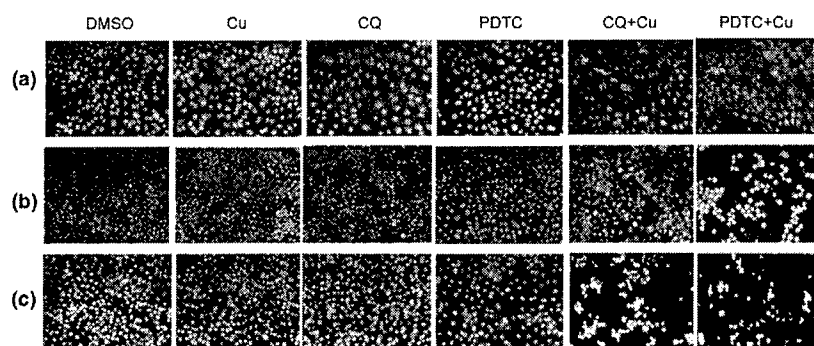
To determine whether the CQ-copper and PDTC-copper complexes have differential effects on normal and tumor breast cells, the MCF10 series of cells [58] were then treated with CQ alone, copper alone, or the product of a 1:1 mixture of each at 20  $\mu$ M (Fig. 5). The 20  $\mu$ M CQ-copper complex

induces apoptotic nuclei within 24 h for both premalignant and malignant-MCF10 cells (10% and 65%, respectively; Fig. 5b, c). The malignant-MCF10 cells fully detached, suggesting that these cells were more sensitive to the complex than the premalignant cells. However, the normal-MCF10 cells demonstrated no apoptotic nuclei after 24 h of treatment with the CQ-copper complex (<2%; Fig. 5a).

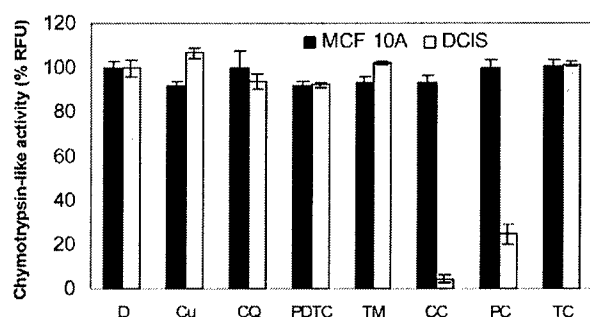
We then tested the effects of the PDTC-copper mixture. The three breast cell lines were treated with PDTC alone, copper alone, or their mixture at 5  $\mu$ M for 24 h. Again, both the premalignant- (63%; Fig. 5b) and the malignant-MCF10 (75%; Fig. 5c) cells showed a dramatic induction of apoptotic nuclei after treatment with the mixture, while the normal-MCF10 cells (<2%; Fig. 5a) showed no apoptosis induction from the mixture. As a control, neither CQ alone, PDTC alone, nor copper alone had effect on any of these cell lines (<2% in all the cases; Fig. 5). These data suggest that CQ and PDTC can spontaneously bind with copper and that the resulting complex is an apoptosis inducer to premalignant and cancerous, but not normal/non-transformed, breast cells, suggesting that such a complex if formed in a normal cell would not be toxic, but would be toxic in tumor cells.

#### **CQ and PDTC in complex with copper do not inhibit proteasome activity in normal breast MCF10A cells**

To better understand the mechanism of resistance in normal breast cells to apoptosis induction by these organic-copper complexes, we treated both normal- and malignant-MCF10 cells with CQ-copper and PDTC-copper complexes and measured changes in the proteasome activity levels. Both cell lines were treated with 20  $\mu$ M Cu, CQ, TM, CQ-copper, and

**Figure 5**

Induction of apoptosis by clioquinol (CQ) + copper and pyrrolidine dithiocarbamate (PDTC) + copper complexes in premalignant- and malignant-MCF10 cells. CQ and PDTC were mixed in a 1:1 molar ratio with  $\text{CuCl}_2$ . Normal-, premalignant, and malignant-MCF10 cells were treated with CQ alone (CQ), copper alone (Cu), or CQ + copper (CQ+Cu) at 20  $\mu\text{M}$ , or PDTC alone or PDTC + copper (PDTC+Cu) at 5  $\mu\text{M}$  for 24 h. Dimethylsulfoxide (DMSO) was used as a control. After treatment, cells were stained with Hoechst for determination of apoptotic nuclei. Nuclei that were punctate or granular and bright were considered apoptotic. (a) The normal MCF10 cell line showed no apoptosis induction from the ligand, copper, or mixture (<2% in all cases). However, both (b) the premalignant MCF10AT1K.cl2 and (c) the malignant MCF10DCIS.com cells showed a dramatic induction of apoptotic nuclei after treatment with the mixture. (b) The percentages of apoptotic nuclei in MCF10AT1K.cl2 cells were: CQ+Cu, 10%; PDTC+Cu, 63%; and others <2%. (c) The percentages of apoptotic nuclei in MCF10DCIS.com cells were: CQ+Cu, 65%; PDTC+Cu, 75%; and others <2%.

**Figure 6**

Clioquinol (CQ) + copper and pyrrolidine dithiocarbamate (PDTC) + copper fail to inhibit proteasome activity in normal MCF 10 (MCF-10A) cells. Normal MCF-10A and malignant mCF-10 (DCIS) breast cells were treated with 20  $\mu\text{M}$  of copper (Cu), CQ, CQ + copper (CC), tetrathiomolybdate (TM), TM + copper (TC), or 5  $\mu\text{M}$  PDTC or PDTC + copper (PC). Dimethylsulfoxide (D) was used as a control. After 24 h, cells were collected and lysed. Lysates were analyzed for chymotrypsin-like activity and showed as released fluorescence units (RFUs). The complexes were capable of eliminating proteasome activity in DCIS cells but not in MCF-10A cells. This strongly suggests that these metal complexes do not inhibit proteasome activity in normal breast cells.

TM-copper, or 5  $\mu\text{M}$  PDTC and PDTC-copper (Fig. 6). We found that PDTC-copper and CQ-copper both strongly inhibited proteasome activity in malignant but not in normal cells (Fig. 6). Again, TM or the TM-copper mixture had no effects on either of the cell lines (Fig. 6). These data suggest that these organic-copper complexes do not inhibit the proteasome and, therefore, do not induce apoptosis in normal breast cells, further protecting normal cells from toxicity.

#### Premalignant-MCF10 cells accumulate copper when cultured in copper-enriched conditions

A difficulty with examining the effectiveness of copper targeting in cell culture models is that cultured cancer cells seem to possess low to trace levels of copper [42]. This differs from the *in vivo* situation where cancer cells and tissues can contain micromolar concentrations of copper. In one study, the serum copper in breast cancer patients was approximately 2  $\mu\text{g}/100\text{ ml}$  (equivalent to 0.3  $\mu\text{M}$ ) [60], while another study showed that the plasma copper levels in the malignant prostate were 124  $\mu\text{g}/100\text{ ml}$  (equivalent to 19.5  $\mu\text{M}$ ) [16].

To simulate the *in vivo* situation, premalignant-MCF10 breast cells were cultured in media enriched with 25  $\mu\text{M}$   $\text{CuCl}_2$  for at least 2 weeks (see Materials and methods). Afterwards, cells were collected and subject to graphite furnace analysis to determine copper content (Table 1). The results of the analysis show that these cells can accumulate at least 16 times more copper when cultured in copper-enriched media (referred to here as copper-enriched cells) than when in a normal culture and an individual enriched cell has at least an order of magnitude more copper than a standard culture cell. Given a volume of 10 ml, this would be equivalent to 6  $\mu\text{M}$ . Previously, we pre-treated prostate cancer PC-3 cells with 100  $\mu\text{M}$   $\text{CuCl}_2$  for 48 h, which resulted in cellular copper levels being increased to 0.2  $\mu\text{M}$  [42]. These data show that, in our enrichment system, premalignant-MCF10 cells can accumulate similar copper concentrations to those found in patients.

Table 1

## Accumulation of copper by MCF10AT1K.cl2 cells

| Cell line | No. of cells in sample | Total copper ( $\mu\text{g}$ ) | Copper per cell           |
|-----------|------------------------|--------------------------------|---------------------------|
| KCL2      | 5,725,000              | ND                             | $(1.75 \times 10^{-8})^a$ |
| KCL2-Cu25 | 1,468,750              | 0.4                            | $2.72 \times 10^{-7}$     |

<sup>a</sup>This is theoretical content assuming no more than 0.1  $\mu\text{g}$  total copper, which is the minimal detection limit. Because cells grown in media without copper enrichment had no detectable copper, the number reported is the highest possible content per cell based on the minimum detection. ND, not detected.

### Copper-enriched breast pre-malignant and cancer cells are sensitive to treatment with CQ or PDTC alone

Fundamental to the strategy we are presenting is the ability of a normally non-toxic ligand to bind with endogenous tumor cellular copper (Fig. 2). Studies in various cancer cells and tissues have found that patients can have copper concentrations in the micromolar ranges in those tissues [16,60]. Similarly, when premalignant-MCF10A cells are cultured in copper they can contain micromolar concentrations of copper (Table 1). We therefore tested the effects of CQ or PDTC alone in copper-enriched breast premalignant or cancer cells.

We first treated the copper-enriched premalignant-MCF10 cells with CQ alone. CQ at 1 to 10  $\mu\text{M}$  caused apoptotic morphological changes of these copper-containing cells (Fig. 7a). Consistent with the morphology study, after 24 h treatment with CQ, these copper-enriched cells underwent extensive apoptosis, measured by the appearance of the PARP cleavage fragment (Fig. 7c). In contrast, premalignant-MCF10 cells that did not contain elevated copper were highly resistant to 25  $\mu\text{M}$  of CQ (Fig. 7c, lanes 1 and 2). Similarly, copper-enriched premalignant-MCF10 cells were also sensitive to treatment with PDTC, but not TM (data not shown; Fig. 7b, d, e).

We also found that copper-enriched breast cancer MDA-MB-231 cells adopt apoptotic morphology after post-treatment with CQ or PDTC, but not TM (Fig. 7b). In the same experiment, lysates of these cells were subjected to western analysis. Both CQ and PDTC were capable of inducing proteasome inhibition and apoptosis in copper-pretreated MDA-MB-231 cells, as measured by accumulation of ubiquitinated proteins and cleavage of PARP, respectively (Fig. 7d, e). This is dramatically different from the behavior of these compounds in the absence of copper or in non-copper enriched cells (Fig. 7d, e versus Fig. 3b, c). In contrast, TM neither inhibited the proteasome activity nor induced apoptosis in these copper-enriched cells (Fig. 7b, d, e). These data support the idea that CQ and PDTC can spontaneously bind with copper in copper-enriched breast cancer cells and form an apoptosis-inducing complex and that cells containing trace or undetectable amounts of copper are resistant to this effect. It is possible, therefore, that CQ and PDTC act as apoptosis inducers through proteasome inhibition in a copper-dependent manner

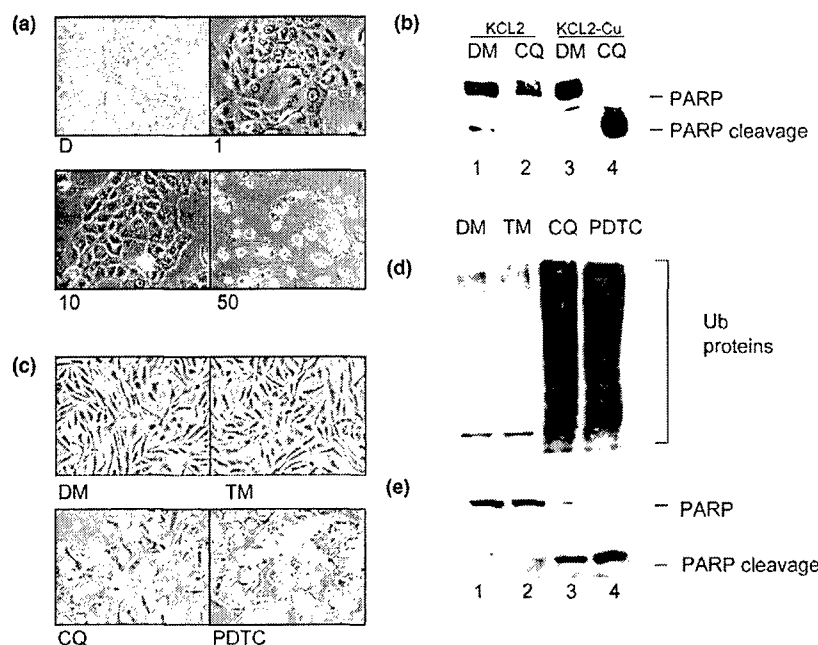
and can do so in cancer cells that contain copper in concentrations similar to those found in patients' tissues and serum.

### Discussion

A difficulty facing most cancer chemotherapy is the inability to discriminate between normal and malignant cells. Anti-angiogenesis and proteasome inhibition may be effective approaches to cancer therapy due to the dependence of cancer on these activities [9,37,61]. Unique among the trace metals, copper is required for angiogenesis [8-11]. Furthermore, it is well documented that cancer cells and tissues accumulate high concentrations of copper [13,18,21,62-64]. We previously reported that certain types of organic-copper complexes are capable of proteasome inhibition that is not a result of oxidative effects [42]. Therefore, the capability of organic copper to inhibit the proteasome, the necessity of copper for angiogenesis, and the accumulation of copper by cancer cells and tissues allows for a novel therapeutic strategy focusing on elevated copper as a selection mechanism against cancer cells and tissues (Fig. 2).

Our previous study [42] Additional file: 1 only briefly looked at an isolated system and examined the phenomenon of organic ligands binding to copper to form proteasome inhibitors and apoptosis inducers. The current study confirms and significantly expands our original findings. Specifically, this study examines a complete breast cancer system, including normal, premalignant and malignant cells. Furthermore, this study examines compounds that have clinical relevance and expands the copper enrichment studies. Several different approaches have been used in the analysis.

CQ and PDTC are two copper-binding compounds [51,53]. CQ has been investigated for use in Alzheimer's disease in regards to its ability to bind to copper found in amyloid plaques [43,47-50]. PDTC is a synthetic copper-binding antioxidant that has been studied for use in AIDS [53,54]. Previously, we have seen that 5,7-DiCl-8-OHQ (an analog of CQ) and PDTC when in complex with copper possessed strong proteasome-inhibitory and apoptosis-inducing abilities [42]. We report here the ability of CQ and PDTC to spontaneously react with copper, and inhibit the proteasome, which is followed by apoptosis, in breast cancer but not normal cells.

**Figure 7**

Induction of apoptosis in premalignant-MCF10 breast cells and proteasome inhibition and apoptosis induction in breast cancer MDA-MB-231 cells cultured to contain elevated copper and post-treated with clioquinol (CQ) and pyrrolidine dithiocarbamate (PDTC). **(a,c)** Premalignant-MCF10 (KCL2) cells were cultured in media containing 25  $\mu$ M copper for two weeks. **(b,d,e)** MDA-MB-231 cells were cultured in 25  $\mu$ M copper for 48 h. After culturing, cells were washed with PBS and treated with media containing: **(a)** 1, 10, or 50  $\mu$ M CQ; **(b-e)** 25  $\mu$ M CQ; 25  $\mu$ M tetrathiomolybdate (TM); or **(b,d,e)** 10  $\mu$ M PDTC. An equivalent volume of DMSO (DM) was used as control. KCL2 and MDA-MB-231 cells were examined for **(a,b)** apoptotic morphology and **(c,e)** PARP cleavage. **(d)** MDA-MB-231 cells were also examined by western blot for accumulation of ubiquitinated proteins. **(a,c)** KCL2 cells containing clinically relevant levels of copper were sensitive to treatment by CQ alone, which induced apoptosis. **(c)** KCL2 cells cultured under standard conditions were resistant to treatment by 25  $\mu$ M CQ. Similarly, MDA-MB-231 cells cultured to contain elevated copper were sensitive to CQ or PDTC and underwent proteasome inhibition as measured by accumulation of **(d)** ubiquitinated (Ub) proteins and apoptosis as evidenced by morphology and **(b,e)** poly (ADP ribose) polymerase (PARP) cleavage. These data suggest that KCL2 and MDA-MB-231 cells cultured to contain clinically relevant levels of copper are sensitive to treatment with CQ or PDTC alone, but not TM.

Our strategy revolves around the idea that a normally inactive or nontoxic organic ligand could bind with copper found in tumor tissues, resulting in a complex capable of proteasome inhibition. It has been shown that cancer cells are more sensitive to proteasome inhibition than normal cells [37,61,65-67]. To that end, we first verified that these two ligands directly interact with copper and form a new metal complex as indicated by dramatic color change (Fig. 1).

Once we verified that these two compounds spontaneously bind with copper and form a new complex we tested these complexes in MDA-MB-231 breast cancer cells to determine whether or not these complexes were proteasome inhibitors. We examined both cellular proteasome activity (Fig. 3a) and accumulation of ubiquitinated proteins (Fig. 3b). We found that treatment with ligand-copper mixtures significantly reduced chymotrypsin-like activity (Fig. 3a) and resulted in accumulation of ubiquitinated proteins (Fig. 3b), indicating proteasome inhibition had occurred. In contrast, ligand alone,

copper alone, or TM mixed with copper did not inhibit the proteasome (Fig. 3). Previously we found that copper-mediated accumulation of ubiquitinated proteins is transient [42]. Therefore, the ubiquitinated protein pattern induced by CQ-copper and PDTC-copper shown in Fig. 3b should be considered transient and relevant only to the time point under consideration.

After determining that these organic-copper complexes could inhibit proliferation in MDA-MB-231 cells (Fig. 4), we examined the apoptosis-inducing abilities of the complexes. The organic-copper complexes were capable of inducing apoptosis strongly in malignant-MCF10 and MDA-MB-231, moderately in premalignant-MCF10, and did not induce apoptosis in normal-MCF10 cells (Figs. 5 and 3c). As a control, CQ, PDTC, TM or copper alone, or TM mixed with copper, were incapable of inducing apoptosis (Figs 5 and 3c). Therefore, the primary concerns of the strategy presented were fulfilled: the compound alone shows no toxic effects, the

compound when mixed with copper becomes toxic, and the toxicity is limited to cancer cells and is associated with proteasome inhibition.

As these complexes have minimal to no effect on our normal cell line and seem to inhibit tumor cellular proteasome activity, we surmise that their toxicity to cancer cells stems from their proteasome inhibitory activity, to which normal cells are resistant. This was verified by examining proteasome activity in breast normal MCF10 cells compared to malignant-MCF10 cells (Fig. 6). We found that normal-MCF 10 cells did not suffer proteasome inhibition when treated with CQ or PDTC in complex with copper, although the concentrations tested inhibited the proteasome activity in malignant-MCF10 cells (Fig. 6), further supporting the argument that these complexes may be non-toxic to normal cells but are toxic to cancer cells through the mechanism of tumor-specific proteasome inhibition.

In a living organism, cancer cells and tissues accumulate high concentrations of copper [13,18,21,62-64]. To simulate this *in vivo* situation, premalignant-MCF10 and cancer MDA-MB-231 breast cells were cultured in copper-enriched media for either 2 weeks (pre malignant-MCF10) or 72 h (MDA-MB-231). Afterwards, premalignant-MCF10 cells were collected and subjected to graphite furnace analysis to determine copper content. We found that premalignant-MCF10 cells were capable of accumulating concentrations of copper similar to those found in patient tissues (Table 1) and contained at least 16 fold more copper than cells cultured in standard media.

Once we had established cultures of premalignant-MCF10 cells enriched with copper, we then treated those cells with CQ or PDTC alone. Both CQ (25  $\mu$ M) and PDTC (1  $\mu$ M) induced apoptosis after treatment (Fig. 7a, c; data not shown). In cells cultured in enriched copper conditions, the compounds at similar concentrations had no effect (Figs 5 and 3c). Similarly, the breast cancer cell line MDA-MB-231, when cultured in elevated copper, is sensitized to apoptosis induction associated with proteasome inhibition with CQ and PDTC alone (Fig. 7b, d, e). This further supports our proposal that the compounds studied can use the increased copper load in cancer cells to form a proteasome inhibitor and an apoptosis inducer, whereas in the absence of this copper load these compounds have minimal to no effect at these concentrations.

The data presented here supports the novel concept of using accumulated copper in breast cancer cells and tissues as a selection method for chemotherapy. Non-toxic organic compounds such as CQ or PDTC can spontaneously bind with copper and form a proteasome inhibitor and an apoptosis inducer that has no effect on normal cells. Cancer cells, containing elevated copper, are sensitive to treatment with these organic compounds. Normal cells, containing trace

amounts of copper, are resistant to these effects (Fig. 2). Both CQ and PDTC have been previously explored for use in other diseases and we believe these data support further investigation of these and other similar compounds in an anticopper/anticancer strategy. Most recently, another group also reported the anticancer activity of CQ [52]. Our data presented here may have provided a mechanistic interpretation for their findings.

The exact mechanisms of the copper-ligand combination are unclear at this time. However, it is apparent that cells cultured to contain elevated copper become sensitive to treatment with the ligands alone. We have future plans to work with the APS at Argonne National Laboratory to determine the final state of the ligand-copper complexes in cells. This should assist in further understanding why copper-enriched cells are sensitive to treatment with ligands that bind copper to form proteasome-inhibiting complexes. It should be noted that the system we have presented in this report is limited by looking at immortalized breast cancer cells rather than true normal primary cell lines. Future experiments should examine not only normal primary lymphocytes in culture but also animal studies to further confirm the effect on normal cells and tissues. Additional studies on cells that naturally contain elevated copper such as kidney, liver, and hematopoietic cells are also warranted.

## Conclusion

A unique feature of cancer cells is to accumulate high concentrations of copper [13,18,21,62-64]. We believe a potential strategy for cancer chemotherapy could involve the use of organic ligands that act as copper sensors and bind with the elevated copper in cancer cells and tissues. These complexes would act as proteasome inhibitors and apoptosis inducers to tumor cells. Because normal cells contain only trace amounts of copper, the organic ligands should form far fewer complexes with copper in them, thus exposing the normal cells to a minimal dose and reducing toxicity. We propose that treatment with copper-binding compounds such as CQ and PDTC will result in these compounds behaving as tumor 'sensors' using copper as a selection criterion. Therefore, this approach may convert the proangiogenic co-factor copper into a cancer-specific killing agent.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

KD and DC contributed equally to this manuscript. KD participated in the design of the study, data collection and interpretation, and manuscript preparation. DC participated in study design, data collection and interpretation, and manuscript preparation. SO and QCC participated in data collection. FRM participated in the design of the study, data interpretation, and manuscript preparation. QPD was responsible for the design of the study, data interpretation, and man-

uscript preparation as well as supervision of this project. All authors have read and approved the final manuscript.

## Additional files

The following Additional files are available online:

### Additional File 1

A PDF file of reference [42].

See <http://www.biomedcentral.com/content/supplementary/bcr1322-S1.pdf>

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# Disulfiram, a Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts via Inhibition of the Proteasome Activity

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## Abstract

**Disulfiram (DSF), a member of the dithiocarbamate family capable of binding copper and an inhibitor of aldehyde dehydrogenase, is currently being used clinically for the treatment of alcoholism. Recent studies have suggested that DSF may have antitumor and chemosensitizing activities, although the detailed molecular mechanisms remain unclear. Copper has been shown to be essential for tumor angiogenesis processes. Consistently, high serum and tissue levels of copper have been found in many types of human cancers, including breast, prostate, and brain, supporting the idea that copper could be used as a potential tumor-specific target. Here we report that the DSF-copper complex potently inhibits the proteasomal activity in cultured breast cancer MDA-MB-231 and MCF10DCIS.com cells, but not normal, immortalized MCF-10A cells, before induction of apoptotic cancer cell death. Furthermore, MDA-MB-231 cells that contain copper at concentrations similar to those found in patients, when treated with just DSF, undergo proteasome inhibition and apoptosis. In addition, when administered to mice bearing MDA-MB-231 tumor xenografts, DSF significantly inhibited the tumor growth (by 74%), associated with *in vivo* proteasome inhibition (as measured by decreased levels of tumor tissue proteasome activity and accumulation of ubiquitinated proteins and natural proteasome substrates p27 and Bax) and apoptosis induction (as shown by caspase activation and apoptotic nuclei formation). Our study shows that inhibition of the proteasomal activity can be achieved by targeting tumor cellular copper with the nontoxic compound DSF, resulting in selective apoptosis induction within tumor cells. (Cancer Res 2006; 66(21): 1-9)**

## Introduction

Copper is an essential trace metal for animals. The amount of copper in an organism is very tightly regulated (1-3). Many critical enzymes and transcription factors require copper for their activities (1-3). Angiogenesis, the growth of a tumor blood supply, is essential for tumor growth, invasion, and metastasis (4, 5). Tumors without an additional blood supply do not grow larger than

1 to 2 mm<sup>3</sup> (6). Molecular processes of angiogenesis include stimulation of endothelial growth by tumor cytokine production (i.e., vascular endothelial growth factor) and the requirement of copper (but not other trace metals) as an essential cofactor (3-6). Consistently, high serum or tissue levels of copper were found in many types of human cancers including breast, prostate, colon, lung, and brain (7-13), although the involved mechanism remains unknown. The above information suggests that copper could be used as a novel selective target for cancer therapies. Along this line, therapies with the strong copper chelator tetrathiomolybdate (TM) are well tolerated and copper elimination can stabilize advanced kidney cancer (14, 15), showing the clinical feasibility of this approach.

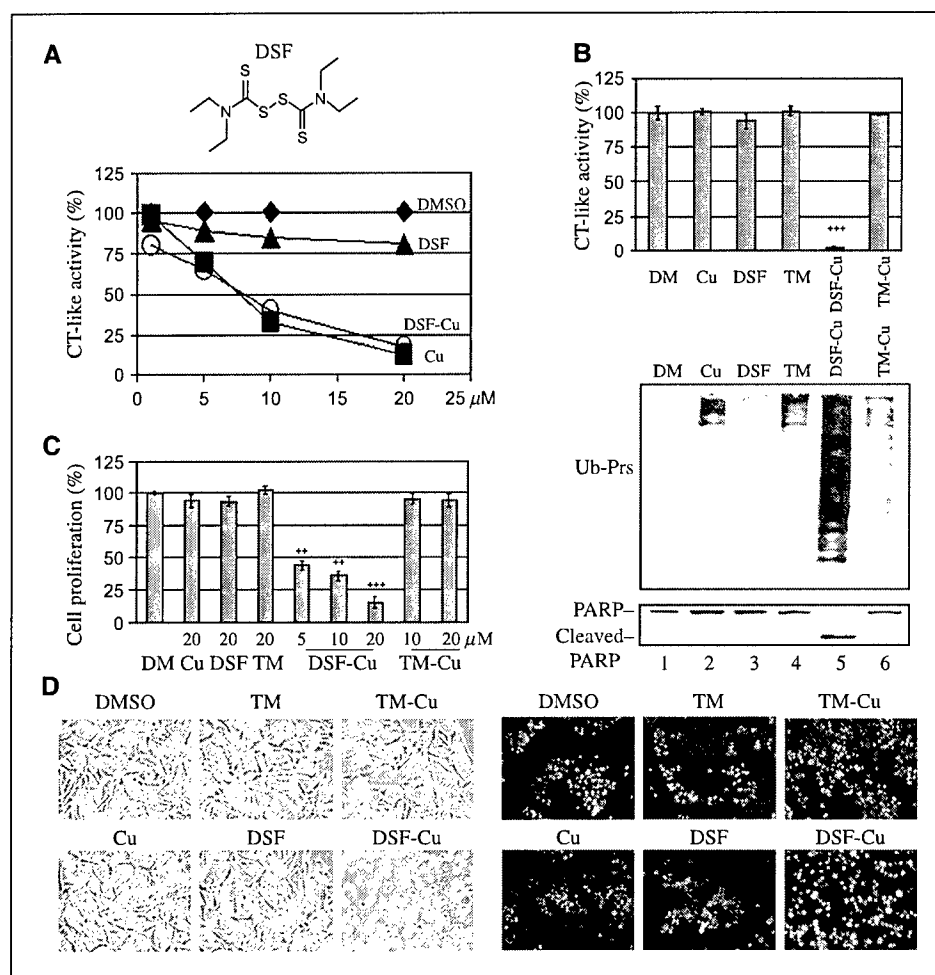
The proteasome-mediated degradation pathway has been considered as an important target for anticancer drug development. The proteasome inhibitor bortezomib (Velcade, PS-341) has been used in clinical trials and its antitumor activity has been reported in a variety of tumor models (16-18). The ubiquitin/proteasome-mediated proteolytic system controls the turnover of critical regulatory proteins involved in several cellular processes such as cell cycle and apoptosis (19-21). This degradation pathway includes two distinct steps: ubiquitination and degradation. Ubiquitination is the step after which the target protein can be selectively recognized from other proteins by the 26S-proteasome complex. Degradation of proteins occurs on the large 26S-proteasome complex in an ATP-dependent manner (19-21). The eukaryotic proteasome contains at least three known catalytic activities: chymotrypsin-like, trypsin-like, and caspase-like or peptidyl-glutamyl peptide-hydrolyzing-like activities (22). Our laboratory and others have reported that inhibition of the proteasomal chymotrypsin-like activity is associated with induction of apoptosis in tumor cells (23-25).

Disulfiram (DSF; Fig. 1A) is a member of the dithiocarbamate family comprising a broad class of molecules possessing an R<sub>1</sub>R<sub>2</sub>NC(S)SR<sub>3</sub> functional group, which gives them the ability to complex metals and react with sulfhydryl groups (26-28). DSF, an irreversible inhibitor of aldehyde dehydrogenase, is one of the two drugs approved by the Food and Drug Administration (FDA) for treatment of alcoholism (28). Clinical trials have shown the efficacy of DSF with no toxicity (28). Several studies have shown that DSF and its metabolites can potentiate the effect of some anticancer drugs (29, 30). However, the precise mechanisms are still unknown.

We have reported that certain classes of copper-containing compounds act as potent proteasome inhibitors (3, 25). Because DSF can also bind copper (31, 32), we hypothesized that the DSF-copper complex is a proteasome inhibitor. We report here that, indeed, DSF is capable of binding copper and forming a new complex, which has proteasome inhibitory and apoptosis-inducing activities when tested in cultured human breast cancer, but not

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**Figure 1.** The effects of DSF-copper (DSF-Cu) complex on purified 20S proteasome and breast cancer cellular proteasome. **A**, inhibition of the chymotrypsin (CT)-like activity of purified rabbit 20S proteasome by  $\text{CuCl}_2$  (Cu) and DSF-copper. *Left*, chemical structure of DSF. To determine whether DSF-copper can inhibit the proteasome activity directly, purified 20S rabbit proteasome was incubated with the peptide substrate for the proteasomal chymotrypsin-like activity in the presence of  $\text{CuCl}_2$ , DSF, and DSF-copper at indicated concentrations, as described in Materials and Methods. **B**, DSF-copper inhibits the proteasome activity and induces PARP cleavage in breast cancer cells. MDA-MB-231 cells were treated with 20  $\mu\text{mol/L}$  of copper (Cu), DSF, TM, DSF-copper complex, or TM-copper complex (TM-Cu) for 24 hours, followed by preparation of cell extracts for the proteasomal activity and Western blot assays. DMSO (DM) was used as vehicle control. *Top*, proteasomal chymotrypsin-like activity in cell extracts. **C**, MTT assay. MDA-MB-231 cells were treated with 20  $\mu\text{mol/L}$  of copper, DSF, or TM, or 5 to 20  $\mu\text{mol/L}$  of DSF-copper, or 10 to 20  $\mu\text{mol/L}$  of TM-copper for 24 hours, followed by MTT assay as described in Materials and Methods. **D**, DSF-copper induces apoptosis in breast cancer cells. As described in (B), treated MDA-MB-231 cells were used for determination of cellular and nuclear apoptotic changes. *Left*, cellular morphologic changes (spherical and detached cells). *Right*, apoptotic nuclear changes were shown by Hoechst 33258 staining (punctuated, granular, and brighter nuclei).

normal/immortalized, cells. In addition, after pretreatment with copper chloride to increase cellular copper concentration, breast cancer MDA-MB-231 cells were sensitive to DSF-induced proteasome inhibition and apoptosis induction. Furthermore, when administered to mice bearing human breast cancer MDA-MB-231 xenografts, DSF potently inhibited tumor growth, associated with *in vivo* proteasome inhibition and apoptotic cell death. Our study further reinforces the idea that inhibition of the proteasome activity can be achieved by targeting tumor cellular copper and suggests the potential use of DSF as a novel anticancer drug.

## Materials and Methods

**Materials.** DSF,  $\text{CuCl}_2$ , ammonium tetrathiomolybdate (TM), Hoechst 33258, and cremophore were purchased from Sigma-Aldrich (St. Louis, MO). Purified rabbit 20S proteasome, fluorogenic peptide substrate Suc-LLVY-AMC, and Ac-DEVD-AMC were obtained from Calbiochem, Inc. (San Diego,

CA). Peptide substrate Z-GGL-AMC was from Biomol International LP (Plymouth Meeting, PA). Apoptag Peroxidase In Situ Apoptosis Detection Kit was from Chemicon International, Inc. (Temecula, CA).

**Cell cultures and whole-cell extract preparation.** MDA-MB-231 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM/F-12 supplemented with 10% fetal bovine serum. MCF10A (normal, derived from benign human breast tissue) and MCF10DCIS.com (human malignant breast cells) cells were obtained and cultured as previously described (33). All cells were maintained at 37°C and 5%  $\text{CO}_2$ . A whole-cell extract was prepared as previously described (23, 25).

**Inhibition of purified 20S proteasome activity by copper chloride and the DSF-copper mixture.** The chymotrypsin-like activity of purified 20S proteasome was measured as previously described (19). Briefly, 17.5 ng of purified 20S proteasome were incubated in 100  $\mu\text{L}$  of assay buffer (50 mmol/L Tris-HCl, pH 7.5) with or without different concentrations of copper chloride, DSF, or the DSF-copper mixture and 10  $\mu\text{mol/L}$  fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like

activity) for 2 hours at 37°C. After incubation, production of hydrolyzed AMC groups was measured with a Wallac Victor3 multilabel counter with an excitation filter of 365 nm and an emission filter of 460 nm.

**Proteasomal chymotrypsin-like and caspase-3 activity assays in cell extracts.** Whole-cell extracts (10 µg) of cells treated as indicated or tumor tissue extracts (10 µg) from human breast tumor xenograft were incubated for 1 hour at 37°C in 100 µL of assay buffer (50 mmol/L Tris-HCl, pH 7.5) with 20 µmol/L fluorogenic substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity in cell extracts) or Z-GGL-AMC (for specific chymotrypsin-like activity in tumor tissues) or Ac-DEVD-AMC (for caspase-3 in tumor tissues) as previously described (34).

**Cell proliferation assay.** MDA-MB-231 cells were seeded in triplicate in a 96-well plate and grown until 70% to 80% confluence, followed by treatment with indicated agents for 24 hours. After that, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done as previously described (34).

**Cellular and nuclear morphologic analysis.** A Zeiss Axiovert 25 microscope was used for all microscopic imaging with either phase contrast for cellular morphology or fluorescence for nuclear morphology after Hoechst 33258 staining as previously described (34). Punctuated, granular, and brightly stained nuclei were considered apoptotic.

**Western blot analysis.** The cell or tissue extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was done using specific antibodies against ubiquitin, p27, Bax, or actin (Santa Cruz Biotechnology Inc, Santa Cruz, CA) or poly(ADP-ribose) polymerase (PARP; Biomol International), followed by visualization with the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

**Human breast tumor xenograft experiments.** Five-week-old female athymic nude mice were purchased from Taconic Research Animal Services (Hudson, NY) and housed under pathogen-free conditions according to Wayne State University animal care guidelines. The protocols of animal experiments were reviewed and approved by Institutional Laboratory Animal Care and Use Committee of Wayne State University. MDA-MB-231 cells ( $5 \times 10^6$ ) were injected s.c. at one flank of the mice. Tumor size was measured every other day. Tumor volume ( $V$ ) was determined by the equation:  $V = (L \times W^2) \times 0.5$ , where  $L$  is the length and  $W$  is the width of the tumor. When xenografts reached volumes of  $\sim 200 \text{ mm}^3$ , the mice bearing tumors were randomly assigned to control or DSF groups ( $n = 10$ ), and administered daily using either solvent control (PBS/cremophore/DMSO/ethanol, 7.5:1.5:0.5:0.5) or 50 mg/kg/d DSF. When the control tumors reached  $\sim 1,500 \text{ mm}^3$  (on day 29), the experiment was terminated and the mice were sacrificed. The tumors were removed and photographed and the tumor tissues were then used for multiple assays to measure proteasome inhibition and apoptotic cell death.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.** Tumor tissues were paraffin embedded and stained according to the instruction of the manufacturer. Briefly, after deparaffinization and hydration, the tissue was incubated with Working Strength TdT Enzyme, Working Strength Stop/Wash Buffer, conjugated with anti-digoxigenin, and then stained with peroxidase substrate. Finally, the tissue was mounted under a glass coverslip in Permount and viewed under a microscope.

**Immunohistochemistry.** Tumor tissues were paraffin embedded. After deparaffinization and hydration, the slide was blocked its endogenous peroxidase by 3% hydrogen peroxide, incubated with primary antibody p27 (1:20; Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), then with biotinylated secondary antibody, antimouse immunoglobulin G (H + L), followed by incubation in ABC reagent (Avidin and Biotinylated horseradish peroxidase Complex, DAKO Labs, Cambridgeshire, United Kingdom). Finally, the slide was mounted with 3,3'-diaminobenzidine and visualized under a microscope.

**H&E staining assay.** Paraffin-embedded sample slides were deparaffinized and hydrated, and then stained with hematoxylin for 1 minute. After rinsing, the slides were then stained with eosin for 1 minute, followed by more rinse, and coverslips were mounted onto slides with Permount.

**Statistical analysis.** Statistical analysis was done with Microsoft Excel software. Student's  $t$  test for independent analysis was applied to evaluate differences between treatment and control.

## Results

**The DSF-copper complex inhibits the chymotrypsin-like activity of purified 20S proteasome and 26S proteasome in breast cancer cells.** It has been shown that DSF is able to bind copper (31, 32). Indeed, when a solution of DSF was mixed with a solution of  $\text{CuCl}_2$  at 1:1 ratio, dramatic color change was observed, from light blue to dark green (data not shown). This indicates that a chemical reaction has occurred that involves formation of a DSF-copper complex.

We have previously reported that certain copper complexes act as proteasome inhibitors in cancer cells (3, 25, 34). To examine whether the complex of DSF-copper is capable of inhibiting the proteasome activity, we incubated  $\text{CuCl}_2$ , DSF alone, or the DSF-copper mixture at various concentrations with a purified rabbit 20S proteasome. The results showed that both  $\text{CuCl}_2$  and DSF-copper could inhibit the chymotrypsin-like activity of the purified 20S proteasome with an  $\text{IC}_{50}$  value of  $\sim 7.5 \text{ µmol/L}$  for both (Fig. 1A). In a sharp contrast, DSF alone at even 20 µmol/L had little effect (Fig. 1A). This is consistent with our hypothesis that DSF is able to carry the copper ion into tumor cells and prevent copper from interacting with many nonspecific proteins, and that it is the copper ion that is responsible for inhibiting the proteasome molecule (see below).

To further evaluate this hypothesis, human breast cancer MDA-MB-231 cells were treated with 20 µmol/L copper alone, DSF alone, the DSF-copper complex, TM, or the TM-copper complex (as a comparison). Cells treated with DMSO were used as control. After 24-hour treatment, the cells were collected and lysates were prepared for measurement of proteasome inhibition by decreased levels of the proteasomal chymotrypsin-like activity and accumulation of ubiquitinated proteins. The proteasomal chymotrypsin-like activity was decreased by >95% in the breast cancer cells treated with the DSF-copper complex, compared with the vehicle control-treated cells (Fig. 1B, top). In contrast, neither copper nor DSF alone has such potent effect (Fig. 1B, top). As previously observed (34), neither TM nor the TM-copper complex can inhibit the proteasomal chymotrypsin-like activity (Fig. 1B, top). Consistent with the inhibition of the proteasomal chymotrypsin-like activity, significantly increased levels of polyubiquitinated proteins were detected in the lysates prepared from the cells treated with the DSF-copper complex, compared with the control or DSF alone-treated cells (Fig. 1B, bottom). Copper alone, TM alone, or the TM-copper complex had some effect, but much less than that of the DSF-copper complex (Fig. 1B, bottom). Collectively, these results show that the DSF-copper complex potentially inhibits the proteasome activity in intact breast cancer cells.

**The growth-inhibitory and apoptosis-inducing effects of the DSF-copper complex in breast cancer cells are associated with inhibition of cellular proteasomal chymotrypsin-like activity.** It has been shown that inhibition of the proteasomal chymotrypsin-like activity is associated with induction of tumor cell growth arrest and/or apoptosis (23–25). To determine whether proteasome inhibition by the DSF-copper complex (Fig. 1B) causes suppression of cell proliferation, MDA-MB-231 cells were treated for 24 hours with copper, DSF, DSF-copper complex, TM, the TM-copper complex, or equal volume of the vehicle DMSO. We found that the DSF-copper complex inhibited proliferation of MDA-MB-231

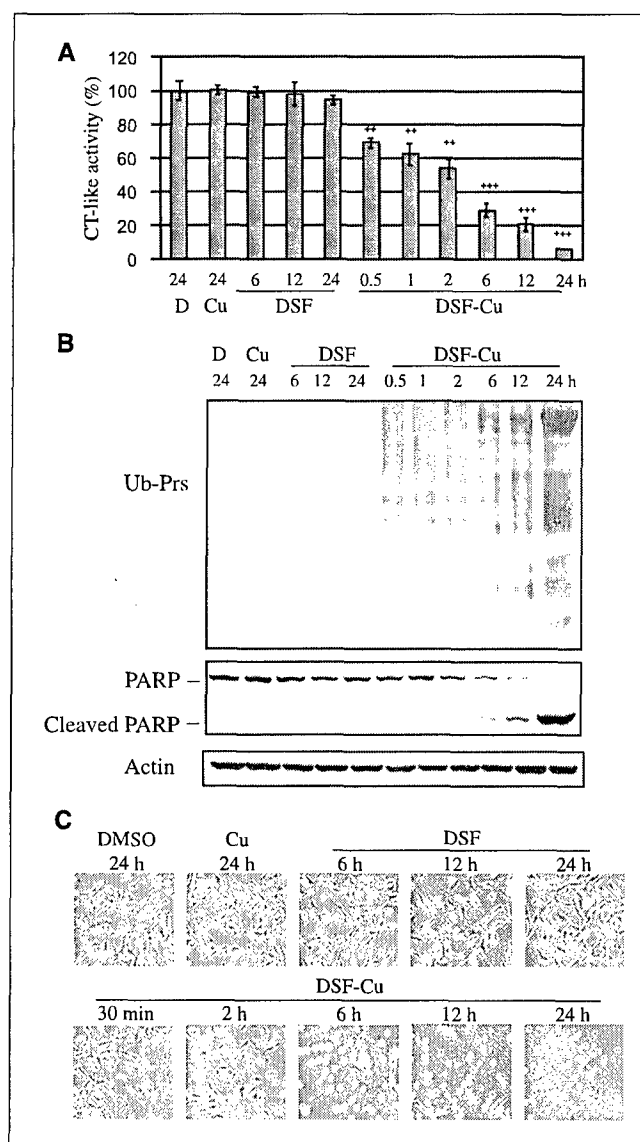
cells in a concentration-dependent manner, by 55%, 65%, and 85%, respectively, when used at 5, 10 and 20  $\mu\text{mol/L}$  (Fig. 1C). In contrast, all the other treatments at up to at 20  $\mu\text{mol/L}$  had very little inhibitory effect on MDA-MB-231 cell proliferation (Fig. 1C).

To determine whether inhibition of growth is due to induction of apoptotic cell death, aliquots of the breast cancer MDA-MB-231 cells after each treatment (Fig. 1B) were subjected to Western blot analysis for measurement of PARP cleavage, an indicator of caspase activation and apoptosis induction (35). PARP cleavage was detected in the breast cancer cells treated with only the DSF-copper complex but not with others (Fig. 1B, bottom), showing that the DSF-copper complex induced apoptosis in breast cancer cells.

To further confirm the apoptosis-inducing ability of the DSF-copper complex, we measured apoptosis-associated cellular and nuclear morphologic changes in the same experiment. Cellular morphology changes (i.e., spherical and detached changes) were visualized by phase-contrast microscope imaging and apoptotic nuclear changes (i.e., punctuated or granular and bright nuclei) were determined after Hoechst dye staining. The apoptotic cellular (Fig. 1D, left) and nuclear changes (Fig. 1D, right) were observed only in the cells treated with the DSF-copper complex, but not with other agents or DMSO. These results further support the conclusion that the DSF-copper complex can induce apoptosis in breast cancer cells.

If proteasome inhibition is responsible for apoptosis induction by the DSF-copper mixture, we would expect that the proteasomal activity would be inhibited before the apoptotic events occur. To test this idea, we did a kinetic experiment in which MDA-MB-231 cells were treated with 15  $\mu\text{mol/L}$  of  $\text{CuCl}_2$ , DSF, or DSF-copper for different hours. The results showed that proteasome inhibition by DSF-copper started at as early as 0.5 hour, because at this time point, the levels of proteasome activity were decreased by ~30% (Fig. 2A) and accumulation of ubiquitinated proteins was significantly increased (Fig. 2B). Importantly, cell death was not observed after 0.5 hour of treatment with DSF-copper, as shown by lack of PARP cleavage (Fig. 2B) and lack of cellular morphologic change (Fig. 2C). From 0.5 to 2 hours, proteasome activity was continuously inhibited while apoptosis had not started yet (Fig. 2). Apoptosis started at 6 hours in the cells treated with DSF-copper and further increased afterwards (Fig. 2B and C). As a comparison, neither  $\text{CuCl}_2$  nor DSF alone was able to induce any of these events (Fig. 2). The results clearly show that the apoptosis induced by DSF-copper treatment is a consequential event of the proteasome inhibition.

**The nontoxic effect of the DSF-copper complex in normal, immortalized breast cells.** The ability to induce apoptosis in tumor, but not normal, cells is an important criterion for novel anticancer drugs. To determine whether the DSF-copper complex could inhibit the proteasome activity and induce apoptosis selectively in breast cancer over normal cells, we used a pair of normal and malignant breast cell lines, MCF-10A and MCF10DCIS.com. MCF-10A cells are normal, immortalized breast cells, whereas MCF10DCIS.com cells are malignant, which were derived from MCF-10A cells (33). Both MCF-10A and MCF10DCIS.com cell lines were treated with 20  $\mu\text{mol/L}$  of copper, DSF, the DSF-copper complex, TM, the TM-copper complex, or DMSO for 24 hours, followed by measurement of proteasome inhibition and apoptosis. We found that the DSF-copper complex, but not others, strongly inhibited the proteasomal chymotrypsin-like activity in breast malignant MCF10DCIS.com cells (97%; Fig. 3A), similar to that observed in breast cancer MDA-MB-231 cells (Fig. 1B). In sharp



**Figure 2.** Kinetic effect of DSF-copper. MDA-MB-231 cells were treated with 15  $\mu\text{mol/L}$  of  $\text{CuCl}_2$ , DSF, or DSF-copper mixture for indicated hours, with DMSO (D) as solvent control, followed by photograph of cellular morphologic changes (C) and preparation of cell extracts for the chymotrypsin-like activity (A) and Western blot (B) analyses. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ . Columns, mean of three experiments; bars, SD. Treatment of Cu or DMSO for 24 hours was chosen and presented.

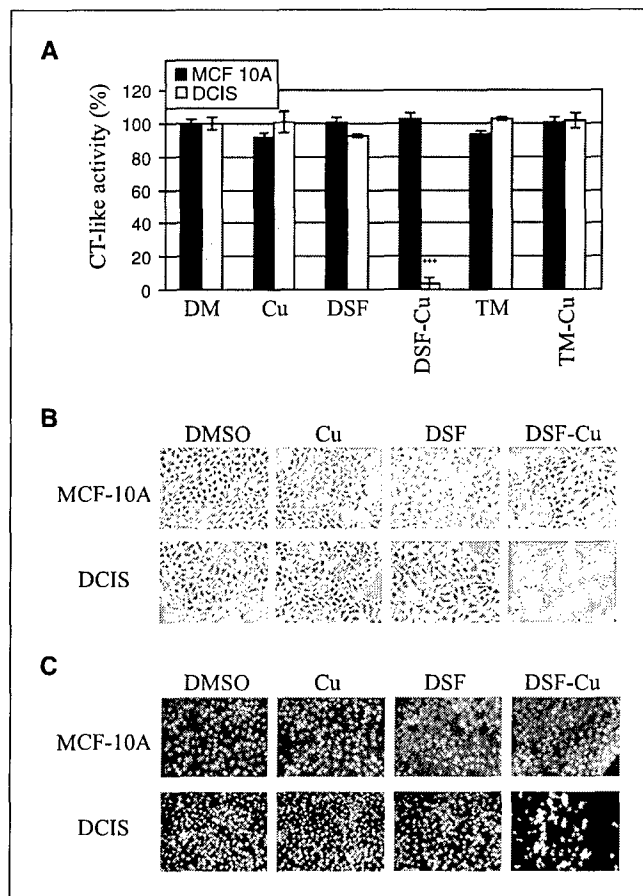
contrast, when lysates of nontransformed MCF-10A cells treated with the DSF-copper complex were analyzed, no proteasome inhibition was detected (Fig. 3A). Other treatments had little or no effects on either MCF10DCIS.com or MCF-10A cells (Fig. 3A).

To determine whether failure of the DSF-copper complex to inhibit the proteasome activity in MCF-10A cells is associated with lack of apoptosis induction in these normal, immortalized breast cells, apoptosis-associated cellular and nuclear morphologic changes were then measured in the aliquots of both cell lines in the same experiment. The MCF10DCIS.com cells treated with the DSF-copper complex, but not others, were fully detached (Fig. 3B) and also showed the apoptotic nuclear changes (Fig. 3C). However, the normal, immortalized MCF-10A cells showed only little, if any, such cell death-related detachment or apoptotic nuclei after

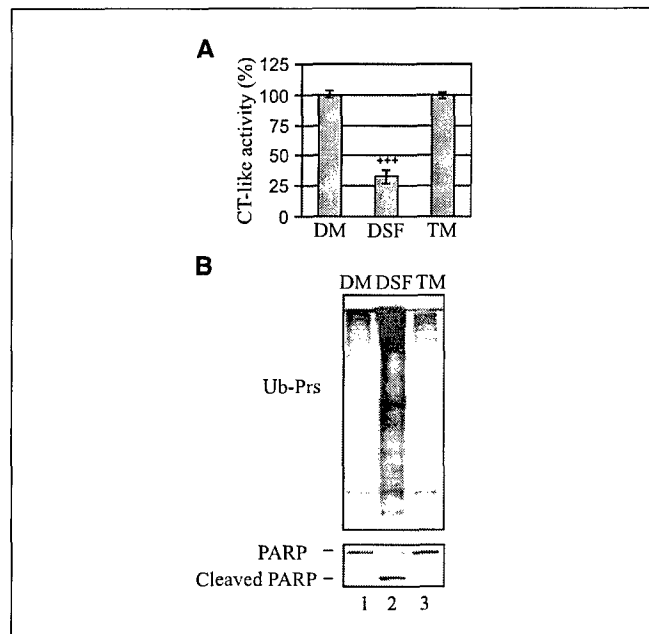
treatment with the DSF-copper complex or others (Fig. 3B and C). Our data suggest that the DSF-copper complex could inhibit the proteasomal activity and induce apoptosis selectively in malignant cells but not in normal, immortalized breast cells.

#### The effects of DSF in copper-enriched breast cancer cells.

Fundamental to the strategy we are using is the ability of the normal nontoxic ligand DSF to bind with endogenous tumor cellular copper. Cancer cells contain high level of copper *in vivo* (7–13). However, we found that the cultured cancer cells possess low to trace levels of copper (25, 34). To mimic the *in vivo* situation, human breast cancer MDA-MB-231 cells were cultured in medium containing 25  $\mu\text{mol/L}$   $\text{CuCl}_2$  for 3 days. Cellular copper concentrations increased severalfold up to micromolar range (25, 34), similar to the copper concentrations found in patients (0.3–20  $\mu\text{mol/L}$ ; refs. 9, 10). Then the  $\text{CuCl}_2$ -precultured MDA-MB-231 cells were incubated with normal growth medium (without detectable copper) and treated with 20  $\mu\text{mol/L}$  of DSF or TM for 24 hours, followed by measurement of proteasome inhibition and apoptosis.



**Figure 3.** The differential effects of DSF-copper complex in normal and malignant breast cells. Normal, immortalized human breast MCF-10A cells and malignant MCF10DCIS.com (DCIS) cells were treated with 20  $\mu\text{mol/L}$  of copper, DSF, DSF-copper complex, TM, or TM-copper complex for 24 hours. DMSO was used as vehicle control. **A**, the inhibition of chymotrypsin-like activity was shown in malignant MCF10DCIS.com cells, but not in normal MCF-10A cells treated with the DSF-copper complex. \*\*\*,  $P < 0.001$ . Columns, mean of three experiments; bars, SD. **B**, cellular spherical and detached changes (indicating apoptosis) were observed only in malignant MCF10DCIS.com cells, but not in normal MCF-10A cells, after treatment with the DSF-copper complex. **C**, punctuated, granular, and brighter nuclei (apoptotic nuclei) were observed in MCF10DCIS.com cells, but not in normal MCF-10A cells, treated with the DSF-copper complex after staining with Hoechst 33258.



**Figure 4.** The proteasome-inhibitory and apoptosis-inducing effects of DSF in copper-enriched MDA-MB-231 cells. MDA-MB-231 cells were cultured in medium containing 25  $\mu\text{mol/L}$  of copper for 3 days and then maintained in normal growth medium (without additional copper). This was followed by treatment with 20  $\mu\text{mol/L}$  of DSF or TM or vehicle DMSO for 24 hours and preparation of cell extracts. **A**, the proteasomal chymotrypsin-like activity was inhibited only in the cells treated by DSF (\*\*\*,  $P < 0.001$ ). No proteasome inhibition was seen in TM-treated cells. Columns, mean of three experiments; bars, SD. **B**, Western blot analysis showed accumulation of ubiquitinated proteins and cleavage of PARP in cells treated with DSF, but not with TM.

The proteasomal chymotrypsin-like activity was inhibited by 67% in the cells treated with DSF compared with the control (Fig. 4A). In addition, accumulation of ubiquitinated proteins and cleavage of PARP were observed in the extract of the cells pretreated with  $\text{CuCl}_2$  and posttreated with DSF (Fig. 4B). In contrast, when the copper-enriched cells were treated with TM, neither proteasome inhibition nor apoptosis induction was observed (Fig. 4). Therefore, DSF is able to induce proteasome inhibition and apoptosis in MDA-MB-231 cells containing increased copper levels (Fig. 4), but not in those with undetectable copper (Fig. 1).

#### The proteasome-inhibitory, apoptosis-inducing, and anti-tumor effects of DSF in human breast tumor xenografts.

Our data described above clearly showed that the DSF-copper complex is a proteasome inhibitor and an apoptosis inducer in cultured human breast cancer (but not normal, immortalized) cells (Figs. 1–3) and that DSF alone can do so in breast cancer cells containing increased cellular copper (Fig. 4). It has been shown that treatment with the strong copper chelator TM inhibits growth of human breast, prostate, and lung tumors in various mouse models (36–38), indicating that human tumor xenografts in mice also contain high levels of copper. If so, DSF alone treatment should be able to induce proteasome inhibition and apoptosis in human tumor xenografts, leading to tumor growth inhibition. To test this idea, human breast cancer MDA-MB-231 cells were implanted s.c. to female nude mice. When tumors became palpable ( $\sim 200 \text{ mm}^3$ ), the mice were randomly grouped (total 10 mice per group from two experiments) and injected i.p. daily with either vehicle control or 50 mg/kg DSF. The injections were kept for 29 days until the control tumors reached  $\sim 1,600 \text{ mm}^3$ . During the

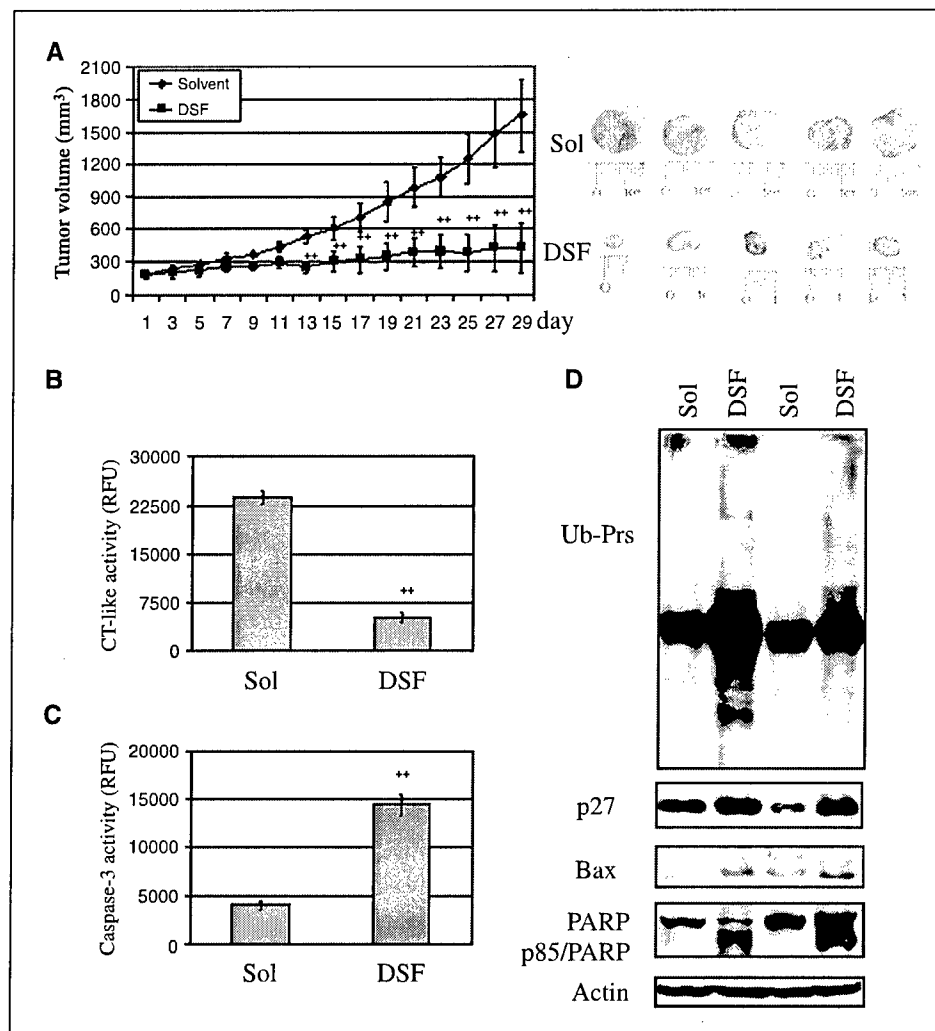
**F5** treatment, the tumor sizes in these two groups were measured and shown in Fig. 5A (left). At the end of the experiment, the mice were sacrificed. The tumors were removed from the mice and photographed (Fig. 5A, right). We found that DSF significantly inhibited tumor growth by 74% ( $P < 0.01$ ) compared with the solvent control (Fig. 5A). Therefore, the data show that DSF possesses potent antitumor effects *in vivo*.

To investigate whether the observed antitumor effects of DSF are associated with proteasome-inhibitory and apoptosis-inducing activities *in vivo*, samples of the control- or DSF-treated tumors were subjected to multiple assays. The proteasomal chymotrypsin-like activity was inhibited significantly (by 87%) in tumor tissues from mice treated with DSF when compared with the control (Fig. 5B), indicating that DSF inhibits the proteasomal activity in MDA-MB-231 tumors. Consistently, accumulation of ubiquitinated proteins and natural proteasome target proteins, p27 (39) and Bax (40, 41), was found in tumors treated with DSF versus control by Western blot analysis (Fig. 5D). Increased accumulation of p27 protein in tumors by DSF treatment was further confirmed by immunohistochemistry assay (Fig. 6A). Accompanying proteasome inhibition, apoptosis was induced in the MDA-MB-231 xenografts treated with DSF, as shown by the increased caspase-3 activity in tumor tissue extracts (Fig. 5C), the appearance of cleaved PARP

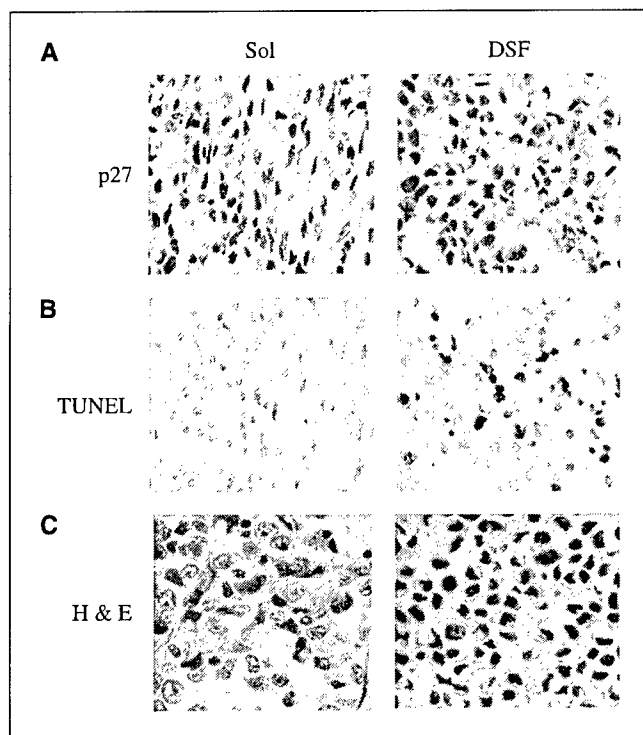
fragment (Fig. 5D), increased terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells (Fig. 6B), and high levels of condensed apoptotic nuclei detected by H&E staining (Fig. 6C). The results clearly show that DSF was able to target the proteasome *in vivo*, resulting in accumulation of natural tumor suppressor proteins (such as p27 and Bax) and induction of apoptotic cell death within the tumor, which may be responsible for the observed potent antitumor activity of DSF (Fig. 5A).

## Discussion

Many anticancer agents are unable to distinguish tumor from normal cells, which is perhaps responsible for their observed toxicity. Therefore, specific and selective targeting of chemotherapeutic drugs to the cancer, but not normal, cells could be of great benefit for cancer patients. Proteasome inhibition and antiangiogenesis have been found to be novel approaches to cancer therapy due to the fact that the cancer cells are much more dependent on these activities/processes than normal cells (3–6, 19–21, 42–44). It is unique that copper, but not other trace metals in the body, is a cofactor essential and requisite for the tumor angiogenesis processes (3–6). Furthermore, it is well documented that cancer



**Figure 5.** Proteasome-inhibitory, apoptosis-inducing, and antitumor effects of DSF *in vivo*. Female athymic nude mice were xenografted by s.c. injection of MDA-MB-231 cells ( $5 \times 10^6$ ) at one flank. When tumor size reached to  $\sim 200$  mm<sup>3</sup>, the mice were divided into two groups and treated with either the control vehicle solvent (Sol) or DSF (50 mg/kg/d;  $n = 10$ ). Tumors were collected after 29-day treatment, and the prepared tissue extracts were used for proteasome activity, caspase-3 activity, and Western blotting assays. **A**, left, tumor growth chart. DSF inhibited tumor growth by up to 74% after 29-day treatment when compared with control (\*\*,  $P < 0.01$ ). Points, mean of tumor volume in each experimental group; bars, SD. Right, comparison of tumor size. Tumor size was significantly decreased in DSF-treated mice when compared with vehicle control. **B**, proteasomal chymotrypsin-like activity assay. The chymotrypsin-like activity was inhibited by 87% in the tissue extract of tumors treated with DSF when compared with control. \*\*,  $P < 0.01$ . Bars, SD. **C**, caspase-3 activity assays. A 2.5-fold increase of caspase-3 activity was found in the tissue extract of tumors treated with DSF when compared with control. \*\*,  $P < 0.01$ . Bars, SD. **D**, Western blot analysis of tumor tissue extract with antibodies of ubiquitin, p27, Bax, PARP, or actin. The accumulation of ubiquitinated proteins p27 and Bax and cleavage of PARP were shown in the tissue extracts of tumors treated with DSF.



**Figure 6.** Immunohistochemistry, TUNEL, and H&E staining assays using mouse tumor samples. Tumors were collected after 29-day treatment (see Fig. 5 legend) and the prepared tissue slides were used for immunostaining with p27 antibody (A), TUNEL assay (B), and H&E staining assay (C). Stronger or/and more p27-positive cells and TUNEL-positive nuclei as well as apoptotic condensed nuclei were found in tumor tissue from mice treated with DSF. Magnification,  $\times 400$ .

cells and tissues accumulate high levels of copper *in vivo* (7–13). Previously, we reported that certain types of organic-copper complexes are capable of proteasome inhibition and apoptosis induction in cancer cells (25, 34). Therefore, the capability of copper-containing complexes to inhibit the proteasome, the necessity of copper for angiogenesis, and the accumulation of copper by cancer cells and tissues allow for a novel therapeutic strategy focusing on elevated copper as a selective mechanism against cancer cells and tissues (3–6, 25, 34).

DSF has been approved by the Food and Drug Administration for the treatment of alcoholism in 1951 by inhibition of aldehyde dehydrogenase (28, 45). It was reported that DSF itself had little effect, but in the presence of Cu(II), it was converted back to the two-electron oxidized form of diethyldithiocarbamate, which is the active form in inducing apoptosis (32). DSF is absorbed as its bis(diethyldithiocarbamate)-copper(II) complex (28), suggesting that a heavy metal-thiolate chelator may be the active drug facilitating mixed DSF formation. It was also reported that DSF could reduce melanoma growth in mice and hepatic tumor in a patient but the involved *in vivo* molecular target was unclear (46). In addition, DSF can effectively protect normal cells in kidney, gut, and bone marrow from the damage of cisplatin and radiation *in vivo* and increase the therapeutic index (47, 48).

Previously, we have reported that complexes of clioquinol with copper and pyrrolidine dithiocarbamate with copper possessed strong proteasome-inhibitory and apoptosis-inducing abilities (25, 34). In the current study, we showed that when complexed with copper, DSF was a potent inhibitor of the proteasomal

chymotrypsin-like activity in cultured breast cancer cells but not in normal, immortalized breast cells (Figs. 1–3). Inhibition of the tumor cellular proteasome activity occurred much earlier than apoptosis induced by DSF-copper (Fig. 2). Furthermore, DSF alone was a potent proteasome inhibitor in copper-elevated breast cancer cells (Fig. 4) and tumor xenografts (Figs. 5 and 6). DSF potently inhibited breast tumor growth in mice (Fig. 5A), associated with *in vivo* proteasome inhibition, as shown by decreased chymotryptic activity and accumulation of ubiquitinated proteins, p27 and Bax, and by apoptotic cell death, as shown by caspase-3 activation, PARP cleavage, TUNEL positivity, and condensed nuclei (Figs. 5 and 6). This finding further supports the conclusion that inhibition of the chymotrypsin-like activity of the proteasome by a specific inhibitor was sufficient to induce apoptosis (23–25).

Our strategy revolves around the idea that an inactive or nontoxic organic ligand (such as DSF) could bind with elevated copper, found in tumors *in vivo*, resulting in formation of a complex capable of proteasome inhibition. We first verified that DSF would directly interact with copper and form a new metal complex, which was indicated by dramatic color change after mixing both of them (data not shown). Once we verified that DSF spontaneously binds with copper and forms a new complex, we then tested whether the complex was a proteasome inhibitor using purified rabbit 20S proteasome. The data showed that, indeed, both DSF-copper and CuCl<sub>2</sub>, but not DSF, inhibited the chymotryptic activity of the purified 20S proteasome (Fig. 1A), showing that copper is responsible for inhibiting the proteasome molecule. This is consistent with the hypothesis that DSF is able to carry the copper ion into tumor cells and prevent copper from interacting with many nonspecific proteins.

Then we tested whether DSF-copper, DSF, or CuCl<sub>2</sub> could inhibit cellular proteasome activity using cultured breast cancer MDA-MB-231 cells. Another copper chelator, TM, was used as a comparison in this experiment. We examined levels of both cellular proteasome activity and ubiquitinated proteins (Fig. 1B) and found that the cells treated with the DSF-copper complex had significantly reduced chymotrypsin-like activity and increased ubiquitinated protein levels (Fig. 1B), indicating that proteasome inhibition had occurred. In contrast, copper, DSF, TM, or the TM-copper complex was incapable of inhibiting the proteasome (Fig. 1B). We also measured effects of these agents on MDA-MB-231 cell proliferation, PARP cleavage, and cellular and nuclear morphologic changes, and found that only the DSF-copper complex inhibited cell proliferation (Fig. 1C), induced cleavage of PARP (Fig. 1B), and caused cell death-associated morphologic and nuclear changes (Fig. 1D).

The following arguments support the idea that proteasome inhibition by DSF-copper is the cause, not the consequence, of apoptotic cell death. First, when MDA-MB-231 cells were treated with DSF-copper, the proteasome activity was inhibited at as early as 0.5 hour, about 5 hours before apoptosis induction (Fig. 2). Second, accumulation of tumor suppressor proteins p27 or Bax was also found in DSF-copper-treated tumors undergoing apoptosis (Figs. 5 and 6).

After determining that the DSF-copper complex could inhibit proteasome activity and cell proliferation and induce apoptosis in MDA-MB-231 cells (Fig. 1), we then examined the possibility that DSF-copper complex could specifically and selectively inhibit proteasome activity and induce apoptosis in breast cancer, but not normal, cells. Indeed, our results showed that the DSF-copper complex was a potent proteasome inhibitor and apoptosis inducer

only in human breast cancer MCF10DCIS.com cells, but not in normal, immortalized human breast MCF10A cells (Fig. 3).

Many cancer tissues contain highly elevated levels of copper (7–13) although cultured tumors cells contain low or undetectable copper content (25, 34). To mimic the *in vivo* tumor environment, MDA-MB-231 cells were cultured in copper-enriched media for 3 days, then maintained in normal growth medium. This treatment caused a significant increase in cellular copper concentrations (up to 0.2–6  $\mu\text{mol/L}$  range; refs. 25, 34), which were similar to the copper concentrations found in patients (0.3–20  $\mu\text{mol/L}$ ; refs. 9, 10). Afterwards, the copper-enriched MDA-MB-231 cells were treated with the ligand DSF or TM. The results support our hypothesis that the organic ligand DSF could interact with tumor cellular copper and form an active, specific proteasome-inhibitory complex, which leads to apoptosis induction (Fig. 4). Although the strong metal chelator TM should bind to copper in cancer cells, the resulted complex seems inactive in such functions (Fig. 4). Most recently, one group reported that DSF alone at  $\sim 0.16 \mu\text{mol/L}$  has proteasome-inhibitory activity under a cell-based screening assay condition (49). However, under our experimental conditions, DSF at 15 to 20  $\mu\text{mol/L}$  was unable to inhibit cellular proteasome activity in cultured breast cancer cells (Figs. 1 and 2). This difference could be due to different cell systems, different levels of cellular copper, and/or different sensitivity of proteasome activity assays. Our study suggests that the proteasome-inhibitory activity of DSF observed under their conditions could be due to formation of an active complex between DSF and cellular copper.

To answer the question of whether DSF can react with copper in tumor tissue and possess antitumor activity, we tested effect of DSF in mice bearing human breast tumor MDA-MB-231 xenografts. Our data showed that DSF treatment caused a significant inhibition of MDA-MB-231 tumor growth in nude mice (Fig. 5A). Also importantly, the antitumor activity of DSF was associated with its proteasome-inhibitory and apoptosis-inducing abilities because DSF treatment resulted in the inhibition of proteasomal chymotrypsin-like activity in tumors (Fig. 5B), accumulation of proteasome target proteins p27 (Figs. 5D and 6A) and Bax (Fig. 5D),

and induction of apoptosis (i.e., increase in caspase-3/caspase-7 activity, PARP cleavage, TUNEL positivity, and condensed nuclei; Figs. 5 and 6). A previous study has suggested the antitumor activity of DSF in melanoma and hepatic tumor, which could be potentiated by  $\text{Zn}^{2+}$  supplementation (46). We have found that a DSF-zinc complex is also a proteasome inhibitor although its potency is weaker than that of the DSF-copper complex.<sup>1</sup> Our results presented here have further confirmed their finding and also showed the requirement of proteasome inhibition for the antitumor activity of DSF.

The data presented here support the novel concept of using accumulated copper in breast cancer cells and tissues as a selective approach for chemotherapy. The nontoxic copper-binding ligands such as DSF can spontaneously bind with copper and form a proteasome inhibitor and an apoptosis inducer in breast cancer, but not normal, cells. Cancer cells and tissues, which contain elevated copper and more dependent on proteasome activity for their survival, should be very sensitive to treatment with DSF and other copper-binding compounds. In contrast, normal cells and tissues, containing trace amounts of copper and having basal level of proteasome activity, should be resistant to these effects. DSF has been previously explored for use in clinic for alcoholism, and our data suggest the potential use of DSF and other similar compounds in cancer therapies.

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<sup>1</sup> Unpublished data.

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**RELATIONSHIPS OF STRUCTURES OF N-METHYLTHIOLATED BETA-LACTAM ANTIBIOTICS TO THEIR APOPTOSIS-INDUCING ACTIVITY IN HUMAN BREAST CANCER CELLS**

**Deborah Kuhn, Yang Wang, Vesna Minic, Cristina Coates, G. Suresh Kumar Reddy, Kenyon, G. Daniel, Jeung-Yeop Shim, Di Chen, Kristin R. Landis-Piwowar, Edward Turos, And Q. Ping Dou, Ph.D.**

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Activation of the cellular apoptotic program is a current strategy for the prevention and treatment of human cancer including breast cancer. The beta-lactam antibiotics have played an essential role in treating bacterial infections without causing toxic side effects for the past 60 years. We hypothesize that active N-thiolated beta-lactams can target a tumor-specific protein(s) and selectively induce apoptosis in human breast cancer but not normal cells.

To test this hypothesis, we designed and synthesized numerous new N-thiolated beta-lactams analogs, evaluated potencies of these synthetic beta-lactams to inhibit proliferation and induce apoptosis in human breast cancer cells, and investigated whether these beta-lactams can induce apoptosis selectively in breast tumor vs. normal cells and the involved molecular mechanisms.

We tested a library of lactam compounds and found that many of them are able to inhibit proliferation and induce apoptosis in human breast cancer cells in a time- and concentration-dependent manner. These active beta-lactams are capable of inducing DNA strand breakage, inhibiting DNA replication, and inducing p38 MAP kinase activation. Consistent with the idea that these beta-lactam antibiotics are potent anti-cancer agents, the active analogs are also capable of inhibiting colony formation potential of breast cancer cells. Furthermore, the active beta-lactams have much reduced effects on human normal or non-transformed cells. Our findings also yielded several important structure-activity relationships (SARs). The N-methylthio group is necessary for the apoptosis-inducing activity. Also observed is the inverse relationship between the number of carbon atoms off the N-thio group and apoptotic activity. Substitutions to C3 reveal that as the substituents increase in size or in polarity, the efficacy of the compound decreases. Therefore, the overall size of the beta-lactam is important, possibly indicating steric hindrance with the cellular target or permeability to the cell membrane. We also found that the stereochemistry of the beta-lactams play an important role in their potency. The 3R,4S enantiomers are more efficacious than the 3S,4R isomers, which may indicate a more favorable configuration for target interaction.

Our future studies will focus on the biochemical target of N-thiolated beta-lactams, whether the N-thiolated beta-lactams can induce apoptosis selectively in breast tumor vs. normal breast cells, and whether the in vivo apoptosis-inducing ability of the N-thiolated beta-lactams is related to their cancer-preventive and anti-tumor activities using nude mice bearing human breast tumors. These studies should provide strong support for proof-of-concept of the potential use of these N-thiolated beta-lactams in breast cancer prevention and treatment.

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## Dou, Ping

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**Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts *via* inhibition of the proteasome activity**

**Short Title:**

Disulfiram/Cu inhibits proteasome

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**Abstract:**

Disulfiram (DSF), a member of the dithiocarbamate family capable of binding copper and an inhibitor of aldehyde dehydrogenase, is currently being used clinically for the treatment of alcoholism. Recent studies have suggested that DSF may have antitumor and chemosensitizing activities although the detailed molecular mechanisms remain unclear. Copper has been shown essential for tumor angiogenesis processes. Consistently, high serum and tissue levels of copper have been found in many types of human cancers, including breast, prostate and brain, supporting the idea that copper could be used as a potential tumor-specific target. Here we report that the DSF-copper complex potently inhibits the proteasomal activity in cultured breast cancer MDA-MB-231 and MCF10DCIS.com, but not normal, immortalized MCF-10A, cells, prior to induction of apoptotic cancer cell death. Furthermore, MDA-MB-231 cells that contain copper at concentrations similar to those found in patients, when treated with just DSF, undergo proteasome inhibition and apoptosis. In addition, when administered to mice bearing MDA-MB-231 tumor xenografts, DSF significantly inhibited the tumor growth (by 74%), associated with *in vivo* proteasome inhibition (as measured by decreased levels of tumor tissue proteasome activity and accumulation of ubiquitinated proteins and natural proteasome substrates p27 and Bax) and apoptosis induction (as shown by caspase activation and apoptotic nuclei formation). Our study demonstrates that inhibition of the proteasomal activity can be achieved by targeting tumor cellular copper with the non-toxic compound DSF, resulting in selective apoptosis induction within tumor cells.

**Author Disclosure Block:** D. Chen, None; Q.C. Cui, None; H. Yang, None; Q. Dou, None.

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Visiting Professor in the Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, 2006-

Honorary Professor, Wuhan Botanical Garden/ Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, Hubei, China, 2006-

#### **MAJOR PROFESSIONAL SOCIETIES:**

American Association for Cancer Research, Inc.

American Association for the Advancement of Science

American Society for Biochemistry and Molecular Biology

American Society for Pharmacology and Experimental Therapeutics

Society of Chinese Bioscientists in America

International Society for Study of Comparative Oncology, Inc.

American Chemistry Society

New York Academy of Sciences (1995-1998)

#### **HONORS AND AWARDS:**

Summer Research Prize in recognition of outstanding accomplishments in research. Rutgers University, 1988

Biochemical Research Support Grant Award. Dana-Farber Cancer Institute, 1991

Barr Program Small Grant Award. Dana-Farber Cancer Institute, 1992

Co-Discussion Leader, University of Pittsburgh Cancer Institute Scientific Retreat, 1995

*NIH Director James A. Shannon Award, 1 R55 AG/OD13300-01, 1995-1997*

*NIH FIRST Award, R29 AG13300-05, 1996-2001*

Q. Ping Dou, Ph.D.

A Predoctoral Trainingship in Breast Cancer Biology and Therapy from the United States Army Medical Research, Development, Acquisitions, and Logistics Command (to Cheryl L. Fattman), 1997-1999

The Best Poster Presentation (An B *et al.*), Scientific Retreat, Department of Pharmacology, University of Pittsburgh School of Medicine, 1997

*Chairman for Session of Clinical Oncology/Apoptosis. 2nd World Congress on Advances in Oncology, Athens, Greece, 16-18 October, 1997*

Award for the Best Abstract, 2nd World Congress on Advances in Oncology, Athens, Greece, 16-18 October, 1997

Cheryl L. Fattman, Ph.D. Graduation with Honor from University of Pittsburgh (mentor: Q. Ping Dou), 1999

*Moffitt's Cancer Center Director's Award (for the article published by Li B and Dou QP in Proc. Natl. Acad. Sci. USA, 2000; 97: 3850-3855). Moffitt Cancer Center & Research Institute, 2000*

*An AACR-AFLAF Scholar-in Training Award* (\$400 to Aslam Kazi/ mentor: QP Dou), for a selected poster (Abstract #788, Kazi A, Smith DM, Zhong Q and Dou QP. Down Regulation of Bcl-X<sub>L</sub> Protein Expression by Green Tea Polyphenols Is Associated with Prostate Cancer Cell Apoptosis. AACR-NCI-EORTC. Molecular Targets and Cancer therapeutics, Miami Beach, FL, October 29-November 2, 2001

Abstract was chosen as one of the selected few for News Briefing (Abstract #788, Kazi A, Smith DM, Zhong Q and Dou QP. Down Regulation of Bcl-X<sub>L</sub> Protein Expression by Green Tea Polyphenols Is Associated with Prostate Cancer Cell Apoptosis. AACR-NCI-EORTC. Molecular Targets and Cancer therapeutics, Miami Beach, FL, October 29-November 2, 2001

Nominee of Team Award (Cancer Control Program), 2002

Kenyon G. Daniel, Ph.D. a Winner of the 2004 Outstanding Dissertation Award from University of South Florida (Major Professor: Q. Ping Dou), 2004

*Invited Visiting Professor in the Department of Urology at the University of California San Francisco and San Francisco VA Medical Center (April 28, 2005), with seminar presentation, "Searching for novel polyphenol proteasome inhibitors for cancer prevention and treatment"*

Training Grant (Kristin Landis; Mentor: Q. Ping Dou). Wayne State University, 2005-2007

First Place, Oral Presentation (Kristin Landis; Mentor: Q. Ping Dou). Wayne State University School of Medicine, Graduate Student Research Day, September 22, 2005. "Molecular Studies for the Regulation of Green Tea Polyphenol Biological Function by the Polymorphic Gene Product Catechol-O-Methyltransferase".

*Awardee (Kristin Landis; Mentor: Q. Ping Dou) for the Honors Recognition Program for Wayne State University School of Medicine, Wayne State University, 2005*

*Invited Visiting Professor in the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China (April 19-20, 2006), with seminar presentation, "Cancer Prevention and the Role of Environmental Factors". Received Honorary Professor Title.*

*Invited Visiting Professor in the Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China (April 24-25, 2006), with seminar presentations, "Nutrient-Gene-Environment Interactions and Molecular Prevention of Cancer" and "Discovery of Novel Small Molecules from Nature and Laboratories for Cancer Therapies"*

The First Place Poster Award (Huanjie Yang, Di Chen, Qiuzhi Cindy Cui, Xiao Yuan, and Q. Ping Dou). The 6<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium,

Q. Ping Dou, Ph.D.

Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006. "Celastrol, A Triterpene Extracted From The Chinese Thunder Of God Vine, Is A Potent Proteasome Inhibitor And Suppresses Human Prostate Cancer Growth In Nude Mice".

**Honorary Professorship, Wuhan Botanical Garden/ Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, Hubei, China (October, 2006), with seminar presentation, "Discovery of Novel Small Molecules for Cancer Therapies".**

**Nominee of the 2007 AACR Landon Prize for Basic Research.**

First Place, Oral Presentation (Kristin Landis; Mentor: Q. Ping Dou). Wayne State University School of Medicine, Graduate Student Research Day, September 21, 2006. "A Novel Pro-drug of Green Tea Catechin (-)-EGCG as a Potential Anti-Breast Cancer Agent".

**Co-Session Chairman.** The 3<sup>rd</sup> International Symposium on Persistent Toxic Substances, Beijing, China, October 22-25, 2006

## **SERVICE:**

### **Professional Consultation**

Tours for University of Pittsburgh Cancer Institute

Tours for Drug Discovery Program Moffitt Cancer Center & Research Institute

Advisor for Project LINK (Leaders In New Knowledge) Students

Advisor for Moffitt Summer Interns

Advisor for Undergraduate Student Honor's Thesis Research

Member, the Scientific Advisory Board, Torrey Pharmaceuticals, LLC, San Diego, CA, 2000-present

Graduate student recruitment faculty for Cancer Biology Program Karmanos Cancer Institute and Wayne State University, April 3, 2004, April 2, 2005

Presentation to Cancer Biology Program Candidates, Karmanos Cancer Institute and Wayne State University, March 25, 2006 (Kristin Landis and Vesna Minic from Dr. Dou's laboratory)

Preferred Consultant, MEDACorp, Boston, Massachusetts, 2004-present

Speaker (Kristin Landis from the laboratory of Q. Ping Dou), "Friends Raising Funds" program, March 5<sup>th</sup>, 2006, the Powerhouse Gym, West Bloomfield, MI.

Invited Speaker, "Green Tea and Cancer Prevention", "The Day of Wellness" Program, September 16, 2006, the Grosse Pointe War Memorial, Grosse Pointe, MI.

Moderator, Population Studies and Prevention Program Scientific Retreat, Lung Cancer session, Karmanos Cancer Institute and Wayne State University, September 15, 2006

Invited Speaker, Green Tea and its Role in Cancer Prevention. Dow and Towsley Foundation Visit, Karmanos Cancer Institute Research, Detroit, MI, October 11, 2006

### **Journal/Editorial Activity**

#### **Editorial Board Memberships**

Invited member of the Editorial Board of the *Oncology Reports*, 1996-present

Invited member of the Editorial Board of *Frontiers In Bioscience*, 1997-present

Invited member of the Editorial Board of *LifeXY* (Currently *International Archives of Biosciences*), 2001-present

Invited panel evaluator of *Current Drugs*, 2001-present

Invited member of *The Science Advisory Board*, 2002-present

Invited managing editor of *Frontiers In Bioscience*, 2003-present

Invited managing editor of *Frontiers In Bioscience* for a special issue of "Potential Molecular Targets for Chemoprevention", 2004-present

Reviewer for Journal Manuscripts

Proceeding of National Academy of Sciences USA

FASEB J

Oncogene

Chemistry & Biology

Cancer Research

Clinical Cancer Research

Molecular Cancer Therapeutics

Cell Death & differentiation

Molecular Pharmacology

Journal of Pharmacology & Experimental Therapeutics

Exp Cell Res.

J Cellular Physiology

Drug Discovery Today

Microbes and Infection

Leukemia

Cancer Letters

FEBS Letters

Carcinogenesis

International J. Oncology

Breast Cancer Research and Treatment

J. Cell. Biochemistry

Biochemical Pharmacology

BMC Cancer

Cancer Chemotherapy and Pharmacology

Lipids

European Journal of Medicinal Chemistry

European Journal of Cancer

Endocrine

Apoptosis

Arch Biochem Biophys

Journal of Pharmacy and Pharmacology

Head & Neck

Journal of Agriculture and Food Chemistry

Obesity Research

Molecular Nutrition and Food Research

Natural Immunity

Molecular Biology Reports

Gene Therapy

Cellular and Molecular Life Sciences

Expert Opinion on Investigational Drugs

Expert Review of Anticancer Therapy

Expert Review of Proteomics

Evidence-Based Integrative Medicine

Life XY (Currently *International Archives of Biosciences*)

The Pittsburgh Undergraduate Review

Reviewer for Grant Applications

Competitive Medical Research Fund (Ad Hoc Reviewer), University of Pittsburgh School of Medicine, 1996

Competitive Medical Research Fund, University of Pittsburgh School of Medicine, 1997

Central Research Development Fund, University of Pittsburgh, 1997

National Science Foundation, 1998

Merit Review Subcommittee for Oncology, the Veterans Affairs Medical Research Programs, US Department of Veterans Affairs, 1999-2005

Member, Cancer Study Section, The Tobacco-Related Disease Research Program, University of California, San Francisco, CA, 2004-present

Invited Reviewer, Biotechnology and Biological Sciences Research Council Research Grant, United Kingdom, 2004, 2006

Invited Proposal Reviewer, The Kentucky Science and Engineering Foundation's R&D Excellence Program, Lexington, KY, 2004, 2005

Invited Reviewer, the Strategic Research Initiative Awards, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2005

Invited Reviewer, the Seed Money Grants, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2006

Invited Reviewer, the European Commission's Research Program on "Combating Cancer" - Apoptosis, Brussels, Belgium, 2006

Other Professional Related Service

Faculty reviewer for applications to University of Pittsburgh and University of Pittsburgh Cancer Institute

Faculty reviewer for applications to Moffitt Cancer Center & Research Institute and University of South Florida

Faculty reviewer for applications to Karmanos Cancer Institute and Wayne State University (2003-)

Faculty reviewer for Deputy Director/Associate Center Director Candidates to Karmanos Cancer Institute and Wayne State University (2004)

Faculty reviewer for Lambert – Webber Endowed Chair, Division Chief, Hematology and Oncology, Department of Internal Medicine, Wayne State University, Program Leader, Developmental Therapeutics, Karmanos Cancer Institute (2005)

National and International Boards and Committees

Ad Hoc Reviewer, National Science Foundation, 1998

Ad Hoc Reviewer, Merit Review Subcommittee for Oncology, the Veterans Affairs Medical Research Programs, US Department of Veterans Affairs, 1999-2001

Member, Department of Veterans Affairs (VA) Medical Research Service Merit Review Subcommittee for Oncology, 2001-2005

Member, the Scientific Advisory Board, Torrey Pharmaceuticals, LLC, San Diego, CA, 2000-present

Council Member, Gerson Lehrman Group's Council of Healthcare Advisors, New York, NY, 2004-present

Member, Cancer Study Section, The Tobacco-Related Disease Research Program, University of California, San Francisco, CA, 2004-present

Invited Reviewer, Biotechnology and Biological Sciences Research Council Research Grant, United Kingdom, 2004, 2006

Q. Ping Dou, Ph.D.

Preferred Consultant, MEDACorp, Boston, Massachusetts, 2004-present

Judge, Cell Biology & Cell Signaling Section, 2nd Annual Research Symposium, Henry Ford Health System Academic Affairs, Detroit, MI, April 15, 2005

Invited Reviewer, the European Commission's Research Program on "Combating Cancer" - Apoptosis, Brussels, Belgium, 2006

### **State and Local Boards and Committees**

#### **Department of Pharmacology, University of Pittsburgh School of Medicine**

Comprehensive Examination Committee, Department of Pharmacology, University of Pittsburgh School of Medicine, 1993-1998

Committee of Graduate Studies, Department of Pharmacology, University of Pittsburgh School of Medicine, 1994-1998

Chairman of Graduate Evaluations, Department of Pharmacology, University of Pittsburgh School of Medicine, 1994-1998

NIH Predoctoral Training Grant Selection Committee, 1995

Director of Departmental Seminar Program, 1997

#### **University of Pittsburgh School of Medicine**

Competitive Medical Research Fund Review Committee (Ad Hoc Reviewer), University of Pittsburgh School of Medicine, 1996-1997

The Graduate Progress Evaluation Committee, University of Pittsburgh School of Medicine, 1997

Central Research Development Fund, University of Pittsburgh, 1997

#### **University of South Florida and Moffitt Cancer Center & Research Institute**

Member, Search Committee for Assistant Professor position in Molecular, Epidemiology Program of Cancer Control Division at Moffitt Cancer Center & Research Institute, 2001

Member, Rb Club, Moffitt Cancer Center & Research Institute, 2001-2002

Member, The Summer Intern Program Committee at Moffitt Cancer Center & Research Institute, 2002

Member, Preliminary Data Club, Moffitt Cancer Center & Research Institute, 2002-2003

Member, Proteomics Task Force Committee, Moffitt Cancer Center & Research Institute, 2002

Member, Search Committee for Structural Biology Faculty position in Drug Discovery Program at Moffitt Cancer Center & Research Institute, 2003

Member, Search Committee for the Cancer Prevention Faculty position in Molecular Epidemiology Program of Cancer Control Division at Moffitt Cancer Center & Research Institute, 2003

Member, Search Committee for Chemistry Faculty position in Drug Discovery Program at Moffitt Cancer Center & Research Institute, 2003

Member, SPARK (Summer Program for the Advancement of Research Knowledge) Selection Committee at Moffitt Cancer Center & Research Institute, 2003

#### **Wayne State University and Karmanos Cancer Institute**

Graduate student recruitment faculty for Cancer Biology Program Karmanos Cancer Institute and Wayne State University, April 3, 2004

Q. Ping Dou, Ph.D.

Graduate student recruitment faculty for Cancer Biology Program Karmanos Cancer Institute and Wayne State University, April 2, 2005

Invited Reviewer, the Strategic Research Initiative Awards, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2005

Invited Judge, the 2nd Annual Research Symposium, Henry Ford Health System, Detroit, MI, 2005

Invited Reviewer, the Seed Grants, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2006

Co-Director and Member of a P01 Group, "AR-mediated cell cycle-dependent anti-apoptotic events in prostate cancer", Wayne State University and Henry Ford Health System, Detroit, MI, 2004-present

Speaker (Kristin Landis from the laboratory of Q. Ping Dou), "Friends Raising Funds" program, March 5<sup>th</sup>, 2006, the Powerhouse Gym, West Bloomfield, MI.

Member, HFHS/WSU Prostate Journal Club, 2003-

Co-Director and Member of a P01 Group, "AR-mediated cell cycle-dependent anti-apoptotic events in prostate cancer", Wayne State University and Henry Ford Health System, Detroit, MI, 2004-present

Dr. Dou has the following responsibilities:

- (1) Assist the Program Leader in the entire Population Studies and Prevention program and provide leadership in the Prevention sub-program, particularly with respect to basic science research, molecular targeting and chemoprevention;
- (2) Participate in Population Studies and Prevention leadership meetings;
- (3) Participate in updating and maintaining strategic planning for the Prevention sub-program;
- (4) Facilitate inter- and intra-programmatic interactions between the Prevention faculty members and members of Population Studies, Communication & Behavioral Oncology, and other cancer center programs;
- (5) Organize and lead the monthly Prevention group meetings;
- (6) Recruit new members into the Prevention sub-program and mentor junior faculty;
- (7) Advocate for shared facilities that meet the needs of the Prevention members;
- (8) Develop the Prevention sub-program into an independent program in the next five years.

## TEACHING:

*Years at Wayne State University:* Since August 1, 2003

*Years at Other Universities:*

Harvard Medical School, 1 year

University of Pittsburgh, 5 years

University of South Florida, 5 years

### Courses Taught at Wayne State University

|       |                                                                                                            |
|-------|------------------------------------------------------------------------------------------------------------|
| 2003- | CB 7250: CANCER CONTROL. 3 credits. 20 students                                                            |
| 2004  | CB 7230: BREAST CANCER. 2 credits. 10-12 students (December 8, 2004, 10:00 AM- 12:00 PM, 1140 Scott Hall). |
| 2005- | CB 7700: RECENT DEVELOPMENT IN CANCER BIOLOGY. 2 credits. ~20 students (April 11, 2005, Oct 24, 2005)      |

### Courses Taught at University of South Florida

1999-2001 BCH 6411: Molecular Biology. Lecture. 3 credits. 25-30 students  
 2001- Cancer Biology I Course Lecture. 3 credits. ~10 students

Courses Taught at University of Pittsburgh

1993-1998 MS MIC 2355: Advanced Molecular Genetics. Lecture and Paper Discussion. 3 credits. 8-16 students  
 1993-1998 PHL 3510: Receptors and Signal Transduction. Lecture and Paper discussion. 3 credits. 10-15 students  
 1993-1998 2563: Cancer Pharmacology. Lecture. 3 credits. ~5 students  
 1993-1998 Medical Student Program: Problem-Based Learning Sessions. 8-10 students  
 1995 Medical Student Program: Pharmacology Conference. ~20 students  
 1997 Medical Student Program: Neoplasia and Neoplastic Disease. 16 students  
 1996-97 The Pennsylvania Governor's School Program. 6-8 students  
 1997 Foundations of Biomedical Science. Small group conference. 3 credits. ~8 students

Undergraduate and Graduate/Medical Student Supervision

1994 Chen Yu, Harvard University, ASPET undergraduate  
 1995 Peggy Lin, Penn State-Jefferson  
 1995 Bill Wang, California University of PA  
 1995 Vivian Lui, Department of Pharmacology, University of Pittsburgh School of Medicine, one lab rotation  
 1996 Toni A. Termin, Saint Vincent College, ASPET undergraduate  
 1996 Kirk E. Dineley, Department of Pharmacology, University of Pittsburgh School of Medicine, two lab rotations  
 1996-1999 **Cheryl Fattman, Department of Pharmacology, University of Pittsburgh School of Medicine. Ph.D., Dissertation Title: "Molecular mechanisms for apoptosis-associated the retinoblastoma protein (RB) internal cleavage". Graduation with Honor (Mentor: Q. Ping Dou). Currently working as a postdoctoral fellow in Department of Pathology, University of Pittsburgh School of Medicine**  
 1997 Lachelle Sussman, University of New York at Buffalo, ASPET Undergraduate, University of Pittsburgh School of Medicine, one lab rotation  
 1997 Kristin S Morrow, Department of Biology University of South Florida, master graduate student  
 1998- Yaser S. Bassel, University of South Florida College of Medicine, medical student  
 1998- Jason A. Evangelista, University of South Florida College of Medicine, medical student  
 1998- Joseph J. Kavanagh, University of South Florida College of Medicine, medical student  
 1998- Alexander Paloma, University of South Florida College of Medicine, medical student  
 1998- Gregory A. Russell, University of South Florida College of Medicine, medical student  
 1999-2002 **David M. Smith, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab (graduated in 2002). Dissertation Title: "Mechanistic Studies on Tumor Cell Cycle Disruption and Apoptosis by**

**Green Tea Polyphenols and N-Thiolated beta-Lactams". Currently working as a postdoctoral fellow in Dr. Fred Goldberg's laboratory at Harvard Medical School)**

- 1999 Lisa Smith, Department of Biology University of South Florida, undergraduate student (currently a graduate student in University of North Carolina)
- 1999 Jessica Hu, Harvard University, undergraduate student
- 1999 Daniel Lorch, University of Florida, undergraduate student
- 1999 Sun Hee Rim, Hillsborough High School, student
- 1999 Alvin Jones, Land O'Lakes High School, student
- 1999 Kristie Main, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation
- 1999-2000 Kenyon Daniel, Department of Biology University of South Florida, undergraduate student. Research for Honor's Thesis
- 2000 Lisa Smith, Moffitt Summer Intern, Department of Biology University of South Florida, undergraduate student (current graduate student at University of North Carolina)
- 2000-2002 Marie Bosley, Project LINK (Leaders In New Knowledge) Student and a Moffitt Summer Intern, Department of Biology University of South Florida, undergraduate student
- 2000 John (Chilu) Chen, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation
- 2001 Jonathan S. Anderson, Moffitt Summer Intern, Zoology, University of Florida, undergraduate student
- 2001 Kyleen Charlton, Moffitt Summer Intern, Boston College, undergraduate student
- 2001-2002 Priyanka Kamath, Volunteer, Hillsborough High School, high school student
- 2000-2004 **Kenyon Daniel, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab(graduated in 2004). Dissertation Title: "Strategies for Cancer Therapy Through Regulation of Apoptotic Proteases". Currently working as a postdoctoral fellow in my laboratory at Karmanos Cancer Institute, Wayne State University**
- 2001-2004 **Deborah Kuhn, Cancer Biology Ph.D. Program (IOP), University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab (graduated in November of 2004). Dissertation Title: "Novel Approaches to Targeting Tumor Cell Apoptotic Signaling Pathways". Currently working as a postdoctoral fellow in University of North Carolina.**
- 2002 Naveen Kumar, Moffitt Summer Intern, New York University, undergraduate student
- 2002 Randy Hill, Moffitt Summer Intern, University of Wisconsin, undergraduate student (currently a graduate student in University of Wisconsin)
- 2002 Seth Pross, Moffitt Summer Intern, Hillsborough High School, high school student (currently a graduate student in University of Pennsylvania)
- 2002-2003 Audrey Burns, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab

Q. Ping Dou, Ph.D.

- 2002-2003 Sam Falsetti, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab
- 2002 Jennelle McQuown, Cancer Biology Ph.D. Program (IOP), University of South Florida College of Medicine, one lab rotation
- 2002-2003 Daniel Urbizu, Project LINK (Leaders In New Knowledge) Student, Department of Biology University of South Florida, undergraduate student
- 2003- Thomas Lendrihas, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation
- 2003 Seth Pross, Moffitt Summer Intern, University of Pennsylvania, undergraduate student
- 2003 Shuojing Song, Moffitt Summer Intern, C. Leon King High School, high school student (currently an undergraduate of MIT)
- 2003 R. Hope Harbach, Summer Student, Department of Chemistry, Eckerd College, undergraduate student
- 2003 Daniel Urbizu, Summer/Project LINK (Leaders In New Knowledge) Student, Department of Biology University of South Florida, undergraduate student
- 2003 Marina Si Chen, Summer Research Volunteer, King High School, high school student
- 2003 Sydnor M. Richkind, Summer Research Volunteer, Hillsborough High School, high school student (currently a graduate student in University of Florida)
- 2004-present Kristin Landis, Cancer Biology Program, Wayne State University, one lab rotation and Ph.D. student in my lab**
- 2004 Marina Si Chen, Summer Research Hourly Technician, Emory University, undergraduate student
- 2005-present Vesna Minic, Cancer Biology Program, Wayne State University, one lab rotation and Ph.D. student in my lab**
- 2005- Joan McCallum, Cancer Biology Program, Wayne State University, one lab rotation in my lab
- 2006- Mike Frezza, Cancer Biology Program, Wayne State University, one lab rotation and Ph.D. student in my lab**
- 2006- Benchamart Moolmuang, Cancer Biology Program, Wayne State University, one lab rotation in my lab
- 2006 Andy Yang, Summer Research Student, Webster Thomas High School, New York
- 2006 Marina Si Chen, Summer Research Hourly Technician, Emory University, undergraduate student
- 2006 Justin Shaya, Summer Research Student, West Bloomfield High School, MI

Theses/ Dissertation or Comprehensive Examination Committees

*Ph.D. Dissertation Committees*

- 1995 Kirti G. Goyal, Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine (Advisor: Dr. Leaf Huang)
- 1997 Jie-Gen Jiang, Pathology, University of Pittsburgh School of Medicine (Advisor: Dr. Reza Zarnegar), graduated in 12/97
- 1997-1998 Marni Brisson, Pharmacology, University of Pittsburgh School of Medicine (Advisor: Dr. Leaf Huang)

Q. Ping Dou, Ph.D.

- 1997-1998 Donald Schwartz, Pharmacology, University of Pittsburgh School of Medicine (Advisor: Dr. John Lazo)
- 1996-1998 Robert Rice, Pharmacology, University of Pittsburgh School of Medicine (Advisor: Dr. John Lazo)
- 1996-1999 Cheryl Fattman, Pharmacology, University of Pittsburgh School of Medicine (Advisor: Dr. Q. Ping Dou)
- 1999-2002 David M. Smith, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (graduated; Ph.D. Advisor: Dr. Q. Ping Dou)
- 2000-2004 Kenyon Daniel, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (Graduated; Ph.D. Advisor: Dr. Q. Ping Dou)
- 2001-2005 Deborah Kuhn, Cancer Biology Ph.D. Program (IOP), University of South Florida College of Arts and Sciences (Advisor: Dr. Q. Ping Dou). Graduated in November, 2004.
- 2002-2003 Audrey Burns, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (Advisor: Dr. Q. Ping Dou)
- 2002-2003 Sam Falsetti, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (Advisor: Dr. Q. Ping Dou)
- 2003 Bonnie Goodwin, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (Advisor: Dr. Duane Eichler)
- 2004 Kristin Landis, Cancer Biology Program, Wayne State University (Advisor: Dr. Q. Ping Dou)
- 2005 Vesna Minic, Cancer Biology Program, Wayne State University (Advisor: Dr. Q. Ping Dou)
- 2006- Mike Frezza, Cancer Biology Program, Wayne State University (Advisor: Dr. Q. Ping Dou)

*Comprehensive Examination Committees*

- 1994 Xiang Gao, Pharmacology, University of Pittsburgh School of Medicine
- 1995 Jeff Phillips, Pharmacology, University of Pittsburgh School of Medicine
- 1995 Chialin Cheng, Pharmacology, University of Pittsburgh School of Medicine
- 1995 Cheryl Fattman, Pharmacology, University of Pittsburgh School of Medicine
- 1996 Marni Brisson, Pharmacology, University of Pittsburgh School of Medicine
- 2003 Deborah Kuhn, Cancer Biology Ph.D. Program, University of South Florida College of Arts and Sciences

*Research Associates and Others*

- 2006-present Guoqing Shi, Ph.D., Visiting Scholar
- 2006-present Nivedita Tiwari, M.S., Sr. Research Assistant
- 2005-present Huanjie Yang, Ph.D., Post-Doctoral Fellow
- 2004-present Cindy (Qiuzhi) Cui, Technician
- 2004-present Marcianna Norris, Administrative Assistant (Supervisor: Dr. Q. Ping Dou)
- 2003-present Di Chen, Ph.D., Research Associate
- 2005-present Jaiwei Ren, Technician
- 
- 2006 Lihua Li, M.D., Visiting Scholar
- 2005-2006 Haiyan Pang, Ph.D., Research Associate

2005 Alejandro Diez, M.D., Physician Intern  
2004-2005 Shirley Adanta Orlu, Research Assistant  
2005-2005 Yezhou Sun, Student Assistant  
2004-2005 Kenyon Daniel, Ph.D., Post-Doctoral Fellow  
2003-2004 MaryAnn Sparkman, Administrative Assistant (Supervisor: Dr. Q. Ping Dou)  
2003-2004 Mohammad Bhuiyan, Ph.D., Research Associate  
2000-2003 Aslamuzzaman Kazi, Ph.D., Research Associate  
2002 Robin Shear, Research Volunteer  
2000-2002 Sherry Zhong, Research Assistant  
2001-2002 Puja Gupta, Research Volunteer  
2000-2001 Hongwei Wang, Research Assistant  
2000-2001 Kenyon Daniel, Research Assistant  
1998-2000 Sangkil Nam, Ph.D., Research Associate  
2000 Gen Wang, Ph.D., Research Associate  
1999-2000 Xiaoxia Zhang, M.S., Research Assistant  
1998-2000 Gui Gao, Ph.D., Research Associate  
1998-2000 Benyi Li, M.D., Research Associate  
1998-1999 Roland Cooper, Ph.D., Research Associate  
1998 Jieliu Tang, Ph.D., Research Associate  
1994-1998 Bing An, Research Associate  
1996-1998 Terence F. McGuire, Ph.D., Instructor  
1997-1998 Yibing Peng, M.S., Research Assistant  
1995-1996 Jia-Rui Jin, Visiting Scholar  
1995 Leilei Zhang, Visiting Scholar

## **GRANT SUPPORT:**

### **Completed support**

American Cancer Society Institutional Research Grant. Cyclins, transcription and defective growth control in cancer. Principal Investigator: Qing Ping Dou. 10/01/93-06/30/95.

Agreement with Beth Israel Hospital. Molecular Biology of Aging. Principal Investigator: Jeanne Y. Wei. 1994.

NIH R01. Molecular Biology of G1/S Regulation in Murine Cells. 07/01/93-06/30/96. Subcontract (Principal Investigator: Arthur B. Pardee)

NIH Shannon Award. Functions of RB-protease(s) in apoptosis. Principal Investigator: Qing Ping Dou. 09/15/95-08/31/97 (replaced by R29 on 04/14/96).

UPCI Breast Cancer Pilot Grant. Induction of p53-independent apoptosis and treatment of human breast cancer. Principal Investigator: Qing Ping Dou. 03/15/96-09/30/97.

Q. Ping Dou, Ph.D.

NIH R29. Functions of RB-protease(s) in apoptosis. Principal Investigator: Q. Ping Dou (50%). 04/15/96-02/28/01.

NIH R01. Growth Inhibition by IL-2 of IL2R+ oral carcinomas. Principal Investigator: Q. Ping Dou (10%). 04/01/98-03/31/01. (a subcontract from University of Pittsburgh)

Department of the Army Advanced Cancer Detection Center Research Grant (Moffitt). Significance of Bax-Dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer. Principal Investigator: Q. Ping Dou. 10/01/00-9/30/01.

Administrative Supplement from Moffitt Cancer control. Co-Principal Investigator: Q. Ping Dou.

Administrative Supplement from Moffitt Foundation. Co-Principal Investigator: Q. Ping Dou.

Agreement from University of North Texas. Co-Principal Investigator: Q. Ping Dou.

NIH R03. Tea Targeting Proteasome: A Role in Cancer Prevention. Principal Investigator: Q. Ping Dou (10%). 07/01/01-06/30/03.

Supplement for Correlative Studies Related to estrogen Receptor Negative (ER-negative) Breast Cancer (Moffitt CCOP Research Base). PI: Krischer; Co-Investigator: Q. Ping Dou.

U10 CA81920. A Clinical Trial of the Action of Isoflavones in Breast Neoplasia: Administration Prior to Mastectomy or Lumpectomy - A Pilot Study. PI: Krischer; Co-Investigator: Q. Ping Dou. 12/01/01-11/30/03

U10 CA81920. The Specific Role of Isoflavones in reducing Prostate Cancer Risks. PI: Krischer; Co-Investigator: Q. Ping Dou. 12/01/01-11/30/03

U10 CA81920. A Randomized Pilot Clinical Trial of the Action of Isoflavones and Lycopene in Localized Prostate Cancer: Administration Prior to radical Prostatectomy. PI: Krischer; Co-Investigator: Q. Ping Dou. 12/01/01-11/30/03

#### **Approved but not funded**

American Cancer Society. Induction of an RB-associated phosphatase and cancer cell apoptosis (**Score: the second decile**). Principal Investigator: Q. Ping Dou. 01/01/97-12/31/99.

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American Institute for Cancer Research. Tea polyphenols target proteasome-mediated Bax degradation pathway: Significance in prostate cancer prevention and treatment (**Score: 2.92**). Principal Investigator: Q. Ping Dou. 02/01/00-01/31/03.

**Present support**

NIH R01. Roles of polymorphic COMT, tea polyphenols and proteasome in cancer prevention. 20% Effort (PI: Q. Ping Dou). 04/01/06-03/31/11.

DOD Breast Cancer Research Program/IDEA Award. Synthetic  $\beta$ -Lactam Antibiotics as A Selective Breast Cancer Cell Apoptosis Inducer: Significance in Breast Cancer Prevention and Treatment. 20% Effort (PI: Q. Ping Dou). 03/01/04-3/31/07.

NIH R01. N-Thiolated  $\beta$ -Lactams. Co-Principal Investigator: Q. Ping Dou (5%) (PI: Ed Turos). 03/01/02-02/28/07. Total Direct Costs (to Dou lab)

DOD Breast Cancer Research Program-Concept Award. Examination of potential anti-tumor activity of N-thiolated  $\beta$ -lactam antibiotics in nude mice bearing human breast tumors. 5% Effort (PI: Q. Ping Dou). 10/01/04-09/30/06.

NIH R03. The Proteasome as Molecular Target of Grape Polyphenols. 5% Effort (PI: Q. Ping Dou). 12/01/04-11/30/06.

Wayne State University President's Research Enhancement Program Proposal. Enhancing chemo- and photodynamic therapy in breast cancer using nanotechnology. (Co-I: Q. Ping Dou; PI: Jayanth Panyam). 06/01/06-05/31/08.

VA Merit. Ubiquitin Protease System in Malignant Pleural Mesothelioma. 10% Effort. (Co-I: Q. Ping Dou; PI: Anil Wali). 09/01/06-08/31/10.

NIEHS P50 ES012395. Center for Urban African American Health. 3.0% Effort (Co-I: Q. Ping Dou; PI: John Flack). 06/01/05-05/31/07.

T32-CA09531-19 NIH Training Grant. "Training Program in the Biology of Cancer" (Ms. Kristin R. Landis-Piwowar; Mentor: Q. Ping Dou) 09/01/05-8/31/07.

NCI/NIH the Cancer Center Support Grant (PI: Ruckdeschel). Population Studies & Prevention Program (Program PI: Schwartz; Co-I: Q. Ping Dou, 5%) 10/01/05-9/31/10.

CellQuest, Inc. CellQuest, A Musaceas Plant Extract: Implications in cancer prevention. Principal Investigator: Q. Ping Dou. 10/01/02-09/30/06. (Approved)

Karmanos Cancer Institute Startup funds. Principal Investigator: Q. Ping Dou. 08/01/03 -.

Q. Ping Dou, Ph.D.

Karmanos Cancer Institute Indirect Account. Principal Investigator: Q. Ping Dou.  
08/01/03.

**Pending support**

NIH R01. Elucidation of molecular mechanisms for the ubiquitin-proteasome pathway in malignant pleural mesothelioma (Co-I: Q. Ping Dou; PI: Anil Wali). 10/01/05-9/30/10.

NIH R21. Molecular Study on Novel NCI Potential Anti-tumor Drugs. 15% Effort (PI: Q. Ping Dou). 04/01/05-03/31/07.

NIH R03. Targeting tumor endogenous copper with clioquinol. 5% Effort (PI: Q. Ping Dou). 07/01/05-06/30/07.

NIH R21. Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/07.

Alliance For Cancer Gene Therapy. Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Pa  
Total Direct Costs: \$449,991; Total Indirect Costs: \$44,999

DOD Breast Cancer Research Program-Concept Award. Determination of potential anti-cancer activity of synthetic acetylated EGCG analog prodrugs in nude mice bearing human breast tumor xenografts. 5% Effort (PI: Q. Ping Dou). 07/01/05-06/30/06.

DOD Breast Cancer Research Program-Concept Award. Synchronized Gene Silencing and Drug Delivery to Overcome Drug Resistance in Breast Cancer. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/06.

NIH R01. Synthesis and evaluation of prodrugs of green tea polyphenol EGCG analogs. 20% Effort (PI: Q. Ping Dou). 12/01/05-11/30/10.

DOD Prostate Cancer Research Program-Idea Development Award. MOLECULAR TARGETS OF NOVEL NCI POTENTIAL ANTICANCER DRUGS IN HUMAN PROSTATE CANCER CELLS. 20% Effort (PI: Q. Ping Dou). 10/01/05-09/30/08.

The Michigan Technology Tri-Corridor Fund, Fiscal Year 2005 Competition. DUAL-AGENT NANOPARTICLES TO OVERCOME DRUG RESISTANCE. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/07.  
(to Dou Lab)

Q. Ping Dou, Ph.D.

DOD Breast Cancer Research Program/IDEA Award. The potential use of the anti-alcoholism drug disulfiram in breast cancer prevention and treatment. 15% Effort (PI: Q. Ping Dou). 01/01/06-12/31/08.

DOD Prostate Cancer Research Program/Physician Research Training Award. Novel organic copper complex PDC-Cu for molecular therapy of prostate cancer facilitated by PET imaging (PI: Fangyu Peng; Mentor: Q. Ping Dou). 10/01/05-09/30/10.

Susan Komen Foundation. Nanoparticle-mediated combination photodynamic and chemotherapy to overcome refractory tumors. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 05/01/06-04/30/08.

Wilson Foundation. Targeting tumor endogenous copper with the antibiotic clioquinol: A novel approach for cancer-specific killing with no or low toxicity. 15% Effort (PI: Q. Ping Dou). 01/01/06-12/31/07.

VA Merit. Ubiquitin Protease System in Malignant Pleural Mesothelioma. 10% Effort. (Co-I: Q. Ping Dou; PI: Anil Wali).

NIH R01. Copper as a novel target for determining fate of AR and prostate cancer cells. 20% Effort (PI: Q. Ping Dou). 07/01/06-06/30/11.

NIH R21 (resubmission). Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/06-06/30/08.

NIH R01. The Chinese Thunder of God Vine: Active Components & Biological Targets in Cancer. 20% Effort (PI: Q. Ping Dou). 12/01/06-11/30/11.

NIH P01. AR-mediated cell cycle-dependent anti-apoptotic events in prostate cancer. (PI: Reddy GPV; Co-Directors: Dou QP and Menon M). Project #3: Targeting 26S proteasome for determining fate of AR and prostate cancer cells. 40% Effort (PI: Q. Ping Dou). 12/01/06-11/30/11.

DOD Breast Cancer Research Program-Concept Award. Chemosensitization of human breast cancer cells by an active compound purified from the Chinese medicine Thunder of God vine. 5% Effort (PI: Q. Ping Dou). 07/01/06-06/30/07.

NIH R01. Maspin in Hormone Refractory Prostate Cancer Intervention (Co-I: Q. Ping Dou, 5%; PI: Shijie Sheng). 12/01/06-11/30/11.

Q. Ping Dou, Ph.D.

NIH R01 (resubmission). Elucidation of molecular mechanisms for the ubiquitin-proteasome pathway in malignant pleural mesothelioma (Co-I: Q. Ping Dou; PI: Anil Wali). 12/01/06-11/30/11.

MICHIGAN ECONOMIC DEVELOPMENT CORPORATION (MEDC). Development of natural pharmaceuticals to protect against low-intensity radiation exposure. 5% Effort (Co-PI: Q. Ping Dou; PI: Michael C Joiner). 10/01/06-09/30/09.

National Natural Science Foundation of China (NSFC). Synthesis and Mechanistic Study of Catechin Glycosides as Proteasome Inhibitors. Co-PI: Q. Ping Dou (PI: Sheng Biao Wan). 10/01/06-09/30/08.

DOD Prostate Cancer Research Program-Idea Development Award. Targeting the proteasome/ NF $\kappa$ B/ Androgen receptor-mediated survival pathway to chemosensitize human prostate cancer cells. 20% Effort (PI: Q. Ping Dou). 12/01/06-11/30/09.

DOD Breast Cancer Research Program/IDEA Award. Overcoming breast cancer drug resistance by a medicinal compound isolated from Indian Winter Cherry. 15% Effort (PI: Q. Ping Dou). 12/01/06-11/30/09.

NIH R21 (resubmission). Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/07-06/30/09.

## **PUBLICATIONS:**

### **Original Observations in Referred Journals**

1. Chen KY and Dou QP NAD<sup>+</sup> stimulated the spermidine-dependent hypusine formation on the 18,000-dalton protein in cytosolic lysates derived from NB-15 mouse neuroblastoma cells. FEBS Letters, 1988; 229: 325-328
2. Chen KY and Dou QP. Isolation and characterization of an 18,000-dalton hypusine-containing protein from NB-15 mouse neuroblastoma cells. Biochem. Biophys. Acts, 1988; 971: 21-28
3. Dou QP and Chen KY. Two hypusine-containing proteins identified by metabolic labeling in chick embryo fibroblasts. J. Chin. Chem. Soc., 1989; 36: 443-450
4. Dou QP and Chen KY. Characterization and reconstitution of a cell-free system for NAD<sup>+</sup>-dependent deoxyhypusine formation on the 18 kDa-eIF-4D precursor. Biochem. Biophys. Acts, 1990; 1036: 128-137
5. Bradley DW, Dou QP, Fridovich-Keil JL and Pardee AB. Transformed and non-transformed cells differ in stability and cell cycle regulation of a binding activity to the thymidine kinase promoter. Proc. Natl. Acad. Sci. USA, 1990; 87: 9310-9314
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7. Fridovich-Keil JL, Gudas JM, Dou QP, Bouvard I and Pardee AB. Growth-responsive expression from the murine thymidine kinase promoter: Genetic analysis of DNA sequences. *Cell Growth. Diff.*, 1991; 2: 67-76
8. Dou QP, Markell PJ, and Pardee AB. Thymidine kinase transcription is regulated at G1/S phase by a complex that contains retinoblastoma-like protein and cdc2 kinase. *Proc. Natl. Acad. Sci. USA*, 1992; 89: 3256-3260
9. Dou QP, Levin AH, Zhao S, and Pardee AB. Cyclin E and cyclin A as candidates for the restriction point protein. *Cancer Res. (Advances in Brief)*, 1993; 53: 1493-1497
10. Dou QP, Zhao S, Levin AH, Wang J, Helin K, and Pardee AB. G1/S-regulated E2F-containing complexes bind to the mouse thymidine kinase gene promoter. *J. Biol. Chem.*, 1994; 269: 1306-1313
11. Dou QP, Molnar G, and Pardee AB. Cyclin D1/cdk2 kinase is present in a G1 phase-specific protein complex Y11 that binds to the mouse thymidine kinase gene promoter. *Biochem. Biophys. Res. Commun.*, 1994; 205: 1859-1868
12. Dou QP, An B and Will PL. Induction of a retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G1 arrest and apoptosis. *Proc. Natl. Acad. Sci. USA*, 1995; 92: 9019-9023
13. Dou QP, An B and Yu C. Activation of cyclin E-dependent kinase by DNA-damaging signals during apoptosis. *Biochem. Biophys. Res. Commun.*, 1995; 214: 771-780
14. Dou QP and Lui VWY. Failure to dephosphorylate retinoblastoma protein in drug resistant cells. *Cancer Res. (Advances in Brief)*, 1995; 55: 5222-5225
15. An B and Dou QP. Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 $\beta$ -converting enzyme-like protease as candidate. *Cancer Res. (Advances in Brief)*, 1996; 56: 438-442
16. Dou QP, Pardee AB and Keyomarsi K. Cyclin E—a better prognostic marker for breast cancer than cyclin D? *Nature Medicine*, 1996; 2: 254
17. An B, Jin J-R, Lin P and Dou QP. Failure to activate interleukin 1 $\beta$ -converting enzyme-like proteases and to cleave retinoblastoma protein in drug-resistant cells. *FEBS Letters*; 1996; 399: 158-162
18. Dou QP, An B, Antoku K and Johnson DE. Fas stimulation induces RB dephosphorylation and proteolysis that is blocked by inhibitors of the ICE protease family. *J. Cell. Biochem.*, 1997; 64: 586-594
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- kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. *Cell Death & Diff.*, 1998; 5: 1062-1075
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32. Nam S, Smith DM and Dou QP. Inhibition of proteasome activity *in vitro* and *in vivo* by ester bond-containing tea polyphenols. *J. Biol. Chem.*, 2001; 276: 13322-13330
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34. Smith DM and Dou QP. Green tea polyphenol epigallocatechin inhibits DNA replication and consequently induces leukemia cell apoptosis. *Intl. J. Mol. Med*, 2001; 7: 645-652
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41. Kazi A, Urbizu DA, Kuhn DJ, Acebo AL, Jackson ER, Greenfelder GP, Kumar NB and Dou QP. A Natural *Musaceas* Plant Extract Inhibits Proteasome Activity and Induces Apoptosis Selectively in Human Tumor and Transformed, but not normal and non-transformed, Cells. *Intl. J Mol Med.*, 2003; 12: 879-887
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- 54 Kuhn DJ and Dou QP. Direct Inhibition of Interleukin-2 Receptor  $\alpha$ -Mediated Signaling Pathway Induces G<sub>1</sub> Arrest and Apoptosis in Human Head-and-Neck Cancer Cells. *J Cell Biochem.*, 2005;95:379-390.
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- 56 Lu M, Dou QP, Kitson RP, Smith DM, and Goldfarb RH. Differential effects of proteasome inhibitors on cell cycle and apoptotic pathways in human YT and Jurkat cell. *J Cell Biochem*; Published Online: 19 Sep 2005
- 57 Chen D, Peng F, Cui QC, Daniel KG, Orlu S, Liu J, and Dou QP. Inhibition of prostate cancer cellular proteasome activity by a pyrrolidine dithiocarbamate-copper complex is associated with suppression of proliferation and induction of apoptosis. *Frontiers in Bioscience*, 2005; 10: 2932-2939.
- 58 Arbiser JL, Li XC, Hossain CF, Nagle DG, Smith DM, Miller P, Govindarajan B, DiCarlo J, Landis-Piwowar KR, Dou QP. Naturally Occurring Proteasome inhibitors from Mate tea (*Ilex paraguayensis*) Serve as Models for Topical Proteasome Inhibitors. *J Investigative Dermatology*, 2005; 125: 207-212.
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- 60 Landis-Piwowar KR, Chen D, Cui QC, Minic V, Becker FF, Banik BK, and Dou QP. Apoptotic-Inducing Activity of Novel Polycyclic Aromatic Compounds in Human Leukemic Cells. *Intl. J Mol Med.*, 2006;17(5):931-5.
- 61 **Yang HJ, Chen D, Cui QC, Yuan X, and Dou QP. Celastrol, a triterpene extracted from the Chinese Thunder of God Vine, is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. (A figure was selected as the cover of the issue) *Cancer Research* 66, 4758-4765, May 1, 2006**
- 62 Wan SB, Dou QP and Chan TH. Regiospecific and Enantioselective Synthesis of Methylated Metabolites of Tea Catechins. *TETRAHEDRON* 62: 5897-5904, JUN 19 2006.
- 63 Daniel KG, Landis-Piwowar KR, Chen D, Wan SB, Chan TH, and Dou QP. Methylation of Green Tea Polyphenols Affects their Binding to and Inhibitory Poses of the Proteasome  $\beta$ 5 Subunit. **(A figure was selected as the cover of the issue)** *Intl. J Mol Med.*, 2006; 18:625-32.
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- 65 Chen D, Cui QC, Yang HJ, and Dou QP. Disulfiram, A Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts *via* Inhibition of the Proteasome Activity. *Cancer Research*, (in press)
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#### **Invited Review Articles and Book Chapters:**

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5. Dou QP. Putative roles of retinoblastoma protein in apoptosis. Apoptosis, 1997; 2: 5-18
6. Dou QP and An B. RB and apoptotic cell death. Frontiers In Bioscience, 1998; 3: d419-430
7. Dou QP and Li B. Proteasome inhibitors as potential novel anticancer agents (invited review). Drug Resistance Updates, 1999; 2: 215-223
8. Dou QP and Nam S. Proteasome inhibitors and Their Therapeutic Potential (invited review). Expert Opinion on Therapeutic Patents, 2000; 10: 1263-1272
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11. Dou QP. Proteasome, tumor cell survival and drug resistance development (invited). Book Chapter, 2003; in press
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15. Dou QP and Goldfarb RH. Bortezomib/ PS341 (millennium pharmaceuticals) (invited review). IDrugs, 2002; 5: 828-834
16. Kazi A and Dou QP. Cell Cycle and Drug Sensitivity. In: CHEMOSENSITIVITY vol. 2, *In Vivo Models, Imaging, and Molecular Regulators*, In **Methods in Molecular Medicine** (edited by RD Bluementhal), Humana Press Inc, USA, 2004; 33-42
17. Daniel KG and Dou QP. Invited Book Review for *Essentials of Apoptosis: A Guide for Basic and Clinical Research* (Edited by: Xiao-Ming Yin, MD, PhD and Zheng Dong, PhD). Cancer Control, 2003; 10: 421
18. Kuhn D, Coates C, Daniel KG, Chen D, Bhuiyan M, Kazi A, Turos E, and Dou QP. Beta-Lactams and Their Potential Use As Novel Anti-Cancer Chemotherapies. Frontiers in Bioscience, 2004; 9: 2605-2617
19. Chen Di, Daniel KG, Kuhn DJ, Kazi A, Bhuiyan M, Li L, Wang Z, Wan SB, Lam WH, Chan T-H and Dou QP. Green Tea and Tea Polyphenols In Cancer Prevention. Frontiers in Bioscience, 2004; 9: 2618-2631
20. Daniel KG, Harbach RH, Guida WC, and Dou QP. Copper Storage Diseases: Wilson's, Menkes and Cancer. Frontiers in Bioscience, 2004; 9: 2652-2662
21. Daniel KG, Chan TK and Dou QP. Computational design, organic synthesis, and biological evaluation of polyphenol proteasome inhibitors: significance in drug discovery and cancer prevention (invited review). Drug Design Reviews-Online, 2005; 2: 227-232
22. Smith DM, Daniel KG and Dou QP. Exploiting the Ubiquitin-Proteasome Pathway for Anticancer Drug Discovery: Unanswered Questions and Future Directions (invited review). Letters in Drug Design & Discovery, 2005; 2: 77-84
23. Kuhn DJ and Dou QP. The Role of Interleukin-2 Receptor Alpha in Cancer. Frontiers in Bioscience, 2005; 10: 1462-1474
24. Daniel KG, Kazi A, Kuhn DJ and Dou QP. Anti-angiogenic and anti-tumor properties of proteasome inhibitors (invited review). Curr Cancer Drug Targets, 2005; 5: 529-541
25. Reddy GPV, Barrack ER, Dou QP, Menon M, Pelley R, Sarkar FH, and Sheng S. Regulatory processes affecting androgen receptor expression, stability and function: potential targets to treat hormone-refractory prostate cancer. J. Cell. Biochem., 2006; 98: 1408-1423.
26. Daniel KG, Chen D, Yan B and Dou QP. Copper-binding compounds as proteasome inhibitors and apoptosis inducers in human cancer. Frontiers in Bioscience, 2007; 12: 135-144
27. Dou QP. Lessons Learned from Art Pardee in Cell Cycle, Science and Life (invited review). J Cell Physiol, 2006; 209: 663-669.

#### **Patents:**

1. "Multicatalytic Protease (Proteasome) Inhibitors for Use as Anti-Tumor Agents", filled in on 12/16/97 (US, Ser. No. 60/069,804) and (International, WO 99/30707)
2. "Bax Degradation Involvement in Tumor Survival and Progression" (Inventors: Dou and Li; United States Letters Patent No. 6,692,927 issued on February 17, 2004)

3. "Tea Polyphenol Esters and Analogs Thereof in Proteasome Inhibition and Cancer Treatment" (Inventors: Dou, Nam and Smith; United States Letters Patent No. 6,713,506 issued on March 30, 2004)
4. "Bax Fragment Induced Tumor Cell Death", filed on 3/16/2000 (Publication Number- 00203921 WO; International Patent Class- A61K; PCT APP.No-PCT/US01/21971)
5. "Novel Beta-Lactams as Potential Anticancer Agents", filed on 4/18/2001 (USF Reference No.: 01A032)
6. "Chemical synthesis and biological activities of the polyphenols GCG (gallicocatechin-gallate) and EGCG (epigallocatechin-gallate)", filed on 8/29/2002
7. "Organic Copper Compounds as Potent Proteasome Inhibitors and Potential Anticancer Agents", filed on 4/17/2002 (USF Reference No.: 02A033)
8. "Computational Docking Model Development of Tea Polyphenol Proteasome Inhibitors: Applications to Rational Drug Design", filed on 12/18/2002 (USF Reference No.: 03A003)
9. "Polyphenol Proteasome Inhibitors, Synthesis, and Methods of Use", filed on 01/26/2004 (with the United States Patent and Trademark Office; US Patent Application No. 20040186167).
10. "(-)-Epigallocatechin Gallate Derivatives for Inhibiting Proteasome" (filed on August 19, 2004) (with the United States Patent and Trademark Office; US Patent Application No. 10/921,332).
11. Peracyloxyl Protected (-)-Epigallocatechin Gallate Derivatives and their Prodrugs as Proteasome Inhibitors and Cancer Cell Apoptosis Inducers" (filed on February 4, 2005) (with the USPTO; patent number to be assigned).
12. The positive feedback loop between proteasome inhibition and CMV-driven expression of a cell death gene significantly improves the efficacy of tumor cell killing: Use in Combinational Cancer Therapies (filed on Oct, 2006) (with the USPTO; patent number to be assigned).

#### **ABSTRACTS FOR POSTER PRESENTATION IN THE LAST FIVE YEARS:**

Fattman CL and Dou QP. Distinct ICE-like proteases mediate cleavage of retinoblastoma protein and poly(ADP-ribose) polymerase during apoptosis. AACR 88th annual Meeting, San Diego, California, April 12-16, 1997.

Dou QP, An B and Fattman CL. Retinoblastoma protein and the regulation of apoptosis. 7th SCBA International Symposium, Toronto, Canada, July 6-11, 1997.

Dou QP, Fattman CL, An B. Putative roles of retinoblastoma protein in apoptosis. 2nd World Congress on Advances in Oncology, Athens, Greece, 16-18 October, 1997

Fattman CL, An B, Zhang LL, Dineley K and Dou QP. Dephosphorylation and cleavage of the retinoblastoma protein during p53-dependent and -independent apoptosis of human breast carcinoma cells. Department of Defense, U.S. Medical Research and Material Command, Breast Cancer Research Program: An Era of Hope, Washington, D.C., October 31-November 4, 1997.

Dou QP. Cell cycle checkpoint proteins as apoptosis therapeutic targets. NATO Advanced Research Workshop at H. Lee Moffitt Cancer Center and Research Institute University of South Florida, Protein-Protein and Protein-Lipid Interactions in Signal Transduction: Use of Small Synthetic Molecules as probes and Therapeutic Agents, Clearwater Beach, Florida, December 5-9, 1997

Johnson DE, Rabinovitz A, Dou QP, Delach SM and Fattman CL. VP-16 treatment of T-leukemic cells results in activation of Cathepsins D and L via a caspase-dependent

- pathway. 1999 Annual Meeting of the American Society of Hematology, New Orland, Louisiana, December 2-7, 1999
- Dou QP and Li B. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. AACR 91<sup>st</sup> Annual Meeting, San Francisco, CA, April 1-5, 2000
- Dou QP, Gao G and Zhang X. Insulin/IGF-I receptor-mediated signal transduction pathway regulates G<sub>1</sub> phase-dependent Bcl-2 expression and tumor chemoresistance. AACR 91<sup>st</sup> Annual Meeting, San Francisco, CA, April 1-5, 2000
- Sun J, Li B, Lee C-S, Nam S, Coppola D, Hamilton AD, Dou QP and Sebti SM. The dipeptidyl proteasome inhibitor LCS-640 inhibits growth and induces apoptosis of the human lung adenocarcinoma A-549 xenografts in nude mice. AACR 91<sup>st</sup> Annual Meeting, San Francisco, CA, April 1-5, 2000
- Gao G and Dou QP. G<sub>1</sub> phase-dependent Bcl-2 expression correlates with chemoresistance of human cancer cells. 29<sup>th</sup> Annual Scientific Meeting of the International Society for Experimental Hematology, Tampa, FL, July 8-11, 2000
- Smith DM and Dou QP. Green tea targets human tumor cell DNA synthesis and consequently induces apoptosis. Poster presentation. New Molecular Targets for Cancer Therapy, St. Petersburg Beach, FL, October 14-17, 2000
- Smith DM, Nam S and Dou QP. Studies on tumor related targets of green tea polyphenols. Poster presentation. 2<sup>nd</sup> International Conference. Mechanisms of Cell Death and Disease: Advances in Therapeutic Intervention, North Falmouth, MA, June 2-6, 2001
- Daniel KG, Zhong Q, Gupta P and Dou QP. Etoposide induces activation of calpain in early stages of apoptosis. Poster presentation. 22<sup>nd</sup> Annual South East Pharmacology Society Meeting (SEPS), Drug Development Symposium (from Bench to Bedside), Clearwater, FL, October 4-6, 2001
- Kazi A, Smith DM, Zhong Q and Dou QP. Down Regulation of Bcl-X<sub>L</sub> Protein Expression by Green Tea Polyphenols Is Associated with Prostate Cancer Cell Apoptosis. Poster presentation. AACR-NCI-EORTC. Molecular Targets and Cancer therapeutics, Miami Beach, FL, October 29-November 2, 2001
- Dou QP, Kazi A, Smith DM and Kuhn DJ. Tea Polyphenols Target Proteasome-Mediated Bax Degradation in Prostate Cancer Cells: A Potential Role in Cancer Prevention. Poster presentation. An AACR Special Conference in Cancer Research. New Discoveries in Prostate Cancer Biology and Treatment, Naples, FL, December 5-9, 2001
- Nam S, Dalton WS, Trotti AM, Dou QP and Calvin DP. Cell adhesion to fibronectin (FN) through  $\beta 1$  integrins results in cell adhesion mediated ionizing radiation resistance (CAM-RR) in human LNCaP prostate cancer cells: the potential involvement of proteasome chymotrypsin-like activity. Poster presentation. AACR 93<sup>rd</sup> Annual Meeting, San Francisco, CA, April 6-10, 2002
- Kazi A, Hill R, Long TE, Turos E, Dou QP. Selective induction of apoptosis in human tumor cells by novel N-thiolated beta-lactams. Poster presentation. Molecular Targets for Cancer Therapy, St. Pete Beach, FL, October 11-15, 2002
- Nam S, Dalton WS, Dou QP, Jove R and Calvin DP. Proteasome chymotrypsin-like activity (PCA) is implicated in LNCaP prostate cancer cell adhesion mediated ionizing radiation (IR) resistance (CAM-RR). Poster presentation. Molecular Targets for Cancer Therapy, St. Pete Beach, FL, October 11-15, 2002
- David M. Smith, Zhigang Wang, Aslamuzzaman Kazi, Kenyon G. Daniel, Lian-hai Li, Tak-Hang Chan, and Q. Ping Dou. Green tea polyphenol proteasome inhibitors as potential cancer-preventative agents: computational design, organic synthesis and

- biological evaluation. Poster presentation. AACR Special Conference, Proteases, Extracellular Matrix, and Cancer, Hilton Head Island, South Carolina, October 9-13, 2002
- Lu M, Dou QP, Kitson RP, Smith DM, and Goldfarb RH. Differential Effects of Proteasome Inhibitors on Cell Cycle Progression and Molecular Modulation in Human Natural Killer Cells and T Lymphocytes. AAI, 2003
- Kazi A, Hill R, Long TE, Turos E, and Dou QP. Selective Induction of Apoptosis in Human Tumor Cells by Novel N-thiolated Beta-Lactams. Poster presentation. Moffitt Scientific Retreat, Tampa, FL, February 15, 2003
- Smith DM, Wang Z, Kazi A, Daniel KG Li LH, Chan TH and Dou QP. Green tea polyphenol proteasome inhibitors as potential cancer-preventative agents: computational design, organic, synthesis and biological evaluation. Poster presentation. Moffitt Scientific Retreat, Tampa, FL, February 15, 2003
- Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of  $\beta$ -Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, Detroit, MI, September 8, 2004
- Di Chen, Marina Chen, Kenyon G. Daniel, Deborah J. Kuhn, Kristin Landis, and Q. Ping Dou. Dietary Flavonoids as Proteasome Inhibitors and Apoptosis Inducers in Human Leukemia Cells and Their Structure-activity Relationships. Poster presentation. Third Annual AACR Cancer Prevention Meeting, Seattle, Washington, October 16-20, 2004
- Deborah Kuhn, Yang Wang, Vesna Minic, Cristina Coates, G. Suresh Kumar Reddy, Kenyon, G. Daniel, Jeung-Yeop Shim, Di Chen, Kristin R. Landis-Piwowar, Edward Turos, And Q. Ping Dou, Ph.D. Relationships of structures of N-methylthiolated beta-lactam antibiotics to their apoptosis-inducing activity in human breast cancer cells. Poster presentation. ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting, Pennsylvania Convention Center, Philadelphia, Pennsylvania, June 8-11, 2005
- Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of  $\beta$ -Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005
- Di Chen, Marina Chen, Kenyon G. Daniel, Deborah J. Kuhn, Kristin Landis, and Q. Ping Dou. Dietary Flavonoids as Proteasome Inhibitors and Apoptosis Inducers in Human Leukemia Cells and Their Structure-activity Relationships. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005
- Kristin R. Landis-Piwowar, Deborah J. Kuhn, Sheng Biao Wan, Di Chen, Tak Hang Chan, and Q. Ping Dou. Evaluation of Proteasome-Inhibitory and Apoptosis-inducing Potencies of Novel (-)-EGCG Analogs and their Prodrugs. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005
- Di Chen, Kenyon G. Daniel, Marina S. Chen, Deborah J. Kuhn, Kristin R. Landis Piwowar, Wai Har Lam, Larry M. C. Chow, Tak Hang Chan and Q. Ping Dou. Dietary and synthetic polyphenols as proteasome inhibitors and apoptosis inducers in human cancer cells. 5<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, Wayne State University School of Medicine, Detroit, MI 48201, April 23, 2005

Q. Ping Dou, Ph.D.

Di Chen, Qiuzhi Cindy Cui, Huanjie Yang, Fazlul H. Sarkar, G. Prem Veer Reddy, Shijie Sheng, Raul A Barrea and Q. Ping Dou. Clioquinol, A Therapeutic Agent For Alzheimer's Disease, Has Proteasome-Inhibitory, Apoptosis-Inducing And Anti-Tumor Activities In Prostate Cancer Cells And Xenografts. 6<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006

**Huanjie Yang, Di Chen, Qiuzhi Cindy Cui, Xiao Yuan, and Q. Ping Dou. Celastrol, A Triterpene Extracted From The Chinese Thunder Of God Vine, Is A Potent Proteasome Inhibitor And Suppresses Human Prostate Cancer Growth In Nude Mice. 6<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006 (The First Place Poster Award)**

Xiaohua Li, Di Chen, Shuping Yin, Yiwei Li, Huanjie Yang, Kristin R. Landis-Piwowar, Fazlul Sarkar, Prem Veer G. Reddy, Q. Ping Dou, Shijie Sheng. Proteasome Inhibition Up-regulates Apoptosis-sensitizing Maspin. 6<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006

Di Chen, Qiuzhi Cindy Cui, Huanjie Yang and Q. Ping Dou. Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts *via* inhibition of the proteasome activity. The AACR International Conference on Frontiers in Cancer Prevention Research, Boston, MA, November 12 - 15, 2006.

#### **INVITED ORAL PRESENTATIONS (IN THE LAST FIVE YEARS):**

Dou QP. Apoptosis control and cancer. Department of Pharmacology, University of Pittsburgh School of Medicine, February 7, 1997

Dou QP. Apoptosis control and cancer. *Cephalon*, Inc., March 11, 1997

Dou QP. RB and apoptosis. University of Pittsburgh Cancer Institute, Molecular Oncology Seminar Series, April 16, 1997

Dou QP. Activation of apoptotic death program in human cancer. H. Lee Moffitt Cancer Center & Research Institute at the University of South Florida, April 28, 1997

**Dou QP. Invited Speaker. Retinoblastoma protein and the regulation of apoptosis. 7th SCBA International Symposium, Toronto, Canada, July 6-11, 1997.**

Dou QP. Cell cycle and Apoptosis. University of Pittsburgh Cancer Institute, FAS-L Club, August 12, 1997.

Dou QP. Apoptosis regulation in breast cancer. Second Annual Pittsburgh Minisymposium on Basic and Translational Research in Breast Cancer, Center for Environmental and Occupational Health and Toxicology, University of Pittsburgh, August 16, 1997

Dou QP. Invited speaker. RB and apoptosis control. Center for Clinical Pharmacology, University of Pittsburgh School of Medicine, September 25, 1997

Dou QP. Invited Speaker. Targeting the Apoptotic Signaling Pathway in Human Cancer. Departments of Biochemistry & Molecular Biology and Microbiology & Immunology, University of North Texas Health Science Center at Fort Worth, September 29, 1997

**Dou QP.** Invited Speaker and Session Chairman. Putative roles of retinoblastoma protein in apoptosis. 2nd World Congress on Advances in Oncology, Athens, Greece, 16-18 October, 1997

**Dou QP.** Invited Speaker. Cell cycle checkpoint proteins as apoptosis therapeutic targets. NATO Advanced Research Workshop at H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Protein-Protein and Protein-Lipid Interactions in Signal Transduction: Use of Small Synthetic Molecules as probes and Therapeutic Agents, Clearwater Beach, Florida, December 5-9, 1997

**Dou QP.** Targeting ubiquitin/proteasom-mediated protein degradation pathway in human cancers. Research Progress Seminar Series at H. Lee Moffitt Cancer Center and Research Institute and University of South Florida, Tampa, Florida, October 29, 1998

**Dou QP.** Invited Speaker. Targeting ubiquitin/proteasom-mediated protein degradation pathway in human cancers. Department of Pharmacology and Therapeutics, University of South Florida College of Medicine, Tampa, Florida, February 17, 1999

**Dou QP.** Invited Speaker. Bax degradation by the proteasome: a survival mechanism used by human cancer cells. Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, Tampa, Florida, October 15, 1999

**Gao G** and **Dou QP.** G1 phase-dependent Bcl-2 expression correlates with chemoresistance of human cancer cells. Oral presentation. 29<sup>th</sup> Annual Scientific Meeting of the International Society for Experimental Hematology, Tampa, FL, July 8-11, 2000

**Smith DM** and **Dou QP.** Green tea targets human tumor cell DNA synthesis and consequently induces apoptosis. Poster presentation. New Molecular Targets for Cancer Therapy, St. Petersburg Beach, FL, October 14-17, 2000

**Dou QP.** Invited Speaker. Proteasome inhibitors as novel anticancer drugs. Cancer Research and Biotechnology in the I-4 Corridor, Moffitt Cancer Center & Research Institute, Tampa, Florida, August 21, 2000

**Dou QP.** Invited Speaker. Therapeutic potential of proteasome inhibitors in cancer prevention and treatment. Moffitt Cancer Center Research Retreat, Saddlebrook Resort, FL, May 19, 2001

**Smith DM** and **Dou QP.** Drug Discovery: Hunting for Cancer-Specific Molecular Targets - from Natural to Synthetic Compounds. Drug Discovery and Developmental Therapeutics Seminar, Moffitt Cancer Center & Research Institute, June 21, 2001

**Dou QP.** Invited Speaker. Proteasome inhibitors. New drugs in hematologic malignancies, Institute Of Hematology and Medical Oncology, "Seragnoli", University of Bologna, Bologna, Italy, November 12-14, 2001

**Dou QP.** Invited Speaker. Proteasome: a novel target for cancer prevention and treatment as well as anti-angiogenic therapy. Moffitt Grand Rounds, Moffitt Cancer Center & Research Institute, Tampa, FL, November 15, 2002

**Dou QP.** Invited Speaker. Identification of A Novel Molecular target for Anti-Copper and Anti-Angiogenic Therapies. Attenuon, L.L.C., San Diego, CA, November 25, 2002

**Dou QP.** Invited Speaker. Natural Proteasome Inhibitors and Chemoprevention. Karmanos Cancer Institute at Wayne State University, Detroit, MI, February 6, 2003

**Dou QP.** Invited Speaker. Proteasome, a novel target for anti-cancer drug discovery. Moffitt Scientific Retreat, Tampa, FL, February 15, 2003

**Dou QP.** Invited Speaker. Proteasome Inhibitors. Sopherion Therapeutics, Inc., New Haven, CT, March 13, 2003

- Dou QP. Invited Speaker. TBN. Department of Pathology, Wayne State University, Detroit, MI, June 25, 2003 (rescheduled)
- Daniel KG and Dou QP. Organic-copper complexes as a new class of proteasome inhibitors: the potential of converting a pro-angiogenic factor to a cancer cell-specific killer. Drug Discovery and Developmental Therapeutics Seminar, Moffitt Cancer Center & Research Institute, May 29, 2003
- Dou QP. Invited Speaker. Chemoprevention: targeting the proteasome. Karmanos Scientific Retreat, Detroit, MI, August 22, 2003
- Dou QP. Invited Speaker. The proteasome: a novel molecular target for cancer prevention and treatment. The Protease Group, Karmanos Cancer Institute, Detroit, MI, September 2, 2003
- Dou QP. Invited Speaker. Proteasome inhibitors and chemoprevention. Great lakes chemoprevention retreat, Maumee Bay Resort, Ohio, September 13, 2003
- Dou QP. Invited Speaker. Prostate Cancer Research Summary. Henry Ford Health System/WSU Prostate Journal Club, Detroit, MI, November 12, 2003
- Dou QP. Invited Speaker. Green tea and cancer prevention. Presentation to Cancer Biology Candidate Students, Karmanos Cancer Institute, Detroit, MI, April 3, 2004
- Dou QP. Invited Speaker. Synthetic beta-lactam antibiotics and a selective breast cancer cell apoptosis inducer: Significance in breast cancer prevention and treatment. The Breast Cancer Group, Karmanos Cancer Institute, Detroit, MI, May 6, 2004
- Dou QP. Invited Speaker. Tea Polyphenols. Karmanos Cancer Institute Research Retreat, Detroit, MI, August 27, 2004
- Dou QP. Invited Speaker. Discovery of Novel Small Molecules: Rational Design, Structure-Activity Relationships, Cellular Targets, and Potential Uses for Cancer Treatment and Prevention. The Developmental Therapeutics Group, Karmanos Cancer Institute, Detroit, MI, September 8, 2004
- Dou QP. Invited Speaker. Proteasome Inhibitors: Killing *via* Tumor-Specific Signaling. Basic and Translational Aspects of Cancer Cell Signaling Research Retreat, Karmanos Cancer Institute, Detroit, MI, January 14, 2005
- Dou QP. Invited Speaker. Searching for Natural and Pharmacological Proteasome Inhibitors for Cancer Prevention and Treatment. Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI, April 13, 2005
- Dou QP. Invited Speaker. Searching for Novel Polyphenol Proteasome Inhibitors for Cancer Prevention and Treatment. Department of Urology at the University of California San Francisco and San Francisco VA Medical Center, San Francisco, CA, April 28, 2005
- Dou QP. Invited Speaker. Roles of polymorphic catechol-O-methyltransferase gene, tea polyphenols and proteasome in cancer prevention. Population Studies and Prevention Joint Meeting, Karmanos Cancer Institute, Detroit, MI, June 14, 2005
- Dou QP. Invited Speaker. Tea polyphenols, Proteasome and Polymorphic Catechol-O-Methyltransferase: Use in Cancer Molecular Diagnosis, Prevention and Treatment. Department of Chemistry at McGill University and American Diagnostica Inc., Montreal, Quebec, Canada, August 22, 2005
- Kristin Landis (Mentor: Dou QP). Selected Speaker. Molecular Studies for the Regulation of Green Tea Polyphenol Biological Function by the Polymorphic Gene Product Catechol-O-Methyltransferase. Wayne State University Graduate Student Research Day, Detroit, MI, September 22, 2005

- Dou QP. Invited Speaker. Copper as a novel target for determining fate of AR and prostate cancer cells. Karmanos Cancer Institute Research Retreat, Detroit, MI, October 7, 2005
- Dou QP. Searching for natural proteasome inhibitors for cancer prevention and anti-cancer drug discovery. Department of Pathology Retreat, Wayne State University School of Medicine, Detroit, MI, August 27, 2005 (canceled)
- Landis-Piwowar KR, Wan SB, Daniel KG, Chen D, Chan TH, and Q. Ping Dou. Computational Modeling and Biological Evaluation of Methylated (-)-EGCG Analogs. Selected as a Minisymposium presentation. The 2006 AACR 97<sup>th</sup> Annual Meeting, Washington DC, April 1-5, 2006
- Dou QP. Invited Speaker. Roles of Diet, Biometals, and Environmental Factors in Cancer Prevention. Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China, April 19-20, 2006
- Dou QP. Invited Speaker. Nutrient-Gene-Environment Interactions and Molecular Prevention of Cancer. The Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, April 24, 2006
- Dou QP. Invited Speaker. Discovery of Novel Small Molecules for Cancer Therapies: - *From Nature to Laboratories and ... back*. The Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, April 25, 2006
- Dou QP. Invited Speaker. A Common Target of Dietary Factors, Traditional Medicine and Chemopreventive Agents in Human Prostate Cancer: the Significance in Molecular Prevention. 6<sup>th</sup> Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006
- Dou QP. Invited Speaker. Molecular Prevention of Human Cancer: An Example of Diet-Gene-Environment Interaction. Institution of Environmental Health Sciences, Wayne State University, Detroit, MI, June 15, 2006.
- Dou QP. Invited Speaker. A Lesson Learned from Thymidine Kinase Transcription at G1/S and later Stories. Symposium Honoring Dr. Pardee on the occasion of his 85th birthday. Boston, MA, June 24, 2006.
- Dou QP. Invited Speaker. Discovery of Novel Natural and Synthetic Compounds for Molecular Prevention of Human Cancer. Henry Ford Health Systems, Detroit, MI, August 3, 2006.
- Dou QP. Invited Speaker. Green Tea and Cancer Prevention. The Day of Wellness, Grosse Pointe War Memorial, Grosse Pointe, MI, September 16, 2006.
- Kristin Landis (Mentor: Dou QP). A Novel Pro-drug of Green Tea Catechin (-)-EGCG as a Potential Anti-Breast Cancer Agent. Wayne State University Graduate Student Research Day, Detroit, MI, September 21, 2006. (Session Winner)
- Dou QP. Invited Speaker. Green Tea and its Role in Cancer Prevention. Dow and Towsley Foundation Visit, Karmanos Cancer Institute Research, Detroit, MI, October 11, 2006
- Dou QP. Invited Speaker. Molecular Cancer Prevention and Therapies. Shandong Institute of Cancer Prevention and Treatment, Jinan, Shandong, China, October 18, 2006
- Dou QP. Invited Speaker. Discovery of Novel Small Molecules for Cancer Prevention and Therapies. Wuhan Botanical Garden/ Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, China, October 20, 2006

Q. Ping Dou, Ph.D.

Dou QP. Invited Speaker. The Proteasome as a Potential Cellular Target of Organic Toxic Metals. The 3<sup>rd</sup> International Symposium on Persistent Toxic Substances, Beijing, China, October 22-25, 2006