

Award Number: DAMD17-98-1-8526

TITLE: An Innovative Strategy for the Prevention and Treatment of Metastatic Prostate Cancer: Modified Tetracyclines as Chemotherapeutics

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

20001019 049

**-REPORT DOCUMENTATION PAGE-**Form Approved  
OMB No. 074-0188

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<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> October 1999	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (01 Oct 98 - 30 Sep 99)	
<b>4. TITLE AND SUBTITLE</b> An Innovative Strategy for the Prevention and Treatment of Metastatic Prostate Cancer: Modified Tetracyclines as Chemotherapeutics			<b>5. FUNDING NUMBERS</b> DAMD17- 98 - 1-8526	
<b>6. AUTHOR(S)</b> Balakrishna Lokeshwar, Ph.D.				
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<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for public release; distributed unlimited			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b>  During the period (10/01/98-09/30/99), we investigated the effects of a novel anti-metastatic drug, CMT-3, on prostate cancer cells <i>in vitro</i> and <i>in vivo</i> . We focused on three main areas: 1) Inhibition of metastases in the Dunning MAT LyLu rat model. Bone and lung metastases were induced in male Copenhagen rats by intravenous injection of tumor cells as per Geldof and Rao. Daily oral administration of CMT-3 began at 7 or 2 days prior or 1 day following implant. CMT-3 treated rats had a significant delay in development of pulmonary morbidity and a subsequent increase in survival which correlated with the predosing period. Paraplegia developed in 83-90% of controls, but was only 10-30% in all treatment groups. These results verify the potential of CMT-3 for use in human skeletal metastasis. 2) Mechanism of CMT-3-induced programmed cell death (PCD). Studies revealed that CMT-3 induces PCD by inhibiting mitochondrial function (suppression of <i>bcl-2</i> and <i>bclx proteins</i> / elevation of <i>bax</i> ) and inducing hydroxyl radicals (rapid activation of caspase-1 and 3 <i>in vivo</i> and <i>in vitro</i> ). 3) Influence of stromal cells on the cytotoxicity of antitumor drugs on prostate cancer cells <i>in vitro</i> . Organ specific stromal cells isolated from bone marrow and lung, but not dermal fibroblasts, inhibited the cytologic effects of CMT-3, doxyrubicin and taxol. This was partially reversed by treatment with hyaluronidase.				
<b>14. SUBJECT TERMS</b> Prostate			<b>15. NUMBER OF PAGES</b> 57	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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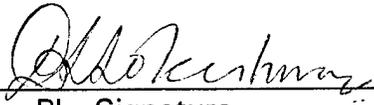
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**Introduction:**

The overall goal of this funded project is to test the utility of a novel orally administrable antimetastatic drug, CMT-3, a chemically modified non-antimicrobial tetracycline, against metastatic prostate cancer (CaP). Our previous studies using the Dunning rat MAT LyLu model showed strong antitumor activity of CMT-3 against both primary (localized) and metastatic tumors. We hypothesized that CMT-3 and its analogues, either alone or in combination with other bone-homing drugs, will inhibit bony metastasis of prostate cancer; the most common, painful and terminal stage of prostate cancer. The mechanism behind the anti-tumor activity of CMT-3 is its ability to inhibit tumor cell-derived matrix degrading metalloproteinases and cytotoxicity. While the anti-MMP activity of CMT-3 was attributable to its calcium chelating activity, the basis of the cytotoxicity was not understood. We had some preliminary data to show that CMT-3 induces apoptosis (programmed cell death, PCD) in proliferating tumor cells, but the pathway of PCD induction by CMT-3 was not discovered. Therefore we proposed to investigate the mechanism of CMT-3 induced cytotoxicity and delineation of CMT-3 induced PCD. The third objective of the project was to investigate the ability of metastatic tumors to resist chemotherapeutic agents, including CMT-3. We hypothesized, that one of the major components of resistance to therapy is the contribution of organ specific stroma. We proposed to study the interaction of organ specific stromal and tumor cells in response to antitumor drugs. We describe below, in the order of the original grant proposal, a detailed summary of experiments performed, results obtained, the implication of those results to the objectives outlined in the project and to the scientific understanding of the disease process.

**Aim. 1. Determine in vivo efficacy of CMT-3 in the prevention and/or elimination of induced or spontaneous skeletal metastasis of prostate tumors in animal models.** We assigned the following specific tasks to fulfil this aim.

***Task 1A. Determine in vivo efficacy of CMT-3 in the prevention and /or elimination of induced skeletal metastasis of prostate tumors in animal models.*** Expected duration: 12 months. We investigated the effects of CMT-3 on the Dunning rat model.

***Experimental Methods (Induction of skeletal metastasis and CMT-3 treatment):*** Bone and lung metastases of Dunning MAT LyLu cells were induced in male Copenhagen rats by injecting tumor cells intravenously while momentarily occluding the vena cava as per the method of Geldof and Rao<sup>1</sup>. We used four groups of six animals each to create this model and to test the efficacy of CMT-3. Adult Copenhagen rats were anesthetized with ketamine, a small midline incision was made, the inferior vena cava isolated and a small surgical "bulldog" clamp was placed on the vena cava. The vena cava was clamped for a maximum of 5 minutes. A bolus of  $5 \times 10^4$  Dunning MAT LyLu cells in 0.2 ml was injected into the lateral tail vein. The clamp was immediately removed to relieve intra abdominal pressure that pushes the cells into lumbar venous plexus. The incision was closed in two layers with 3-0 silk. The animals withstood the procedure well and usually recovered within two hours, without any noticeable discomfort. Post-operative posture, food and water intake was similar to that observed prior to the procedure. A suspension of CMT-3 (40 mg/kg) in 2% carboxy methyl cellulose (vehicle) was administered by oral gavage daily up to 4 weeks with no sign of systemic toxicity. Four groups of six animals each were treated with CMT-3 as follows: group I: pre dosed daily for 7 days; group II: predosed daily for 2 days; Group III: one day post implant; group IV (control): 2% carboxymethyl cellulose in water (vehicle) only. Treatment in all groups continued until the animals were euthanized. The criteria for euthanasia was either complete hind limb paralysis or acute respiratory distress. At necropsy the lungs, lumbar vertebrae and femurs were excised. Marrow plugs, from the femurs of paralyzed rats, were collected and cultured in MAT LyLu culture medium (RPMI containing 10% fetal bovine serum and 250 nM dexamethasone and gentamicin). The lungs and vertebrae were fixed in Bouin's and formalin respectively. The overall health of the animals was monitored by recording their weight two times a week. Urine was collected daily from 3 - 6 animals per group before drug dosing and blood was collected at necropsy. Spot urine

samples were analyzed for the urinary excretion of the bone matrix collagen cross link product deoxypyridoniline (DPD) using an ELISA (Pyrilynks-D, Metra Biosystems, Mountain View, CA).

**Results:** All animals that received tumor cell injection survived the initial surgery but began to develop acute pulmonary distress and paralysis. There was a significant delay in the development of pulmonary morbidity and subsequently an increase in survival in rats treated with CMT-3. We observed a 10.5% to 29.2% increase in the length of survival depending on the length of the predosing period. Significantly, while 83% of the animals developed paraplegia in the control group only 10% to 30% of the CMT-3 treated animals developed paraplegia regardless of the length of treatment. (**Fig 1**). Tumor cells were recovered from 50% of the marrow plugs of animals with paraplegia, nonparaplegic animals had no tumor cells in their marrow. There was also a significant difference in the extent of lung metastasis in animals treated with CMT-3 as compared to the controls. Although all animals suffered pulmonary distress, the animals in Group 1 and 2, predosed with CMT-3, had significantly smaller and fewer tumor foci in lungs (**Fig 2**). There was no evidence of tumor in any other organs such as liver, kidney or bladder using histological examination and /or primary tissue culture to recover metastatic tumor cells. Tumor foci were confined to lung, pleura, vertebrae and femoral bones<sup>2</sup> (**Appendix 1**). Analysis of urine samples for the bone collagen cross-link product DPD from tumor bearing rats yielded mixed results. All urine samples had detectable and significant amounts of DPD when normalized to total creatinine levels (**Fig 3**). However, there was no significant difference in the levels of DPD in treated vs control animals. Furthermore, there was no significant trend in the urinary DPD levels from the day of tumor cell injection to the day animals were euthanized. These observations contrast with that observed in humans with skeletal metastasis where there is an increasing trend with the progression of bony metastasis.<sup>3</sup>

**Task 1B. Study the effect of CMT-3 on the spontaneous skeletal metastasis model ARCAP orthotopic tumor implant model:** We are currently in the process of testing CMT-3 or other CMT-3 analogues in the treatment of the *spontaneous skeletal metastasis* in a human tumor xenograft model. We used the androgen repressed CaP model of Zhao et al<sup>4</sup>. We injected 12 nude mice with ARCAP cells orthotopically as described by Stephenson et al<sup>5</sup>. Four of the 12 animals injected with cells died within two weeks apparently due to viral infection. Of the remaining 8 mice, although all developed abdominal swelling and tumor of the prostate within 68 to 97 days after tumor cell injection, none developed skeletal metastasis. None of the animals developed hind limb paralysis. Bone marrow plugs cultured from the femurs of all the tumor cell injected animals did not contain any clonogenic tumor cells.

**Aim 2. Investigate the mechanism of CMT-3 induced cytotoxicity and programmed cell death (PCD).**

**Task 2: Investigate the mechanism of CMT-3 induced cytotoxicity and PCD in established cell lines, primary human prostate cancer cells, and bone metastasis of tumor cells co-cultured with organ specific stroma. Expected duration: Months 12-24.**

We have accomplished significant progress in identifying pathways of CMT-3 induced cytotoxicity and PCD. We have established the kinetics of induction of PCD and identified the mechanism by which PCD is executed.

**Experimental methods: Cell culture:** Human prostate tumor cell lines (LNCaP, PC-3, and DU 145) and FHS 738, a human fetal lung fibroblast cell line, were from ATCC, Rockville MD., Baltimore, MD. Cultures were maintained in complete medium composed of RPMI 1640 with 10% FBS and gentamicin (10 µg/ml). MAT LyLu cells were maintained in the complete medium with added 250 nM dexamethasone.

**Assay for anti-proliferative activity:** A colorimetric methyl tetrazolium reduction assay (MTT assay) was used to assay the cytotoxic effects of CMT-3 and doxycycline (DC). DNA synthesis activity, as an indicator of cell proliferation activity, was assayed by pulse labeling the cell cultures with <sup>3</sup>H-thymidine. Amount of macromolecular incorporation of <sup>3</sup>H-thymidine was quantitated by counting acid precipitable radioactivity. All experiments were repeated at least three times for each cell line.

**Cell death assay to measure chromatin fragmentation:** Drug induced apoptosis was assayed using the Cell Death

detection ELISA plus kit (Boehringer Mannheim Biochemicals). The assay uses an one-step sandwich immunoassay to detect nucleosomes released into the culture media of drug treated cells. Manufacturer supplied positive and negative controls and medium blank were used to compare the results from individual experiments.

*Determination of Phosphatidyl serine (PS) translocation:* Drug induced translocation of PS was assayed by cell surface binding of FITC labeled Annexin V using the ApoAlert Kit CLONTECH, Palo Alto, CA). Annexin V (or FITC-Annexin V) has a high affinity for PS and its binding to intact cells is indicative of PS translocation. Cells treated with the drug for 0-24 hours were harvested by a nonenzymatic method and labeled with FITC-Annexin V. Labeled cells were analyzed by flow cytometry using a Coulter EPICS XL instrument. Median FITC fluorescence channels were compared from sample to sample. Median fluorescence channels of untreated cells were within one S.D. of unlabeled cells.

*Determination of Mitochondrial permeabilization:* The uptake of the mitochondria specific-dye (rhodamine-123, Sigma Chemicals) was used to measure the changes in mitochondrial permeabilization. Greater the uptake of rhodamine-123 (rh123) the greater the permeabilized status of the mitochondria. Once inside the mitochondria, rh123 emits an intense green fluorescence that can be measured by flow cytometry. Cultures were incubated with CMT-3 or DC for periods of time ranging from 15 min to 24 h. At the end of the incubation, 10 µg/ml rhodamine-123 was added to the culture. Fifteen minutes later cells were harvested and mitochondrial fluorescence activity was measured in a flow cytometer. Median fluorescence channels of untreated and treated samples were compared. Cells with permeabilized mitochondria will have a higher median fluorescence channel.

*Determination of Caspase-3 activation:* Drug induced activation kinetics of Caspase-3 (CPP-32), one of the downstream caspases, was assayed using the ApoAlert-CPP32 fluorescent assay kit. The kit detects the shift in the fluorescent emission of 7-amino-4-trifluoromethyl coumarin (AFC). AFC is conjugated to a specific tetrapeptide sequence DEVD as a substrate of activated CPP-32. Upon hydrolysis of the DEVD-AFC conjugate the liberated AFC emits an intense yellow-green fluorescence at 505 nm, this is quantitated using a spectrofluorometer. Cells were exposed to drugs for periods of time ranging from 5 min to 4 hours. Cytosolic extracts of the treated cells were incubated with DEVD-AFC for 15 min and the fluorescent activity was measured in a spectrofluorometer.

*Detection of  $bcl_2$ ,  $bcl_x$  and  $bax$  by immunoblotting:* Cultures were treated with CMT-3 or DC for various periods of time. Drugs were removed by washing and fresh culture medium was added. At 24 hours after treatment, cells were lysed in SDS-gel sample buffer and boiled for 3 min under reducing conditions. Samples were fractionated by SDS-Polyacrylamide (12%) electrophoresis and electrophoretically blotted to PVDF membrane. Blotted samples were immunohistochemically detected after successive incubations with primary antibody ( $bcl_2$  and  $bcl_x$  from Transduction Labs, and  $bax$  antibody from Calbiochem Corporation) and peroxidase labeled second antibody. Bound antibody was visualized on x-ray film using the ECL detection technique (Amersham Life Sciences, Skokie, IL). Supplier provided positive controls were run in parallel lanes to ascertain corresponding protein bands. Blots were photographed with the Kodak DC20 digital camera and analyzed with gel analysis software (Kodak 1DAS software).

**Results:** CMT-3 and doxycycline both inhibited cell proliferation as determined by  $^3\text{H}$  thymidine assay. CMT-3 was at least 10x more potent than doxycycline (DC) on all the CaP cell lines tested. This is a confirmation of our earlier studies using MTT assay<sup>6</sup>. Although the results shown in **Fig 4** are for three CaP cell lines, similar results showing higher antiproliferative activity was observed for all the prostate and other cell lines tested to date. We next examined the ability of CMT-3 to induce PCD in both CaP and non-CaP cells. As shown in **Fig 5**, CMT-3 but not DC was able to induce PCD and nucleosomal degradation in all the four CaP cell lines we routinely maintain in the laboratory. A minimum of 4 hour incubation with the drug at the 50% cell proliferation inhibition dose (e.g., 1.5 µg CMT-3/ml) was essential to observe a significant levels of nucleosome release into the medium. As shown in **Fig 6**, a significant decrease in PCD activity was observed in a normal fibroblast cell line FHS when they were made quiescent by serum starvation. We next examined the effect of CMT-3 and DC on early events associated with PCD, namely translocation of phosphatidyl serine (PS) using cell surface FITC-annexin V binding analysis by flow cytometry. As shown in **Fig 7**, there was a rapid increase in the translocation of PS indicating a rapid induction of

PCD by CMT-3. Conversely DC did not induce significant translocation of PS as indicated by lower binding of FITC-annexin V. Since a common mechanism of PCD involves alteration of cell death regulating proteins, notably *bcl2*, *bax* and *bclx*, we investigated the synthesis and accumulation of these proteins in cells treated with CMT-3. As shown in **Fig 8**, we observed a significant decrease in the levels of *bcl2* and *bclx* (both suppressors of PCD) however, the PCD promoting protein *bax* showed a steady increase in cells treated with CMT-3, at nonlethal dose. Next we examined whether CMT-3 and DC induce down-stream events in PCD such as the death-affecting caspases, e.g. caspase-3 (CPP32). As shown in **Fig 9**, we observed a rapid activation of CPP32. Again, the activity peaked within 90 min of treatment with CMT-3, but not with DC. Rapid induction of the down stream PCD events such as caspase-3 activation indicated that CMT-3 might affect mitochondria of tumor cells. CMT-3-induced mitochondrial permeability changes were assayed by rhodamine 1243 (Rh123) uptake<sup>7</sup>. Uptake of Rh 123 is controlled by mitochondrial potential. An increase in the uptake of Rh 123 is indicative of mitochondrial permeability. As shown in **Fig 10**, we observed a time and dose -dependent increase in the RH123 fluorescence as indicated by an increase in the median channel fluorescence. A rapid irreversible increase in Rh 123 was observed in CaP cells incubated with CMT-3. Interestingly, DC did not increase the Rh123 fluorescence. Change in mitochondrial permeability is also correlated with the depolarization of mitochondrial potential. This is measured by a shift in the fluorescent emission spectra of the dye JC1<sup>8</sup>. JC1 fluorescence shift, in cells treated with CMT-3, was as rapid as it was for Rh123 fluorescence indicating, it is indeed, due to an alteration of mitochondrial potential (data not shown). A common mechanism by which cytotoxic drugs induce mitochondrial permeability changes is by the generation of reactive free radicals e.g. hydroxyl radical ([OH]). Production of hydroxyl radicals in cells treated with CMT-3 or DC was investigated using a fluorescent dye 2,7 dichlorocarboxy fluorescein-diacetate (DCF-DA)<sup>9</sup>. Cells were loaded with DCFDA and treated with CMT-3. Flowcytometric analysis was used to detect the fluorescein levels in the cells, an increase in the median fluorescence channel is indicative of an increase in hydroxyl free radical. As shown in **Fig 11**, a rapid increase in the median fluorescence channel, indicative of the increase in the cellular accumulation of fluorescein, was observed in cells treated with CMT-3 but not DC. The specificity of the increase in fluorescent intensity was verified by measuring fluorescence intensity of cells treated with both DCF-DA and 0.1 mM hydrogen peroxide. Rapid production of hydroxyl radicals and resulting lipid peroxides result in profound damage to mitochondria leading to the initiation and progression of apoptosis. Based on these results we conclude that the basis of cytotoxicity of CMT-3 is its mitochondrial toxicity as evidenced by the increase in mitochondrial permeabilization and generation of hydroxyl radicals. These findings, however, do not rule out cell surface initiation of apoptosis (ligand mediated apoptosis signal transduction). We are currently investigating other pathways of PCD induction by this interesting and potent antitumor drug.

**Aim 3. Investigate the role of stromal contribution in drug induced cytotoxicity on tumor cells. Task3. Co-culture stromal cells isolated from bone and lungs with tumor cells and investigate the cytotoxic effects of at least two antitumor drugs including CMT-3. Expected duration: 12-24 months.**

At the cellular and molecular level how CaP cells develop resistance to chemotherapy, following androgen ablation, is an intriguing phenomenon. Among several non mutational mechanisms that regulate chemosensitivity to anticancer drugs, cell-cell and cell-extracellular matrix (ECM) interactions are important<sup>10</sup>. Normal prostatic epithelial cells rely on their interactions with both stromal cells and the extracellular matrix (ECM) for their survival<sup>11</sup>. Prostate tumor cells preferentially metastasize to lumbar and vertebral bones, lungs and liver. Whether tumor cells interact with the organ specific stroma or ECM to grow and develop resistance to anticancer drugs is not known at present. It may be argued that stromal cells, or the factors released by them, may alter the response of tumor cells to anticancer drugs. If indeed, the stromal cells or the ECM secreted by the normal cells alter the response of tumor cells to cytotoxic drugs, we should be able to demonstrate such activity *in vitro* using appropriate co-culture systems. Our first objective in this aim was to determine whether the sub-population of tumor cells, isolated from various organ metastasis, respond differently to cytotoxic drugs such as doxorubicin (**dxr**) or CMT-3. Our second objective was to examine, whether the presence of organ specific stroma influences the response to

tumor cells to these cytotoxic drugs. Evidence is presented to show that stromal cells and stromal-ECM interaction modulate CaP cell response to two anticancer drugs.

**Experimental methods:** *Cell cultures:* In addition to established CaP cell lines and a human lung fibroblast cell line (FHS 388) several primary human epithelial and fibroblasts were established. All primary cultures were established in the authors' laboratory. Human prostate specimens, obtained after patients' informed consent, were used to set up primary prostatic epithelial, fibroblast and smooth muscle cell cultures. The identity of each individual batch of primary cultures was histologically confirmed by immunocytochemical staining using anti-cytokeratin antibody (for epithelial cells), anti-vimentin/alpha-actin (for fibroblasts) and factor VIII antibody (endothelial cells). Primary cultures cells that were >90% positively stained with a marker antibody were used in co-cultures with tumor cells.

*Isolation of tumor cells and stromal cells:* MAT LyLu cells were induced to form bone and lung metastasis in 90-100 day old male Copenhagen rats by injecting  $5 \times 10^4$  cells into a tail vein while momentarily occluding the vena cava following a midline incision [15]. About 50% of the animals developed hind limb paralysis within 16 days of tumor cell injection. Animals were euthanized and tumor cells were isolated from the femurs and the lungs by enzymatic dissociation. Cell suspensions, thus obtained, were cultured in a complete medium (RPMI + 10% fetal bovine serum with gentamicin (0.02%) and 250 nM dexamethasone). The tumor cells outgrew other lung or marrow tissue cells in one passage, and could be easily detached from the culture flask for further passaging. The tumor cells from femurs were named MAT-Bone (MAT-B) and those from the lung, MAT-Lung (MAT-L). Both MAT-B and MAT-L cells were plated in multi-well culture plates to examine the cytotoxicity of CMT-3 and Dxr

*Preparation of ECM from organ specific stromal cells:* Stromal fibroblasts from lung and bone marrow were cultured in 24-well culture plates. When the cultures were confluent, the cells were dissolved in 0.015% ammonia and then the plates were washed extensively, to neutralize the ammonia. This process resulted in a thin coating of ECM in culture wells. To test the effect of this organ specific ECM on CMT-3 (CollaGenex Pharmaceutical Inc., New Town, PA) or Dxr (Sigma Chemicals, Inc., St. Louis, MO) induced cytotoxicity, we used MAT-B and MAT-L cells cultured *in vitro* for over five passages, so that their sensitivity to CMT-3 or Dxr was comparable. After five passages, the 50% growth inhibition dose ( $GI_{50}$ ) of MAT-B and MAT-L cells were comparable to that of the parent MAT-LyLu cells ( $2.36 \pm 0.86 \mu\text{g/ml}$ ). The MAT-L and MAT-B cells were plated on the ECM-coated wells and cultured for 24 hours. The cells were then exposed to CMT-3 and dxr and the cytotoxicity was measured 48 hours later by the methyl-tetrazolium reduction (MTT) assay. As a control, human tumor cells were also cultured in wells coated with Matrigel (Collaborative Research Inc., Bedford, MA). Matrigel is a mouse fibrosarcoma tumor-derived solubilized basement membrane. We compared the cytotoxic effect of CMT-3 and dxr on tumor cells cultured with or without Matrigel coated ( $10 \mu\text{g/ml}$ ) tissue culture wells.

*Assay of drug-induced cytotoxicity:* DU145 cells were plated on ECM prepared from either human lung fibroblasts or prostatic fibroblasts and tested for CMT-3 and dxr cytotoxicity. DU145 and prostatic fibroblasts (PF-98-1) were cultured in Transwell plates (Costar-Coring, Boston, MA) with 3 micron filter inserts. DU145 cells were cultured in the top chamber and the prostatic fibroblasts were cultured in the bottom chamber. In this setting, there is free flow of diffusible molecules between PF-98-1 and DU145 cultures. After two days of co-culture, the cells were exposed to CMT-3 or dxr for 48 hours. The MTT assay was used to measure cell viability at the end of the incubation.

**RESULTS:** The cytotoxicity of CMT-3 on cells (MAT-B or MAT-L), cultured on ECM from rat lung fibroblasts, was not significantly different from that obtained for the same cells cultured without the ECM ( $GI_{50}$ :  $3.1 \pm 0.7 \mu\text{g/ml}$ ). On the other hand, the cells cultured on the ECM of bone marrow fibroblasts showed a significant decrease in cytotoxicity to CMT-3. (**Fig 12**). The human prostate cancer DU145 cells cultured on ECM showed less sensitivity to CMT-3 induced cytotoxicity. The  $GI_{50}$  for CMT-3 was 3.7, 1.7 x higher when cultured on ECM from either from prostate or lung fibroblasts than when they were cultured alone [**Fig 13**]. The  $GI_{50}$  of CMT-3 for MAT-

B or MAT-L cells, cultured on bone marrow fibroblast ECM, was significantly higher ( $GI_{50}$   $7.2 \pm 1.2$   $\mu\text{g/ml}$ ) than that of the same cells cultured without ECM ( $GI_{50}$   $2.5 \pm 0.7$   $\mu\text{g/ml}$ ). Thus CMT-3 was less cytotoxic to both normal stromal and Prostate cancer cells when co-cultured. In a parallel experiment when DU145 cells (or MAT-LyLu cells) were co-cultured with normal prostatic epithelial cells no down modulation of CMT-3 induced cytotoxicity was observed (data not shown). Similarly, MAT-LyLu cells plated on ECM prepared from normal prostatic epithelial cells did not show an appreciable decrease in CMT-3 induced cytotoxicity (data not shown). We found that MAT-LyLu cells stimulated a 4- to 5-fold increase in MMP-2 production by HMVE cells, under co-culture condition. We could not detect MMP-9 levels in the culture conditioned media using the commercially available MMP-9 ELISA kit, as it was not sufficiently sensitive. Interestingly, ECM from either fibroblasts or HMVE cells did not induce MMP-2 production in MAT LyLu or DU145 cells as determined by zymography (data not shown).

**KEY ACCOMPLISHMENTS:**

1. The results obtained in Task 1 strongly suggest the utility of CMT-3 in treating skeletal metastasis in patients. Oral bioavailability, relative non-toxicity (at least in rodents) at the pharmacologically achievable dose and site specific accumulation are the advantages of CMT-3 to treat prostate cancer metastatic to bone.
2. Results accomplished under Task 2 demonstrated that (a) cytotoxic activity of CMT-3 is largely due to its ability to induce rapid programmed cell death. (b) CMT-3 induced a series of cell surface (PS translocation), cytoplasmic (caspase-3 activation), and nuclear events (chromatin fragmentation), associated with apoptosis. (c) Only CMT-3, not doxycycline, was able to induce significant apoptotic activity at pharmacologically achievable doses ( $\leq 5 \mu\text{g/ml}$ ). (d) Induction of apoptosis by CMT-3 is dependent upon the proliferation status of the cells. (e) CMT-3 treatment permeabilized mitochondria and affected the synthesis of the apoptosis associated proteins, bax, bcl<sub>2</sub>, and bcl<sub>x</sub>. Therefore, CMT-3 is a potent anti-cancer drug due to its pro-apoptotic activity and antiproliferation activity
3. Rapid permeabilization of mitochondria either as a result of or due to the production of reactive hydroxyl radical is the molecular basis of the cytotoxic activity of CMT-3.
4. The results obtained in task 3 show that it is the stromal-tumor epithelial cell interaction, and not the tumor-normal epithelial cell interaction, that protects both cell types from drug-induced cytotoxicity.

## REPORTABLE OUTCOMES

### Manuscripts:

**Bal L. Lokeshwar** MMP Inhibition in prostate Cancer. *Annal NY Acad Sci.* 1999, 878:271-289.

Marie G. Selzer, Baoqian Zhu, Norman L. Block and **Bal. L. Lokeshwar**. CMT-3, a chemically modified tetracycline, inhibits bony metastases and delays the development of paraplegia in a rat model of prostate cancer. *Annal NY Acad Sci.* 1999, 878:678-682.

Baoqian Zhu, Norman L. Block, and **Bal L. Lokeshwar**. Interaction between stromal cells and tumor cells insuces chemoresistance and matrix metalloprotease secretion. *Annal NT Acad Sci.* 1999, 878:642-646.

### Abstracts:

**Bal L Lokeshwar**, Eva Escatel, Heather L. Houston-Clark and Baoqian Zhu. (1999) Rapid induction of apoptosis signalling as a mechanism of cytotoxic activity by a chemically modified tetracycline, a novel antitumor drug. *In Signal Transduction and Therapeutic Strategies*. Miami Nature Biotechnology Short Report **10**: Adv. in Gene Technology: Ed: Whelan, W J *et al.* Oxford U Press London, (U.K).

**Lokeshwar, B.L.**, Zhu B-Q., Block, N.L. Organ-specific stromal cells and ECM modify chemoresistance of prostate tumor cells. (1999). *Proc. Amer. Assoc. Cancer Res.* **40**: 2829A

**SUMMARY OF FINDINGS:** The results obtained in Aim 1, using the induced metastasis model (Dunning MAT LyLu), clearly showed that CMT-3 prolongs development of paraplegia and lung morbidity when given orally, without causing systemic toxicity. Results obtained under Aim 2 showed that the cytotoxic activity of CMT-3 was largely due to its ability to induce rapid programmed cell death. CMT-3 induced a series of cell surface (PS translocation), cytoplasmic (caspase-3 activation), and nuclear events (chromatin fragmentation), associated with apoptosis. Only CMT-3, not doxycycline, was able to induce significant apoptotic activity at pharmacologically achievable doses ( $\leq 5 \mu\text{g/ml}$ ). Induction of apoptosis was dependent upon the proliferation status of the cells, CMT-3 did not induce significant apoptotic activity in non-proliferating (quiescent) cells. CMT-3 treatment permeabilized mitochondria and affected the synthesis of the apoptosis associated proteins, bax, bcl<sub>2</sub>, and bcl<sub>x</sub>. It up-regulated bax but down regulated bcl<sub>2</sub>, indicating its strong pro-apoptotic function. The basis of mitochondrial toxicity and induction of apoptosis by CMT-3 is likely to be its ability to induce hydroxyl radical formation and mitochondrial permeabilization. Therefore, CMT-3 is a potent anti-cancer drug due to its pro-apoptotic activity and antiproliferation activity. The results obtained under Aim 3 showed that there is a *bi-directional communication* between tumor cells and the stroma. These results also show that during tumor-stroma interactions, the tumor cells can activate endothelial cells to become invasive by inducing MMP secretion, or the excess MMP-2 thus secreted may aid further angiogenesis and tumor cell infiltration. The outcome of this communication depends closely on the organ specific origin of the stroma. The bi-directional communication may be established through organ-specific, stroma-derived, soluble stromal factors and ECM. The tumor-stromal interaction can potentially alter the response of tumor cells (as well as stromal cells) to cytotoxic drugs, suggesting it to be a key factor in the failure of chemotherapy for Prostate cancer. In addition, the tumor-stroma interaction possibly regulates the secretion of matrix-degrading enzymes and, therefore, may also regulate the invasion and metastasis of Prostate cancer

**FUTURE DIRECTIONS:** One potential problem with our analysis could be that we measured DPD levels in spot urine instead of 24 hour urines. Our discussions with other scientists in the bone disease area convinced us that we need to measure DPD levels in 24 pooled urines. We plan to use this 24 hour urine collection and analysis protocol in the next experiment, on this and other models of skeletal metastasis.

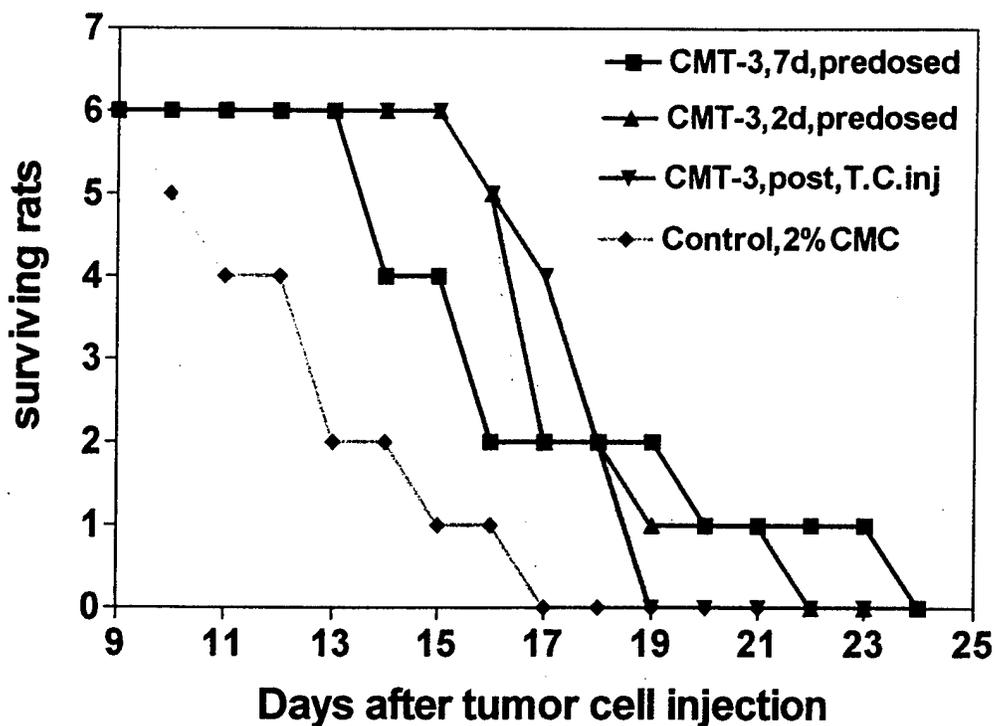
We are currently in the process of generating a newer bone metastatic prostate tumor cell line, using the PC-3 ML line of CaP cells (obtained from Dr. Mark Sterns, Medical College of Pennsylvania, Philadelphia, PA) by repeated injection and enrichment of tumor cells from nude mouse femurs. We will initiate the drug treatment in the PC-3ML, orthotopically generated, xenograft nude mice once the incidence of paraplegia in the test animals exceeds 20%.

At present, it is unclear why both conditions (e.g., ECM and co-culture situations diminish the response of tumor cells to CMT-3 to the same degree. It is possible that both diffusible (co-cultures) and contact-mediated factors (ECM) protect tumor cells from cytotoxic drugs via similar pathways (i.e., induction of a multidrug resistance phenotype). In any event, a 2-3 fold increase in the GI<sub>50</sub> of a cytotoxic drug due to stromal influence, may be enough to cause failure of chemotherapy with that drug *in vivo*. Due to the poor therapeutic index (ratio of tumor toxicity to normal tissue damage) of anticancer drugs, quite often the recommended clinical dose is within range of the *in vitro* cytotoxicity. Thus a combination treatment involving both cytotoxic drugs and agents that disrupt the stromal protection of drug-induced cytotoxicity, may be a preferred modality for the treatment of Prostate Cancer

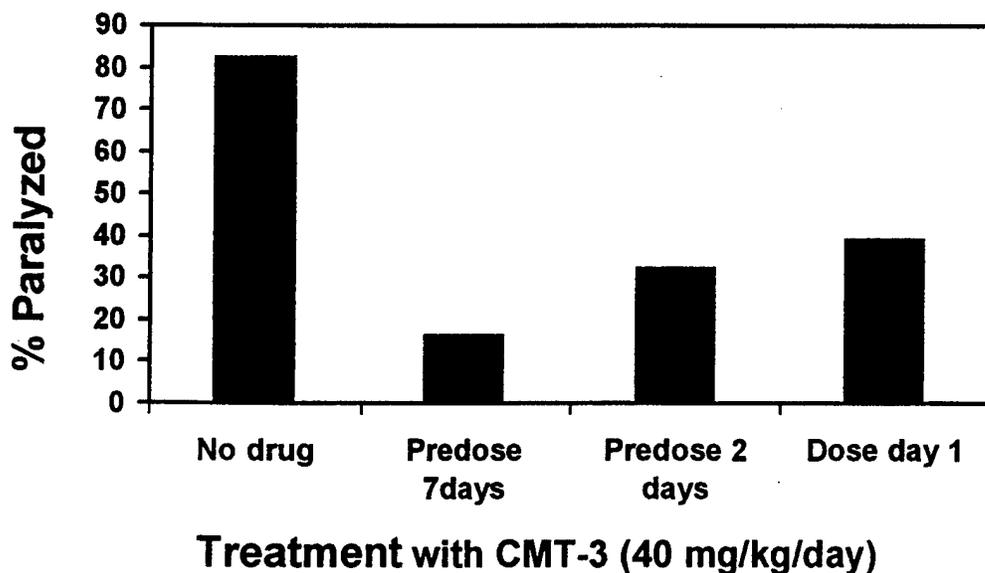
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### CMT-3 Prolongs Survival of Rats with MAT-LyLu Tumor



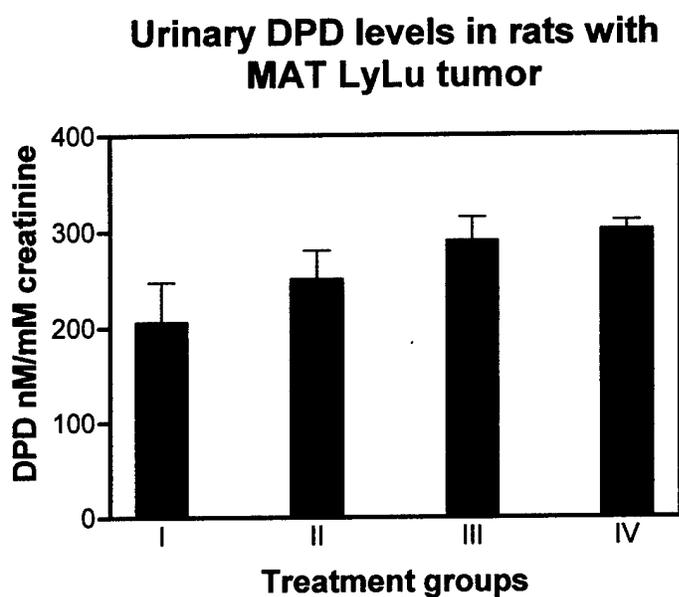
### Tumor induced paralysis



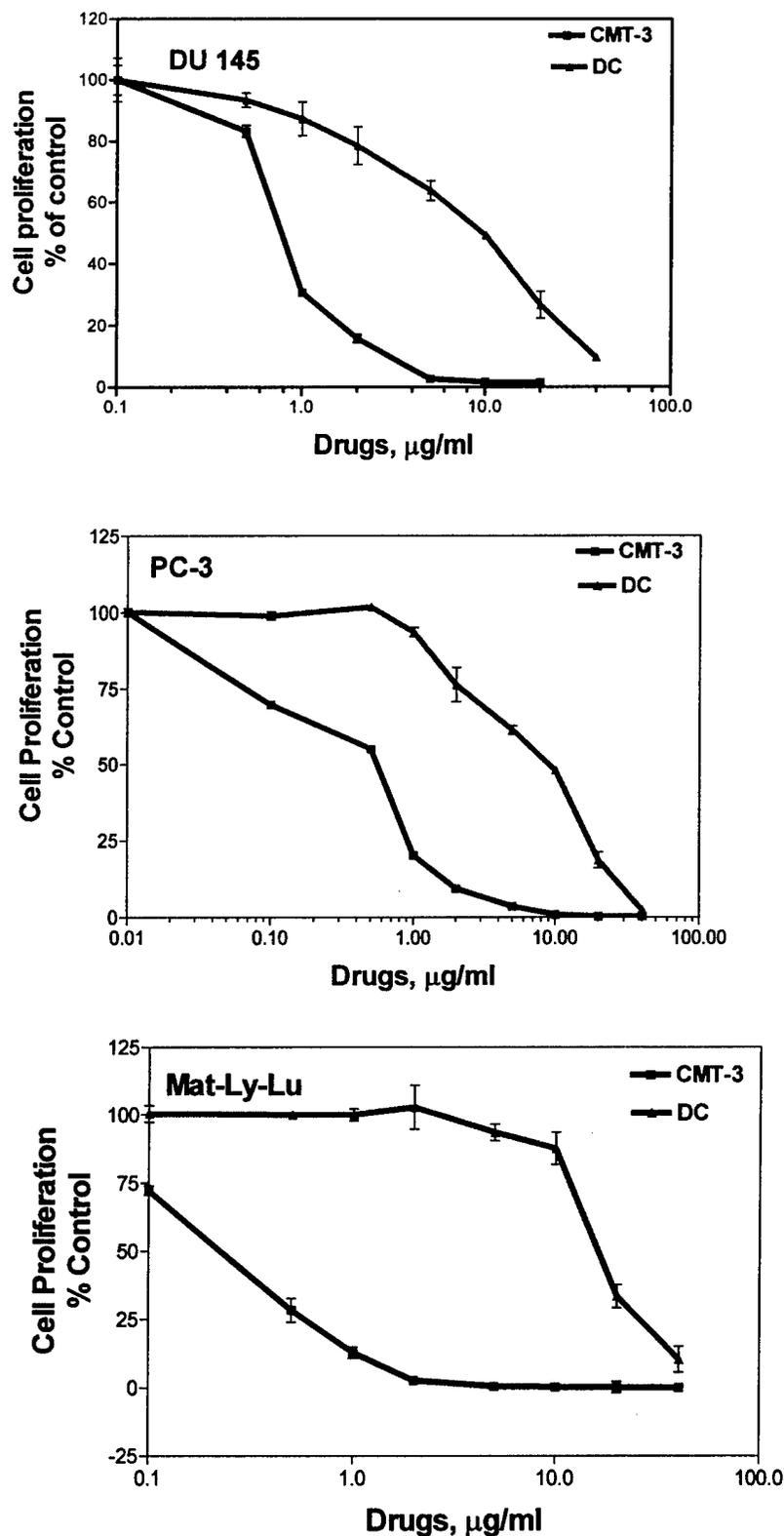
Tumors were induced by a tail vein injection of  $5 \times 10^4$  MAT LyLu cells with simultaneous caval vein occlusion. Rats were gavaged with CMT-3 or vehicle starting on pre- or post-tumor cell injection as indicated in the top panel. Rats that developed acute pulmonary distress and/or paraplegia were euthanized.



Rat lungs with metastases of MAT LyLu tumor resulting from intravenous injection of tumor cells. Each nodule is a metastatic focus. Rats were predosed with CMT-3 (40 mg/kg/d) for 7 days (Gr.1), predosed two days (Gr. 2) prior to tumor cell injection, or one day following injection (Gr. 3). Rats in Group 4 were gavaged with the drug vehicle only (Control).



**Fig 3. Levels of urinary DPD in rats bearing metastatic prostate tumors:** Urinary DPD was measured using the Pylinks-D assay kit and normalized to total creatinine. Data represent measurements from spot urine collected on the last day of treatment (mean  $\pm$  SEM, N=6). Group I: 7day predose with CMT-3, group II; 2 day predose, Group III: CMT-3 dosing started 1 day after tumor cell injection, group IV: Control, no CMT-3 treatment.



**Fig 4: CMT-3 and DC Inhibit Cell Proliferation.** Cells were treated for 48 h and proliferation activity was estimated. Results presented are from a single experiment. Similar results were observed in other experiments. .

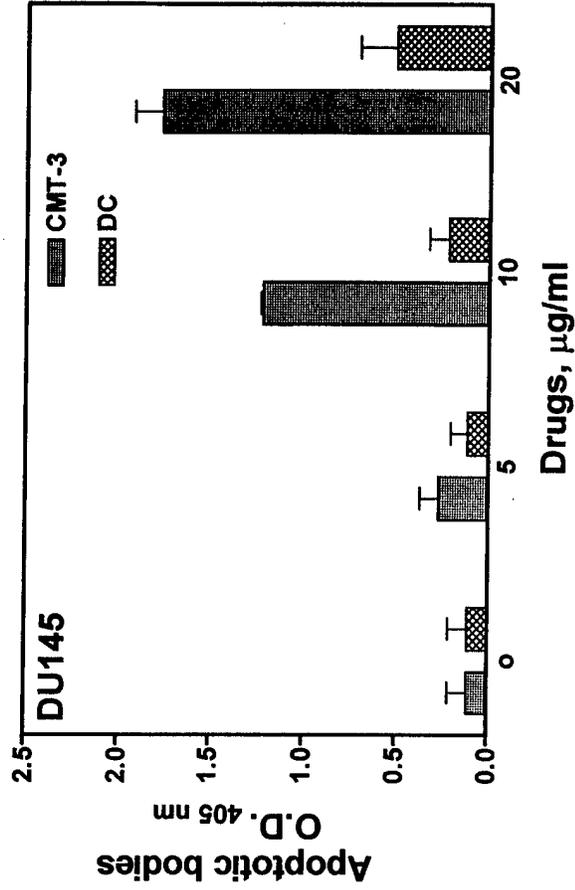
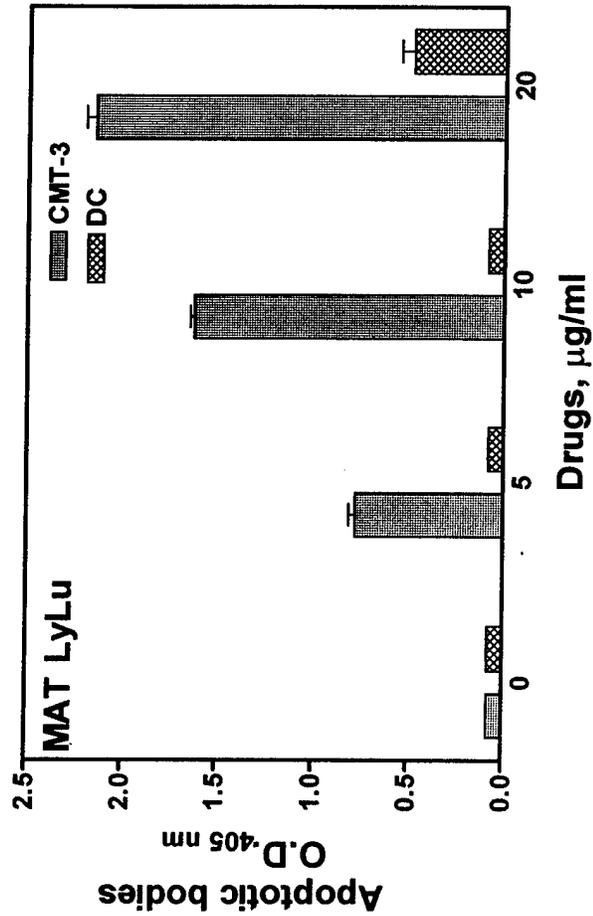
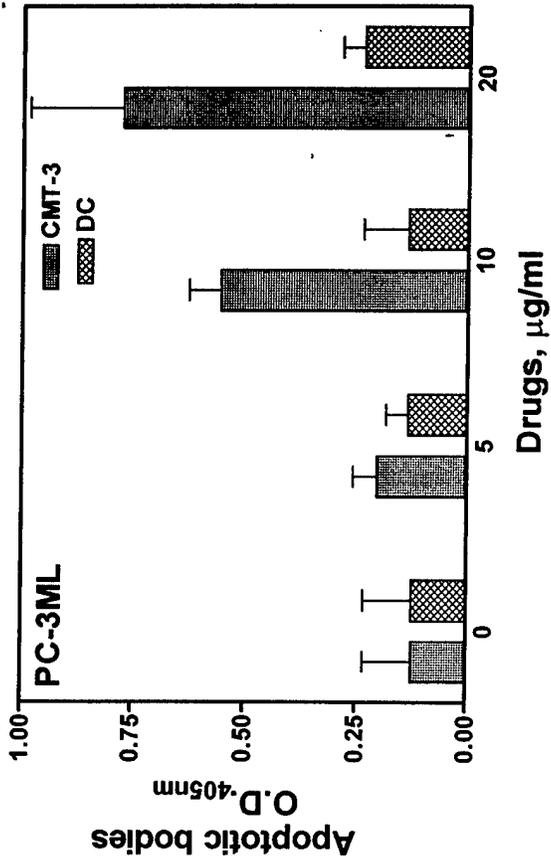
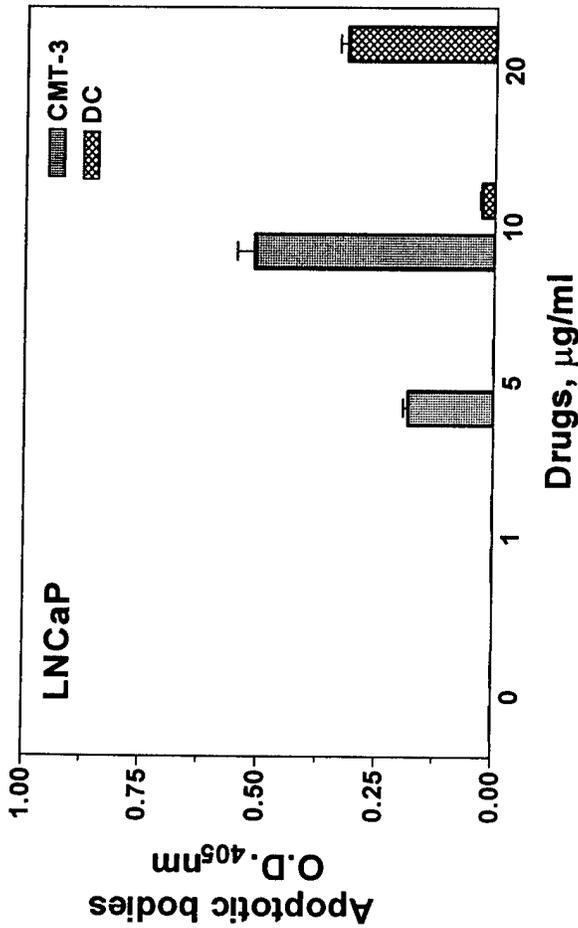
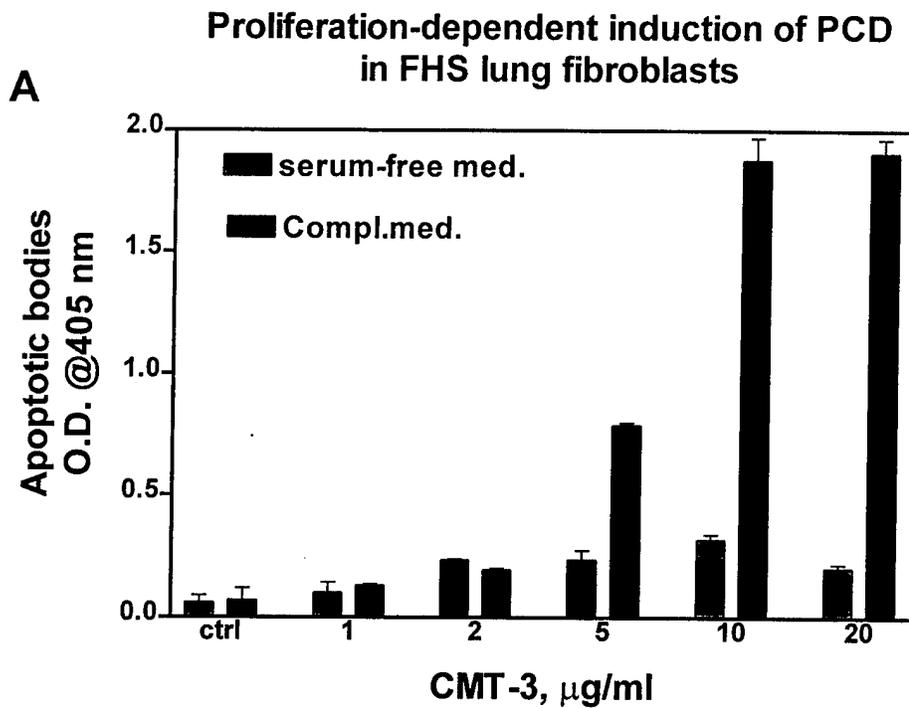
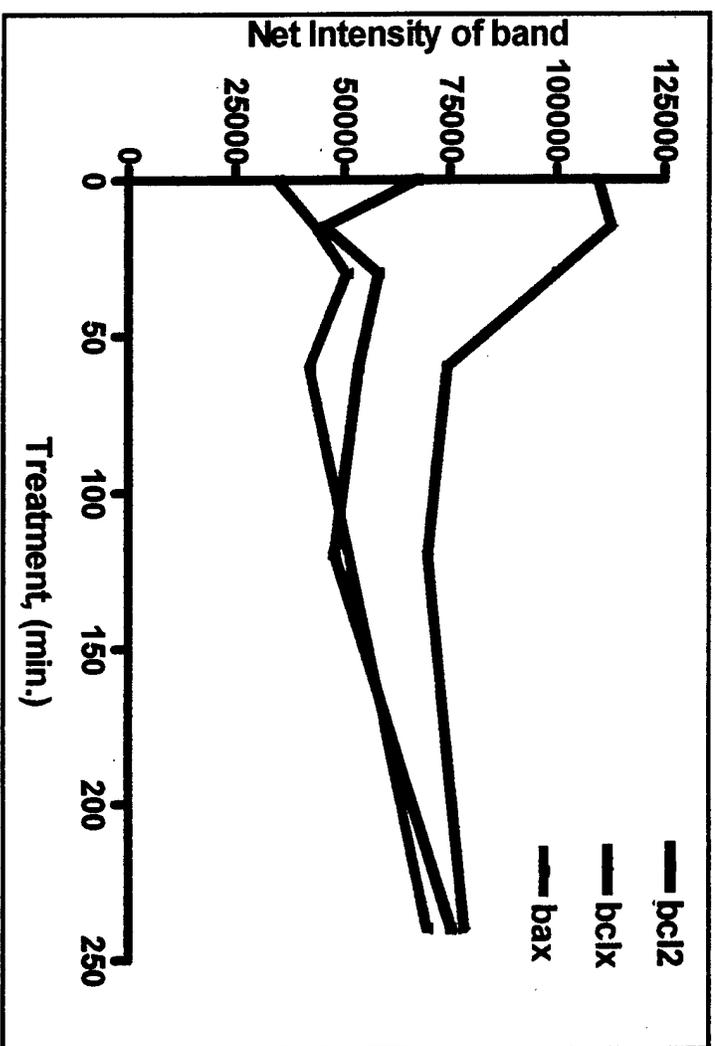
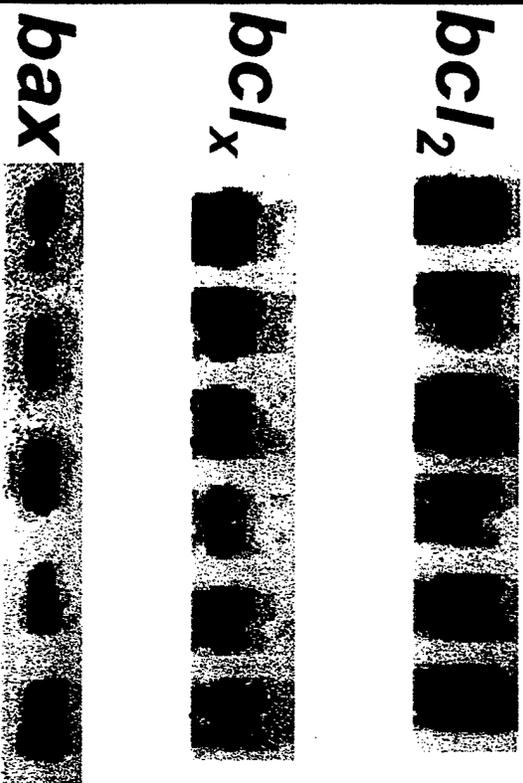


Fig 5. Induction of apoptosis by CMT-3 and Doxycycline (DC). CaP cells (LNCaP, PC-3ML, MAT LyLu and DU 145) were incubated with CMT-3 or DC for 48 hours. The culture conditioned media were analyzed for soluble nucleosomes (apoptotic bodies) resulting from DNA fragmentation (apoptosis). Relative levels of apoptotic bodies were quantified using an ELISA (Cell Death ELISA Plus kit, BM Corp., Indiana) according to the supplied instructions. Note that CMT-3 but not DC induced a strong apoptotic response in CaP cells. Similar results were also obtained using cell lysates (data not shown)



Proliferation-dependent induction of apoptosis by CMT-3: Normal lung fibroblast cells were cultured with or without serum for 48 hours and then treated with CMT-3. Cell proliferation activity was assayed by  $^3\text{H}$ -thymidine incorporation assay. Apoptosis was determined by assaying culture supernatants after the drug treatment. Panel A shows apoptotic activity of cells. Panel B (bottom) shows the DNA synthesis activity in cells incubated with complete medium or in serum-starved medium, with or without CMT-3. Data presented are from two independent experiments. C: control, no drug.

# CMT-3 alters *bcl<sub>2</sub>*, *bcl<sub>x</sub>* and *bax* expression in PC-3 ML-2 Cells



Expression of *bcl<sub>2</sub>*, *bcl<sub>x</sub>* and *bax* proteins were analyzed using the cell lysates from CMT-3 treated PC-3 ML-2 cells. Proteins blotted electrophoretically on to PVDF membrane were separately probed with specific monoclonal antibodies. Bound antibody was visualized by ECL technique using X-ray film. The identity of *bcl<sub>2</sub>* (Mr. 27,000) *bcl<sub>x</sub>* (Mr. 26,000) and *bax* (Mr. 21,000) were confirmed using purified standards (Transduction Labs., Louisville, Ky). The density of protein blots were digitized and plotted against treatment period.

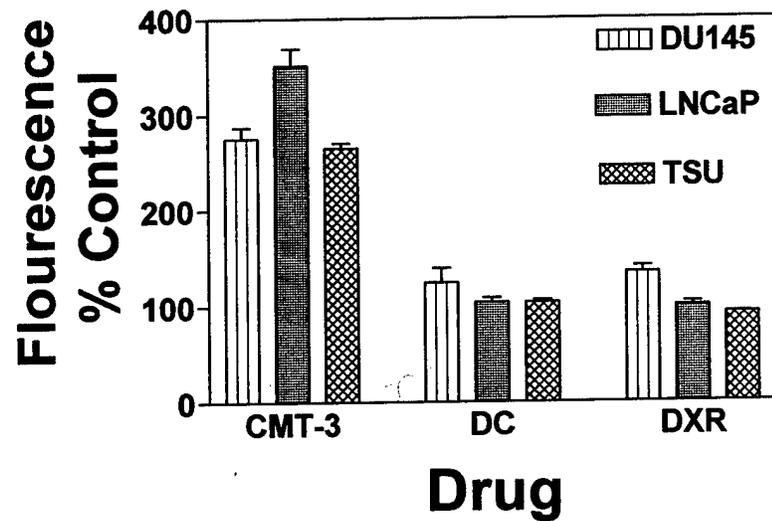
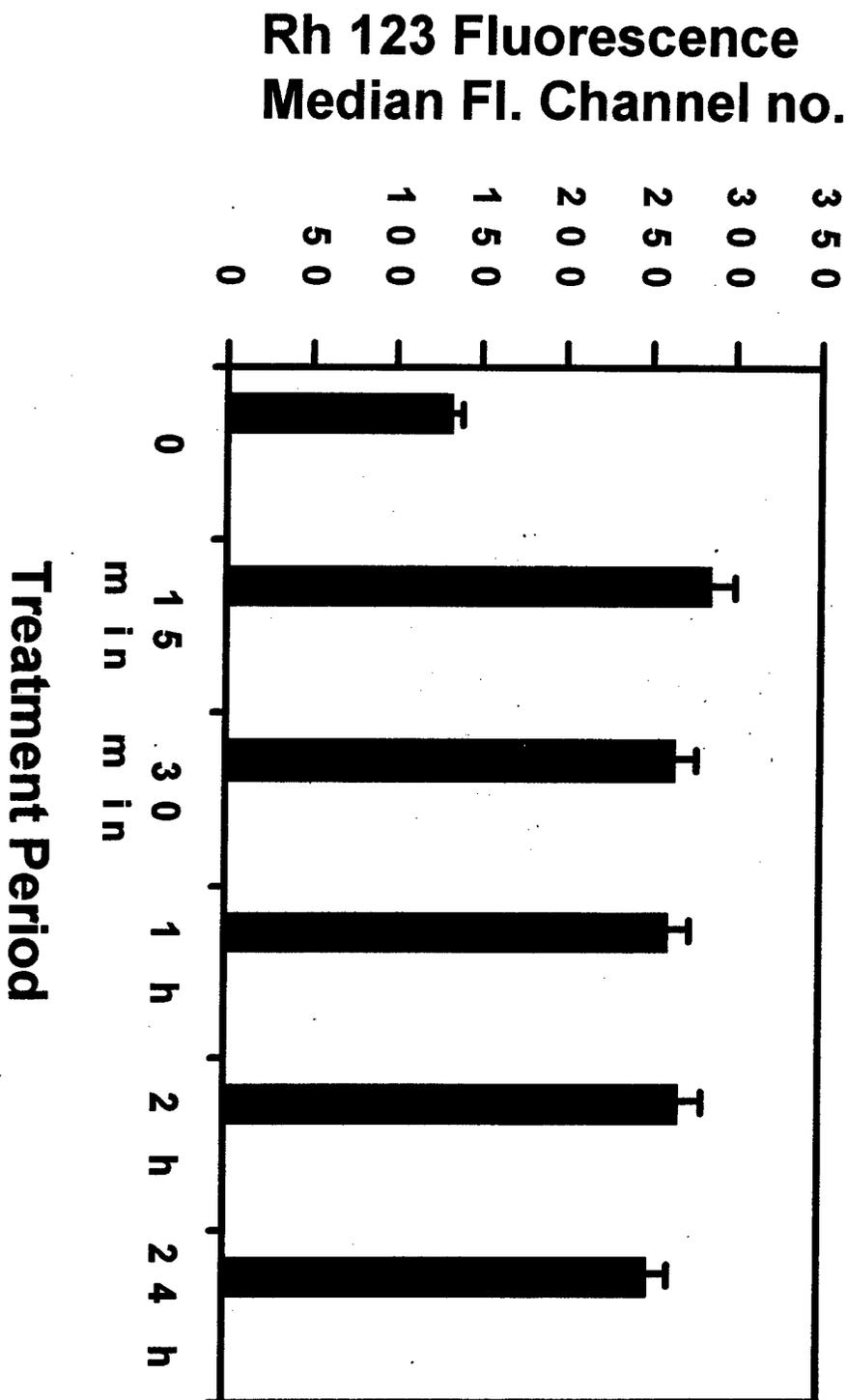
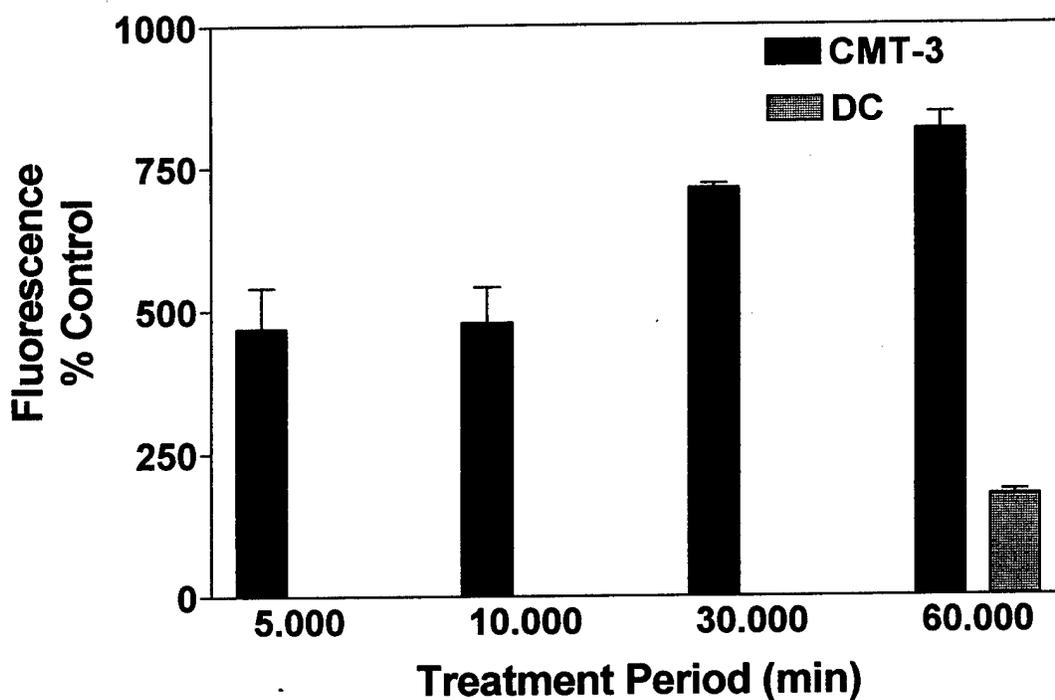


Fig 9. *in vivo* activation of Caspase-3. Cells were treated with various drugs for 60 min and cell lysates were prepared to assay caspase-3. Cell lysates were assayed for activated enzyme using a fluorometric assay (ApoALert kit, Clontech, Palo Alto, CA). The results presented are means  $\pm$  SEM from three experiments from each cell line.

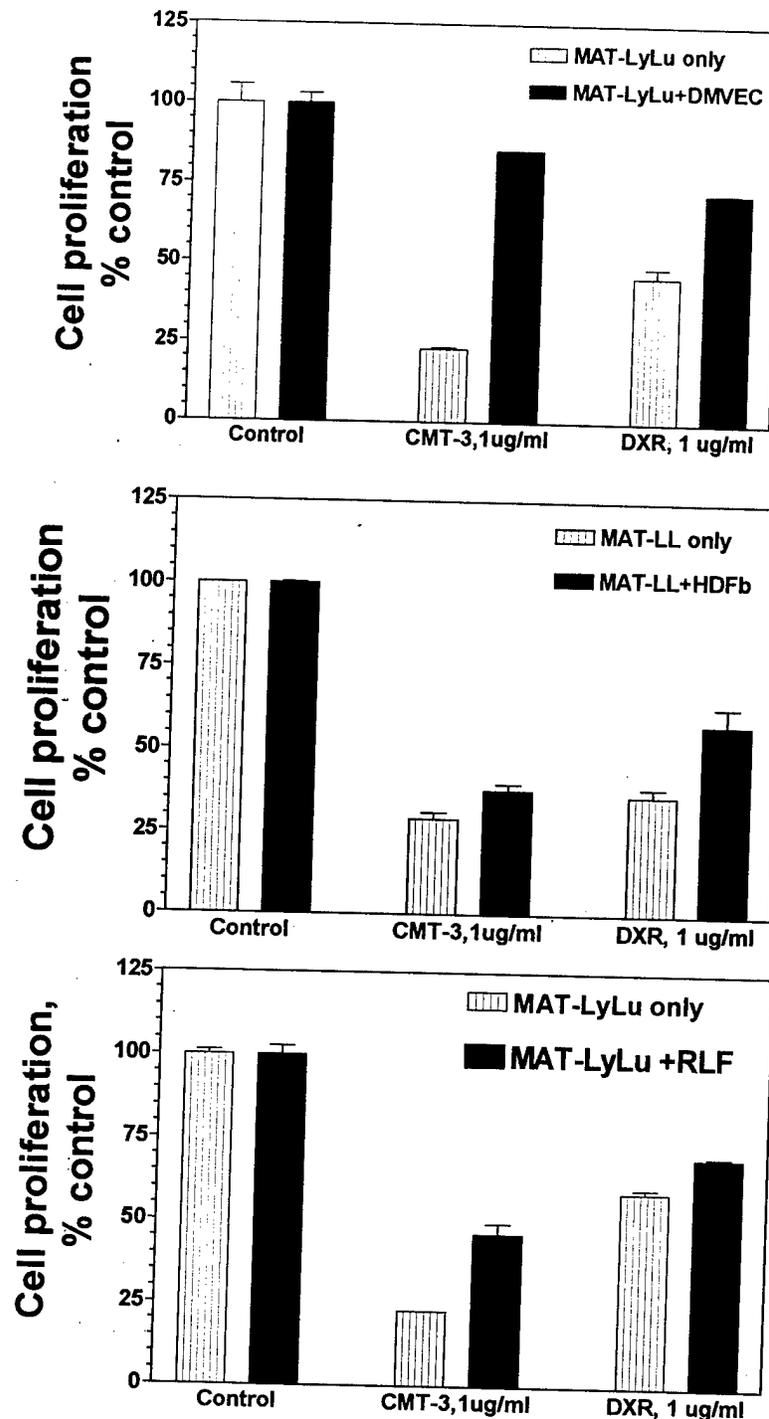
# CMT-3 Permeabilizes Mitochondria



DU 145 cells were treated with CMT-3 (10 µg/ml) for the indicated period. Cells were rinsed and pulsed with Rhodamine 123 (10 µg/ml) for further 30 min before harvesting the cells for analysis. Single cell suspensions were immediately analyzed in a Coulter EPICS XL flow-cytometer. Fluorescence intensity (FL1 filter set) is directly correlated with mitochondrial uptake of RH 123. Data shown is from a typical experiment, similar results were obtained for additional Cap cell lines.

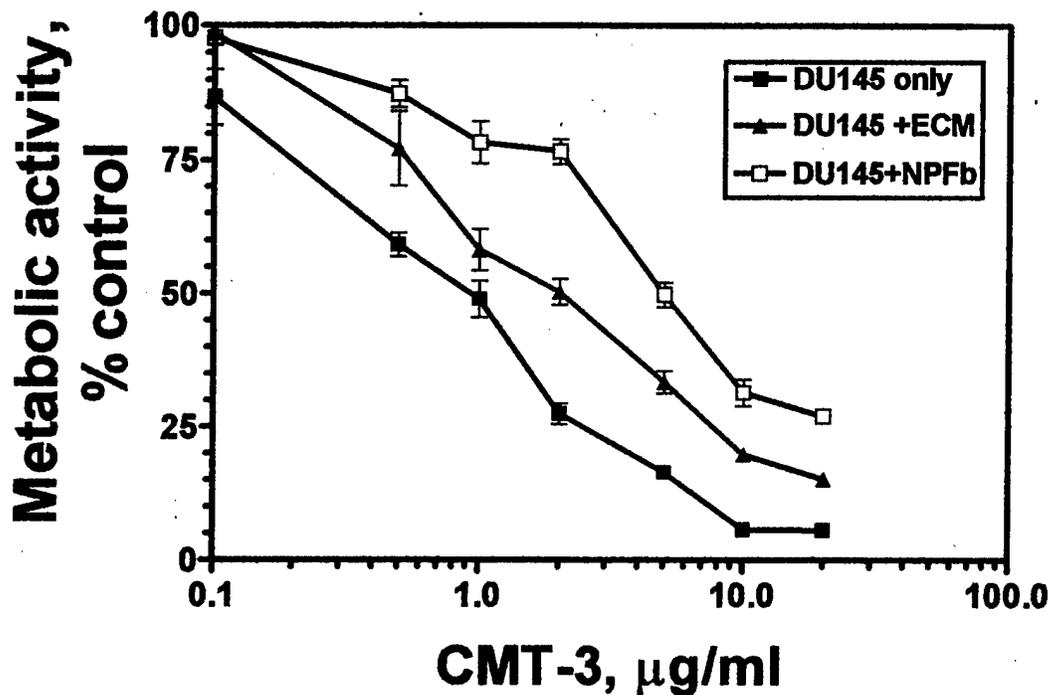
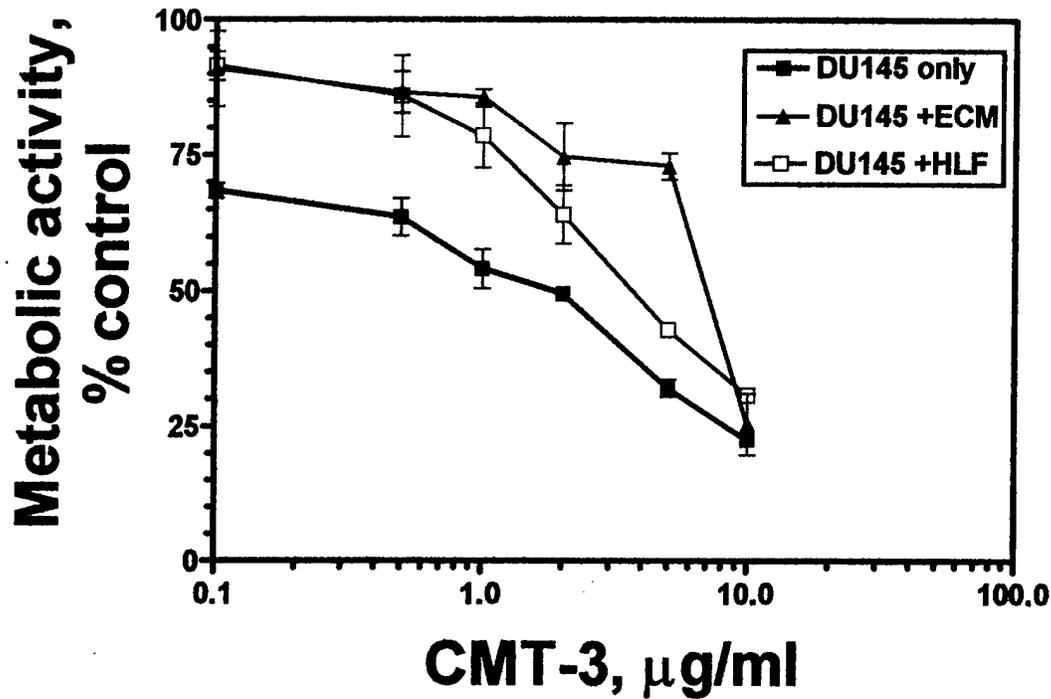


**Fig 11. Hydroxyl radical generation by CMT-3 in CaP cells.** DU145 cells were treated with 2',7'DCF-DA for 60 min followed by incubation with CMT-3 (5  $\mu\text{g/ml}$ ) or DC (10  $\mu\text{g/ml}$ ). Cells were harvested at indicated intervals and the cellular fluorescence was measured by flow cytometry. Results are from a typical experiment. Note, CMT-3 but not DC increased cellular fluorescence due to oxidation of DCF-DA.



Dermal microvessel endothelial cells (DMVEC) and fibroblasts induce chemoresistance in Dunning rat MAT LyLu (MAT-LL) tumor cells. MAT LyLu tumor cells isolated from subcutaneous or metastatic sites were cultured in the presence of dermal DMVEC, dermal fibroblasts or lung fibroblasts. Cells were incubated with DXR or Cmt-3 at  $IC_{50}$  concentration for 48 hours. Cytotoxicity was estimated using the MTT assay. Note that among the three different stromal cell populations, endothelial cells offered maximum protection against the chemotherapeutic agents that were tested. Continuous presence of the stromal cells was necessary to induce the chemoresistance. Two tested drugs were also cytotoxic to stromal cells and caused a 20-40% reduction in stromal cell proliferation.

**Stromal cells and ECM decrease chemosensitivity of tumor cells to CMT-3, a cytotoxic drug.**



Stromal cells and their ECM induce chemoresistance in DU 145 cells. DU145 cells cultured in a 24-well Transwell plate with or without the 3μ inserts containing normal human prostatic fibroblasts or lung fibroblasts were exposed to a cytotoxic drug, CMT-3. Cell proliferation was estimated 48 hours later using methyl-tetrazolium bromide (MTT) reduction assay. Cells were also plated on the ECM prepared from lung or prostatic fibroblasts and then exposed to CMT-3. Results presented is from a typical experiment.

**THE p42-ETS1 RESCUES DEFECTIVE FAS-INDUCED APOPTOSIS IN COLON CARCINOMA CELLS BY INDUCING ICE/CASPASE-1**

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**INTRODUCTION.** ETS1 is a cellular homologue of the viral *ets* oncogene of the E26 virus and functions as a tissue-specific transcription factor. It plays an important role in cell proliferation, differentiation, lymphoid cell development, transformation, angiogenesis and apoptosis (1). ETS1 controls the expression of critical genes involved in these processes by binding to *ets* binding sites present in the transcriptional regulatory regions (2, 3). The ETS1 gene generates two proteins, p51, and a spliced variant, p42, lacking exon VII. We have previously established a model system (4) in epithelial cancer cells showing that the two major ETS1 iso-types have overlapping (both repress the tumorigenic phenotype of epithelial cells) and unique (only the p42-ETS1 protein promotes apoptosis) biological properties. Understanding the mechanism of p42-ETS1 induced apoptosis in epithelial cells will provide information about its function in T-cells in which it is normally expressed. This can have a profound medical significance in that it may open new insights into the potential role of the p42-ETS1 variant in the induction of apoptosis in epithelial cancers and may provide a rationale for its use for potential gene therapy experiments to initiate cell death in cancer cells.

**METHODS.** All methodologies included in this report are described in Ref 5. **RESULTS.** In this report we show that p42-ETS1 expression bypasses the damaged Fas-induced apoptotic pathway in DLD1 colon carcinoma cells by up-regulating ICE/caspase-1 and causes these cancer cells to become susceptible to the effects of the normal apoptosis activation system. ICE/caspase-1 is a redundant system in many cells and tissues, and here we demonstrate that it is important in activating apoptosis in cells where the normal apoptosis pathway is blocked. Blocking ICE/caspase-1 active using specific inhibitors of this protease prevents the p42-ETS1 induced apoptosis from occurring, indicating that the induced ICE/caspase-1 enzyme is responsible for killing the cancer cells.

**DISCUSSION.** In summary, we have shown that p42-ETS1 bypasses the damaged Fas-induced apoptotic pathway in colon cancer cells by up-regulating ICE/caspase-1 and causes these cancer cells to become susceptible to the

effects of the normal apoptosis activation system. ICE/caspase-1 is a redundant system in many cells and tissues, and it becomes important in activating apoptosis in cells where the normal apoptosis pathway is blocked. A hypothetical model of p42-ETS1 mediated apoptosis is shown in Figure 1. p42-ETS1 activates a critical alternative apoptosis pathway in cancer cells which are resistant to normal immune attack, and thus may be useful as an anti-cancer therapeutic.

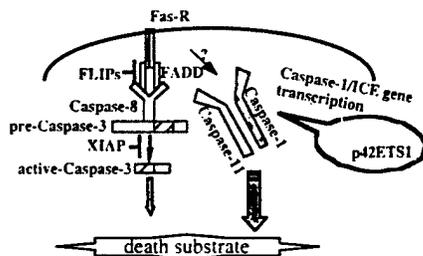


Figure 1

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This work was funded by the American Cancer Society Grant (GN-164) to Takis S. Pappas.

**RAPID INDUCTION OF APOPTOSIS SIGNALING AS A MECHANISM OF CYTOTOXIC ACTIVITY BY A CHEMICALLY MODIFIED TETRACYCLINE, A NOVEL ANTITUMOR DRUG.**

Bal L. Lokeshwar, Eva Escatel, Heather L. Houston-Clark, and Baoqian Zhu Department of Urology (M-800), University of Miami, School of Medicine, Miami, FL 33101

**INTRODUCTION** Chemically modified tetracyclines (CMTs) are a class of synthetic tetracyclines that lack antimicrobial activity<sup>1</sup> CMTs were initially characterized for their anti-collagenase activity and as possible drugs for the treatment of periodontal and rheumatoid diseases<sup>2,3</sup>. We and others have recently reported that some of the CMTs inhibit cell proliferation *in vitro* and show potent antitumor and antimetastatic activity *in vivo*<sup>4,5</sup>. Here we present evidence that the selective anti-proliferation and cytotoxic activity exhibited by a CMT, CMT-3 (6-deoxy, 6-demethyl, 4-de-dimethylamino tetracycline) on proliferating cells may be due to its unique ability to induce rapid downstream apoptotic signaling.

**MATERIALS AND METHODS:** Established (LNCaP, PC-3, DU 145 (ATCC Inc., Rockville MD)), and primary prostatic carcinoma cells were used in the assays to measure the effect of CMTs (CollaGenex Inc., Newtown PA) and doxycycline. Cell proliferation was quantitated by the MTT assay. Nuclear fragmentation resulting from apoptosis was assayed using the Cell Death ELISA Plus kit (Behringer Mannheim Corp). Membrane translocation of phosphatidyl serine was assayed by cell surface annexin V binding using the ApoAlert-Annexin V assay kit (Clontech, Palo Alto, CA). Annexin labeled cells were analyzed by flow cytometry and median fluorescence channels of treated cell samples were compared. Down stream apoptosis signaling in affected cells were quantitated by measuring caspase-3 activation assay using an ApoAlert CPP32 kit (Clontech). The drug induced expression of *bax* and *bcl<sub>2</sub>*, two proteins that promote or suppress apoptosis, respectively, were detected and quantitated by Western blotting and densitometry.

**RESULTS AND DISCUSSION:** Both doxycycline and CMT-3 were cytotoxic to proliferating normal and tumor cells. However, the potency varied greatly, the 50% growth inhibition dose for doxycycline (10 - 40 µg/ml) was typically three to five fold higher than that of CMT-3 (2.0 -10.0 µg/ml) CMT-3 but not doxycycline, induced apoptosis in five prostate cancer cell lines tested. As shown in Fig 1 A-B, the induction of apoptosis was both dose and time dependent. An analysis of cell-surface binding of annexin V revealed a rapid translocation of phosphatidyl serine from inside of the cell to outside that peaked in 60 min. Similarly, down stream apoptosis signaling cascade, initiated by caspase-3 (CPP32), was evident following

a 15 min exposure to CMT-3 that peaked in 1 h (Fig 2A & B). Such responses were absent in cells treated with doxycycline or other CMTs. Adding CMT-3 to tumor cells led to a transient increase and a steady decrease in the apoptosis suppressing protein *bcl<sub>2</sub>*, and an increase in the apoptosis promoting *bax* protein (Fig 3). Changes in mitochondrial permeability, measured by rhodamine 123 labeling, were also rapid and irreversible. Normal cells that were made quiescent by serum-starvation or contact inhibition showed reduced response to CMT-3, indicating cell-cycle progression is an essential step in CMT-3 induced apoptosis.

**CONCLUSIONS:** CMT-3 could be a candidate chemotherapy drug that is capable of selectively inducing apoptosis in proliferating cells. These results provide additional support to our previous observation<sup>6</sup> that oral administration of CMT-3 can suppress tumor growth and metastasis in a prostate tumor model by dual mechanisms: induction of apoptosis, and inhibition of activity of matrix metalloproteinases. Because Drugs that are capable of a strong apoptosis induction in tumor cells by transient exposures may have an advantage over other drugs chemotherapy drugs that are poor inducers of apoptosis, as continuous peak plasma drug concentration is not required to kill tumor cells. (Supported by a grant from NIH (R29 CA 61308), and L. Austin Weeks Endowment).

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Figure 1



Figure 2a



Figure 2b



Figure 3

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activities to anticancer drugs. Thus, these results suggest that COX-2 may be closely related to sensitivities of lung cancer cells to anticancer drugs and that specific inhibitor of COX-2 expression may be useful for augmenting chemotherapeutic effect against lung cancer.

**#2825 Modulation of metal sensitivity and glutathione content of human cells by overexpression of the arsenite-stimulated ATPase (hASNA-1).** Buran Kurdi-Haidar, Dennis Heath, Doreen K. Hom, and Stephen B. Howell. *UCSD Cancer Center and School of Medicine, University of California, San Diego, La Jolla, CA, 92093-0058.*

The hASNA-1 is a novel human arsenite-stimulated ATPase whose cDNA was cloned based on homology to the ArsA, the ATPase component of the arsenite efflux pump in *E. coli*. Northern and Western analyses had shown it to be overexpressed in DDP-resistant human malignant melanoma T289 cells. Here, we present the effect of overexpression of the hASNA-1 on metal and drug sensitivity and on steady-state glutathione (GSH) level. A highly infectious hASNA-1 retroviral vector and a eukaryotic expression vector were constructed and used to transduce the melanoma T289 and human embryo kidney 293 cells, respectively. Populations of empty retroviral vector and hASNA-1-transduced T289 cells were examined for their sensitivity to DDP, NaAsO<sub>2</sub>, ZnCl<sub>2</sub>, and CdCl<sub>2</sub> using the Sulforhodamine B growth inhibition assay. Results showed that overexpression of hASNA-1 resulted in 2-fold hypersensitivity to NaAsO<sub>2</sub> and ZnCl<sub>2</sub>, and did not impact sensitivity to DDP and CdCl<sub>2</sub>. In addition, a GSH level of 265 ± 44 (SD) nmoles/mg protein, in empty vector transduced human 293 cells, was increased by 30% (p=0.005) upon overexpression of hASNA-1. A similar increase of 22% in GSH level was observed in the human T289 cells overexpressing hASNA-1. Together, these results suggest that hASNA-1 influences some cellular metal detoxification pathway and glutathione metabolism.

**#2826 Effects of Raf-1 on drug resistance to daunorubicin in breast and prostate cancer cells.** Weinstein-Oppenheimer, C.R., Steelman, L.S. and McCubrey, J.A. *East Carolina University, School of Medicine, Department of Microbiology and Immunology, 600 Moye Blvd, Greenville, NC 27858.*

The precise molecular events involved in the development of drug resistance remain largely unknown. Raf is an intermediate in signal transduction cascades initiated by growth factors. The hypothesis of this research is that Raf has a role in the development of drug resistance. A positive correlation was observed between increased Raf-1 activity and increased values for inhibitory concentration 50% (IC<sub>50</sub>) for daunorubicin. The cell lines, PC-3 and MCF-7/Adr exhibited both the highest Raf activity and the highest IC<sub>50</sub> values for daunorubicin. On the other hand, the cell lines MCF-7 and DU-145 exhibited both lower Raf activity and IC<sub>50</sub> values for daunorubicin. Geldanamycin, a drug that induces Raf degradation, produced a decrease in IC<sub>50</sub> values for the MCF-7/Adr cell line. PD98059, an inhibitor of MEK, a kinase that is a direct downstream substrate of Raf, also caused a decrease in IC<sub>50</sub> values for MCF-7/Adr. In addition, MCF-7 cells, transfected with constitutively active Raf-1, displayed increased IC<sub>50</sub> values for daunorubicin. The above observations suggest a role for Raf protooncogene in drug resistance.

**#2827 Intrinsic resistance to rapamycin in human tumor cells may correlate with c-MYC protein levels.** Dilling, Michael, Hosoi, Hajime, Liu, Linda N., Germain, Glen, S., and Peter J. Houghton. *Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis TN 38105.*

Rapamycin, a macrolide antibiotic, is a potent suppressor of cell growth that functions by inhibiting the 289 kD protein kinase mTOR. Tumor cells vary widely in sensitivity to the drug and we seek to determine the underlying mechanisms. We compared the effect of rapamycin on two pairs of rhabdomyosarcoma (Rh1 and Rh30) and glioblastoma (SJ-G2 and SJ-G3) cell lines that differ markedly (>1000 fold) in sensitivity to rapamycin. We determined that p70<sup>S6</sup> kinase levels did not correlate with sensitivity, with enzyme activity being similarly suppressed by rapamycin in both sensitive and resistant cells. Resistance also did not correlate with phosphorylation of PHAS-I (4E-BP1) as serum stimulated phosphorylation was inhibited in both sensitive and resistant cells. Binding of PHAS-I to e-IF4E was similar in sensitive and resistant cells (Rh30 vs. Rh1), with rapamycin equally abrogating the effects of IGF-I stimulation in both lines. These data indicate that resistance to rapamycin is not due to mutations in mTOR that prevent FKBP-rapamycin binding. Interestingly, in an Rh30 derivative (Rh30/rapa-10K) selected for growth in the continuous presence of 10,000 ng/ml rapamycin (20,000 fold greater than the IC<sub>50</sub> value), we detected elevated levels of c-MYC (10-fold greater than parental line). The elevated c-MYC levels were unaffected by rapamycin treatment, in contrast with the parental Rh30 cells where rapamycin blocks c-MYC induction by serum. The elevation of c-MYC in Rh30/rapa-10K appears to be an adaptive response to culture in high concentrations of rapamycin, as sensitivity to the drug returns when selective pressure is removed. Supported by CA23099, and ALSAC.

**#2828 Effects of c-erbB2 overexpression on drug sensitivities of normal human mammary epithelial cells.** Orr, M.S. *Laboratory of Molecular Pharmacology, Division of Basic Sciences, National Cancer Institute, Bethesda, MD 20892.*

Clinical results indicate that c-erbB2 overexpression is associated with poor prognosis. However, c-erbB2 overexpression has been associated with either increased or decreased response to various therapies. Overexpression of exogenous c-erbB2 in breast cancer cell lines sometimes alter drug sensitivities, but sometimes has no effect. In order to circumvent the innate genetic complexities associated with established cancer cell lines, normal human mammary epithelial cells (HMEC) were studied to determine if c-erbB2 overexpression by itself would alter chemosensitivity. The lifespan limitations of the HMEC cells were overcome through the use of retroviral gene transfer. Flow cytometric analysis of c-erbB2 protein levels on the cellular surface were determined using a FITC labeled monoclonal antibody to c-erbB2. Interestingly, HMEC cells overexpressing high levels c-erbB2 protein, as indicated by western blot analysis, had only 11% of the c-erbB2 protein present on the cellular surface as compared to the total population of cells. These cells failed to show any alterations in chemosensitivity to cisplatin, adriamycin, 5-fluorouracil, paclitaxel, methotrexate, or flavopiridol. Using FACs (Fluorescent activated cell sorting) to enrich for HMEC cells overexpressing c-erbB2 on their surface, an 85% pure population of cells were isolated and the chemosensitivity to cisplatin, adriamycin, 5-fluorouracil, paclitaxel, and methotrexate were evaluated. Again, the cells failed to display any alterations in chemosensitivity. These results suggest that overexpression of c-erbB2 is not sufficient by itself to induce changes in chemosensitivity. Furthermore, the results indicate that other as yet unidentified genetic changes inside a cancer cell work in concert with c-erbB2 to illicit alterations in chemosensitivity.

**#2829 Organ-specific stromal cells and ECM modify chemoresistance of prostate tumor cells.** Lokeshwar, B.L., Zhu, B-Q. Block, N.L. *Department of Urology (M-800), University of Miami, Miami, FL 33101.*

Androgen refractory metastatic prostate cancer (CaP) is resistant to most anticancer drugs. It is believed that resistance to chemotherapy is mediated by conditions that exist only *in vivo*. The protective function of organ-specific stromal cells might be one such condition. We tested this hypothesis *in vitro* using CaP cells and organ-specific stromal cells or their extracellular matrix (ECM). We assayed the response of human CaP cells (DU145, PC-3) to two cytotoxic drugs, doxorubicin, and CMT-3, a non-antimicrobial tetracycline, in the presence or absence of human stromal fibroblasts isolated from prostate, bone and lung. Tumor cells co-cultured with prostate and lung fibroblasts showed decreased (50-300%) sensitivity to either drug. Next, we tested whether tumor cells (Dunning MATLyLu) isolated from different metastatic sites (bone and lung) differ in their chemoresistance. The tumor cells isolated from lung or bone, cultured alone, responded similarly to the drugs. However, co-culturing in corresponding organ-derived fibroblasts significantly reduced the drug-induced cytotoxicity (25-75%). Increased chemoresistance was also observed when tumor cells were cultured on ECM prepared from organ-specific stromal cells. A soluble laminin fragment and hyaluronidase, both antagonists of ECM components, reduced the protection of ECM and increased cytotoxicity. These results demonstrate that cell-cell interactions between organ-specific stromal cells and tumor cells may be the significant factors affecting chemotherapy, some of which can be duplicated *in vitro*. (Funded by NIH Grant No. CA 61038-R29; L. Austin Weeks Endowment).

## PREVENTION/BASIC SCIENCE AND CLINICAL STUDIES 5: Biomarkers and Their Application in Clinical Cancer Prevention

**#2830 Validation of biomarkers of benzene exposure: Urinary metabolites.** Qu, Q.S., Melikian, A.A., Kagan, M., Li, H., Meng, M., Shore, R., Chen, L.C., Cohen, B., Jin, X., Winnik, W., Li, G., Yin, S., Li, Y., and Wu, R. *Dept. Environ. Med. NYU School of Medicine, Tuxedo, NY 10987; American Health Foundation, Valhalla, NY 10595 Beijing Inst. Occup. Med; Tianjin Inst. Occup. Med., China.*

This study was conducted among Chinese workers with an average personal benzene exposure of 31 ± 25.9 ppm (mean ± SD). The metabolites monitored were S-phenylmercapturic acid (S-PMA), t,t-muconic acid (t,t-MA), hydroquinone (HQ), catechol (CAT), 1,2,4-trihydroxybenzene (BT), (quantified by HPLC-tandem mass spectrometry) and phenol (analyzed by GC-MS). With the exception of BT, levels of benzene metabolites measured in urine samples collected at the end of each workshift were significantly higher than those measured in unexposed subjects (p < 0.001). The big increases in urinary metabolites from before to after work were strongly correlated with benzene exposure. The Spearman rank-order correlations with current day benzene exposure were 0.78 for HQ, 0.78 for t,t-MA, 0.73 for S-PMA, 0.72 for CAT, but only 0.38 for phenol and 0.24 for BT. The median half-lives of S-PMA, t,t-MA, HQ, CAT, and phenol from the time course study were 13.8, 11.1, 17.9, 27.6, and 17.8 hrs, respectively. Concentrations of these metabolites in urine samples collected from exposed workers before work were significantly higher than those from unexposed subjects (S-PMA and t,t-MA p < 0.01, HQ and CAT p < 0.05), indicating that the previous days' exposure made contributions to the levels of metabolites measured in the spot samples. (Supported by the Health Effects Institute).

## MMP Inhibition in Prostate Cancer

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**ABSTRACT:** Matrix metalloproteinases (MMPs) play a significant role during the development and metastasis of prostate cancer (CaP). CaP cells secrete high levels of MMPs and low levels of endogenous MMP inhibitors (TIMPs), thus creating an excess balance of MMPs. Established CaP cell lines that express high levels of MMPs frequently metastasize to the bone and the lungs. Drugs such as Taxol and alendronate that reduce cell motility and calcium metabolism reduce bony metastasis of xenografted CaP tumors. We tested several synthetic, nontoxic inhibitors of MMPs that can be administered orally, including doxycycline (DC) and chemically modified tetracyclines (CMTs) on CaP cells *in vitro* and on a rat CaP model *in vivo*. Among several anti-MMP agents tested, CMT-3 (6-deoxy, 6-demethyl,4-de-dimethylamino tetracycline) showed highest activity against CaP cell invasion and cell proliferation. Micromolar concentration of CMT-3 and DC inhibited both the secretion and activity of MMPs by CaP cells. When tested for *in vivo* efficacy in the Dunning rat CaP model by daily oral gavage, CMT-3 and DC both reduced the lung metastases (> 50%). CMT-3, but not DC, inhibited tumor incidence ( $55 \pm 9\%$ ) and also reduced the tumor growth rate ( $27 \pm 9.3\%$ ). More significantly, the drugs showed minimum systemic toxicity. Ongoing studies indicate that CMT-3 may inhibit the skeletal metastases of CaP cells and delay the onset of paraplegia due to lumbar metastases. These preclinical studies provide the basis for clinical trials of CMT-3 for the treatment of metastatic disease.

### INTRODUCTION

Carcinoma of the prostate (CaP) is a major malignant disease in developed countries. In the United States alone an estimated 186,500 Americans are expected to be diagnosed with CaP in 1998, and about 39,200 fatalities are expected.<sup>1</sup> Approximately 50% of prostate cancer patients have extra-prostatic disease at the time of diagnosis.<sup>2</sup> Furthermore, the disease relapses in a majority of the patients despite treatment of the primary tumor.<sup>3,4</sup> Surgery and radiation are the two common treatment modalities for patients with localized (stage A and B) or locally extensive disease (stage C).<sup>5</sup> Patients with inoperable conditions due to age are treated with "hormonal" therapy or radiation.<sup>6</sup>

The most common hormonal therapy for prostate cancer is either neo- or adjuvant androgen ablation.<sup>7</sup> Most prostate tumors originate from the glandular epithelial cells of the peripheral region of the prostate. The glandular epithelium of the prostate is dependent on androgens, the common male steroid hormones—testosterone and dihydrotestosterone—for survival and proliferation.<sup>8</sup> Depriving the prostate tumor

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cells of androgens by castration or by suppressing androgen production in the gonads with pituitary gonadotropin (LH-RH) analogue (e.g., Lupron, TAP Pharmaceuticals, Chicago, IL), inhibitor of steroid  $5\alpha$ -reductase (e.g., Proscar, Merck Pharmaceuticals, Rahway, NJ), or treatment with anti-androgens (e.g., Flutamide) almost always results in temporary tumor remission.<sup>9</sup> However, deprivation of androgens to prostate (androgen ablation) almost always leads to the onset of a more aggressive, metastatic, hormone-refractory incurable phase of the disease.<sup>10</sup>

Since most instances of prostate cancer initially respond well to androgen ablation (which is more selective than cytoreductive chemotherapy), chemotherapy is not currently a first line of therapy during any stage of this disease. Several unique features of CaP contribute to this. For example, very low growth fraction in the primary tumor, lower than most proliferating tissues of the body, precludes treatment of early or localized disease with cytoreductive chemotherapy.<sup>11</sup> Drug-induced morbidity, or the co-morbidity of other ailments, or simply advanced age of the patient preclude aggressive chemotherapy of patients with advanced prostate cancer. Nevertheless, after the failure of "hormone"-related treatments, the disease is usually treated palliatively with radiation or cytoreductive chemotherapy, as its metastatic growth leads to acute bone pain, spinal compression, and often paraplegia.<sup>12</sup>

Targeting the metastatic growth of prostate cancer with site-directed, nontoxic drugs that disable tumor cells from establishing metastatic colonies is an alternative therapeutic option. Drugs that inhibit metastatic process, but not necessarily cell proliferation, are not likely to discriminate between androgen-dependent and independent prostate tumor cells. Nontoxic inhibitors of MMPs that inhibit tumor cell invasion, angiogenesis, and adhesion-dependent interactions of tumor cells with the host tissue are drugs with such potential. Although a number of laboratories are engaged in developing such therapeutic agents for prostate cancer, currently few drugs exist in clinical use that achieve desired efficacy. In the next few pages I will summarize what is known about the role of matrix metalloproteinases in prostate cancer. I will also summarize current efforts to develop therapeutic avenues using MMP inhibition as a tool.

#### *Basic Mechanism of Cancer Metastasis*

The cellular basis of cancer metastasis is explained by the three-step model proposed by Liotta *et al.*<sup>13</sup>: (1) adhesion of tumor cells to basement membrane; (2) local proteolysis that leads to the invasion of cancer cells into stroma; and (3) tumor cell proliferation. Thus, according to this model, the process of invasion begins by adhesive interactions between tumor cells and the extracellular matrix (ECM), leading to the proteolysis of the basement membrane. This is followed by the migration of tumor cells through stroma, invading the capillary wall to enter blood circulation. After entering the circulation the tumor cells are embolized throughout the circulatory system and migrate to distant organs by hemodynamic principles. In this process, the tumor cells adhere to the capillary wall and begin to extravasate in a sequence the reverse of the invasion process. Tumor cells then interact with the stromal components of the new organ, which results in either the elimination of tumor cells or their colonization owing to stimulation of cell proliferation and angiogenesis. Taken together, the entire metastatic process is known to be extremely inefficient, where less than 0.01% of tumor cells that enter circulation are capable of establishing a meta-

static colony.<sup>14</sup> Chambers *et al.*<sup>15</sup> have recently challenged the earlier concept that all steps in metastasis are equally inefficient. They showed, by direct observation of intravenously injected tumor cells, that early steps in metastasis, such as hemodynamic destruction of circulating tumor cells and extravasation, may contribute less to metastatic inefficiency than the interaction of tumor cells with the surrounding environment. Interaction with the host environment is likely to dictate either the proliferation or death of tumor cells at the metastatic site.

#### *Mechanism of Prostate Cancer Metastasis*

At the organ level, extraprostatic spread of prostate tumors, especially to lumbar bones, is explained by two mechanisms. The first mechanism is based on the principles of hemodynamics, where the prostate cancer cells enter the circulation to reach lumbar vertebra (bone metastasis) under increased intra-abdominal pressure (Bateson's hypothesis<sup>16</sup>). Studies have shown a direct correlation between the size of the primary prostate tumor and the incidence of capsular invasion and distant metastasis.<sup>17</sup> In addition, experimentally induced bone metastasis that approaches the frequency found in prostate cancer patients can be achieved by increasing the intra-abdominal venous pressure at the time of tumor cell injection. Shevrin *et al.*<sup>18</sup> and later Geldof and Rao<sup>19</sup> showed that tail vein injection of prostate tumor cells with simultaneous vena cava clamping causes skeletal and lumbar metastases at unusually high frequency.

The second mechanism of extraprostatic spread of tumors is explained by Paget's "seed and soil" hypothesis.<sup>20</sup> This hypothesis states that tumor metastasis is dependent on both the tumor cells (the "seed") and the microenvironment of the organ (the "soil"). Many studies support the "soil and seed" hypothesis as a mechanism of prostate cancer metastasis. For example, although several highly aggressive CaP cell lines have been established, few are spontaneously metastatic to bone.<sup>21</sup> One requirement that determines organ-specific metastasis may be the tumor cells' potential to establish a bi-directional communication ("inductive interaction") with the marrow cells.<sup>22</sup> Very likely, the inductive interactions result in the secretion of both tumor cell- and host cell-derived factors that support tumor growth and angiogenesis. These factors include diffusible growth factors that support osteoblast growth, capillary bed formation, and inducers of matrix-degrading enzymes (e.g., MMPs, urokinase-like plasminogen activator [uPA], etc.)<sup>23,24</sup> that are involved in bone matrix remodeling.

#### *Matrix-Degrading Enzymes and Tumor Cell Invasion*

The dissolution of basement membrane components such as type IV collagen, laminin, fibronectin, and proteoglycans is a critical step in the multistep cascade that leads to metastasis.<sup>25</sup> Tumor cells dissolve these components using a variety of matrix-degrading enzymes, including aspartyl, cysteine, serine, and matrix metalloproteinases.<sup>26</sup> Both stromal and epithelial cells of the prostate secrete aspartyl proteases (e.g., cathepsin D<sup>27</sup>), serine proteases (e.g., uPA, PSA<sup>28</sup>), and metalloproteinases (MMPs<sup>29</sup>).

Several studies have shown an association of increased production of MMPs (MMP-2, -3, -7 and -9) with malignant progression of prostate cancer.<sup>28</sup> For example, both our work and that of others has shown, by analyzing the primary cultures

of human prostate tumor tissues, that epithelial cell cultures of malignant prostate secrete high levels of MMP-2 and MMP-9 and low levels of their inhibitors (TIMP-1 and TIMP-2).<sup>30,31</sup> Stearns *et al.*<sup>32</sup> have reported that, among these, MMP-2 expression is associated with Gleason sum 7 or higher. (Gleason sum is the current pathology index to evaluate malignant status of prostate tumors.<sup>33</sup>) Wood *et al.*<sup>34</sup> have reported that levels of both MMP-2 and MMP-9 are low in normal prostate and organ-confined tumors with Gleason sum 5 or lower, whereas they were highly expressed in high Gleason sum (8–10) tissues. A particularly significant observation about the role of MMP in CaP progression is that of increased expression of *activated* MMP-2.<sup>32</sup> The expression of activated MMP-2 in high-grade tumor tissues suggests that in addition to higher levels of MMP-2, MMP-2 activators such as uPA, membrane type-MMP (MMP-14), and matrilysin may also be associated with malignant progression and metastasis.<sup>35</sup>

#### *Association between MMP Expression and Metastatic Potential*

Probably, the strongest evidence as yet for the role of MMPs in prostate cancer metastasis has come from studies on animal models. Stephenson *et al.*<sup>39</sup> showed that only those sublines of prostate cancer cells that produce high levels of MMPs are capable of distant metastasis when tumors are generated by intraprostatic tumor cell injection. Powell *et al.*<sup>37</sup> reported significant induction of metastatic activity associated with high-level expression of matrilysin. Studies have also been reported that androgen ablation, which frequently results in more aggressive and metastatic cancer, can lead to increased expression of MMPs. Two independent studies on rats have shown that castration leads to involution of ventral prostate with a significant increase in MMP-2, matrilysin, and uPA.<sup>29</sup> These studies, if confirmed in patients, raise the spectre of inadvertent promotion of metastatic potential of residual tumor cells in patients undergoing androgen-ablation therapy.

#### *Role of TIMPs in Prostate Cancer*

The tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) have been recognized as the “balancing” factors in MMP-induced invasion and metastasis.<sup>38</sup> Several studies using tumor tissue specimens and primary explant cultures have shown decreased TIMP-1 expression (mRNA) and secretion (protein) in malignant prostate cancer. For example, stimulation of a highly metastatic prostate cancer cell line (PC-3ML) with interleukin-10 (IL-10) results in upregulation of TIMP-1 and downregulation of MMP-2 and MMP-9, with concomitant decrease in liver and bone metastases.<sup>39</sup> Jung *et al.*<sup>40</sup> reported, however, increased levels of TIMP-1 in the plasma of patients with metastatic prostate cancer compared to the those of patients with organ-confined cancer or those with benign prostatic hyperplasia. One should be cautious, though, in comparing the significance of the levels of MMPs and TIMPs in circulation with cancer aggressiveness rather than the corresponding tissue levels of TIMPs and MMPs in active metastatic sites.

Owing to the large body of evidence suggesting the association of MMP activity and promotion of metastasis, inhibition of MMP activity by either natural or synthetic inhibitors, with relatively mild systemic toxicity, may be an important avenue to treat hormone-refractory prostate cancer.

### *Inhibitors of Matrix Degrading Enzymes as Cancer Therapeutics*

DeClerck and others<sup>41</sup> have shown that excess production of TIMP-1 or TIMP-2 in invasive tumor cells can reduce their metastatic potential.<sup>42,43</sup> The strategy of using the superinduction of TIMPs to control metastasis, although exciting, is not very practical at present, because TIMPs are large glycoproteins (23 kDa and 21 kDa) and require a significant portion of the molecule for biological activity.<sup>44</sup> To overcome this limitation, smaller synthetic MMP inhibitors have been developed (e.g., BB-94 [batimastat] and BB2516 [marimastat], British Biotech. Inc., Oxford, England). The hydroxamate group in these compounds reversibly chelates the zinc atom at active site of MMPs, thereby potently inhibiting MMPs.<sup>45</sup> Batimastat and marimastat are forerunners in the area of MMP inhibitors used for clinical application, especially for treating metastatic tumors.<sup>46</sup> Preclinical studies using several animal models and human xenograft models of a variety of solid tumors have established the efficacy and possible clinical application of these compounds.<sup>47,48</sup> Initially batimastat and recently marimastat have been introduced in clinical trials on patients with advanced, treatment-refractory aggressive cancer. Results of the orally administrable marimastat are encouraging, with only a few dose-limiting cases of toxicity reported in clinical trials.<sup>49,50</sup>

### *Preclinical Studies of Anti-MMP Agents in Prostate Cancer*

Although extensive studies have been conducted on the potential clinical use of MMP inhibitors in other kinds of carcinoma, only a handful of such studies have been reported on prostate cancer. Among the limited number of studies reported so far, notable ones are by Stearns and Wang,<sup>51</sup> who showed that paclitaxel (Taxol, Bristol-Myers, Raritan, NJ), a potent cytoreductive chemotherapeutic drug, inhibits both MMP secretion and synthesis in PC-3 ML cells. Taxol reduced metastases of PC-3 ML cells to bones, liver, and lungs in SCID mice. Although Taxol is not an inhibitor of MMP-2 enzyme activity, it interfered with the synthesis and secretion of MMP-2, but not TIMPs. In another study,<sup>52</sup> using the same tumor model, PC-3 ML, these investigators showed that alendronate (a bisphosphonate used for treating osteoporosis) together with Taxol blocks the establishment, growth, and metastasis of PC-3 ML tumors. Treatment with alendronate alone, however, increased soft-tissue metastases, and only partially blocked bone metastasis.

### *Clinical Studies*

So far one clinical study has reported using MMP inhibitor against prostate cancer,<sup>53</sup> a dose-finding study using marimastat.<sup>54</sup> Eighty-eight patients with aggressive stage C and D prostate cancer with poor prognosis were given marimastat orally for 4 weeks. The therapeutic response, as measured by decrease in the rate of rise of serum prostate-specific antigen (PSA) levels from 53% to 29%, was considered "encouraging." The response was dose-dependent: at a higher dose more than 50% of the patients showed a significant fall in the rate of increase in PSA. A decline in serum PSA is currently the most common surrogate marker for evaluating treatment efficacy for prostate tumors.

In the remainder of this article I will present evidence to demonstrate the efficacy of a class of nontoxic MMP inhibitors on a prostate cancer model with potential clinical application.

### TETRACYCLINES AND THEIR NON-ANTIMICROBIAL ANALOGUES ARE POTENT INHIBITORS OF COLLAGENASE

Golub and his colleagues discovered that the common antibiotic, tetracycline, is a potent inhibitor of gingival fibroblast-derived collagenase.<sup>55</sup> In subsequent studies, the gelatinolytic, cytolytic, and anti-angiogenic activities of tetracyclines were discovered.<sup>56,57</sup> This group also showed that chemical modifications of tetracycline, such as removal of the dimethyl amino group from the carbon 4 of the A ring, resulted in the loss of antimicrobial activity, but did not abolish anti-collagenolytic activity.<sup>58</sup> Several of the chemically modified tetracyclines (CMTs) available at present, are non-antimicrobial and inhibit the activities of collagenases and MMPs.<sup>59</sup> We have tested a number of CMTs for their potential as anti-metastatic agents and found that one CMT, 6-dedimethyl, 6-deoxy, 4-dedimethylamino tetracycline (CMT-3), is superior to the others.<sup>60</sup> The potential advantages of CMTs over conventional tetracycline with antimicrobial activity include long-term systemic administration without gastrointestinal toxicity, higher plasma accumulation, and a longer plasma clearance time and therefore lower drug dose requirement.<sup>61</sup>

#### *Investigation of Antitumor and Antimetastatic Activities of CMTs and Doxycycline(DC)*

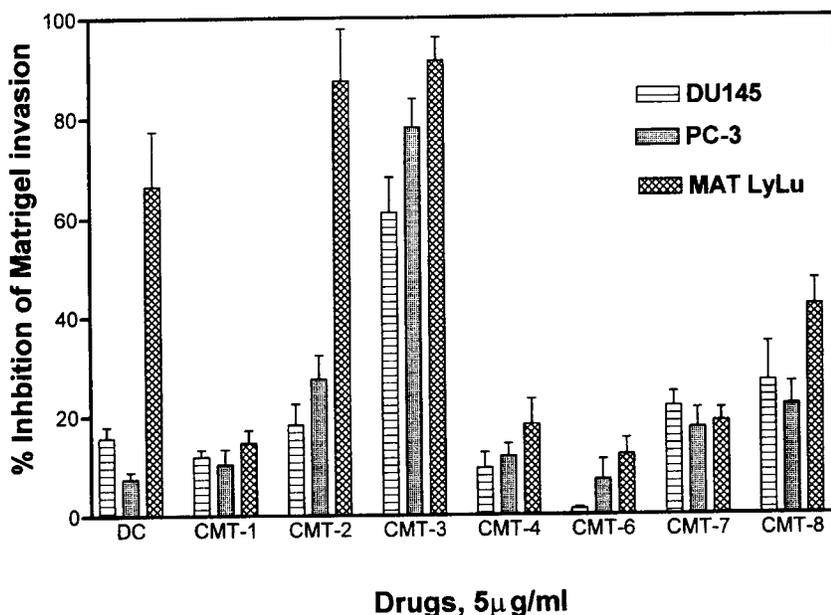
##### *In Vitro Studies*

Our objectives for testing these drugs against CaP cells *in vitro* were two-fold: (1) to investigate whether the CMTs or DC inhibit basement membrane invasive activity of CaP cells at physiologically achievable serum concentration levels (e.g., 5 µg/ml), and (2) to test whether the CMTs and DC are cytotoxic to tumor cells, as some studies have shown earlier.<sup>57</sup>

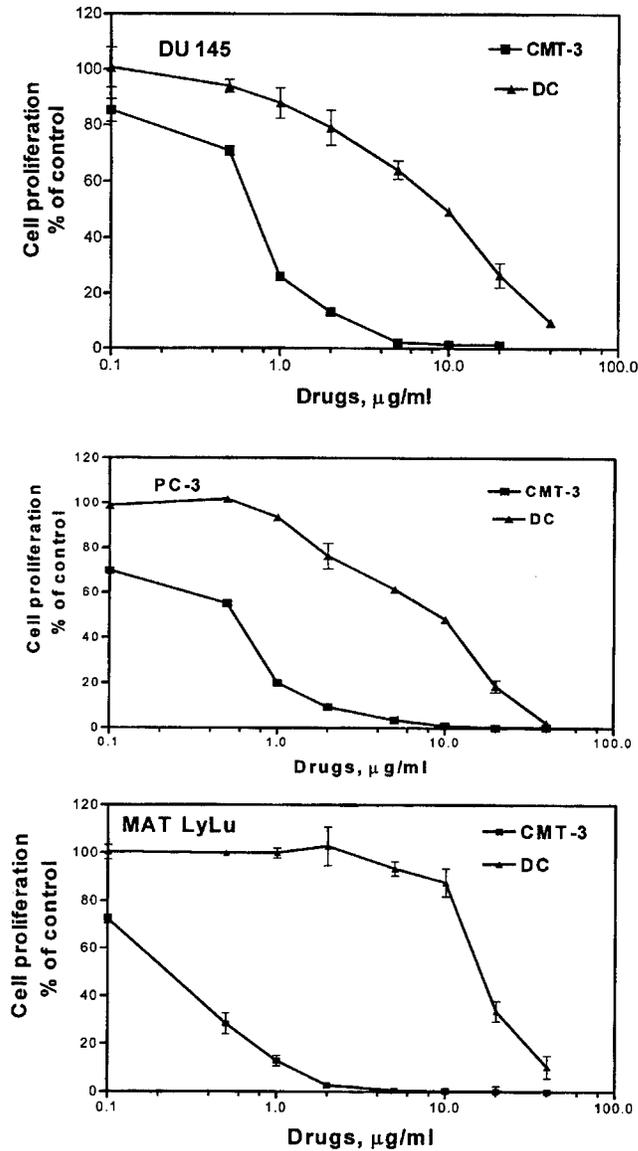
The ability of CMTs and DC to inhibit invasive activity of CaP cells *in vitro* was tested by plating cells on the top chamber of the Boyden Chemotactic chambers (Transwell plates, Costar-Corning Corporation, Cambridge, MA). Before plating the cells, the porous (12-µ) filter of the top chamber was coated with a soluble preparation of basement membrane, Matrigel (Collaborative Research/B-D Systems, Bedford MA). The bottom chamber contained human fibroblast culture-conditioned medium as a chemoattractant. Others have shown that cells invade the bottom chamber by dissolving Matrigel and migrating through the pores in the filter.<sup>62</sup> Various CMTs or DC were added to both top and bottom chambers at the time of plating cells. Invasive activity was quantified by estimating the fraction of invaded cells on the underside of the filter and in the bottom chamber. As we reported before, we find CMT-3 to be the most effective inhibitor of Matrigel invasion by CaP cells among the various CMTs tested. Using two CaP cell lines, PC-3 ML and DU 145, we found that CMT-3 was the most potent inhibitor of invasive activity and CMT-6 the least. Interestingly, although DC was minimally effective as an inhibitor of invasion of hu-

man CaP cells, it significantly inhibited the invasive activity of the Dunning rat MAT LyLu prostate cancer cells (FIG. 1). In subsequent studies we determined the drug concentration at which Matrigel invasion was inhibited by 50% ( $IC_{50}$ ). CMT-3 had the lowest  $IC_{50}$  among the seven CMTs tested ( $IC_{50} = 1.5 \mu\text{g/ml}$ ). The  $IC_{50}$  for DC was  $7.9 \mu\text{g/ml}$  in DU 145 cells and  $5.3 \mu\text{g/ml}$  for MAT LyLu cells.

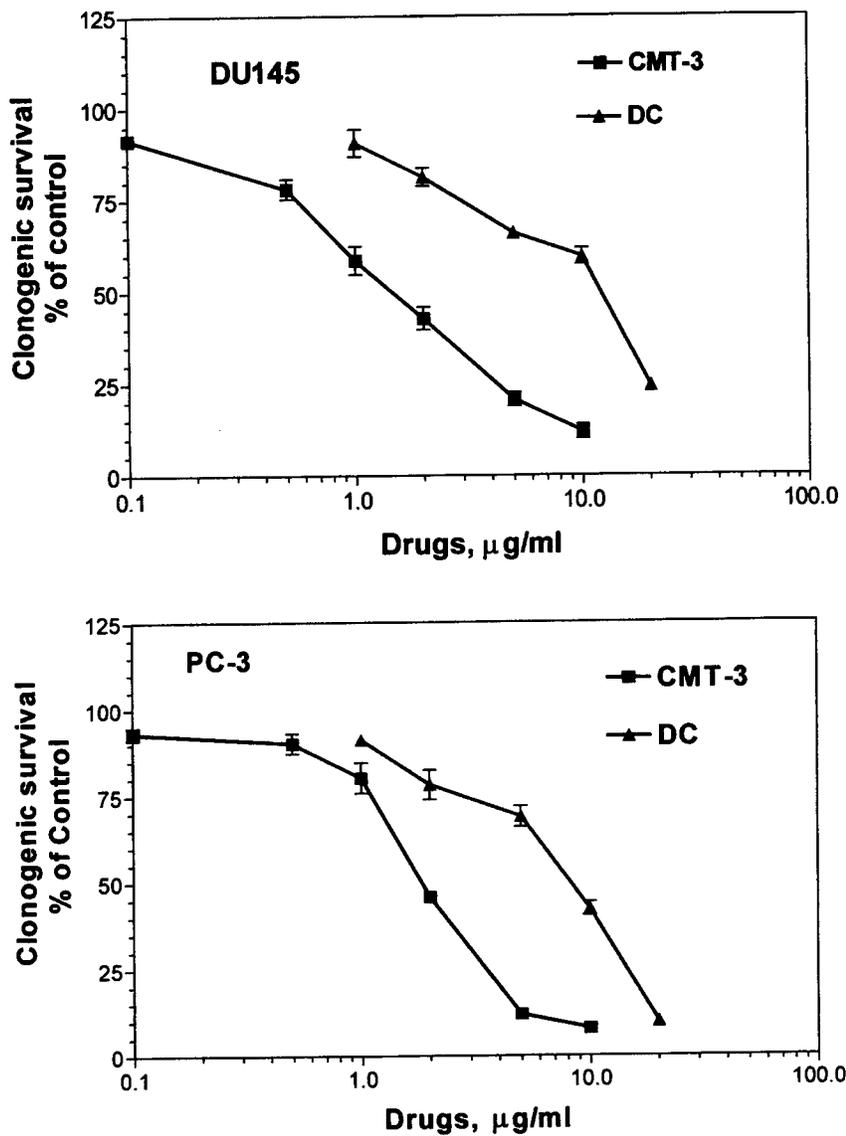
We also investigated whether the ability of CMTs and DC to inhibit invasive activity against CaP cells is associated with their ability to inhibit MMP activity. We reported previously that both CMT-3 and DC inhibit not only the activity of MMPs secreted by tumor cells, but also the synthesis of MMPs by human CaP cell lines and MAT LyLu cells.<sup>63</sup> Interestingly, while CMT-3 and DC strongly inhibited the synthesis of MMP-2, the synthesis of MMP-9 was weakly inhibited by DC in human CaP cells. Furthermore, while CMT-3 inhibited MMP synthesis in a dose-dependent manner, the synthesis of TIMP-1 and TIMP-2 were weakly inhibited (Lokeshwar, Selzer, Zhu *et al.*, submitted for publication).



**FIGURE 1.** Inhibition of invasive potential of tumor cells by DC and CMTs. Invasion of tumor cells through the Matrigel-coated filters was assayed following 48 hours of exposure to  $5 \mu\text{g/ml}$  of each drug. Only the drug diluent (0.1% dimethyl sulfoxide) was added to control wells. Percentage of cells that invaded in the control (0.1% DMSO) wells varied from  $12.5 \pm 6.4\%$  for DU 145 cells to  $17 \pm 4.2$  for MAT LyLu cells. 0.1% DMSO had negligible effect on invasion. Results presented are from three independent experiments.



**FIGURE 2.** Effect of DC and CMT-3 on proliferation of prostate tumor cell lines. Tumor cells were incubated with various concentrations of DC or CMT-3 for 48 hours in complete culture medium. Cell proliferation activity, defined as synthesis of [ $^3\text{H}$ ]-thymidine-labeled DNA, was assayed by 2-hour pulse-labeling the cells with [ $^3\text{H}$ ]-thymidine as described in the text. Data presented are for three CaP cell lines. Similar results were obtained for other cell lines. Vertical bars represent mean  $\pm$  SEM from four independent determinations.



**FIGURE 3.** Clonogenic survival of CaP cells exposed to CMT-3 or DC. Cells were exposed to CMT-3 or DC for 24 hours and plated out for clonogenic survival. Colonies of cells that survived the drug treatment were counted after 7–10 days. Note that the 50% clonogenic inhibition dose is similar to that obtained using the DNA synthesis inhibition assay as described in FIGURE 2.

### *Inhibition of Cell Proliferation by CMTs and DC*

There are several reports in literature regarding cytotoxic and cytostatic actions of tetracycline, although there are none at present on CMTs.<sup>64,65</sup> We report here the effects of CMT-3 and DC on cell proliferation. Antiproliferative effects of CMT-3 and DC were assayed quantitatively using the [<sup>3</sup>H]thymidine assay as described before.<sup>67</sup> Cells cultured in 48-well clusters were incubated with a range of concentrations of DC or CMT-3 for 48 hours. Cell proliferation activity was determined by pulse labeling the cells with [<sup>3</sup>H]thymidine for 2 hours. [<sup>3</sup>H]thymidine incorporation into proliferating cell DNA was stopped by adding cold 10% trichloroacetic acid to the culture wells. [<sup>3</sup>H]thymidine-labeled DNA was extracted, and incorporated radioactivity was measured in a liquid scintillation counter. The results are presented in FIG. 2, which shows that the inhibition of cell proliferation by CMT-3 or DC was dose dependent. CMT-3 was significantly more potent than DC in inhibiting cell proliferation. The concentration of the drug that decreased cell proliferation by 50% (IC<sub>50</sub>) was at least 5-fold lower for CMT-3 than that of DC.

We next examined the cytotoxic effects of DC and CMT-3 on clonogenic cell survival in two CaP cell lines, PC-3 ML and DU 145. Cells were incubated with various concentrations of drugs for 24 hours. Drugs were then washed off from the culture plates, fresh culture medium was added, and cell culture continued for 10 days in the absence of the drugs. Resulting cell colonies were fixed and stained with 0.1% crystal violet. Colonies of cells containing  $\geq 50$  cells were enumerated. As shown in FIGURE 3, the clonogenic cell survival was significantly inhibited by CMT-3 at concentrations  $\geq 1$   $\mu\text{g/ml}$ , when exposed for 24 hours. The 50% clonogenic inhibition doses for CMT-3 and DC for both CaP cell lines were  $1.4 \pm 0.2$   $\mu\text{g/ml}$  and  $10.8 \pm 0.3$   $\mu\text{g/ml}$ , respectively. Thus, CMT-3 was about 10-fold more potent inhibiting clonogenic survival of two prostate cancer cell lines tested than was DC.

### *Inhibition of Dunning Tumor Growth and Metastasis*

Encouraged by the activity of CMT-3 and DC on cancer cells *in vitro*, we investigated whether these drugs inhibit tumor growth and metastasis *in vivo*. We chose an androgen-unresponsive spontaneously metastatic prostate tumor model, the Dunning MAT LyLu rat tumor.<sup>68</sup> This tumor grows rapidly upon subcutaneous injection of as low as  $5 \times 10^4$  cells in Copenhagen rats. Palpable tumor growth appears in 7 to 9 days post tumor implant, with a medium tumor growth rate of 1.7 days in untreated animals. Spontaneous metastasis to lymph nodes and lungs are observed from 12 days after tumor cell injection.

The tumor-bearing rats were treated for 21 days with a daily oral gavage of CMT-3 or DC suspended in 2% carboxymethyl cellulose (2% CMC) at the dose of 20 or 40 mg/kg. Groups of seven to ten rats were dosed either 7 days before or immediately after tumor cell injection ( $1 \times 10^5$  and  $1 \times 10^6$  cells/site/animal, respectively). The rats in the control group were gavaged with 2% CMC. The results of two experiments where the tumors were generated by injecting  $1 \times 10^6$  cells/injected site or  $1 \times 10^5$  cells/injected site are summarized in TABLE 1: when a high tumor cell inoculum ( $1 \times 10^6$  cells/injected site) was used to generate tumor, tumor incidence, latency, or growth rate of tumors was not affected by oral administration of CMT-3 or DC. However, the tumor latency, defined as the duration between tumor cell injec-

TABLE 1. Growth and metastasis of MAT LyLu tumor in rats<sup>a</sup>

Experiment	Treatment group <sup>b</sup>	Tumor growth <sup>c</sup> (days to 3-cc tumor [mean ± sd])	Tumor growth <sup>d</sup> (days to 10-cc tumor [median])	Number of tumor foci in lungs (% of control)
(1) $1 \times 10^6$ cells/site, s.c. (10 per group)	Control	13.2 ± 2.4	17	59.5 ± 13.9 (100)
	DC (40mg/kg)	15 ± 1.3	17	43.6 ± 18.8 (73.2)*
	CMT-3 (40 mg/kg)	16 ± 1.6	19	28.9 ± 15.4 (48.5)*
(2) $1 \times 10^5$ cells/site, s.c. (7 per group)	Control	15.9 ± 2.0	21	77 ± 12.5 (100)
	DC (pre-dose, 7 days)	16.7 ± 1.9	23	38.3 ± 12.1 (49.7)*
	CMT-3 (pre- dose, 7 days)	20.2 ± 3.5	23	31.8 ± 4.7 (41.2)*

<sup>a</sup> Adult male Copenhagen rats, 90–100 days old and weighing approximately 250 g, were injected s.c. with MAT LyLu cells grown in culture. Tumor growth was monitored by palpating the site of cell injection and by using vernier calipers as described.<sup>60</sup>

<sup>b</sup> Drugs were orally administered by daily gavage, starting from the day of tumor cell injection (experiment No. 1), or a pre-dose was given 7 days before tumor cell injection (experiment No. 2) to a total of 21 days. Animals in the control group were gavaged with vehicle (2% carboxymethyl cellulose).

<sup>c</sup> Tumor growth rate was determined as described<sup>60</sup> by the slope of log-linear regression of individual tumor volume measurements for each animal and then time to reach tumor growth to 3 cc was determined by interpolation of the regression line.

<sup>d</sup> Median time to a growth of 10-cc tumor was when >50% of the animals per group had tumors ≥10 cc.

\*Values are significantly different than those for control ( $p < 0.05$ ; *t*-test).

tion and appearance of a palpable tumor mass, significantly increased if the tumor cell inoculum was lowered from  $1 \times 10^6$  cells/site to  $1 \times 10^5$  cells/site. Tumor growth rate, defined as the time duration to reach a 3 cubic cm tumor, was comparable between the low and high inoculum groups in untreated animals. Tumor growth was significantly affected, however, by CMT-3 gavage in the experiments when reduced tumor cell inoculum was used. The tumor growth was significantly slowed down in CMT-3-gavaged rats, but not to an appreciable extent in the DC-treated group. Tumor growth rate was  $20.2 \pm 3.5$  days in rats gavaged with CMT-3 (40 mg/kg, daily for 21 days) versus  $16.7 \pm 1.9$  days (DC, 40 mg/kg) or  $15.9 \pm 2.0$  days in the control group. In addition, in two independent studies, we observed a regression or disappearance of palpable tumor in CMT-3-treated groups, but not in the control or DC-treated groups. Tumor regression was observed in about 40% (3/7 and 4/10). Furthermore, we also observed a significant reduction in tumor incidence ( $55 \pm 9\%$ ) in rats which were pre-dosed with CMT-3 (40 mg/kg), but not in those treated with DC. Animals with no tumor incidence lived tumor-free for up to a year, at which time they were euthanized. At necropsy we observed a scar at the site of tumor cell injection, but no live tumor cells.

Regardless of the inhibition of local tumor growth, spontaneous metastasis to the lungs was reduced significantly in groups of rats treated with DC or CMT-3. We observed, in four separate experiments, that the reductions in the number of metastatic foci were  $33 \pm 12.3\%$  (DC) to  $51 \pm 7.4\%$  (CMT-3).

A remarkable observation during the course of these experiments was the lack of adverse reaction (systemic toxicity) of the drugs on the animals. As a measure of toxicity, we monitored the body weights of the animals during all the experiments. The animals were weighed before injecting the tumor cells, during gavage, and until the animals were euthanized because of a large subcutaneous tumor. As shown in FIGURE 4, the all three groups treated gained weight. Interestingly, animals treated with DC or CMT-3 gained a mean weight by  $8 \pm 3.4\%$  during the 3 to 4 weeks of the measurement period. These weight gains were comparable to those of the naive animals. We did not observe any other adverse effects of the two drugs during the course of this study (it spanned three years and 177 animals). Only six animals, never more than one animal per treatment group, died prematurely. The animals had no visible signs of distress or common signs of chemotherapy-induced lethargy, alopecia, or gastrointestinal abnormalities, indicating the very safe nature of the drugs.

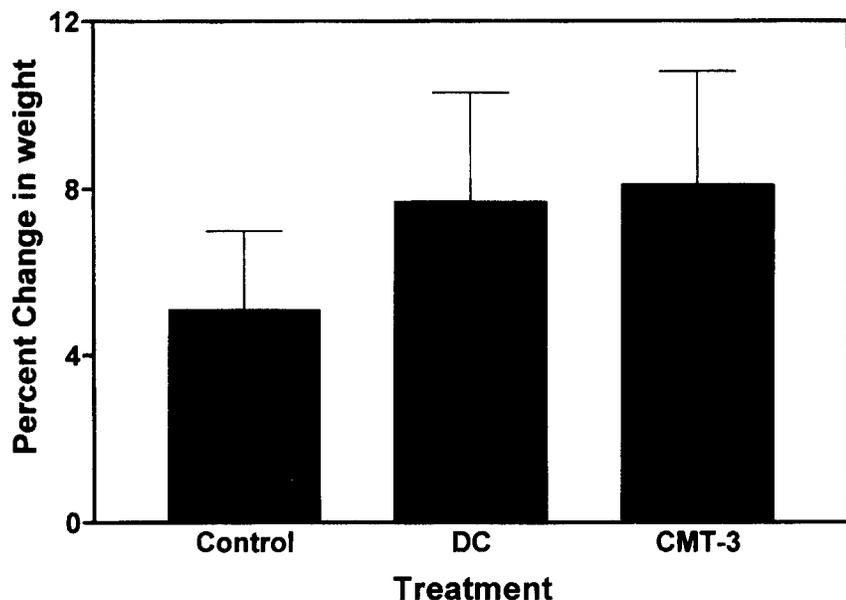


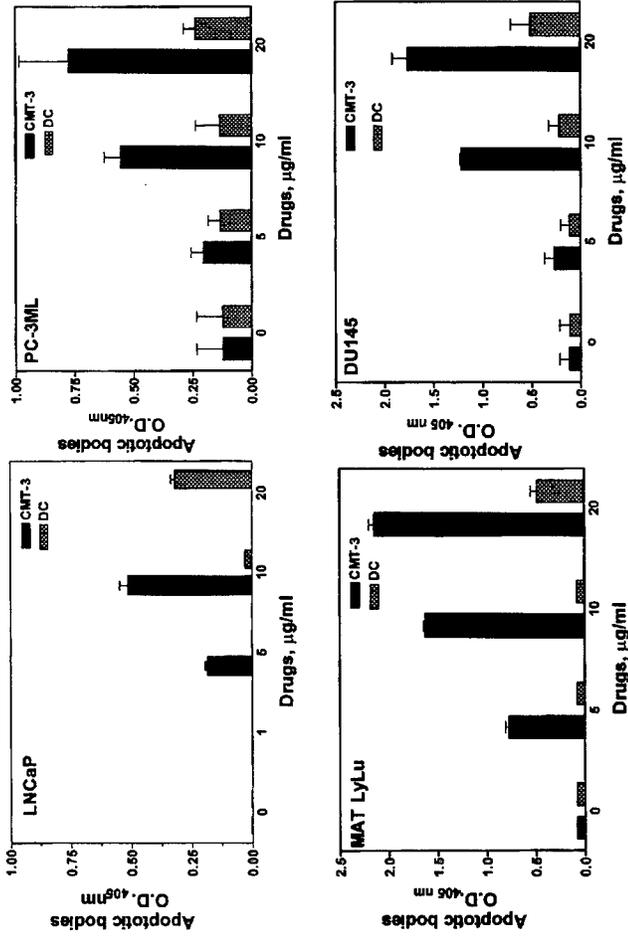
FIGURE 4. Change in body weight of rats treated with oral gavage of CMT-3, DC or the drug vehicle. Pooled measurements for each group before after the treatment were averaged. Data represent mean changes in weight after the treatment, just before the animals were euthanized. Data presented are from a single experiment with 10 animals per group, and similar results were observed in other experiments.

### *Effect of CMT-3 on Induced Skeletal Metastasis in the MAT LyLu Model*

Lumbar and vertebral bones are the common sites of prostate cancer metastases.<sup>69</sup> Degradation of bone matrix collagen is an essential step in skeletal invasion and skeletal remodeling during metastatic growth. We reasoned that inhibitors of MMPs should be able to reduce or eliminate bony metastasis and thus prolong the survival of rats bearing tumors that are metastatic to bone. Furthermore, in view of the classical finding that tetracyclines accumulate in bone tissues,<sup>70</sup> these agents may have an added advantage in inhibiting skeletal metastasis. To facilitate MAT LyLu tumor metastasis to lumbar and skeletal bones we followed the procedure of Geldof and Rao,<sup>16</sup> injecting MAT LyLu tumor cells into tail vein while momentarily occluding the caval vein with surgical clamps in the inferior vena cava of anesthetized rats. This procedure results in hematogenous spread of tumor cells into lumbar regions via lumbar venous plexus. Once the cells enter the lumbar region, they colonize lumbar and vertebral bones, which eventually leads to hind-limb paralysis in affected animals. The details of the experiment are described in a separate article, by Selzer *et al.* in this volume. In brief: four groups of six animals were injected with MAT LyLu tumor cells and were gavaged with CMT-3 (40 mg/kg), starting 7 or 2 days before tumor cell injection or 1 day after tumor cell injection. Animals in the control group were gavaged with 2% carboxymethyl cellulose (vehicle). The animals in all groups injected with MAT LyLu cells developed lung metastasis before they were moribund. In the group treated with vehicle alone, 5/6 (83%) of the animals had also developed hind-limb paralysis. The hind-limb paralysis might have resulted from spinal chord compression or have been due to the growth of tumors in the femur. Indeed, we were able to recover tumor cells from the cultures of marrow plugs obtained from femurs of paralyzed animals. However, in the group gavaged with CMT-3 starting 7 days pre-dose and continued until the animals were euthanized, only 1/6 (17%) developed hind-limb paralysis, and in another group treated with CMT-3, starting 2 days before tumor cell injections, none of the animals developed paralysis. In the group treated with CMT-3 one day after tumor cell injection, only 2/6 (33%) developed paralysis (see FIGURE 1 of the article of by Selzer *et al.* in this volume) and the survival in groups treated with CMT-3 increased significantly. Thus, in summary, treatment with CMT-3 results both in increase in survival and in decrease in skeletal and soft tissue metastasis. Further studies are under way in our laboratory to determine a noninvasive method of monitoring skeletal metastasis using this tumor model.

### *Investigation of the Mechanism of Action for CMT-3 Induced Cytotoxicity*

Since CMT-3 strongly inhibited cell proliferation *in vitro*, tumor growth, metastasis and tumor incidence *in vivo*, without much systemic toxicity, we investigated its mechanism of selective cytotoxic action. We found that the cytotoxicity of CMT-3 was limited to actively proliferating cells. For example, the IC<sub>50</sub> (>20 µg/ml) of CMT-3 on quiescent cells, such as serum-starved fibroblasts or confluent cultures of nontransformed cells, was at least 5- to 10-fold more than that found for continuous prostate cancer cell lines. Since many of the anti-tumor drugs which inhibit cell proliferation induce programmed cell death (PCD), we investigated whether CMT-3 also induces PCD. In these experiments, MAT LyLu cells were treated with various concentrations (1–20 µg/ml) of either CMT-3 or DC. The culture-conditioned media



**FIGURE 5.** CMT-3 and DC induced programmed cell death (PCD) in prostate cancer cells. Cells were exposed to DC or CMT-3 for 48 hours. Culture supernatants were assayed for the presence of apoptotic bodies (free nucleosomes) using the Behring-Mannheim cell death ELISA-Plus kit. The kit provided both positive and negative controls for the assay for comparing inter-assay consistency and specificity. The absorbance at 405 nm (OD) was directly proportional to the amount of free nucleosomes. Note that LNCaP and PC-3 cells were less apoptotic to CMT-3 or DC than were DU145 and MAT LyLu CaP cells. Induction of PCD was also independently confirmed using other parameters of cell death such as caspase-3 activation and cell surface translocation of phosphatidyl serine in drug-exposed cells (data not shown). Results presented are from at least three independent determinations.

from the drug-treated cells were assayed for PCD. Cells undergoing PCD release soluble nucleosomes into the culture medium. These nucleosomes contain H1 histone which is detected by means of an indirect-ELISA (Cell Death Detection ELISA Plus kit, BM Biochemicals, Indianapolis, IN). As we have reported before,<sup>60</sup> induction of PCD by CMT-3 exposure was both dose and time dependent. CMT-3 was able to induce PCD in all the seven permanent prostate cancer cell lines tested. As reported before,<sup>60</sup> a brief exposure ( $\leq 4$  hr) to CMT-3 (10  $\mu\text{g/ml}$ ) led to nuclear fragmentation in  $> 80\%$  of the cells. Of interest, DC induced PCD at 5–10-fold higher concentrations ( $\geq 20$   $\mu\text{g/ml}$ ) than that of CMT-3 (FIG. 5) in our assays. The ability to induce PCD is a desirable property of an orally administrable anticancer drug. Rapid and irreversible induction of PCD should make the drug more effective, even if the peak concentration of the drug is achieved only briefly. Thus, our results and those of others<sup>71</sup> clearly demonstrate that CMT-3 could be an effective therapeutic drug for metastatic prostate cancer.

### SUMMARY

Results obtained by us and others support the hypothesis that MMP inhibition could be an effective approach to reduce tumor growth and metastases. Prophylactic administration of some anti-MMP agents (e.g., DC or CMT-3) may delay clinical manifestation of prostate cancer metastatic to bone. Results obtained using CMT-3 support the concept that MMP inhibition, combined with cytotoxic properties, but little systemic toxicity, could be an effective therapy for advanced cancer. We demonstrated a unique property of CMTs, an induction of apoptosis with cell-type specificity.

### ACKNOWLEDGMENTS

This work is funded in part by the National Institute of Health Grant R29 CA 61038, Department of the U.S. Army Prostate Cancer Research Program Ideas Development Grant No. DAMD 17-98-272, and Austin L. Weeks Endowment to the Department of Urology.

I am grateful to Marie G. Selzer, Bao-qian Zhu, Heather L. Houston-Clark, and Eva Escatel for generating most of the data reported here. I am also indebted to my mentor, Professor Norman L. Block, for many years of financial support, advice, and encouragement.

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## **Interaction between Stromal Cells and Tumor Cells Induces Chemoresistance and Matrix Metalloproteinase Secretion**

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### **INTRODUCTION**

Metastatic prostate cancer is resistant to most cytotoxic chemotherapeutic drugs.<sup>1,2</sup> At the cellular or molecular level the development of resistance to chemotherapeutic drugs by cancer cells is a complex phenomenon. Among the many non-mutational mechanisms that regulate chemosensitivity to anticancer drugs, cell-cell and cell-extracellular matrix (ECM) interactions are believed to be important.<sup>3-5</sup> Because the normal prostatic epithelial cells rely on their interactions with stromal cells and the ECM for their survival, it may be argued that stromal cells or the factors released by them may also alter the response of tumor cells to anticancer drugs.<sup>6</sup> Moreover, the role of tumor cells in modifying the healthy stroma to facilitate tumor cell growth, invasion, and metastasis is also just being unraveled.<sup>7</sup> Furthermore, it has long been known that tumors growing in different metastatic sites are not equally sensitive to cytotoxic actions of chemotherapy drugs.<sup>8,9</sup>

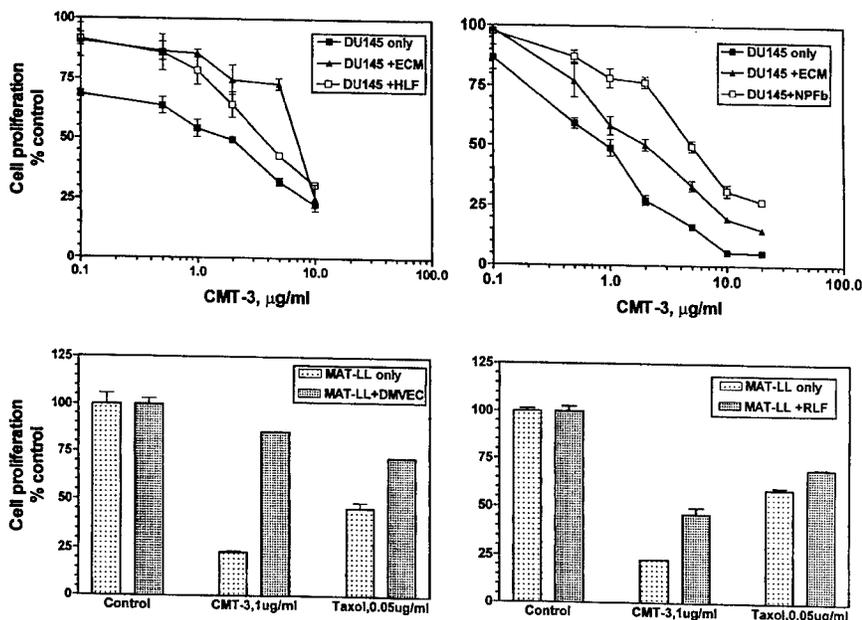
We tested two hypothesis: (1) The differences in the responses of the metastatic tumors to anticancer drugs *in vivo* is due to the interaction of tumor cells with the normal cells that surround them; and therefore, by creating these conditions *in vitro* we should be able to reproduce some of the observed differences *in vitro*. (2) Tumor cells modify the stromal cells and endothelial cells to their advantage for invasion and metastasis by inducing them to secrete matrix-degrading enzymes. These hypotheses were tested on prostate tumor cells by coculturing tumor cells with stromal cells isolated from both the primary tumor site (prostate) and a distant metastatic site (e.g., lung). The drugs used were Taxol and CMT-3 (6,deoxy, 6-dimethyl, 4-dedimethylamino tetracycline). CMT-3 is a novel cytotoxic compound with multiple cellular targets.<sup>10-12</sup> The effect of tumor cells on the pattern of MMP secretion by stromal cells and endothelial cells was examined in culture-conditioned medium using species-specific enzyme immunoassays (ELISA).

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## RESULTS AND CONCLUSIONS

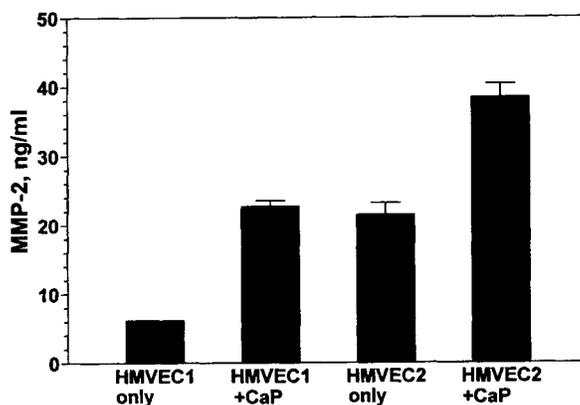
*Effect of Stromal Cells on Cytotoxicity of Anticancer Drugs*

We tested the effect of CMT-3 and Taxol on both the human prostate tumor cell line DU145 and on the Dunning rat MAT LyLu prostate tumor cells. Tumor cells were cultured in the bottom wells of Transwell plates with 3 $\mu$  filter inserts (Corning-Costar Cat. no. 3415). Cells were cultured with or without the normal cells in the inserts. The inserts included complete medium (control), or cultures of normal human prostate epithelial cells, prostate stromal cells, or dermal microvessel endothelial cells (DMVEC). Drugs were added to both top and bottom cultures two days after plating cells. Cytotoxicity was determined after 48 hours by a colorimetric assay (methyl tetrazolium bromide reduction assay, the MTT assay). The 50% inhibition dose (IC<sub>50</sub>) for each drug was estimated for each culture condition. Cells were considered chemoresistant if the ratio between the IC<sub>50</sub> under coculture condition and tumor cells cultured alone was greater than one.



**FIGURE 1.** Effect of stromal cells and ECM on the inhibition of prostate tumor cell proliferation by CMT-3 and Taxol. Tumor cells (DU145 or MAT-LL) were cultured separately using the Costar Transwell inserts in the presence of a human lung fibroblast cell line (HLF), rat lung fibroblasts (RLF), or on their ECM, prepared as described.<sup>13</sup> Both tumor cells and the fibroblasts were exposed to CMT-3 or Taxol for 48 hours. The cell proliferation (as a measure of cell viability) was quantified by the MTT assay as described.<sup>12</sup> Results presented are for two cell lines from three experiments (mean  $\pm$  SE). Similar results were obtained for several other prostate cancer cell lines.

Tumor cells cocultured with prostate fibroblasts showed significant chemoresistance to all the three cytotoxic drugs. The resistance was dependent on both tumor cells and the normal cell combination (FIG. 1). Prostate fibroblasts and DMVEC rendered both DU145 and the MAT LyLu cells resistant to CMT-3, and Taxol by 3.0- and 1.7-fold, respectively. Tumor cells cocultured with the normal prostatic epithelial cells did not show any chemoresistance (data not shown). We next examined the effect of ECM prepared from the stromal cells on drug sensitivity of tumor cells. The results shown are for CMT-3 only, although other drugs were tested. The human prostate cancer (DU145) cells cultured on ECM prepared from both lung fibroblasts and prostate fibroblasts showed significant resistance to CMT-3-induced cytotoxicity. The  $IC_{50}$  for CMT-3 was 3.7  $\mu\text{g/ml}$ , 1.5  $\times$  higher than that for tumor cells cultured alone (FIG. 1A, B). Similarly, the  $IC_{50}$  of CMT-3 for MAT-LyLu cells, cultured on rat lung fibroblast-derived ECM, was significantly higher ( $IC_{50}$  7.2  $\pm$  1.2  $\mu\text{g/ml}$ ) than that of the same cells cultured without the ECM ( $IC_{50}$  2.5  $\pm$  0.7  $\mu\text{g/ml}$ ). These findings show that it is the *stromal cell-tumor (epithelial) cell interaction*, and not the tumor-normal epithelial cell interaction, that protects both cell types from drug-induced cytotoxicity. Interestingly, cell-cell contact was not necessary to induce chemoresistance. The ECM prepared from the specific stromal components alone was also capable of inducing significant chemoresistance. Furthermore, our experiments demonstrate for the first time that conditions observed only *in vivo* can also be reproduced *in vitro*, under an appropriate organ-specific metastasis-dependent microenvironment.



**FIGURE 2.** Tumor cells induce MMP-2 production in microvessel endothelial cells. HMVECs were cultured in the bottom well of the Transwell plates until the cells cover 50% of the growth surface. Tumor cells (MAT-LyLu) cultured in the top inserts separately were transferred to HMVEC culture wells. Culture-conditioned medium from the bottom wells was collected and assayed for MMP-2 or MMP-9 by ELISA. The assay did not detect any MMP-2 or MMP-9 produced by the MATLyLu cells, as the primary antibodies used in the assay kit were specific to human MMPs. Results presented are for MMP-2 only, as the amount of MMP-9 was below the detection limit of the assay. Results presented are pooled from two independent experiments each with triplicate wells.

### *Tumor Cells Induce MMP Secretion*

We analyzed the MMP content in the conditioned medium from cocultures. To distinguish the MMP secreted by stromal cells or endothelial cells from that of tumor cells, we used an immunoassay to analyze MMPs. The anti-MMP antibody used to detect MMP in the conditioned medium was specific to human cell-derived MMP (Biotrack human MMP ELISA kits, Oncogene Sciences/Calbiochem, Cambridge, MA). Conditioned media collected from cocultures of rat MAT LyLu prostate tumor cells and human prostate fibroblasts, human lung microvessel endothelial cells (HMVEC-1), or dermal human microvessel endothelial cells (HMVEC-2) were analyzed for both MMP-9 and MMP-2. Normal lung MVEC or dermal MVECs did not produce significant amounts of MMP-2 in our culture systems. However, there was a two- to fivefold increase in MMP-2 produced by these cells under coculture conditions (FIG. 2). Very little MMP-9 was secreted and therefore could not be quantitated using the ELISA system. These results indicate that tumor cells modulate invasive enzyme production by host cells (stromal cells). This modulation plausibly enhances the invasive and angiogenic potential of tumor cells.

### ACKNOWLEDGMENT

This work was funded by a NIH grant (1R29-CA-61038), the U.S. Army Prostate Cancer Research Program (DAMD 17-98-272), and the L. Austin Weeks Endowment (U. Miami).

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## **CMT-3, a Chemically Modified Tetracycline, Inhibits Bony Metastases and Delays the Development of Paraplegia in a Rat Model of Prostate Cancer**

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### **INTRODUCTION**

In 1998 an estimated 186,500 Americans will be diagnosed with prostate cancer.<sup>1</sup> At the time of diagnosis approximately 50% of prostate cancer patients will have some form of extraprostatic disease—e.g., metastasis to lymph node, lung, and bone.<sup>2</sup> Carcinoma of the prostate is, by far, the most common of the neoplasms that produce osteoblastic metastases.<sup>3</sup> These metastases result from tumor cell invasion into the bony matrix by the enzymatic degradation of collagen and other matrix components.<sup>4</sup> Key enzymes in collagen degradation are the matrix metalloproteinases (MMPs), secreted by tumor and stromal cells, during tumor-induced bone remodeling.<sup>5</sup> Existing therapies to control this painful disease are only palliative, not curative.<sup>6</sup> We reported, some years ago, that primary cultures of human prostate tissue secrete high levels of activated MMP-2 and MMP-9, but low to undetectable amounts of their natural endogenous inhibitors (TIMPs).<sup>7</sup> In this study we examined whether CMT-3 (6-demethyl, 6-deoxy, 4-dedimethylamino tetracycline) a nontoxic, non-antimicrobial, orally bioavailable MMP inhibitor, with a strong affinity to bone,<sup>8</sup> could inhibit prostate cancer skeletal metastasis.

### **MATERIALS AND METHODS**

Male Copenhagen rats, 90–100 days old, were purchased from Harlan Labs, Indianapolis, IN. CMT-3 was a gift from CollaGenex Pharmaceuticals Inc., Newtown, PA. Tissue culture reagents and supplies were from Life Technologies, Inc., Gaithersburg, MD. All other reagents were from Sigma Chemical Corp, St. Louis, MO.

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### *Cell Cultures*

An androgen-independent, highly metastatic rat prostate tumor model, the Dunning MAT LyLu, was obtained from Dr. John T. Isaacs, Johns Hopkins Oncology Center, Baltimore, MD. MAT LyLu cells were cultured in RPMI containing 10% fetal bovine serum, gentamicin (0.2 mg/L) and dexamethasone (0.25  $\mu$ M). Cultures were routinely tested and found negative for common mycoplasma. Cultures were frequently inoculated into rats for tumor production and were found to retain their stable phenotype, capable of producing tumor nodules at cell concentrations  $\geq 10,000$  cells/site.

### *Induction of Skeletal Metastasis*

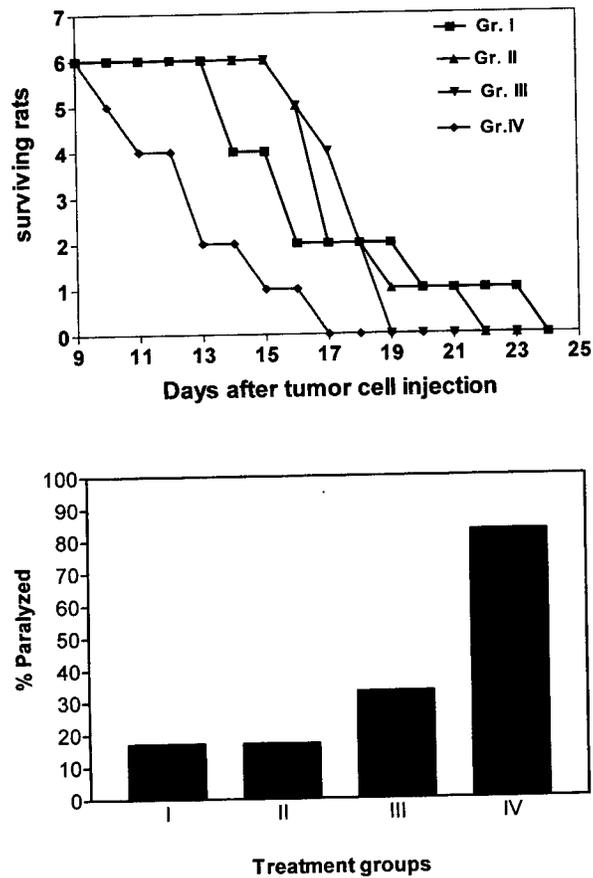
Tumors metastatic to lumbar bones were induced in Copenhagen rats by the method of Geldof and Rao.<sup>9</sup> Rats were anesthetized by ketamine injection and the lower abdomen was shaved and cleaned with alcohol. A small midline incision was made and the inferior vena cava isolated; a small surgical "bulldog" clamp was placed on the vena cava, followed immediately by injection of  $5 \times 10^4$  Dunning MAT LyLu cells into the lateral tail vein. The vena cava was clamped for a maximum of 5 minutes. The clamp was then removed and the incision closed in two layers with 3-0 silk. The animals withstood the procedure well and usually recovered within two hours, without any noticeable discomfort. Postoperative posture—e.g., conduct, food and water intake—was similar to that observed prior to the procedure.

### *Agent Administration*

CMT-3 (40 mg/kg) was administered by oral gavage daily for up to 4 weeks with no sign of systemic toxicity. Four groups of six animals were treated with CMT-3 as follows: group I were predosed daily for 7 days; group II were predosed daily for 2 days; group III were dosed one day postimplant; group IV (control) were given 2% carboxymethyl cellulose in water (vehicle) only. Treatment in all groups continued until the animals were euthanized. The criterion for euthanasia was either complete hind limb paralysis or acute respiratory distress. At necropsy the lungs, lumbar vertebrae, and femurs were excised. Marrow plugs, from the femurs of paralyzed rats, were collected and cultured in MAT LyLu culture medium. The lungs and vertebrae were fixed in Bouin's fixing fluid and formalin, respectively.

## RESULTS

Most animals developed acute pulmonary distress with or without paraplegia starting from 12 days after tumor cell injection, and therefore were euthanized. There was a significant delay in the development of pulmonary morbidity and subsequently an increase in survival in rats treated with CMT-3. For example, the median survival was increased by 29.2% (group I, CMT-3 predosed for 7 days), 22.7% (group II, CMT-3 predosed 2 days), and 10.5% (group III, CMT-3 dosed from one day after tumor cell injection) (FIG. 1, top). Upon necropsy, induction of hematogenous metastases, by tail vein injection of tumor cells, was evident as visible pleural tumors, and in the control group the lung surface was completely covered with tumor



**FIGURE 1.** (Top) Oral administration of CMT-3 prolongs survival of rats injected with Dunning MAT LyLu tumor. Tumors were induced and treated with CMT-3 or vehicle alone as described. Animals with acute pulmonary distress or paraplegia were euthanized on the day indicated in the figure. Results are from a single experiment with six animals per group (Bottom) Tumor-induced paralysis in the Dunning MAT LyLu tumor-bearing rats. Tumor growth in the vertebral and lumbar bones were induced by iv injection of  $5 \times 10^4$  MAT LyLu cells with simultaneous vena cava clamping. All animals survived surgery. Groups of rats were gavaged with CMT-3 (40 mg/kg) starting from 7 days before tumor cell injection or as indicated in the figure. Animals that could not stand on hind limbs or that dragged their limbs during forward motion were considered paraplegic and were euthanized.

foci. A majority of the animals (83%) in the control group (IV) also developed paraplegia at about the same time as they developed pulmonary distress. Tumor cells were recovered from the marrow plugs in about 50% of the animals that had developed paraplegia, and none from nonparaplegic animals. There was no evidence of tumor in any other organ. There was a significant and remarkable reduction in the number of animals that developed paraplegia before they developed pulmonary distress, an end point in our experiment. Compared to the 83% of the animals that developed paraplegia in the control group, only 17% developed paraplegia in groups I and II, and 33% in group III (FIG. 1, bottom). Furthermore, one animal in group IV also developed enlargement of the urinary bladder due to urinary retention.

### DISCUSSION

An experimentally induced bone metastasis model, using the MAT LyLu tumor cells, provided the opportunity to test the potential of an anti-MMP drug to affect prostate tumor metastasis to bone. This complemented our earlier study, where we demonstrated the potential of CMT-3 against tumor metastasis to lungs. Our observation of a significant reduction in the size and number of pleural tumors in those animals treated with CMT-3 demonstrates the principle that prostate cancer metastasis is associated with increased MMP activity. An increase in longevity and the delayed onset, or total lack, of paraplegia demonstrate that bony metastasis can be countered with an administration of strong inhibitor of MMPs, such as CMT-3. CMT-3 could potentially be a potent new, site-directed (bone), orally administrable, and safe drug against prostate cancer metastatic bone, a highly painful and debilitating stage of the malignant disease.

### ACKNOWLEDGMENTS

This work is funded by grants from the National Institutes of Health (1R29-CA 61038), the U.S. Army Prostate Cancer Research program (DAMD 17-98-272), and the L. Austin Weeks Endowment (University of Miami).

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