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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: April 2002

TYPE OF REPORT: Annual, Phase II

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2002	Annual, Phase II (1 Apr 01 - 31 Mar 02)

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

5. FUNDING NUMBERS
DAMD17-98-1-8526

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Two non-antibiotic forms of tetracycline (CMT-3 and CMT-308) and a collagen/collagenase inhibitor halofuginone (Stenerol) were tested either alone or in combination in vitro on human prostate cancer (CaP) cell lines and in vivo on a rat model of metastatic prostate cancer (MAT LyLu). Studies during previous grant period had shown the potent activity of CMT-3 against skeletal metastasis and cytotoxic effect against all CaP cell lines. CMT-3 induced changes in gene expression and alteration of its effect by stromal cells (endothelial cells, bone marrow osteoblasts (OB), and lung fibroblasts (LFb)) were investigated in vitro. Studies revealed, endothelial cells decrease efficacy of CMT-3 but OB and LFb sensitize CaP cells to CMT-3. Increase production of IL-1b, IL-6 by CaP cells were linked to sensitivity where as arrest in G1 phase linked to resistance. Changes in gene expression in CaP cells resulting from CMT-3 treatment are documented. In vivo characterization of CaP and MAT LuLu cells expressing enhanced green fluorescent protein (EGFP) was done. Studies showed oral CMT-308 (less phototoxic CMT-3 derivative) treatment was more effective than taxol+CMT-308 combination. Stenerol did not show enhanced efficacy. Studies on other models of metastatic CaP with radiation plus drugs are ongoing.

14. SUBJECT TERMS	15. NUMBER OF PAGES
prostate cancer, chemically modified tetracycline, metastasis, stenerol	35
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Title: Title: An innovative strategy for the prevention and treatment of metastatic prostate cancer: chemically modified tetracycline as chemotherapeutics-Phase II.

A. Introduction and background: The overall goal of this funded project is to determine the utility of non-antibiotic forms of chemically modified tetracycline (CMT) against metastatic prostate cancer, with specific emphasis on prostate cancer-metastasis to bone. During the first phase of this project after screening about 10 analogues of CMTs, we identified a non-antimicrobial CMT, 6-deoxy, 6-demethyl, 4--dedimethylamino tetracycline (CMT-3, COL-3) as a potent orally bioavailable anti-neoplastic and anti-metastatic agent. Although we had initially hypothesized CMTs as anti-metastatic agents due to their strong anti-metalloproteinase (anti-collagenase) activity in several systems [1-3], we discovered some of the CMTs have activities similar to cytotoxic chemotherapeutic agents. We found, CMTs inhibit cell proliferation via mitochondrial permeabilization, induction of apoptosis and by arresting cells at G1/S boundary [4-6]. These properties combined with their strong affinity for bone matrix led us to test the efficacy of CMT-3 on bone metastasis. We reported that when tested on a highly aggressive prostate cancer model, induced to form skeletal/lumbar bone metastasis, CMT-3 was able to inhibit development of paraplegia and cause a significant delay in development of morbidity associated with lung metastasis [7].

The compound was tested in the clinic (Phase I) independent of this research group and was recommended for Phase II and III trials. However, its clinical efficacy was modest (28% disease stabilization, and 13% remission [8]. The major dose-limiting toxicity was development of photosensitivity at doses above 70 mg/day, and drug-induced lupus (7%). Several derivatives of CMT-3s, with modifications to Carbon-7 and carbon-9 positions of the CMT-3 was developed and tested for similar activities. We reported earlier that functionally, CMT-303 (9-Nitro CMT-3), and CMT-308 (9-aminoCMT-3) resemble CMT-3 activity without photo toxicity [5]. We had three objectives in this second phase of the project. These are: (1) Test the efficacy of CMT-3 and CMT-308 on tumor growth and metastasis, either alone or combined with other therapeutic modality; such as cytotoxic chemotherapeutic drugs (e.g., docetaxel or doxorubicin) or radiation, (2) Test the cytotoxic and antimetastatic activity of halofuginone - a novel collagenase and collagen synthesis inhibitor halofuginone (Stenorol) [9-11] - and (3) Determine how stromal cells modify the cytotoxic response of tumor cells to CMT-3 and other chemotherapeutic drugs (chemodrugs).

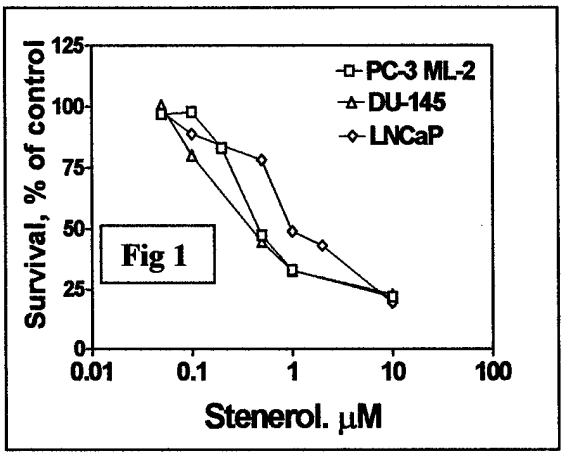
Since halofuginone is a new agent, recently identified as a potential therapeutic agent for bladder and prostate cancer [11-13], the following is a brief background for this compound as it relates to this report. Halofuginone (HF) is a quinazoline alkaloid (M W 495 Da) isolated from the plant Dichroa febrifuga (Merck Index No. 4509, p724,25th Edition, 1993]. HF is routinely used in poultry feed as a coccidistat, including cryptosporidium, drug under the trade name Stenerol (Hoecsht-Roussell-Vet, Clinton, NJ). Recent studies have demonstrated that HF is a specific inhibitor of MMP-2 transcription, type I collagen synthesis and extracellular matrix (ECM) deposition [11]. Furthermore, a single treatment of HF reduced lung metastasis of a mouse bladder cancer line MBT2t50 [12]. A preliminary report at the time of submission of this research project had suggested that HF might be a potential antitumor drug against CaP [12]. We were excited in HF as a potential antimetastatic drug, capable of complementing CMTs because, (1) HF is orally administrable, as a diet supplement, (2) it is non-toxic at pharmacologically effective dose ($\leq$1.5 mg/kg, I.V.) [14], and (3) it may inhibit invasion and metastasis by inhibiting MMP-2 transcription [14]. MMP-2 is the predominant MMP produced by MAT LyLu, PC-3ML and other CaP cells [9]. Since the HF inhibits MMP-2, type I collagen gene expressions and ECM synthesis, all three important in bone remodeling, we reasoned HF might have additive or synergistic effect with CMT-3 in a combination therapy involving CMT-3/CMT 308 and HF. In addition, like CMT-3, if HF inhibits CaP lung metastasis, the combined therapy may further improve the length of survival of patients with late stage (D1) CaP. We first wanted to demonstrate such activity in vitro and in vivo using metastatic CaP cells and the Dunning MAT LyLu rat model [15].

The following is a succinct report of the progress in achieving our objectives in the first year of the two-year project.

B. Progress related to Aim 1: Compare and contrast the efficacy of orally bioavailable inhibitors of metastasis (CMT-3, CMT-308 and HF), either alone or in combination against metastatic prostate cancer.

B1. Determination of cytotoxicity of HF and HF+CMT-3:

Initially we tested the efficacy of HF alone on common CaP cell lines (DU 145, LNCaP, PC-3ML and MAT LyLu). Since HF in pure form was not commercially available, we obtained its poultry feed formulation Stenorol (Hoechst-Roussel Vet, Clinton, NJ). Stenorol is halofuginone hydrobromide (6g/Kg; 0.6%) formulated with insoluble poultry feed material. Upon dissolving in water, the active ingredient of Stenorol, halofuginone hydrobromide (HF), forms a pale brown solution, which was used for all cell culture assays, after clarifying and sterilizing the solution with 0.2-micron filter. HF was added to CaP cell cultures in 48-well plates and cell viability was estimated using the tetrazolium blue reduction assay (MTT assay) as described before [6, see Appendix 2, for this and all other procedures in detail]. HF induced cytotoxicity in CaP cells is profiled in **Fig 1**. HF was cytotoxic to all three human prostate cancer cell lines tested. It is clear from Fig 1, the 50% growth inhibition dose, at 0.8 ± 0.3 μM did not differ significantly among various cell lines tested. HF was also cytotoxic to MAT LyLu rat prostate tumor cell line with a comparable 50% growth inhibition dose (data not shown). We also examined the clonongenic survival of CaP cells, PC-3 and DU 145, treated with Stenorol for 48 hours or continuously for 8 days. Treated cells were allowed to grow into colonies containing 50 cells or more. Clonogenic survival profile of cultures treated with HF for 48 h were similar to that observed for the growth inhibition assays (clonal 50% growth inhibition dose (CIC_{50}): 1.2 ± 0.33 μM for both cell lines). Continuous incubation of cell cultures with Stenerol showed significantly more toxicity (CIC_{50}: 0.6 ± 0.4 μM).



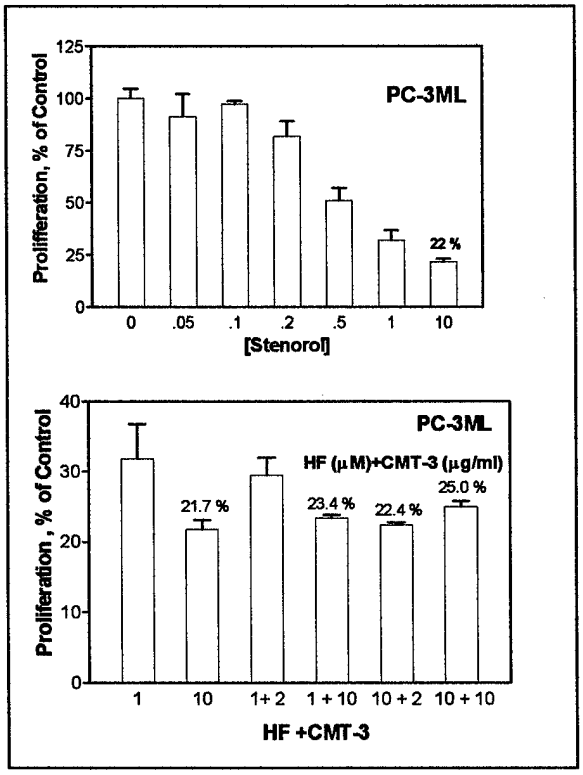


Fig 2. Combined cytotoxicity of CMT-3 and HF on PC-3 ML cells incubated with the drugs for 48h. Top panel, HF alone. Results presented are from three experiments.

We next examined whether a combination of CMT-3 and CMT-308 with Stenerol results in higher cytotoxicity. Cell cultures in 48-well plates were incubated with various concentrations of HF, CMT-3 or HF+CMT-3 at the same time. Cytotoxicity was determined by a MTT assay following exposure for 48 h. The results of a series of experiments to determine possible additive or synergistic cell proliferation-inhibitory activity of HF with CMT-3 on PC-3ML cells is shown in **Fig 2**. The experiment did not demonstrate an additive or synergistic effect of the HF+CMT-3 combination in PC-3ML cells. Similar results were also obtained using HF and CMT-308 (COL-308) in PC-3ML and MAT LyLu cells.

B. 2. Effect of HF, CMT-3 and HF+CMT-3 combination on Matrigel invasion: We investigated the anti-invasive activity of HF alone, or HF+CMT-3 against the invasive potential of MAT Ly Lu cells. Since MAT LyLu cells are relatively more invasive in Matrigel invasion assays than other CaP cells [6], we first tested these drugs on the MAT LyLu cells. For this test, MAT LyLu cells plated in 60-mm culture dishes were incubated with CMT-3 (10 μg/ml) or HF (1 and 10 μM each) for 6 hours. At the end of incubation, cells were detached from culture dishes using trypsin/EDTA dissociation method, washed and

plated on the top chamber of 12 μ membrane inserts, coated with a 0.5mm thick layer of Matrigel. Cells were induced to migrate to the bottom wells through the Matrigel coated barrier with the addition of prostate fibroblast culture-conditioned medium in the bottom chamber, as a chemoattractant. Extent of invasion was estimated with a MTT assay of live cells in the bottom and top chamber [6]. Amount of formazan generated by live cells in the bottom and top chamber was dissolved in DMSO and read in a plate reader, the OD was taken as proportional to number of live cells In respective chambers. Invasion activity was expressed as the percent ratio of OD of the bottom wells to that of total (top+bottom). The ratio was 23 ± 7.4 % in the two-chambered transwells containing untreated cells (Control). OD was equal or less than the controls in all other wells. As shown in **Fig 3**, where the invasion activity is expressed as % of control, HF did not appear to cause any significant alteration in chemoinvasive activity in MAT LyLu cells. However, CMT-3 was able to inhibit invasive activity as much as 50%. IN addition, the invasion activity was not significantly less for cells treated with both CMT-3 and HF, indicating a lack of additive response for the CMT-3+HF combination treatment. *Thus in summary, although HF alone had significant cytotoxicity against CaP cells, when combined with CMT-3 or CMT-308 (data not shown), no significant enhancement in cytotoxicity or invasive activity was observed. We are reexamining these results and will conduct additional experiments before concluding there is no additive or synergistic effect on CaP cell invasion. Furthermore, the situation may be quite different in vivo for CaP metastatic to bone, as MMPs are known to play more significant role in establishing and colonizing bone tissue [16, 17]. We will conduct in vivo studies on bone metastatic model of CaP during the coming year after careful assessment of in vitro findings.*

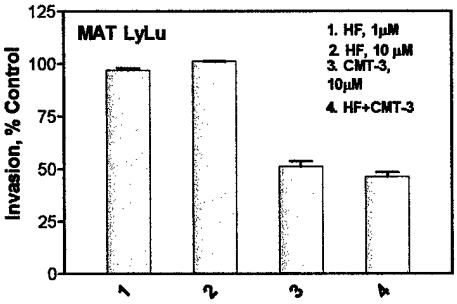


Fig.3. Effect of CMT-3 and HF against invasive potential of MAT LyLu cells.

B3: In vivo efficacy of combined chemotherapeutic drug (docetaxel) and anti-metastatic drug (CMT-3 or CMT308): Although we had planned to combine CMT-3 (COL-3) and the cytotoxic drug (taxol or docetaxel (Taxotere)), a clinical outcome of a Phase I trial of CMT-3 (COL-3) made us change our plans. As mentioned earlier, in this recently concluded clinical trial, involving 35 patients, Rudek et al reported that COL-3 treatment resulted in disease stabilization in about 28% of patients up to 26 months [8]. However, the major dose-limiting toxicity was photosensitivity. Patients who took COL-3 developed severe photosensitivity reaction to both UV-A and UV-B (normal sunlight). Less photosensitizing (determined experimentally, not in patients) derivatives of COL-3 were synthesis zed and we tested their in vitro cytotoxicity and anti-invasive activity. Preliminary studies showed that COL-308 (9-Amino, 6-deoxy, 6-dimethyl, 4-dedimethly amino tetracycline) is a comparable alternative to COL-3 for *in vivo* studies [Lokeshwar et al, Appendix 1]. The only major difference was COL-308 did not show as much antiproliferative activity as that of COL-3. However, it has significant anti-invasive activity as that of COL-3. We tested the activity of COL-308 against MAT LyLu tumor growth in rats, both as a single treatment and as an adjuvant to docetaxel, the second generation Paclitaxel drug (Taxotere, Aventis Pharmaceuticals Products Inc, Collegeville, PA). In this first experiment, tumors were induced in 90-100 day old male copenhagen rats by subcutaneous injection of 2×10^5 tumor cells into dorsal flanks. Animals were divided into 4 groups of six each. Treatment with COL-308 and Taxotere began on the same day we injected tumor cells. The test groups were: Group 1: Control (no treatment, oral gavages with 2% carboxyl methyl cellulose, the drug vehicle), Group 2: Taxotere (injected, 4 mg/kg intra-muscular(IM) three times a week). Group 3: COL-308 (oral gavages, 40 mg/kg, daily for three weeks), Group 4:COL308+Taxol (daily gavages and injection 3x /week). Tumors were palpable from day 8, post implant in most of the rats in Group 1. Tumor growth was monitored over time, by measuring the tumor volume three times a week with calipers. Body weight was monitored weekly to assess possible systemic toxicity of the drugs. Tumor bearing animals were euthanzied once the tumor volume reached ≥ 10 CC, or the animals showed visible signs of respiratory distress, due to excessive tumor burden in the lungs. Lungs and tumor tissue were fixed for histology at necropsy. All rats in control group 1(100% incidence) developed tumors that grew rapidly with a median time of three weeks to reach 10 CC. Number of animals developing tumor in Groups 2-4 varied. Tumor incidence was 50% in Group 4 (COL-308+taxol), 67% in Group 2 (COL-308 only) and 84% in Group 3 (taxol

only). However, the tumors that were incident in the remaining animals grew rapidly, resulting in median survival of 21 days in-group No. 2, 3 and 4. Although there was a significant decrease in tumor incidence, probably due to tumor cell killing and lack of angiogenesis, the incident tumors grew at comparable rate in all the three groups. In addition, the animals sustained an average of 5.7% weight loss in Group 3 and 4, during the course of the treatment. Rats that did not develop tumors were euthanized two weeks after the last animal from the group that had developed tumor. Except a scar no tumor was visible at the site of tumor injection. Mean number of tumor foci in the lungs for each group were 18 ± 4.2 (Gr.1); 12 ± 3.7 (Gr.2), 21 ± 5.0 (Gr.3), 17 ± 4.7 (Gr.3). The number tumor foci in treated versus that in control was not significantly different, as tested by Tukey-Kramer multiple comparison test (Graph-Pad Software, San Diego, CA). Other than a significant reduction in tumor incidence, there was no appreciable enhancement of tumor growth inhibition in combination group. It should be noted however, that animals in treated groups were killed about one week later than that of control group, which allowed additional time for metastatic growth in the lungs, that may account for the lack of difference in tumor foci in lungs among the groups. **The conclusion to be drawn from this study is that the combination of two drugs may not decrease the tumor growth rate but might decrease the incidence.**

We next examined whether CMT-3 alone can reduce tumor incidence and slow of tumor growth. We used a MAT Ly Lu subline that expresses enhanced green fluorescent protein, EGFP-MAT LyLu that were first tested in this model. We are investigating this further in other CaP models including human cells lines (e.g., EGFP-PC3) that are genetically transformed to produce enhanced green fluorescent protein. Tumor incidence and metastatic pattern will then be monitored more efficiently in these models. In addition, this combination of taxol and COL-308 did not result in significantly enhanced tumor growth inhibition; we are in the process of testing other combinations. These include COL308+halofuginone, COL-308+DXR (or cis-platin) in this and other CaP models

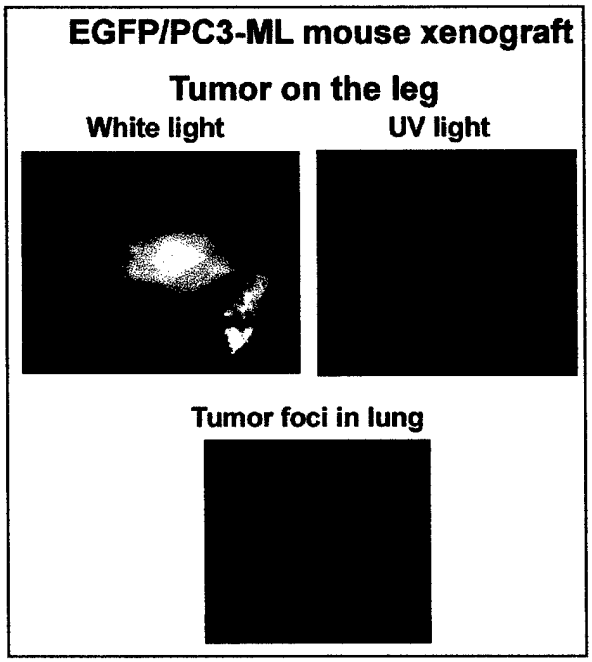


Fig 4. Metastasis of EGFP-PC-3ML cells in balb/c nude mice 90 days after s.c injection in the thigh. Fluorescent tumor foci in the lungs are shown.

Study the effect of CMT-3 on a spontaneous skeletal metastasis model: we had limited success in testing the efficacy of CMT-3 on the LNCaP C4-2, or ARCaP spontaneous bone metastasis models in xenografted nude mice as originally proposed [18]. The models did not produce any detectable bone metastasis upon orthotopic implantation in male balb/c mice in two attempts, each with 12 mice. Although the tumors grew to obstruct bladder, leading to bladder distention, no tumor foci were found in the histology of the vertebral bone. We plan to use alternate CaP cell lines (e.g., PC-3ML) that are more likely to produce visible and measurable signs of bone metastasis. Towards this objective, we have generated stable PC-3ML cell line that expresses Enhanced Green Fluorescence Protein (EGFP); created by stable transfection using an EGFP-expression plasmid (pIREShygro/EGFP plasmid; B-D-Clontech, Labs, Palo Alto, CA). As shown in **Fig. 4**, EGFP/PC-3ML cells form tumors in nude mouse xenografts and spontaneously metastasize to tibia and lung, as revealed by the presence of fluorescent foci in these sites. In addition we recently obtained newly established CaP tumor lines, either derived from vertebral metastasis or spontaneously metastatic lines [19,20]. These include, ***v-cap*** and ***DuCaP*** lines from Dr. Ken Pienta's lab, (Karmanos Cancer Center, University of Michigan) and LAPC-4 and LAPC-9 tumor lines from Dr. Charles Sawyer's lab from UCLA [21]. We will continue to enrich these human CaP derived tumor cells that metastasize to bones and lungs and then characterize their response to CMT-3, CMT-308 and combination modality, such as HF+CMT-308.

C1. Progress related to Specific Aim 2: Investigate alteration of therapeutic response of tumor cells by tissue-specific Stromal cells: Since tumors that are metastatic to distant organs respond to chemotherapeutic drugs differently [22], the role of stromal cells specific to organ or tissues may play a significant role in this acquired chemoresistance or chemosensitization [23-24]. We have examined this hypothesis in depth to estimate the extent of modification by stromal cells such as smooth muscle fibroblasts from prostate tissue explants, lung fibroblasts, endothelial cells and bone marrow derived osteoblastic cell lines. The following is a brief report on this in-depth study.

C1.1. Primary and established cell cultures: Initially, we established 18 primary cultures of tumor derived epithelial cells, fibroblasts and endothelial cells from the explant tissues obtained from patients undergoing radical prostatectomy for clinically localized CaP (Gleason sum 5 to 7). Stromal fibroblast (vimentin and β-actin positive), epithelial cells (cytokeratin 8, 18 and 5 positive) and endothelial cell (vimentin and Factor VIII-positive) cultures were characterized by immunostaining for cell-specific markers. In addition, for a constant supply of fibroblast or endothelial cells we used several established lines, including a human lung fibroblastic line (HLF, ATCC Cat No. FHSS733.1), lung (HMVEC-L; Clontech/BioWhitekar, San Diego, CA) and an SV40-tsT-antigen transformed human embryonic osteoblastic cell line (hFOB, ATCC Cat No. CRL-11372, ATCC, Rockville, MD). We also studied the response of established androgen sensitive (i.e., LNCaP) and androgen resistant (i.e., PC3ML, DU145) CaP lines to chemotherapeutic drugs in the presence and absence of stromal cells.

For investigating the interaction between stromal-derived factors and metastatic CaP cells, tumor cells were either co-cultured with stromal cells in a two chambered system, or they were cultured in a medium containing a 1:1 mix of stromal cell conditioned medium (CM) and normal growth medium. The latter procedure was adopted after observing identical results between direct cell co-culture and culture with CM, indicating that stromal cells release diffusible substances that modulate tumor cells' response to chemotherapeutic drugs.

C.1.2. Response of CaP cells to chemotherapeutic drugs: For this study, we used the three drugs, CMT-3, DXR and Taxol. CaP Cells were incubated in 1:1 mix of growth medium and CM from PrFb, HLF, NIH 3T3 cells, hFOB or HMVEC-L, Following a 48 h incubation, CaP cells were exposed to drugs for the next 48 h. Cytotoxicity in CaP cells was estimated by MTT assay and [3H]-thymidine incorporation assay [6]. Determination of cytotoxicity by two independent methods and both yielding same results assured cytotoxicity was not associated with cell cycle arrest. As shown in **Table 1**, IC_{50} of taxol and DXR to inhibit proliferation of PC-3ML cells increased in the presence of HMVEC-L, hFOB and NIH 3T3 CM by 3.5 and 1.7-fold, respectively. However, PrFb CM was the least effective in inducing chemoresistance to DXR and taxol. Interestingly, stromal cell CM increased the sensitivity of CaP cells to CMT-3, and thus, decreased IC_{50} by 1.6-fold. We also observed similar results with DU145 and LNCaP cells and primary cultures. The clinical implication of this finding could be that a 2- to 3-fold increase in IC_{50} of common chemotherapeutic drugs due to stromal influence might be enough to cause failure of chemotherapy. This is because due to the poor therapeutic index of the anticancer drugs, quite often the recommended drug dose is normally within the range of *in vitro* cytotoxicity. Thus a combination treatment involving both cytotoxic drugs and agents that disrupt stromal protection of drug-induced cytotoxicity should be a preferred modality for treating metastatic CaP.

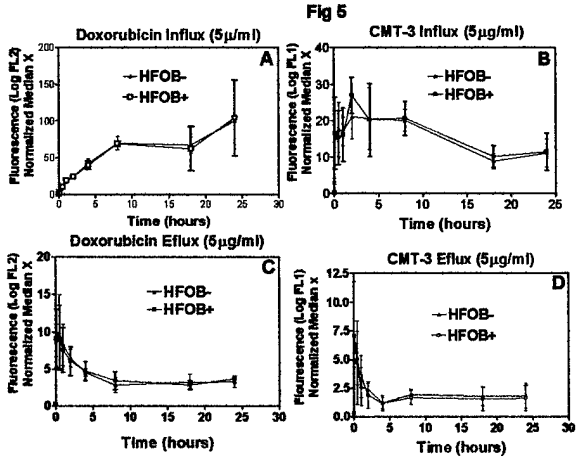
Medium	CMT-3 (COL-3), μM	DXR, nM	Taxol, nM
Complete Med.(Ctrl)	10.9 ± 0.9	27.5 ± 3.5	18.4 ± 3.1
PrFb CM	12.8 ± 0.75	32.8 ±5.3	21.4 ± 4.2
HFOB CM	6.8 ± 0.5	44.5 ± 3.0	52.1 ± 6.2
NIH 3T3 CM	7.2 ± 1.3	39.4 ± 4.1	31.5 ± 4.9

Table 1: Summary of stromal modulation of cytotoxicity inPC-3 cells. The IC_{50} values given in the table are from 3 separate experiments.

C.2. Investigating the mechanisms of modulation of cytotoxicity: We were intrigued how stromal cells or their culture CM regulate cytotoxic response of CaP cells to chemotherapeutic drugs. We reasoned that stromal factors may modulate response to chemotherapeutic drugs by 2 mechanisms: (1) Uptake or efflux of drugs into CaP cells, and (2) Changes in drug-induced apoptosis. We investigated both possible mechanisms.

First we investigated whether exposure to CM alters drug accumulation in tumor cells. For this study we studied accumulation and retention of CMT-3 and DXR in PC-3ML and DU 145 cells. Drug accumulation in the cells was measured using flow cytometry. Since both CMT-3 and DXR autofluoresce at 515 nm and 580 nm respectively, when excited at 480 nm. Flow cytometry was the logical choice. PC-3ML cells cultured singly or together with HFOB CM for 3 days, were incubated with CMT-3 or DXR for 5 min to 4 h. Single cell suspensions of these cells were analyzed by a Beckman-Coulter EPICSXL flow cytometer and the median fluorescence intensities (MFI) at 515 nm and 580 nm for various treatments, were compared. As determined by increase in the MFI, drug uptake was fairly rapid and saturated quickly, within 90 min.

Fig 5



As shown in **Fig 5 A** and **B**, accumulation of both drugs could be detected in cells exposed to the drugs for as little 5 min. However, as shown in Fig 5 A and B, there was no significant difference in drug accumulation when cells were cultured either singly or in the presence of HFOB CM. We next examined drug-efflux by incubating PC-3ML cells with the DXR or CMT-3 for 90 min at 37°C. Following incubation, the cells were washed to remove unincorporated drug and cell aliquots were analyzed by flow cytometer at different time intervals. As shown in **Fig. 5 C** and **D** showed that drug retention rate was comparable between PC-3ML cells cultured singly and in presence of HFOB CM, for both the drugs. However, DXR retention was more evident than CMT-3 retention, as the MFI remained constant over an 18-hour period for DXR. It is not known however, that the drug is bound to the proteins or organelles in these cells. In summary, we did not detect any statistically significant differences in drug accumulation or retention in the presence and absence of HFOB CM. Similar results were obtained when HMVEC-L, HLF and NIH3T3 CM were used.

Gene	***Left primer***	**Right primer**	Size
MMP-2	CTCAGATCCGTGGTGAGATCT	CTTTGGTTCTCCAGCTTCAGG	496
MMP-1	GGAGGGGATGCTCATTTTGATG	TAGGGAAGCCAAAGGAGCTGT	541
MMP-7	GGAGTGCCAGATGTTGCAGAA	GTTTCCTGGCCCATCAAATGG	261
IL-1β	TGAACTGAAAGCTCTCCACCA	CTGATGTACCAGTTGGGGAA	297
IL-6	CTT CGG TCC AGT TGC CTTCT	CCAGATTGGAAGCATCCATC	291
MDR1	TTGGTGTGGTGAGTCAGGAAC	AGCTATCACAATGGTGGTCCG	350
MRP	GGACCTGGACTTCGTTCTCA	CGTCCAGACTTCTTCATCCG	291

C 2.1. Effect of stromal cells on the expression of mdr1 and mrp1: We also examined the alteration in the expression of drug efflux pump proteins GP180 (mdr1 product) and MRP-1 antigen mRNA expression using semi quantitative reverse transcriptase–PCR (RT-PCR). PC-3ML cells were cultured in the presence or absence of HFOB CM for 3-days. Cells were then exposed to the drugs, CMT-3 and taxol for 4 hours and the cells were allowed to

recover in the absence of the drug for 24 h, but in the presence of HFOB CM. Total RNA was isolated (RNeasy Mini kit, Quiagen Inc. Valencia CA) and was reverse transcribed. The first strand cDNA was PCR amplified using the GeneAmp Kit (Applied Biosystems, NJ) and specific primers. The primers for all PCR reactions mentioned in section C are listed in **Table 2.**

Semi quantitative RT-PCR was established by terminating reactions at intervals of 20, 24, 28, 32, 36 and 40 cycles for each primer pair to ensure that PCR products formed were within the linear portion of the amplification curve. The RT-PCR products were visualized on 1.5% agarose gels with ethidium bromide staining. Comparing the size of the amplified product to the expected cDNA bands and sequencing the PCR products verified the fidelity of PCR products. The analyses of mdr1 mrp1 and GSTP1 PCR products, after 32 cycles and GAPDH PCR product after 20 cycles, are presented in **Fig 6**. We found significant differences in the levels of *mdr1* and *mrp* expression, but not in the levels of GSTP1 expression, among various treatment groups. Treatment with CMT-3 and taxol significantly elevated the expression of mdr1 and mrp1. However, the presence of HFOB CM did not significantly enhance further, the increase in *mdr1* and *mrp* expression.

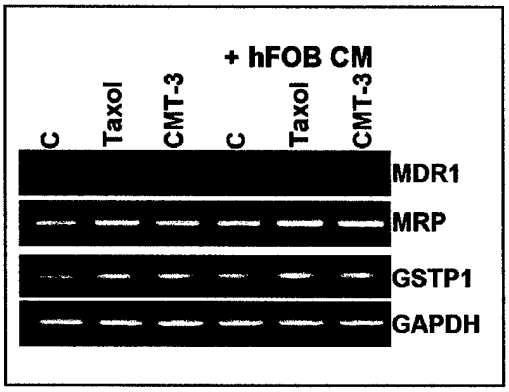


Fig 6. SQ-RT-PCR analysis of multidrug resistance genes in CaP cells cultured with or without bone –marrow osteoblastic cells.

Summary: The modulation of CaP cells' response to chemotherapeutic drugs in the presence of stromal factors does not involve either drug-influx or drug-efflux or elevation in *mdr1* and *mrp* expression.

C 2.2. Changes in the drug-induced apoptotic activity in PC-3ML cells by stromal cells and their culture CM: We examined whether the CaP cells' cultures treated with chemotherapeutic drugs (CMT-3, DXR and Taxol) with or without co-incubation with stromal cell CM show altered apoptotic cell death. We used the Cell Death Plus ELISA kit (Roche) to quantitate drug-induced apoptosis in CaP cells, when cultured alone or with stromal cell CM. As shown in **Table 3**, apoptotic activity was detected just after treatment for 4 hours with various drugs. However, while stromal CM seem to protect PC3-ML cells from DXR-induced apoptotic activity and CMT-3-induced apoptotic activity, the stromal CM sensitized the cells for CMT-3 induced apoptosis. These results show stromal factors may induce resistance to conventional cytotoxic drugs by inhibiting drug-induced apoptosis. None-the-less, CMT-3 seems to be a better drug for overcoming the stromal effect.

Condition	Control	CMT-3	DXR
Growth medium	0.0	0.48 ± 0.07	0.81 ± 0.15
HFOB CM	0.21 ± 0.04	0.88 ± 0.11	0.46 ± 0.17
HLF	0.0	0.66 ± 0.08	0.32 ± 0.05
PrFB	0.15 ± 0.03	0.72 ± 0.21	0.92 ± 0.03

Table 3: Apoptotic activity of PC-3ML cells. The numbers indicate specific O.D. using growth medium as the baseline.

C.3. Analyses of gene expression changes by Panorama cytokine gene array: In order to compile a meaningful yet comprehensive list of mRNA species whose expression is altered by chemotherapeutic drugs in the presence or absence of stromal CM, we used Sigma-Genosys Panorama human cytokine gene expression array (Sigma, St Louis). This array includes cDNAs for most known cytokines, chemokines and cell adhesion molecules and matrix component proteins, nine positive controls (e.g., house-keeping gene sequences such as that for GAPDH) and 5 negative controls (e.g., pUC19 or E.Coli specific genes). We compared the gene expression pattern in 4 mRNA samples isolated from PC3-ML cells (1) cultured in growth medium, (2) treated with CMT-3 (5 µg/ml) for 3 hours and recovered for 24 hours (3) cultured in the presence of HFOB CM, noted as SC-CM in the figure (4) cultured in the presence of SC-CM and treated with CMT-3 (5 µg/ml) for 3 hours and

recovered for 24 hours. Following various treatments, mRNA was isolated from PC-3ML cells using a polyA$^+$ RNA isolation kit (Ambion Inc, Austin, Texas) and reverse transcribed in the presence of α-^{32}P-dCTP using primers and protocol provided with the array membrane. Radiolabeled cDNA products were hybridized to the cytokine gene array membranes, followed by Phosphor Image analysis. Digitized signals were then compared to each of the spots using the ImageQuant analysis software. A typical pair of cytokine expression-arrays, representing the mRNA expressions in PC3ML under various treatment conditions is shown in **Fig 7**. A difference >2 in the digitized signal intensity of a selected gene expressed in the comparable samples were considered significant. Based on this analysis we identified certain genes which are over expressed (~ 3-fold) in cells treated with CMT-3; these over genes include TIMP1 (>3.8), CXCR2, IL-4 receptor, IL-1β, IL-6, IL-10 integrin a-3, TNFRII. However, mRNA expression of CXCR-1, IL-8, TIMP-3, FGF-3 and CD-28 was **inhibited.** In the presence of SC-CM, IL-1β, IL-6 and IL-8 expression was up regulated, and in the presence of CMT-3 it did not change significantly. We further characterized genes of interest (i.e., IL-1β, IL-6 and IL-8) by semi-quantitative RT-PCR, Quantikine mRNA ELISA (R&D systems, MN), protein ELISAs.

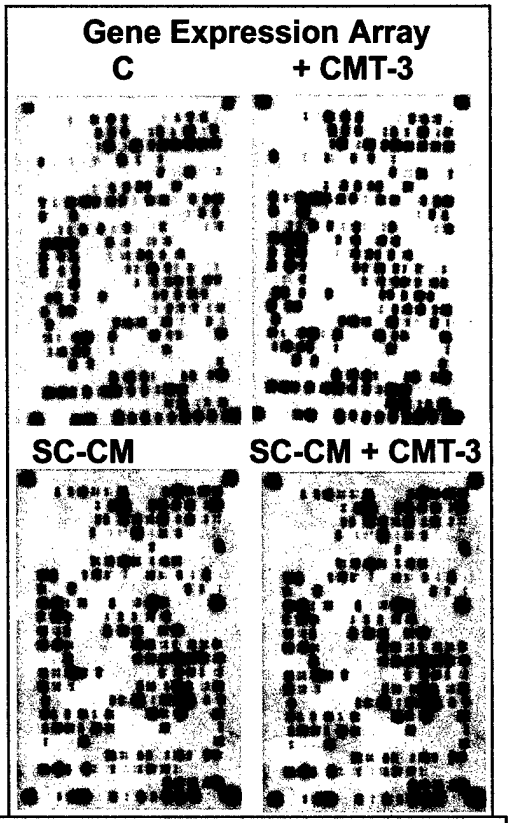


Fig 7. *A panel of autoradiograms of Panorama cytokine expression array, hybridized against cDNA prepared from mRNA of CMT-3 treated PC-3ML cells in the presence or absence of HFOB culture-conditioned medium is shown.*

Summary: Organ-specific stromal cells or their culture CM increase the invasive properties of CaP cells. This alteration is associated with increase in MMP-1, MMP-2 levels. DXR and taxol decrease MMP expression and increase TIMP-1 expression, however, this effect is abrogated in the presence of stromal CM. Interestingly, CMT-3 is effective in decreasing the invasive phenotype of CaP cells, regardless of the presence of stromal factors. The gene expression array experiment revealed differential expression of proinflammatory cytokine and chemokines, in the presence of stromal CM. Since CMT-3 did not significantly decrease proinflammatory cytokine expression in the presence of stromal CM, **use of anti-inflammatory drugs** may compliment therapeutic efficacy of CMT-3.

2. Key Accomplishments:

- **Three new drugs Stenorol (halofuginone, HF), COL-3 and COL-308, were tested in vitro and in vivo on metastatic prosatate cancer cells in vitro.**
- **The anti-metastatic drugs: halofuginone, known to inhibit Type I synthesis and MMP-2 also proved to be potent inhibition of tumor cell proliferation.**
- **HF did not show additive or synergistic effect on CaP cells when combined with other inhibitors of cell growth and metastasis (CMT-3).**
- **HF did not show anti-invasive activity in vitro models.**
- **A new derivative of COL-3, COL-308 (CMT-308) was tested on the rat model of metastatic both as a single agent and as an adjuvant to the chemotherapeutic drug Taxotere. CMT-308 alone was found effective in inhibiting metastasis and tumor incidence.**
- **An extensive investigation of acquired chemosensitivity and/or chemoresistance of tumor cells in the presence of organ specific stromal cells were undertaken. Biochemical and gene expression studies (expression arrays) showed that inflammatory cytokines specifically secreted by stromal endothelial cells and fibroblasts induce chemo resistance in tumor cells were as osteoblastic cells and primary prostatic fibroblasts induce chemo sensitivity by excessive secretion of IL-6.**

3. Reportable outcomes:

A. **Publications**: 1. Lokeshwar BL, Escatel E, and Zhu B-q. Cytotoxic activity and inhibition of tumor cell invasion by derivatives of a chemically modified tetracycline CMT-3 (COL-3) ***Current Med Chem. 8:271-279, 2001.***

2. Lokeshwar BL, Selzer MG, Zhu B-q, Block NL, and Golub LM. Inhibition of cell proliferation, invasion, tumor growth and metastasis by an oral non-antimicrobial tetracycline analog CMT-3 (COL-3) in a metastatic prostate cancer model. ***Int. J. Cancer. 98:297-309, 2002.***

B. **Patents:** No patents were filed or issued.

C. **Clinical Translational research. CMT-3:** No new clinical trials of Phase II or Phase III have been reported.

D. **Personnel:** One full time Research Associate (technician), a part time Research Associate, a post-doctoral associate (October2001-present) and the Principal investigator worked on this project.

4. Conclusions: Results derived from experiments outlined under the Specific Aim 1 show that HF alone is cytotoxic to CaP cells and therefore has significant clinical potential. Experiments planned in the current year will fully explore the potential of HF as an anti-tumor drug in vivo in two models of metastatic prostate cancer. In our studies we could not demonstrate anti-invasive activity of HF in the in vitro Matrigel invasion assay. Furthermore, combining HF and CMT-3 or HF and CMT-308 did not significantly enhance cytotoxicity or anti-invasive activity of either agent alone. Combination had limited additive or synergistic anti-proliferative or anti-invasive activity. However, the combination may have enhanced activity in vivo, especially in the bone metastasis model. Experiments performed to investigate in vivo efficacy of CMT-308, the new derivative of CMT-3 with less photosensitization effect (unpublished), either alone or combined with taxol, revealed limited enhancement of efficacy with cytotoxic drug. However, in one experiment, CM-308 alone significantly decreased tumor incidence (57%) compared to either untreated group or the group treated with Taxotere. Results derived from experiments proposed under Specific Aim 2 to investigate the effect of tissue-specific stromal cells suggest that stromal cells of fibroblastic origin and that of osteoblastic cells increase drug induced cytotoxicity in stromal cells, endothelial cells increase the resistance to drug induced cytotoxicity in CaP cells. The mechanism of drug induced cytotoxicity and its modification by stromal cells were explored using analysis of gene-expression by panorama cytokine expression arrays. Stromal modulation of drug sensitivity in tumor cells was mediated by over expression of cytokines, IL-1β, IL-6 and IL-8. Members of the inflammatory pathway cytokines were found to be responsible for chemoresistance. Further inquiry into these mechanisms is slated for the current year.

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Cytotoxic Activity and Inhibition of Tumor Cell Invasion by Derivatives of a Chemically Modified Tetracycline CMT-3 (COL-3)

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Abstract: Tetracyclines such as chlortetracycline and doxycycline with antimicrobial activity were reported to possess cytostatic and cytotoxic activity against mammalian tumor cells, often at high doses. Non-antimicrobial chemically modified tetracyclines (CMTs), with limited systemic toxicity but with significant tumor cell toxicity and anti-metastatic activity, are attractive for long term treatment for cancer. We recently reported one such CMT, 6-deoxy,6-demethyl 4-dedimethylamino tetracycline (CMT-3) is a potent anti-tumor and anti-metastatic drug. Here we report on the anti-cell proliferation and anti-invasive activity of five nitro derivatives of CMT-3 (CMT-3N).All the five CMT-3Ns (CMT-302, CMT-303, CMT-306, CMT-308 and CMT-316) inhibited *in vitro* cell proliferation of prostate cancer cells. The 50% growth inhibition concentration (IC_{50}) of CMT-3Ns was similar to that of CMT-3. Although CMT-3 was by far the most potent anti-cell proliferation drug, all CMT-3Ns except CMT-303 and CMT-308 had similar anti-cell proliferation activity (IC_{50}: 2.5 -5.7 μg/ml). IC_{50}s for CMT-303 and CMT-308 were ~ 8.1 and -12.4 μg/ml, respectively. Activity against tumor cell invasion was tested *in vitro* using the Matrigel invasion assay. All CMT-3Ns had similar anti- invasive activity. While cytotoxic activity of CMT-3 was strongly associated with cell death-effector caspase activation, mitochondrial permeablization and apoptosis, the CMT-3Ns weakly induced apoptosis and did not activate Caspase-3. However, the CMT-3Ns were able to induce mitochondrial permeabilization. This dichotomous mechanism of cytotoxic activity of CMTs may have significance in their selection for clinical application.

INTRODUCTION

Regardless of the tremendous progress in the treatment and care of cancer patients, cancer is still a major disease that defies effective cure. While most cancer patients are treated with chemotherapy towards the terminal stages of many types of cancers, new therapeutics are still needed for effective treatment and management of this disease. A singular drawback of many existing cancer chemotherapeutics is their unwanted systemic toxicity, often themselves a cause of fatality than the disease they are meant to control. Newer therapeutic with limited systemic toxicity, easier administration, longer efficacy, and ability to act synergistically with existing chemotherapeutic drugs should not only improve the survival and quality of life for patients, but also be economical. Drugs that already have well established use to treat other diseases but with properties that are relevant to other cancer chemotherapeutics are attractive candidates to explore.

Kroon *et al.* [1] reported for the first time that continuous infusion of tetracyclines such as chlortetracycline and oxytetracycline inhibit a variety of tumor growth. They postulated that the mechanism of this inhibition may be through inhibition of mitochondrial protein synthesis [1,2]. Further studies, many years later, showed that doxycycline inhibits tumor cell proliferation at high concentrations [3]. In addition, it was shown that doxycycline inhibits tumor cell invasion *in vitro*, and tumor-derived metalloproteinases (MMPs) [4]. The idea of the potential use of a well established antibiotic to control tumor growth captured our attention and motivated us to investigate their potential further. We were more attracted to the possibility of using a synthetic tetracycline such as doxycycline not only for treating localized tumor but also for inhibition of metastasis. Golub and his colleagues showed, for the first time, that tetracyclines inhibit collagenase and gelatinases (MMPs) at doses achievable by oral administration, at concentration of 5μg/ml [5-8]. Since MMPs are the major enzymes that degrade basement membrane and their inhibition leads to reduced metastasis, we embarked on testing the anti-tumor and antimetastatic activity of doxycycline and several CMTs. It was also reported that some CMTs are more potent inhibitors of MMPs than doxycycline [9-12].

CMTs are more attractive candidates as cancer chemotherapeutic than tetracyclines with antibiotic property. Prolonged treatment with antimicrobial tetracyclines results in significant gastrointestinal toxicity and opportunistic fungal infection [13]. CMTs without antibacterial activity may not induce such pathological conditions. In addition,

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0929-8673/01 $28.00+.00

due to the increased anti-MMP activity some of the CMTs may be efficacious at lower doses than doxycycline. At least two groups have reported the anti-tumor and anti-metastatic activity of CMTs, mainly 6-deoxy,6-demethyl,4-dedimethylamino tetracycline (CMT-3) on two tumor models, a melanoma model [14] and a metastatic androgen unresponsive prostate cancer model [15,16]. In either models, CMT-3 was reported to exhibit anti-cell proliferation and anti-invasive activity in vitro. It strongly inhibited tumor growth at the site of inoculation (if administered prophylactically) and inhibited lung and bone metastasis [17,18]. More importantly, CMT-3 was effective by daily oral gavage. This led to its first phase I clinical trial being conducted at the National Cancer Institute, Bethesda Md., under the brand name COL-3. To better understand the structure -activity relationship of CMT-3 toward inhibition of tumor cell proliferation, invasion and tumor metastasis, we compared CMT-3 to several variants (CMT-3N) in our assay systems.

In the present report we present data on the anti-cell proliferation activity and anti-invasive activity of CMT-3 derivatives. Using the same *in vitro* model systems, we investigated the chemotherapeutic properties of CMT-3. Our results suggest that some of the CMT-3 derivatives may potentially be candidates for further tests in experimental model systems and eventually, for clinical applications.

MATERIALS AND METHODS

Reagents

The following non-antimicrobial CMTs were used in this study: CMT-3 [6-demethyl, 6-deoxy, 4-dedimethylamino tetracycline],CMT-302 [7-nitro CMT-3], CMT-303 [9-nitro CMT-3], CMT-306 [CMT-3-9-dimethylamino HCl], CMT-308 [9-aminoCMT-3], CMT-316 [9-hexanolaminoCMT-3], all obtained from Collagenex Pharmaceuticals Inc., Newtown, PA. Apoalert Annexin V and Apoalert CPP32 (caspase-3) assay kits were from CLONTECH, Inc. Palo Alto, CA. Cell death Plus-ELISA kit was from Roche Molecular Biochemicals, Indianapolis, IN. All other reagents were from Sigma Chemicals Inc. St. Louis, MO, USA.

Cells and Tumor Lines

Human prostate tumor cell lines (LNCaP and DU 145), were from ATCC, Rockville MD. Dunning rat MAT LyLu prostate cancer line was from Dr. John T. Isaacs, Johns Hopkins Oncology Center, Baltimore, MD. PC-3ML2, a metastatic subline of PC-3 was obtained from Dr. Mark Stearns, MCP Hahnemann University, Philadelphia, PA. 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 supplemented with dexamethasone (250 nM).

Cytotoxicity Assay

A colorimetric assay (the MTT assay [19]) was used to assay the cytotoxic effects of CMTs. This assay measures the amount of an insoluble chromogen, formazan, generated by live cells from the substrate 3-(4,5-dimethylthiozol-2yl)-2,5-diphenyltetrazolium bromide (MTT). The formazan crystals are dissolved in dimethyl sulfoxide (DMSO) and read at 515 nm in a plate reader. To determine the cytotoxicity of CMTs, multi well plates were seeded with 2 x 10^4 cells/well incomplete medium and cultured for 24 hours. CMTs prepared as 100x stock solution in DMSO was added to replicate culture wells and incubated for 48 hours. MTT (0.5 mg/ml) was added during the final 2 hours of incubation. Amount of MTT conversion was determined by reading the optical density (O.D.) Of DMSO-solubilized formazan at 515 nm. Wherever applicable, the O.D. of cultures incubated with 0.1% DMSO was used as controls. Under these conditions, the O.D. was directly proportional to the number of viable cells.

In vitro Tumor Cell Invasion Assay

Effect of CMTs on tumor cell invasion through Matrigel coated porous membrane was assayed using two CaP cell lines, PC-3ML2 and MAT LyLu as described before [15]. In brief, we plated 4 x 10^5 cells into the top wells of Transwell plates. The top wells were supported by 12 μm-pore polycarbonate filters that were coated with a thin layer of Matrigel (0.5 mm, BD Systems Inc., Bedford, MA). The bottom well contained a growth factor enriched-serum-free culture-conditioned medium from human lung fibroblasts. The drugs were added to both top and bottom wells just before plating cells. After 48 hour incubation, MTT was added to both top and bottom wells (0.5 mg/ml) and the incubation was continued for 4 more hours. The wells were then emptied and the cells from the undersides of the filter were pooled with those in the bottom wells with a filter tip. The reduced MTT (formazan) from top and bottom wells was dissolved in DMSO overnight, and their O.D. was read at 515 nm. The ratio of the O.D. from the bottom wells to that of the total O.D. (O.D. of bottom plus top wells) multiplied by 100 was taken as percent of invaded cells (invasive potential). We used this method to normalize the invasive activity against cytotoxic activity of the these drugs.

Cell Death Assay to Measure Chromatin Fragmentation

Drug induced apoptosis was assayed using the cell death detection ELISA -plus kit. The assay measures the amount of free H1 histone in cell lysates as a measure of nucleosomal fragmentation [20]. Assays were performed on cells cultured in multi well plates and exposed to drugs for 24 to 48 hours. Assays were conducted as per the instruction provided in the kit. Manufacturer supplied positive and negative controls and medium blank were used to compare the results from several experiments.

Determination of Phoshatidyl Serine (PS) Translocation

Drug induced translocation of PS was assayed by cell surface binding of annexin V, labeled with either green fluorescent protein or FITC-labeled Annexin V, using the Apoalert Kit from Clontech, Inc., 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 [21]. Cells treated with the drug for 90 min were harvested and labeled with FITC-annexin V as per the instructions provided in the kit. Labeled cells were analyzed by flow cytometry on the Coulter EPICS XL instrument. Median FITC fluorescence channels were compared from sample to sample. Median fluorescence channels of untreated cells was within one standard deviation of that of unlabeled cells.

Determination of Caspase-3 Activation

Drug induced activation kinetics of Caspase-3 (CPP-32), one of the down-stream caspases [22], was assayed using the

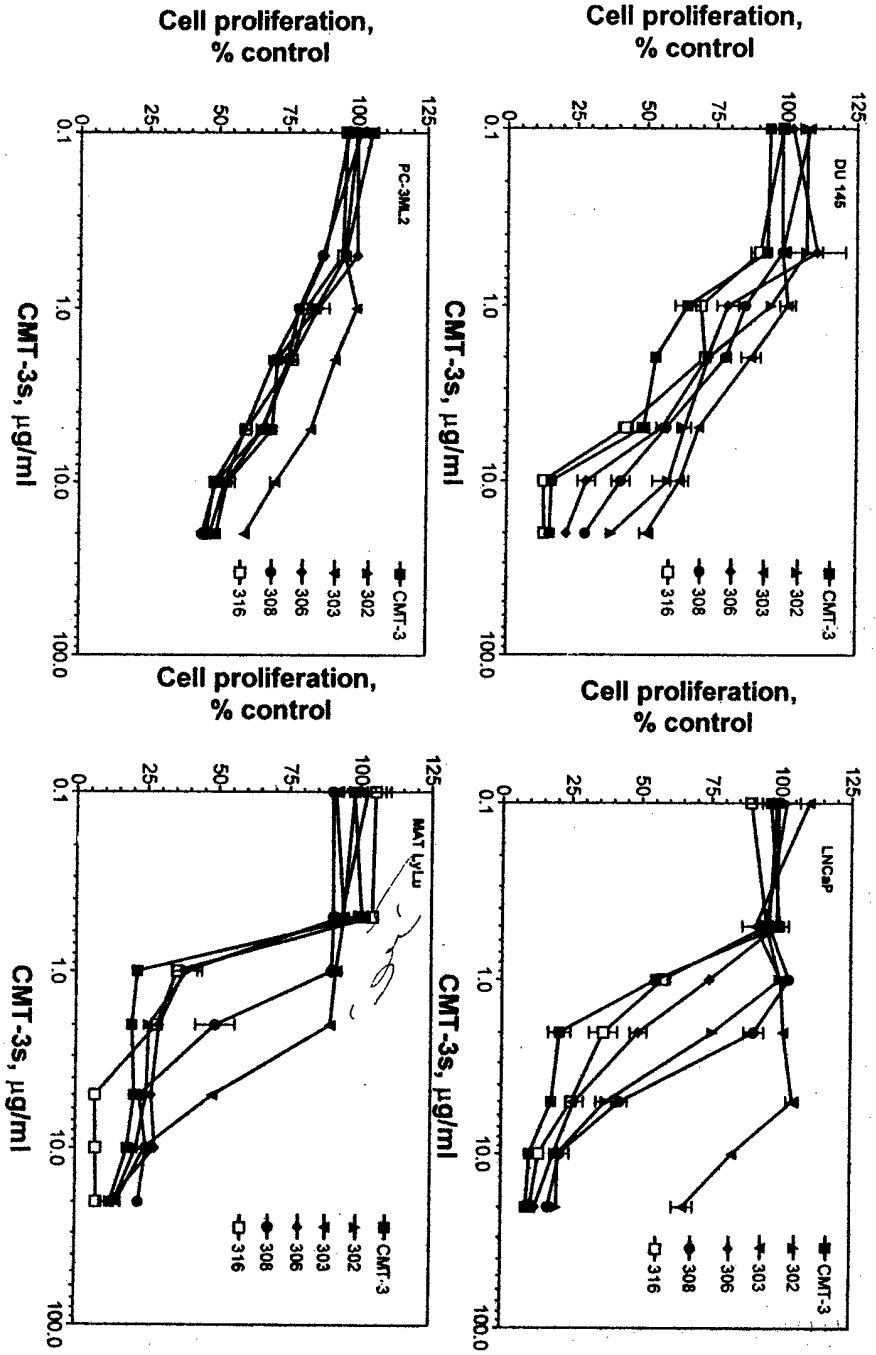

Fig (1). Inhibition of prostate cancer cell proliferation by CMT-3Ns. Cytotoxic activity of CMT- 3Ns were tested using the MTT assay on three established human prostate cancer cell lines (DU145, LN CAP, PC-3ML2) and one cell line from the Dunning MAT LyLu model. Amount of MTT converted to insoluble formazan by treated cells in proportion to untreated cells (i.e., % of control) is plotted against concentration of CMT-3Ns. Data presented are mean ± SEM from three separate experiments for each cell line.

Apoalert-CPP32 fluorescence assay kit. The kit detects the shift in the fluorescence 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, that is quantitated using a fluorescence multi well plate reader (Cytofluor II). Cells were exposed to drugs for 90 min. Cytosolic extracts prepared by centrifugation of cell lysates at 30,000xg and the cytosolic fractions were incubated with DEVD-AFC for 15 min and the fluorescence intensity was read in the Cytofluor II plate reader.

Determination of Drug-induced Permeabilization and Depolarization of Mitochondria

We used a mitochondria specific, depolarization-sensitive green fluorescent drug JC-1 (5',5'6',6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazoleylcarbocyanine iodide) [22]. This drug exists as a monomer at low concentrations or at low membrane potential with the emission maximum at 527 nm. It selectively enters mitochondria and accumulates at high concentration dependent on changes in membrane permeability. A change in membrane potential results in change in emission maximum to 590 nm due to the formation of J-aggregates. This shift in fluorescence is measured in individual cells in a flow cytometer. We added JC-1 to cell cultures 30 min before treating cells with CMT-3 or CMT-3N. Following incubation with the drugs and JC-1, cells were harvested and fluorescence intensity was determined by flow cytometric analysis. Median fluorescence channels of untreated and treated samples were compared. Cells with permeabilized mitochondria will have higher median fluorescence channel. In addition, the fluorescence intensity of the labeled samples were determined using the fluorescein longpass optical filter in Coulter EPICS XL flow cytometer.

RESULTS AND DISCUSSION

Inhibition of Cell Proliferation by CMTs

Incubation of proliferating tumor cells with CMT-3 and CMT-3Ns resulted in inhibition of cell proliferation in a dose dependent manner. As shown in **Fig 1**, *in vitro* cell proliferation of CaP cells was significantly inhibited by incubation with CMT-3 and all the five CMT-3Ns. As low as 1 μg/ml (2.2 μM) of CMT-3 inhibited MTT conversion in two cell lines LNCaP and MAT LyLu cells by 40%. The dose of the drug that reduced the number of viable cells by 50% (IC_{50}) varied between 2.5 μg/ml for CMT-3 in LN CaP and MAT LyLu cells and 5.7 μg/ml for PC-3ML2 cells.

Similarly the IC_{50} for CMT-308 varied between 8.2 μg/ml and 12.5 μg/ml in the four cell lines. Cell proliferation inhibition activities of other CMT-3Ns were in the neighborhood of CMT-3, not significantly different from that of CMT-3. CMT-302 and CMT-306, and CMT-316 also had similar cytotoxicity as measured in this assay.

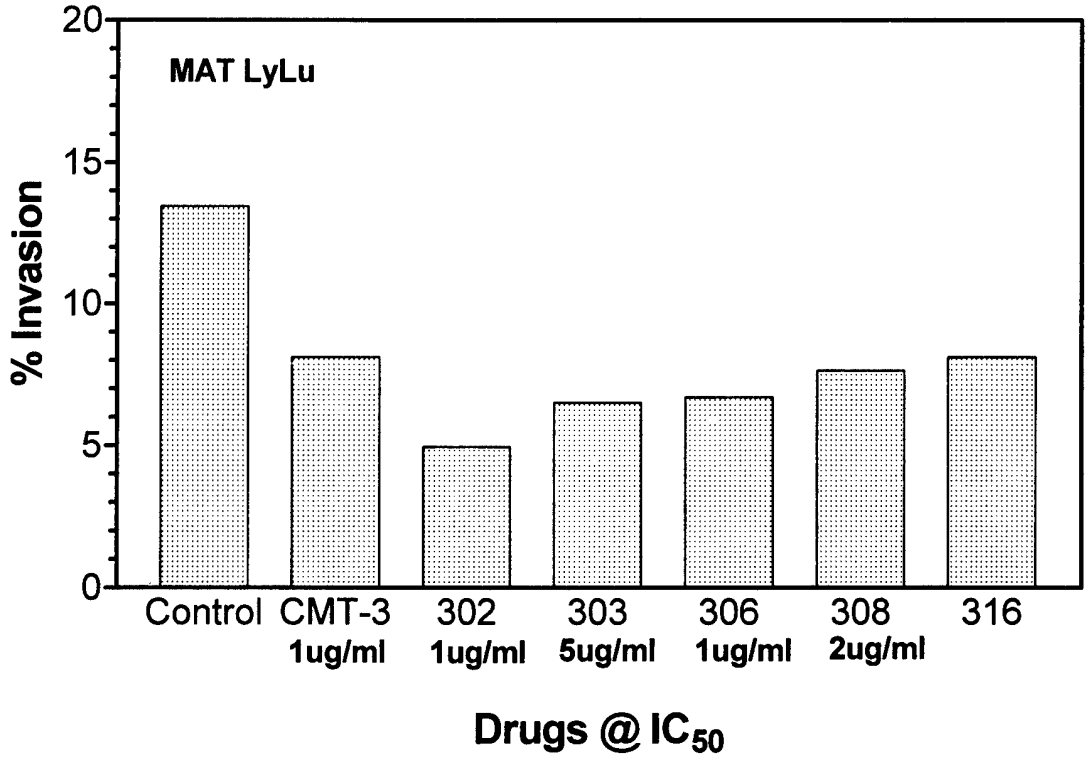

Fig (2). Inhibition of Matrigel invasion of MAT LyLu cells by CMT-3 and CMT-3Ns. Invasion activity of MAT LyLu cells was assayed as described in Methods. Invasive activity is expressed as % ratio of number of cells in the bottom (invaded) wells to total number of cells (bottom plus top wells) pooled from three wells of the chemoinvasion Transwell chambers. Results are from a single experiment (therefore, no error bars), comparable results were obtained in other experiments.

CMT-303 has lowest cytotoxicity on all the four cell lines tested.

Inhibition of *In vitro* Invasive Activity

Since we showed previously that CMT-3 is a potent inhibitor of tumor invasion and metastasis in a rat prostate cancer model [16], we investigated CMT-3Ns for similar activities. Using a reproducible in vitro assay, the Matrigel chemoinvasion assay, we estimated the chemoinvasive activity of MAT LyLu cells treated with CMT-3Ns. As shown in **Fig 2**, all the CMTs, including CMT-3, showed a significant anti-invasive activity in this tumor cell line. As the data present in **Fig 2** indicate, we observed between 23% to 60% inhibition in chemoinvasive activity by CMT-3Ns. All the five analogues of CMT-3 exhibited similar but significant anti-invasive activity. Interestingly, although CMT-303 and CMT-308 had low cytotoxic activity, they inhibited invasive activity of tumor cells. Although the data presented in Fig 2 is for MAT LyLu cells, similar activity was observed also when PC-3ML2 cells were used (data not shown).

Induction of Apoptosis by CMT-3Ns

A common mechanism of cytotoxic action of anticancer drugs is induction of apoptosis. We have shown previously that CMT-3 triggers apoptosis in prostate cancer cells [16]. We investigated whether the CMT-3 analogues also trigger similar pathway in the exposed cells. The results of our investigation is illustrated in **Fig. 3**. We observed that the analogues were weakly capable of triggering apoptosis in prostatic cancer cells (**Fig 3**). Apoptosis was triggered rapidly in CMT-3 treated cells (5 and 10 μg/ml) for as short as 30 min, and peaked in sample incubated with CMT-3 for 18 hours or later (**Fig 3**). Although data presented are for the DU145 cells, similar results were obtained when other CaP cells were used. CMT-3Ns caused very little apoptosis at 50% growth inhibition concentrations. However, at 90% cell growth inhibition concentration of CMT-302, CMT-306 and CMT-316 free-H1 histones were detectable. However, the optical density of CMT-3N treated-cell lysates assayed by H1 histone ELISA was 20% to 40% of that of CMT-3 treated-cell lysates. At this time, we are not sure whether this increase in H1 histone levels are indicative of DNA fragmentation due to apoptotic death or necrotic death. As shown in **Fig 3**, CMT-3 was at least 10-fold more potent inducer of apoptosis compared to its closest analogue CMT-302 at the same level of cytotoxic concentration.

Effect of CMT-3 and CMT-3Ns on Phoshatidyl Serine (PS) Translocation (Cell Surface Annexin V Binding) in Prostate Cancer Cells

It is well established now that PS translocation from inner cell membrane to the cell surface is an early indicator of apoptotic signaling [21]. Since it is an earliest event in the apoptotic signaling pathway one can determine the kinetics of drug induced apoptosis signaling in this assay. We

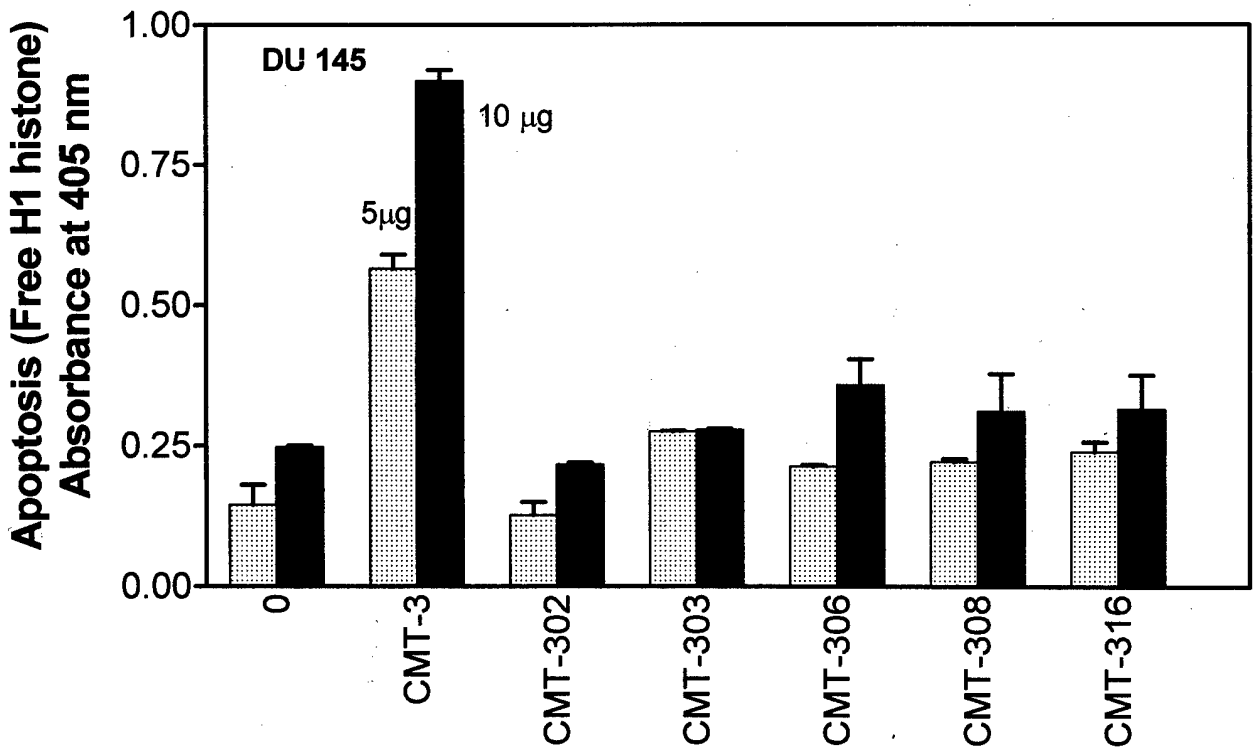

Fig (3). Effect of CMT-3 and CMT-3N on apoptosis of CaP cells. Cells were incubated with CMT-3 or CMT-3Ns at 5 μg/ml or 10 μg/ml for 90 min. Cultures were incubated with fresh culture medium for 2 days following which cell lysates were prepared and assayed for free H1 histones using the Cell death ELISA plus kit. Data shown are mean ± SD from a single experiment performed in triplicate. Similar results were obtained from assays performed on LN CAP and MAT LyLu cells.

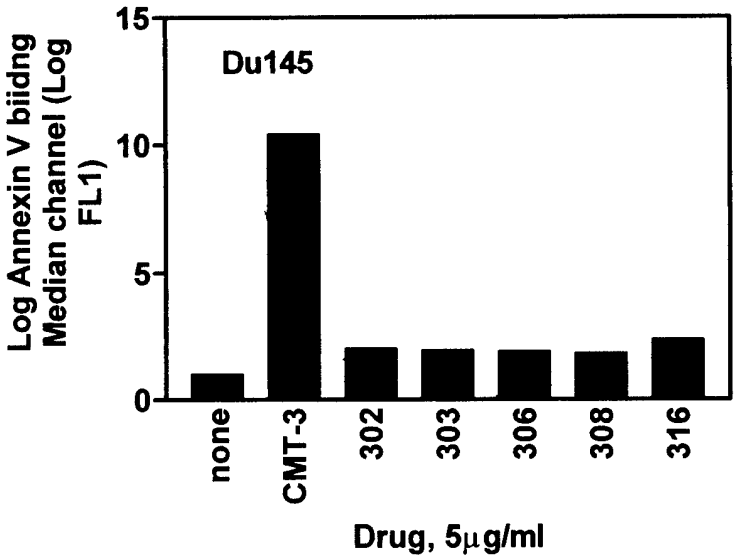

Fig (4). Cell surface annexin V binding in CMT-3 and CMT-3N treated cells. DU 145 cells treated with CMT-3 or CMT-3N for 90 min were labeled with Clontech Apoalert-FITC Annexin V for 30 min and fluorescence intensity of individual cells was analyzed on a Coulter EPICS XL flow cytometer. Data presented are the median fluorescence channel of each labeled sample. Data presented are for a single experiment, similar results were obtained in three independent experiment and for other three cell lines tested.

detected and measured using flow cytometry, the relative amount of PS translocation in prostate cancer cells incubated with CMT-3 and CMT-3Ns by labeling the cells with fluorescent Annexin V binding to the cell surface. As shown in **Fig 4**, there was an 8 fold increase on the cell surface levels of PS in cells treated with CMT-3 for 90 min. However, there was no significant increase in the levels of cell surface PS in cells treated with CMT-3Ns. Only CMT-316 triggered a small increase in Phoshatidyl serine translocation. These results are consistent with the relative levels of apoptotic activity triggered by CMT-3 and CMT-3Ns.

Activation of Caspase-3

Caspases are intracellular aspartyl-serine proteases that are activated in a proteolytic cascade triggered by early apoptotic

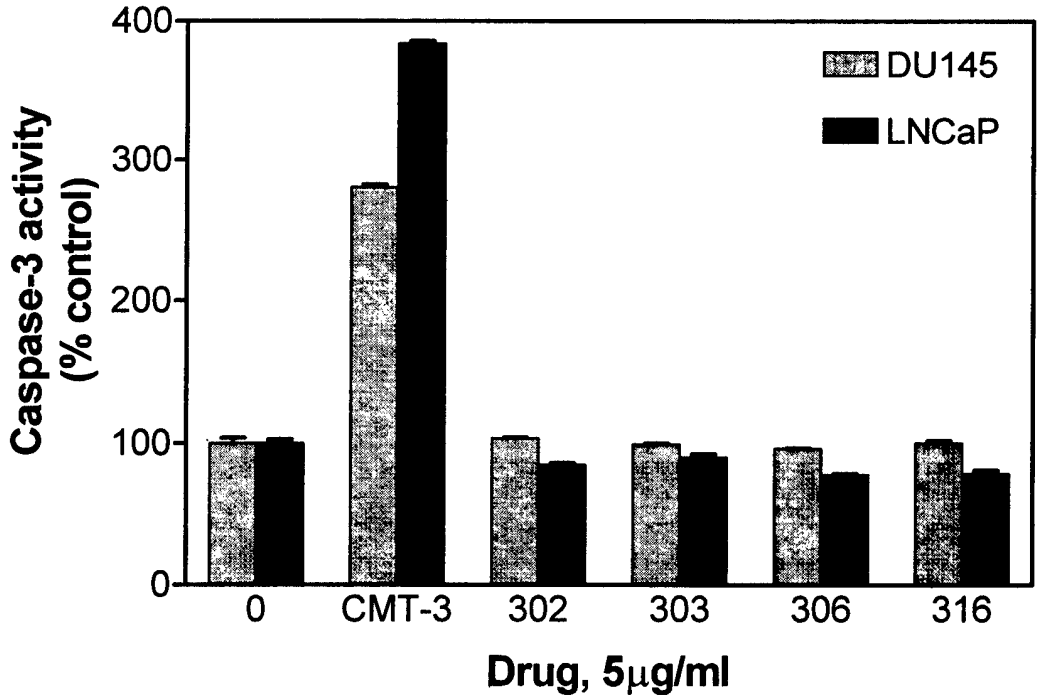

Fig. (5). Caspase 3 activation by CMT-3. Cells were treated with indicated amount of drugs for 60 min and cell lysates prepared from these cells were assayed for caspase-3 (CPP32) activity using Clontech ApoalertCPP32 kit. Fluorescence intensity of the samples resulting from the conversion of the DEVD-AMC to 8-AMC was measured in a fluorescence plate reader, Cytofluor II. Results presented are mean Å SEM from three indipendent experiment for each of the two cell lines.

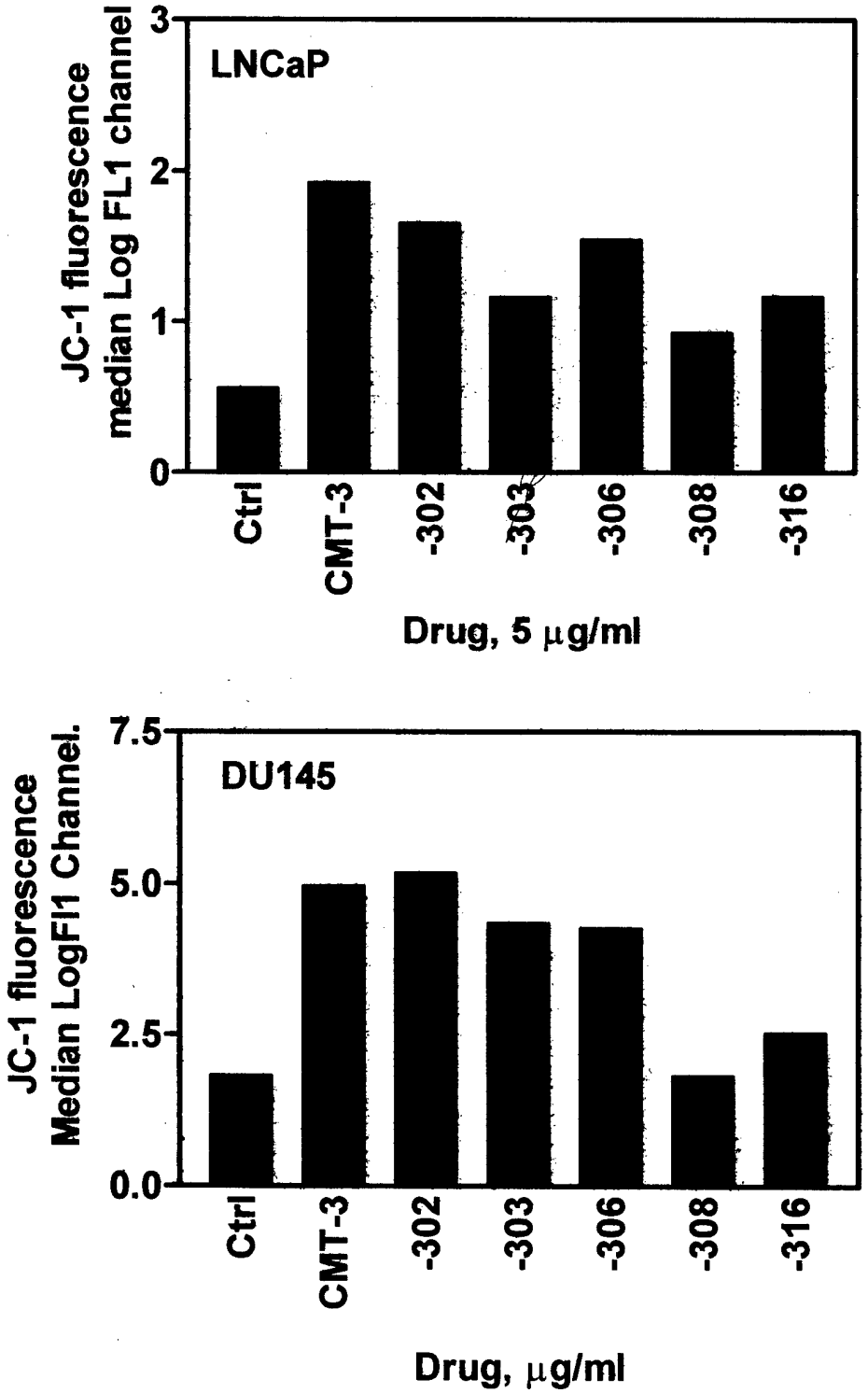

Fig (6). Measurement of depolarization of mitochondria by CMTs. Cells were preloaded with JC-1 and treated with CMT-3 and CMT-3Ns. Fluorescence intensity of cells at 575 nm was measured by flow cytometry. Data presented are from a single experiment for each cell line. The scale of the ordinate in the two panels are different because Du 145 cells take up more JC-1 (control) than LNCaP.

signaling. Activation of caspase is considered a significant event in the execution of apoptotic activity [23]. Although PS translocation is an early indication of apoptotic activity, caspases, especially caspase-3, are executioners of apoptosis signaling [23]. Therefore, we next examined whether CMT-3 and CMT-3Ns are capable of activating caspases. We observed significant caspase activity in CMT-3 treated samples but not in those cell lysates treated with CMT-3Ns. We detected very little caspase-3 activity in cell lysates prepared from untreated samples as well as those treated with CMT-3Ns for up to 90 min (**Fig 5**). These results show that cytotoxic activity of CMT-3Ns is not associated with

induction of cell death associated caspase activation. We are currently investigating whether the CMT-3 and CMT-3Ns are capable of activating other caspases, upstream of caspase-3, in the protease activation cascade.

Permeablization and Depolarization of Mitochondria by CMT-3 and CMT-3Ns

A mechanism of cytotoxic action of chemotherapeutic drugs is through the inactivation of mitochondrial function, especially altering the permeability of mitochondrial membrane and depolarization [22]. This function of cytotoxic drugs may be independent of their ability to trigger apoptosis. In order to distinguish between necrotic and apoptotic pathway we investigated the effect of these cytotoxic drugs on mitochondrial permeability. Analysis of JC-1 labeled cells by flow cytometry revealed a significant permeabilization of mitochondria as an increase in the median fluorescence channel. As shown in **Fig 6**, we saw an increase in the levels of fluorescence at 575 nm in cell samples treated with CMT-3 and CMT-3Ns. Interestingly, the accumulation and fluorescence emission of cells treated with all CMT-3s were comparable to their cytotoxic activity and not the weak apoptosis inducing activity. This investigation revealed a common site of action of these antitumor drugs. This also suggested to us that although not all CMT-3 analogues are capable of inducing apoptosis in our prostate model system, they are capable of disruption of mitochondrial activity.

Our results demonstrate that a single substitution in the CMT-3 structure can lead to completely different biological action although, almost all of them advantageous for the treatment of diseases. Although CMT-3 is the most cytotoxic in its class, two derivatives 7-nitro CMT-3 (CMT-302) and 9-hexonylaminoCMT-3 (CMT-316) are equally cytotoxic. All of the derivatives however, seem to exert their cytotoxic activity by acting as poisons for tumor mitochondria. Although CMT-3 has been shown to be cytotoxic and capable of inducing apoptosis in non-tumor cells such as monocytes [24], the mechanism of this activity is not yet been elucidated. Our results presented here suggest that it is through caspase mediated nuclear breakdown, In addition, CMT-3 is also capable of permeablizing mitochondria, a property shared by other CMT-3Ns. Interestingly, many cytotoxic drugs that are capable of inducing apoptosis do permeabilize and alter the membrane potential, here we found a closely related drug that alters these mitochondrial properties but does not increase apoptotic activity. There are several reports now in literature that these two actions may not be linked, agents exist that can induce one without causing the other [25-27]. Thus the CMT-3Ns exert their cytotoxicity by divergent mechanisms, CMT-3 induced apoptosis and the typical cellular signaling cascade, the derivatives apparently act by disrupting mitochondria, but not the death effector caspase (CPP32). Thus, results presented in this report suggest, that CMT-3 derivatives are capable of anti-proliferation and anti-invasive activity, a potent combination of anti-tumor functions which may have significance in selecting a proper analogue of CMT-3Ns for further therapeutic development.

ACKNOWLEDGMENT

This work is supported by grants from the U.S.Army Prostate cancer program (DAMD17- 98-1-8526) and the NIH (R01 CA 61038).

ABBREVIATIONS USED

CMT	=	Chemically modified tetracycline,
CaP	=	Carcinoma of the prostate,
MMP	=	matrix metalloproteinases,
MTT	=	(3-(4,5-dimethylthiozol-2yl)-2,5-diphenyltetrazolium bromide)

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Int. J. Cancer: **98,** 297–309 (2002)

DOI 10.1002/ijc.10168

Publication of the International Union Against Cancer

INHIBITION OF CELL PROLIFERATION, INVASION, TUMOR GROWTH AND METASTASIS BY AN ORAL NON-ANTIMICROBIAL TETRACYCLINE ANALOG (COL-3) IN A METASTATIC PROSTATE CANCER MODEL

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Antibiotic forms of tetracycline exhibit antitumor activity in some tumor models. However, their low *in vivo* efficacy and associated morbidity limit their long-term application in cancer therapy. This report appraises the efficacy of doxycycline (DC) and non-antimicrobial, chemically modified tetracyclines (CMTs) against prostate cancer. Both DC and several CMTs inhibited prostate tumor cell proliferation *in vitro*. Some of the CMTs were significantly more potent than DC. One of the CMTs, 6-deoxy, 6-demethyl, 4-de-dimethylamino tetracycline (CMT-3, COL-3), was the most potent inhibitor (50% inhibition dose [GI_{50}] ≤ 5.0 μg/ml). Exposure of tumor cells to CMT-3 induced both apoptosis and necrosis. Mitochondrial depolarization and increased levels of reactive hydroxyl radicals were also observed in cells treated with CMT-3. Cell cycle arrest at the G_0/G_1 compartment was observed in CMT-3- and DC-treated cells. DC and CMTs also inhibited the invasive potential of the tumor cells *in vitro*, from 10% (CMT-6) to >90% (CMT-3). CMT-3 and DC decreased matrix metalloproteinase (MMP)-2, tissue inhibitor of MMP (TIMP)-1 and TIMP-2 secretion in treated cultures and inhibited activity of secreted MMPs, CMT-3 was a stronger inhibitor. Daily oral gavage of DC and CMT-3 inhibited tumor growth and metastasis in the Dunning MAT LyLu rat prostate tumor. Decreases in tumor growth (27–35%) and lung metastases were observed (28.9 ± 15.4 sites/animal [CMT-3-treated] *versus* 43.6 ± 18.8 sites/animal [DC-treated] *versus* 59.5 ± 13.9 [control]; $p < 0.01$]. A delay in tumor growth (27 ± 9.3%, $p < 0.05$), reduction in metastases (58 ± 8%) and decrease in tumor incidences (55 ± 9%, CMT-3-treated) were also observed, when rats were predosed for 7 days. No significant drug-induced morbidity was observed in any of the animals. These results, along with a recently concluded clinical trial, suggest a potential use of CMT-3 as an oral, nontoxic drug to treat metastatic prostate and other cancers.

Key words: *tetracycline; chemically modified tetracyclines; COL-3; prostate cancer; metastasis; metalloproteinase inhibitors; therapeutics*

Metastasis of initially localized tumor causes much of the pain, suffering and eventual death in about one-third of patients diagnosed with prostate cancer.[1] At present, metastatic prostate cancer is incurable and is poorly palliated. This has created an urgency to find an effective therapy.[2]

An avenue to prevent the spread of prostate tumor cells is inhibition of their invasive potential. Degradation of basement membrane, which leads to the invasion of tumor cells into the surrounding stroma and blood capillaries, is a critical step in metastasis.[3] A repertoire of cell adhesion molecules, proteinases, their activators and their inhibitors participate in this process.[4–6] The matrix metalloproteinases (MMPs), including stromelysin and gelatinases that degrade the extracellular matrix, are the important enzymes involved in invasion and metastasis.[7] An imbalance between MMPs and their endogenous inhibitors, the tissue inhibitor of MMPs (TIMPs), has been observed, with the enzymes more active at the invasive front of the tumors.[8] The paradigm proposed by Liotta *et al.*[8] that this imbalance may favor invasion and metastasis of many solid tumors, if not all, has guided many investigations since its proposal.[9]

We previously reported that primary cultures of human prostate tumors secrete high levels of gelatinases (MMP-2 and MMP-9) and low levels of TIMPs.[10] Other studies have also shown high levels of mRNAs for MMPs in prostate tumor tissues.[11,12] Natural or synthetic inhibitors of MMPs may therefore inhibit or slow tumor metastasis in general and prostate cancer in particular.[13,14] The antibiotic tetracycline and its chemically modified non-antimicrobial analogs (CMTs) are such agents because they are potent inhibitors of MMPs.[15–18]

The common tetracyclines such as doxycycline (DC) and minocycline have a variety of antitumor properties such as inhibition of protein synthesis in the mitochondria,[16,20] collagenolysis[15] and angiogenesis.[18] Because of these activities tetracyclines may be useful as antitumor agents. However, systemic administration of the antibiotic tetracycline over prolonged periods has limitations due to potential emergence of antibiotic-resistant, systemic microbial flora. Furthermore, long-term exposure to tetracycline may also cause additional gastrointestinal and nutritional toxicity due to the destruction of normal beneficial microbial flora. In addition, common tetracyclines have a limited *in vivo* efficacy due to their short life in circulation and rapid elimination, thus requiring relatively large continuous dosing.[20]

A series of chemically modified tetracyclines (CMTs) have been synthesized and characterized in the hope of overcoming the limitations associated with the antimicrobial forms of tetracycline.[21–23] Golub *et al.*[21] reported that removal of the dimethylamino group (CMT-1) from the carbon-4 position of the A-ring of the 4-ringed tetracycline structure (*e.g.*, CMT-1) eliminates the antimicrobial activity of the tetracycline, while retaining the anticollagenolytic activity. Further modifications of the de-dimethylamino tetracycline have resulted in CMTs with more potent anti-

Abbreviations: CM-DCFDA, carboxyl methyl-2′,7′-dichloro-dihydro carboxyl fluorescein diacetate; CMT, chemically modified tetracycline; DC, doxycycline; GI_{50}, 50% inhibition dose; MMP, matrix metalloproteinase; OD, optical density; TIMP, tissue inhibitor of matrix metalloproteinase.

Grant sponsor: Department of Defense, U.S. Army Medical Research Program; Grant sponsor: DAMD 17-98-272; Grant sponsor: PHS; Grant numbers: 1R29-CA 63108, 5R01 CA 63108; Grant sponsor: L. Austin Weeks Endowment Fund of the Department of Urology, University of Miami.

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Received 5 July 2001; Revised 5 October 2001; Accepted 12 October 2001

Published online 18 December 2001

collagenolytic activity, oral bioavailability and long-serum half-life.[22–24] Previous reports of cytostatic activity and strong MMP inhibition by the antimicrobial and non-antimicrobial forms of tetracyclines motivated us to investigate further the efficacy of these drugs on prostate cancer cells *in vitro* and on a rat model of androgen-insensitive metastatic prostate tumor *in vivo*.

The objective in our study was 2-fold. The first was to test the cytotoxic or cytostatic and antiinvasive activities of a common tetracycline antibiotic, DC, and 1 or more CMTs *in vitro* and *in vivo*. The second objective was to test whether oral administration of 1 or more CMTs with their enhanced anti-MMP activities and longer *in vivo* retention would be therapeutically more effective than that reported before. We report here that although both DC and 1 of the CMTs (CMT-3) show strong antimetastatic activity by oral administration, CMT-3 is significantly more effective as a cytotoxic antitumor and antimetastatic drug.

MATERIAL AND METHODS

Reagents

The following non-antimicrobial CMTs were used in our study: CMT-1 (4-dedimethylamino tetracycline), CMT-2 (tetracycline nitrile), CMT-3 (6-demethyl, 6-deoxy, 4 de-dimethylamino tetracycline), CMT-4(7-chloro 4-de-dimethylamino tetracycline), CMT-6 (4-hydroxy-4-de-dimethylamino tetracycline), CMT-7 (12-α deoxy 4-de-dimethylamino tetracycline) and CMT-8 (6-α deoxy, 5-hydroxy 4-de-dimethylamino tetracycline). All the CMTs were from the laboratory of 1 of the authors (L.M.G.). Purified, GMP-grade COL-3 (CMT-3) was a generous gift from CollaGenex Pharmaceuticals (Newtown, PA). The characterization of anti-MMP activities of CMTs has been previously described.[21–25] TIMPs and MMP ELISA kits were from Oncogene Sciences/Calbiochem (San Diego, CA). The Cell Death ELISA-Plus kit was from Roche Molecular Biochemicals (Indianapolis, IN). Fluorescent dyes (propidium iodide, JC-1: a membrane potential-sensitive fluorescent dye [5′,5′6′,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazoleyl carbocyanine iodide] and the hydroxyl free radical-reactive dye carboxyl methyl-2′,7′-dichloro-dihydro carboxyl fluorescein diacetate [CM-DCFDA] were from Molecular Probes (Eugene, OR). All other reagents were from Sigma (St. Louis, MO), or as indicated.

Cells and tumor lines

Established cell lines used in our study were the Dunning rat prostate tumor line MAT LyLu[26] and the human prostate cancer cell lines PC-3ML, LNCaP, DU 145 and TSU PR1. The procurement, culture and maintenance of these cell lines in the authors' laboratory have been described previously.[26,27] In addition, a non-tumorigenic human prostate epithelial cell line, BPH-1,[28] was a gift from Dr. Simon Hayward (University of California, San Francisco, CA). All cultures were maintained in complete medium (RPMI-1640 basal medium with 10% FBS [Atlanta Biological, Atlanta, GA] and 10 μg/ml gentamicin [Life Technologies, Gaithersburg, MD]). MAT Ly Lu cells were cultured in the complete medium containing 250 nM dexamethasone.[26]

Cytotoxicity assays

Initially, 2 methods were evaluated for estimating cytotoxicity of CMTs and DC. These included the cellular [^{3}H]thymidine incorporation assay[27] and the colorimetric thiazolyl blue (MTT) reduction assay (tetrazolium bromide: [3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide).[29] The details of the procedures for these methods, adapted in the authors' laboratory, have been previously published.[27,30] Values of growth-inhibitory doses of DC and CMTs evaluated from [^{3}H]thymidine incorporation or the MTT assay were very similar. Subsequently, the nonradioactive MTT assay was used. Drug-induced cytotoxicity was determined in cultures with initial plating density of 2×10^4 cells/well, in 48-well plates as described previously.[30] Stock solutions of DC (0.1–5 mg/ml) were prepared in sterile Dulbecco's PBS, pH 7.2. The CMT stocks (0.1–5 mg/ml) were prepared in sterilized DMSO. The final concentration of DMSO in cultures did not exceed 0.1%, a nontoxic concentration. Cultures incubated with drugs for 48 hr, in replicate wells, were assayed for viability by the MTT assay. All experiments were repeated at least 3 times.

Determination of apoptotic activity

Drug-induced apoptosis was assayed using the Cell Death ELISA-Plus kit (Roche Molecular Biologicals). The assay measures the amount of free nucleosomes in cell lysates and culture supernatants resulting from programmed cell death and necrosis, respectively.[31] Cell cultures were exposed to drugs in a dose- and time-dependent manner; culture supernatant and cell lysates were assayed separately for apoptotic bodies per the supplier's instructions. Triplicate samples were used for each time- and dose-dependent apoptosis induction experiment. Manufacturer-supplied positive and negative controls and medium blank were used to compare the results from replicate experiments.

Determination of depolarization of mitochondria

We used a mitochondria-specific, lipophilic cationic probe, JC-1,[32] to detect changes in mitochondrial membrane potential ($\Delta\Psi$). Polarized mitochondrial membrane (inside negative) permits more accumulation of JC-1 aggregates (J-aggregates) emitting greenish orange fluorescence ($\lambda_{max} \sim 590$ nm) when excited at 488 nm. Decrease in membrane potential (depolarization) results in increase in green fluorescence (527 nm). This shift in cell fluorescence is measured in a flow cytometer. Cultures were incubated with JC-1 for 30 min after their incubation with CMT-3 or DC for up to 48 hr, as detailed previously.[33] Fluorescence intensity was profiled in a flow cytometer equipped with both a narrow bandpass green filter (FL1) and a long-pass green filter (>575 nm; FL2; EPICS XL, Beckman-Coulter, Palo Alto). Median fluorescence intensity of untreated and treated samples was compared in the green fluorescence channel (log FL1) instead of FL2, as the fluorescence associated with CMT-3 also interfered at the FL2 channel. Increase in green fluorescence intensity (log FL1) was approximated as a decrease in $\Delta\Psi$.

Determination of hydroxyl free radical production

Alteration in the levels of hydroxyl free radicals in drug-treated cells were estimated using oxidation of a nonfluorescent analog of fluorescein, CM-DCFDA. This moderately polar, cell-permeable dye is oxidized by oxygen and hydroxyl free radicals, resulting in oxidized carboxyl fluorescein diacetate. Cytoplasmic esterases convert the carboxyl-fluorescein diacetate to hydrophilic carboxyl-fluorescein.[34,35] The hydrophilic carboxyl fluorescein is trapped inside intact cells, which now emit bright green fluorescence upon excitation at 488 nm.[36] Cellular fluorescence is then quantified by flow cytometry. Cultures treated with CMT-3, DC or 0.1 mM hydrogen peroxide for various periods were incubated with CM-DCFDA (1 μg/ml) for 60 min. Unreacted DCFDA was rinsed off from culture after 60 min and treated cells were suspended as a single-cell suspension for analysis in a flow cytometer. Median fluorescence intensity of samples treated with drug alone, CM-CFDA alone or both were compared to estimate the levels of hydroxyl free radicals relative to untreated controls. Relative levels of the free radicals produced by CMT and DC, indicated by the positive shift in median fluorescence channel (log FL1), were also compared against that observed with cells treated with hydrogen peroxide alone.

Determination of cell cycle phase fractions

Drug-induced alteration in cell cycle phase progression was analyzed by determining the percent of cells in each cell cycle phase compartment by flow cytometry as described before,[27] with modifications.[37] Briefly, CaP cell cultures were treated with various concentrations of CMT-3 or DC for 48 hr, cells were lysed and the nuclei were stained with 50 μg/ml propidium iodide (PI) simultaneously in a cell lysis buffer (PBS, 0.4% Nonidet p40 (NP40) detergent, 50 μg/ml PI). DNA contents of the stained

nuclei were profiled in a Coulter XL flow cytometer. The MODFIT LT program (Verity Software House, Topsham, ME) was used for cell cycle analysis of the DNA histograms.[38] Experiments were repeated 3 times for each of the 2 cell lines analyzed.

In vitro *tumor cell invasion assay*

The effects of CMTs and DC on the invasive potential of TSU PR1 and Dunning MAT LyLu cells were tested using the Matrigel assay as described earlier.[30] Briefly, the procedure used was a modification of Albini *et al.*[39] by Hussain *et al.*[40]: tumor cells, incubated with various drugs for 6 hr, were deposited (4×10^5 cells/filter) in the top wells of Transwell plates (Corning/Costar, Boston, MA). The top wells, made from 12 μm pore polycarbonate filters, had been previously coated with a layer of Matrigel (0.5 mm, 500 μg/cm^2; Collaborative Research-BD Bioscience, Bedford, MA). The bottom well contained a chemoattractant, a serum-free culture-conditioned medium of human lung fibroblasts (1 ml/well). Percent of cells in each treatment group that invaded through the Matrigel-coated filter in 48 hr was determined by MTT assay as described.[30] Invasive activity (% invasion) was defined as the ratio of optical density (OD) from the bottom wells to that of the total OD (OD of bottom plus top wells), multiplied by 100. Use of the MTT assay allowed us to normalize the coincident cytotoxicity of the drugs. All assays were repeated at least 3 times.

Determination of secreted MMP-2 and TIMPs

Culture conditioned medium from a primary culture of a human prostate tumor tissue (Gleason sum 8, preoperation prostate specific antigen [PSA] > 40) was collected and assayed using ELISA kits according to the supplier's instructions (Oncogene Sciences/Cal-Biochem). We have previously reported the establishment and propagation of primary prostate epithelial cells.[30] The ELISA was specific for human cell culture-derived MMP-2, TIMP-1 and TIMP-2. Levels of MMP-2 and TIMPs were normalized against total cell proteins, estimated using the Pierce BCA protein assay kit (Pierce Chemicals, Rockford, IL).

Gelatinase activity assay

The activity of gelatinase in the MAT LyLu cell culture-conditioned medium was determined by a modified [^{3}H]gelatin digestion assay of Dean and Woessner.[41] Serum-free culture-conditioned medium, collected from MAT LyLu cells, was chemically reduced (1 mM dithiothreitol) and alkylated (1 mM iodoacetamide), for 30 min at 37°C and dialyzed to destroy any endogenous TIMPs, which could interfere with the MMP assay.[41,42] The dialyzed medium was assayed for gelatinase activity after activating the latent MMPs with 1 mM p-aminophenyl mercuric acetate (APMA) for 30 min at 37°C. MMP enzyme activity was assayed with 1 or 10 mM $CaCl_2$, with 2 μM $ZnCl_2$ in the assay buffer. We chose 1 mM $CaCl_2$ because it is closer to the physiologic concentration of [Ca^{2+}][43] and compared it with the activity obtained using 10 mM $CaCl_2$, which was reported to be the optimum concentration for assaying MMP activity.[41]

SDS-PAGE and zymography

MMPs secreted into the culture medium by cells treated with DC and CMT-3 were identified by electrophoresis and zymography as described previously.[10] Conditioned medium collected from cultures treated with DC or CMT-3 for 2 days were incubated with SDS-gel sample buffer for 30 min at 21°C and analyzed by gel electrophoresis on a 1 mg/ml gelatin-embedded SDS-polyacrylamide gel (8%).[10]

In vivo *studies*

Tumor generation. Dunning MAT LyLu cells harvested from culture flasks in 0.5 ml suspension, containing 2×10^5 to 2×10^6 cells/ml (see Results), were implanted in the dorsal flank of adult male Copenhagen rats (Harlan Sprague-Dawley, Indianapolis, IN). The rats weighed 250–300 g and were 90–100 days old at the time of implanting. Tumor growth was examined by palpating the injection site 5 days after the implant.

Drug treatment. DC and CMT-3 were dissolved in a 2% aqueous solution of carboxymethyl cellulose (Sigma, cat. no. C-5678); a fresh solution was made daily. Rats were dosed by oral gavage with a 4-inch gavage needle daily, with either 1 ml of the drug solution or vehicle (2% carboxymethyl cellulose). Tumor growth was recorded 3 times a week and the rats' weights weekly. The effect of the various treatments on tumor growth was monitored over time by measuring tumor volumes with a caliper; the volume was approximated to an ellipsoid (*i.e.*, volume = length × width × height × 0.5236), as previously reported.[27] Tumor growth rate was estimated from a regression analysis of log-transformed tumor volumes *versus* time. The mean tumor growth rates (time to reach a fixed volume) of control and different treatment groups were then compared. The statistical significance of the differences in tumor growth rates was tested by Tukey-Kramer multiple comparisons test. Rats were euthanized when the tumors reached a volume > 10 ml. Necropsy was done, and tumors and lungs were removed and fixed in formalin (tumor tissue) or in Bouin's fixative (lungs). Macroscopic tumor foci on the lungs were counted under a dissecting microscope in a blinded fashion. Fixed tumor and lung specimens were randomly selected for histology. Tissues were sectioned and stained with hematoxylin and eosin solution (Fisher Scientific, Fair Lawn, NJ). A veterinary pathologist examined the slides.

RESULTS

Effect of DC and CMTs on prostate cancer cell proliferation

The effect of CMTs on cell proliferation or viability varied greatly. Some CMTs significantly inhibited cell proliferation, but others did not. As shown in Figure 1, all but CMT-7 was significantly cytotoxic in all 3 cell lines. CMT-3 was the most cytotoxic among all the tetracyclines tested (GI_{50} 2.3 ± 0.9 μg/ml to 6.7 μg/ml). CMT-7 was nearly nontoxic to all cell lines except the LNCaP, in which it was slightly toxic (GI_{50} 120 ± 14.7 μg/ml). We could not test the toxicity of CMTs at concentrations higher than 50 μg/ml as they precipitated in the culture medium. DC inhibited the proliferation of LNCaP cells (GI_{50} 6.3 μg/ml), but it was much less potent in other cell lines tested (Fig. 1A and Table I). Presence or absence of serum in the culture medium did not alter the cytotoxicity of any of the CMTs or DC in the 3 cell lines tested (data not shown).

Since CMT-3 was the most potent of all CMTs, we compared the cytotoxicity of CMT-3 with that of DC against primary culture CaP 139, derived from a prostate tumor specimen, and the MAT LyLu rat prostatic carcinoma cell line. Cytotoxicity was measured using the MTT assay. As shown in Table I, CMT-3 was up to 8-fold more cytotoxic than DC to these cell cultures. The cellular basis of CMT-3- and DC-induced toxicity was further investigated by examining cytotoxic mechanisms common to other chemotherapeutic drugs.

Cell death induction by CMTs and DC

Due to the contrasting cytotoxic actions of CMT-3 and DC, we chose these 2 drugs for further investigation. We reasoned that observed cytotoxicity may be due to necrotic or apoptotic cell death induced by 1 or more of these agents. Cell death ELISA revealed that cells incubated with CMT-3, and much more weakly with DC, underwent both necrotic and apoptotic cell death in a dose- and time-dependent manner. As shown in Figure 2, cells underwent apoptosis in cultures exposed to CMT-3 for 4 hr and longer. Among the various prostate cell lines tested, the MAT LyLu cell line was the most sensitive. Table II summarizes the results for CMT-3-induced apoptosis in various cell lines. Necrotic cell death was apparent in cultures treated with high concentrations of CMT-3 or DC (>10 μg/ml for CMT-3 and >20 μg/ml for DC; Fig. 3). Free nucleosomes were detected by ELISA in both cytoplasmic and culture supernatants at these concentrations, indicating that these drugs induce necrosis and apoptosis at high concentrations (CMT-3 > 5 μg/ml and DC > 20 μg/ml). CMT-3-induced

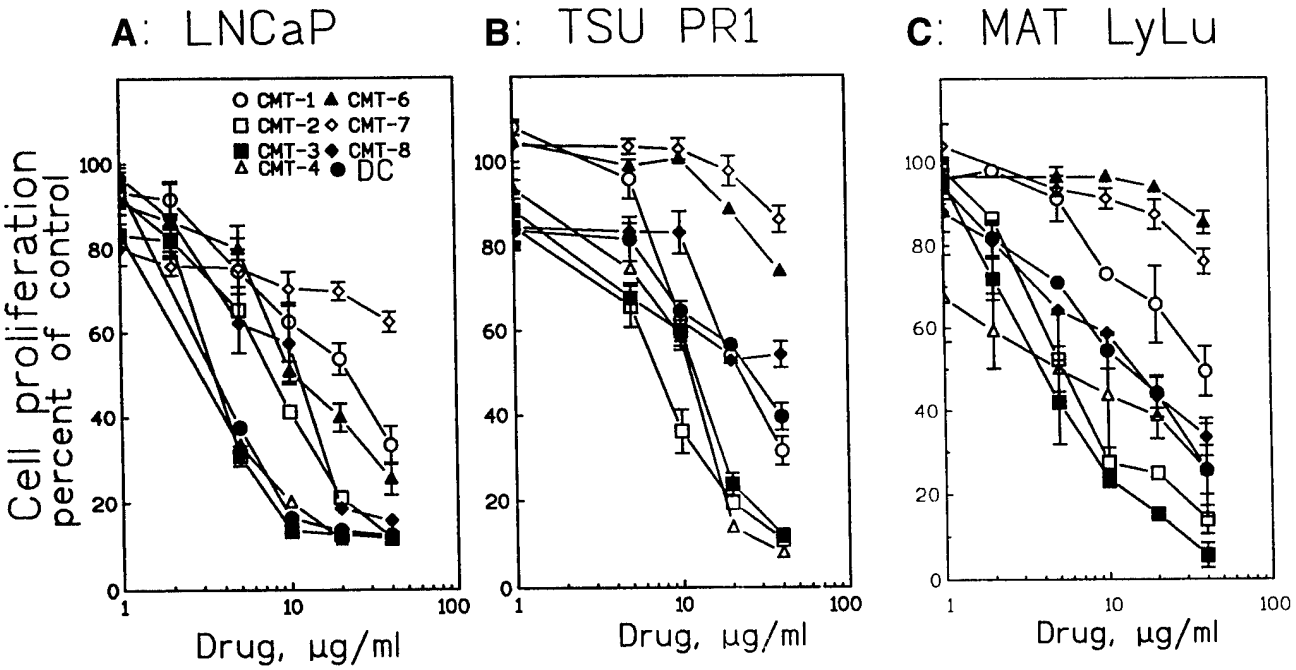

FIGURE 1 – Cytotoxicity of DC and CMTs in prostate tumor cell lines. Two cell lines, an androgen-sensitive, low metastatic potential cell line (LNCaP; *a*), and an androgen receptor-negative, metastatic cell line (TSU PR1; *b*) were exposed to DC or to various CMTs in complete medium for 48 hr. The Dunning MAT LyLu cell line was similarly treated (*c*). Cell proliferation was measured using the MTT assay. Vertical bars represent the mean ± SEM from 4 independent determinations. Symbols for various CMTs are the same in all panels.

TABLE I – CYTOTOXICITY OF DC AND CMT-3 IN PROSTATE CELLS

Cell line[1]	GI_{50}[2]	
	DC	CMT-3
ALVA 101 (4)	16.67 ± 1.3[b]	3.1 ± 0.34
BPH-1 (3)	9.68 ± 2.45	4.78 ± 1.68
CaP 139 (1)	18.7 ± 3.7	9.3 ± 2.11
DU 145 (8)	19.8 ± 4.25	2.3 ± 0.53
LNCaP (5)	6.3 ± 1.35	2.29 ± 0.96
MAT Lylu (7)	9.09 ± 2.95	2.36 ± 0.86
PC-3 (5)	16.55 ± 1.06	4.8 ± 0.96
TSU PR-1 (5)	18.64 ± 5.1	6.7 ± 1.2

[1]Numbers of replicate experiments are given in parentheses.– [2]Growth inhibition was calculated from linear regression of the dose-response curves generated for each experiment using log (dose) *vs.* cell proliferation (% of control), as shown in Figure 1*a–c*. Correlation coefficient (r) was always ≥0.95 (negative). Results are presented as mean ± SEM µg/ml (1 µg/ml = 2.2 µm) of at least 3 GI_{50} values calculated from each experiment.

apoptotic activity was significantly higher than that induced by DC at concentrations at which their cytotoxic activity was comparable (Figs. 1,2). A possible pathway to drug-induced apoptosis, commonly mediated by alteration in mitochondrial polarity and permeability was examined next.

Effect of CMT-3 and DC on mitochondrial membrane polarity ($\Delta\psi$) and production of reactive hydroxyl free radical [OH•]

As shown in Figure 4, a significant accumulation of JC-1 was observed in cells treated with CMT-3. In addition, cells treated with CMT-3 for >90 min exhibited an increase in green fluorescence, indicative of lack of J-aggregation (red fluorescence, FL-3), as found in control (Fig. 4). In addition, a stabilization of green-orange fluorescence, accumulated in the long-green fluorescence channels (log FL2), was also observed (data not shown), indicating a decrease in $\Delta\Psi$ and leading to increased permeabilization. We next investigated a plausible cause of mitochondrial permeabilization. Free radical-induced cellular damage, leading to both necrotic and apoptotic cell death, is 1 of the most common mechanisms of cell kill by antibiotics in bacteria and anticancer drugs in tumor cells.[45,46] Free-radical-increased generation of hydroxyl free radicals [OH•], in cells exposed to CMT3 and DC, was examined by measuring the oxidative conversion of CM-CFDA to fluorescein. As shown in Figure 5, flow cytometric analysis of cells incubated with the drug revealed elevated production of [OH•] in cells exposed to CMT-3. Cells exposed to DC, however, did not show any appreciable increase in fluorescence intensity (Fig. 5).

CMT-3 and DC affect cell cycle progression

We observed an arrest of cell cycle progression in CaP cells incubated with CMT-3 or DC. Distribution of cells into various cell cycle phase compartments at the end of treatment with DC or CMT-3 is summarized in Table III. Both CMT-3 and DC induced arrest of cells at the G_0/G_1 phase in both the cell lines, DU 145 and LNCaP. There was a significant increase in percent of cells at the G_0/G_1 compartment, from ~50% in the control cell population to up to 85% in cells treated with CMT-3 (10 µg/ml). Similarly, treatment with DC (≤20 µg/ml) also increased the percent of cells in G_0/G_1 phase up to 79.2% based on the DNA content of the cells. This increase was mainly due to decrease in S-phase fractions, indicating block at the G_1/S boundary. In contrast, there was no significant change in G_2/M fractions in LNCaP cells incubated with CMT-3 or DC. However, incubating DU 145 cells with CMT-3, but not DC, resulted in a significant decrease (from 17.6 ± 1.8% to 5.6 ± 5.6% [at CMT-3 of 5µg/ml] and 7.0 ± 5.5% [at CMT-3 of 10 µg/ml], $p < 0.003$ for both treatment concentrations) in both S-phase and G_2/M phase fractions. Similar results were obtained for another androgen-independent CaP cell line, PC-3 (data not shown).

Effect of DC and CMTs on invasive/metastatic potential of prostate cancer cells

Since tetracycline is a known inhibitor of MMPs, including those associated with invasion and metastasis,[17] we next tested antiinvasive activity of CMTs and DC on a highly invasive human

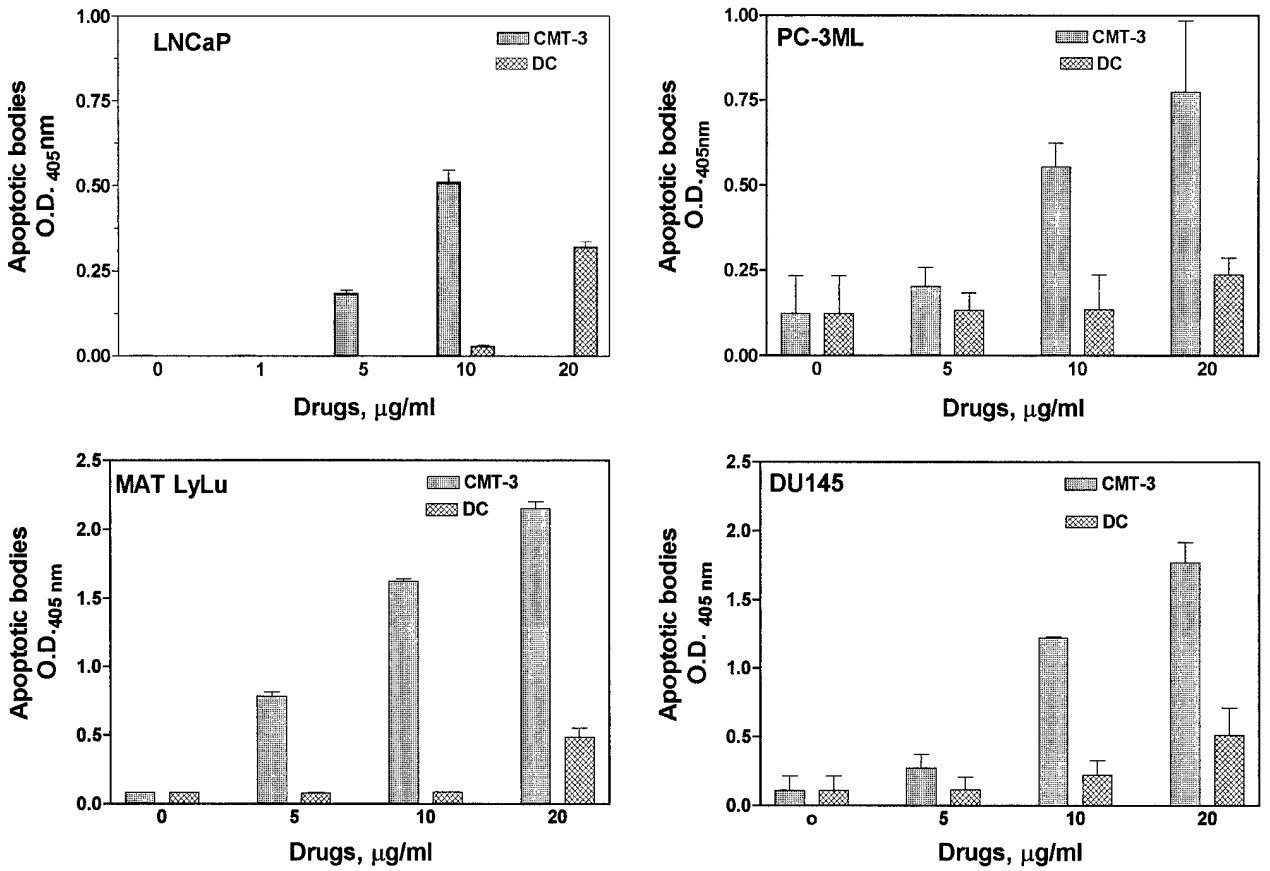

FIGURE 2 – Induction of apoptosis in prostate cancer cells by CMTs. Relative quantities of free nucleosomes in the culture supernatant of CaP cells, incubated with or without CMT-3 and DC for 48 hr, were estimated using the Cell Death ELISA-Plus kit. The ordinate represents specific OD. Note that apoptotic bodies in the culture supernatants of treated LNCaP and PC-3 cells were less than that for DU 145 and MAT LyLu cultures. The data presented for supernatants represent the accumulated free nucleosomes, resulting from apoptosis *and* necrotic cell death. Data presented are mean ± SEM (error bars) from 3 independent experiments. [Reprinted from Lokeshwar 1999,[77] with permission from the publisher.]

TABLE II – CMT-3- AND DC-INDUCED PROGRAMMED CELL DEATH

	Apoptosis dected			
	MAT LyLu	DU 145	PC-3	LNCaP
Range of concentration[1] (μg/ml)	1–10 (CMT-3) 5–20 (DC)	5–10 (CMT-3) ≥20 (DC)	5–10 (CMT-3) ≥20 (DC)	2.5–10 (CMT-3) ≥20 (DC)
Minimum duration of exposure (hr)[2]	4 (CMT-3) 24 (DC)	4 (CMT-3) 24 (DC)	4 (CMT-3) 24 (DC)	4 (CMT-3) 24 (DC)
Replicate experiments	8	4	4	4

[1]Free nucleosomes were detected (specific OD > 0.1) in the cell lysates when assayed 48 hr after treatment.–[2]Cultures were exposed to CMT-3 (5 μg/ml) or DC (10 μg/ml) from 1 to 24 hr, and cell lysates were assayed 24 hr after exposure. An OD of 0.1 from untreated control was taken as the specific due to apoptosis activity.

(TSU PR1) and a rat cell line (MAT Ly Lu) using an *in vitro* (Matrigel) invasion assay.[39] As shown in Figure 6, DC and CMTs inhibited invasive activity of these 2 cell lines. However, once again the potency of various CMTs varied greatly (Fig. 6). For example, CMT-3 was the most potent and CMT-7 was the least potent inhibitor of invasive activity of TSU PR1 cells. The 50% inhibition dose (IC_{50}) calculated for various CMTs varied from 1.7 ± 0.31 μg/ml (CMT-3) to >100 μg/ml for CMT-7. DC was not significantly inhibitory in TSU PR1 cells (IC_{50} = 27 ± 4.3 μg/ml). The IC_{50}s of CMT-3 and DC for 3 other invasive human prostate cancer cell lines (DU 145 and PC-3) were also in the same concentration range as that for the TSU PR1 cells (data not shown). CMTs affected the invasive potential of MAT LyLu cells similarly (Fig. 6*b*). However, compared with the TSU PR1 cells, DC was significantly more effective in the MAT LyLu cells (68 ± 4.2% inhibition at 5 μg/ml; Fig. 6*b*).

Effect of CMT-3 and DC on gelatinase activity

First we examined whether CMT-3 and DC inhibit the activity of MMPs secreted by the cells into the culture medium. TSU PR1 and MAT LyLu cells were cultured in a serum-free medium for 48 hr. Culture-conditioned media were then assayed for MMP activity using [^{3}H]gelatin as the substrate, in the presence of DC or CMT-3. As shown in Table III, both CMT-3 and DC inhibited MMP activity *in vitro*. At 1 mM $CaCl_2$, the IC_{50}s of CMT-3 and DC were ~0.5 and 2.25 μM, respectively. At 10 mM $CaCl_2$, the IC_{50} for CMT-3 was ~5.5 μM and that of DC was 10.0 μM (Table IV).

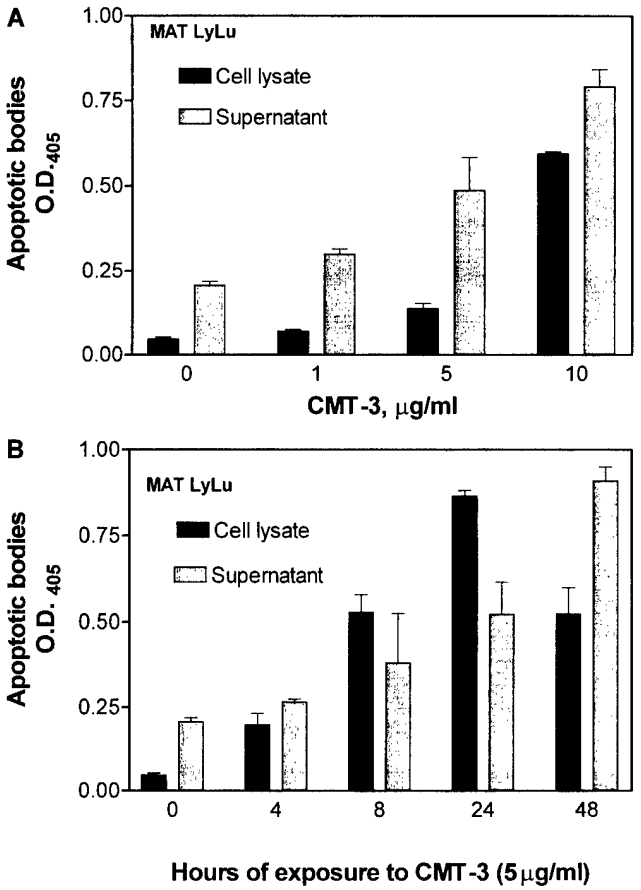

FIGURE 3 – CMT-3-induced apoptosis and necrosis in MAT LyLu cells. To distinguish between drug-induced apoptosis and necrotic cell death in tumor cells, cell lysates and culture medium were both assayed for free nucleosomes. (*a*) Drug-dependent induction of apoptotic or necrotic cell death, after incubation for 24 hr. Note: The supernatant contained the sum of free nucleosomes due to both necrotic and apoptotic cell death, as a result of cell lysis. (*b*) Analysis of time-dependent induction of apoptosis or necrosis. Bars represent mean ± SEM from 3 independent experiments.

Inhibition of MMP secretion/production by CMT-3 and DC

Next we examined whether CMT-3 and DC also affect the levels of production (synthesis and/or secretion) of 2 gelatinases, MMP-2 and MMP-9 by prostate tumor cells.[10] MMP levels in serum-free conditioned media from drug-treated cultures were analyzed by SDS-PAGE and zymography.[10] A typical zymogram is shown in Figure 7. As shown in this figure, TSU PR1 cultures predominantly secreted latent forms of MMP-2 and MMP-9 (Fig. 7*a,b*), whereas the MAT LyLu cells secreted activated MMP-2 (62 kDa form) and little MMP-9 (Fig. 7*c,d*). Incubation with CMT-3 or DC decreased the secretion of MMPs in a dose-dependent manner. As appears in Figure 7, cells treated with CMT-3 secreted significantly less MMP than the cells treated with DC. Moreover, MMP-9 levels did not decrease significantly even at the highest concentration of DC tested (50 μg/ml). Presence of the drug in the conditioned medium used in the gel did not interfere with the digestion of gelatin after electrophoresis; most likely all the bound CMT-3 or DC had diffused out of the gel during electrophoresis and washing steps.

To establish further that the observed decreases in MMP levels in conditioned medium are indeed due to the drug-induced inhibition of MMP production/secretion, protein levels of MMPs were measured by an ELISA that uses a monoclonal anti-MMP-2 antibody. Initial measurement of MMP-2 protein levels in TSUPR1

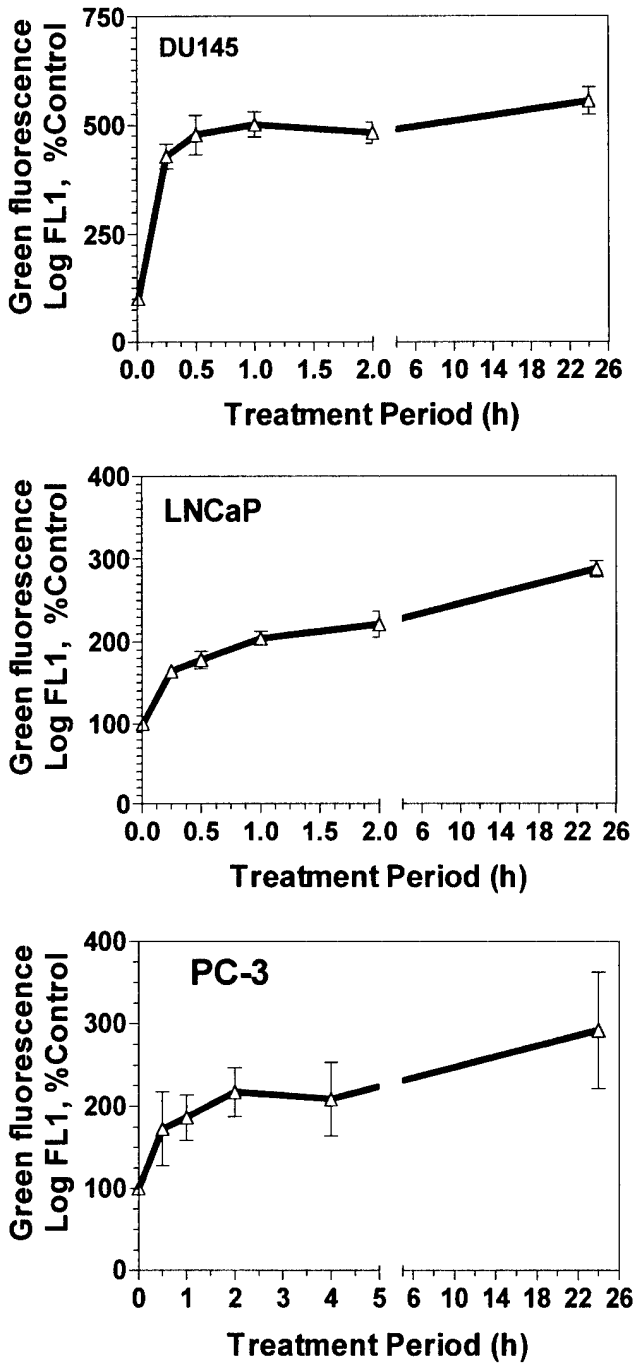

FIGURE 4 – Decrease in mitochondrial membrane potential in cells treated with CMT-3. Fluorescence intensity of CaP cells incubated with CMT-3 (5 μg/ml) for various periods and with JC-1 for 30 min was estimated by flow cytometry as described previously.[31] Median fluorescence intensity, log FL1 (green fluorescence, narrow pass filter λ = 530 nm) is taken as that proportional to the amount of JC-1 present inside the cells in an unaggregated form. Unaggregated JC-1 accumulation is indicative of a decrease in mitochondrial membrane potential, $\Delta\Psi$.[32] Bars represent mean ± SEM from 3 independent determinations.

conditioned medium exhibited levels of MMP below the detection limits (<10 ng/ml). However, MMP-2 protein levels were measurable in a primary prostate tumor-derived culture, CaP 139. As we have reported before, the primary cultures typically secrete a 10-fold higher amount of MMP-2 than that of common established

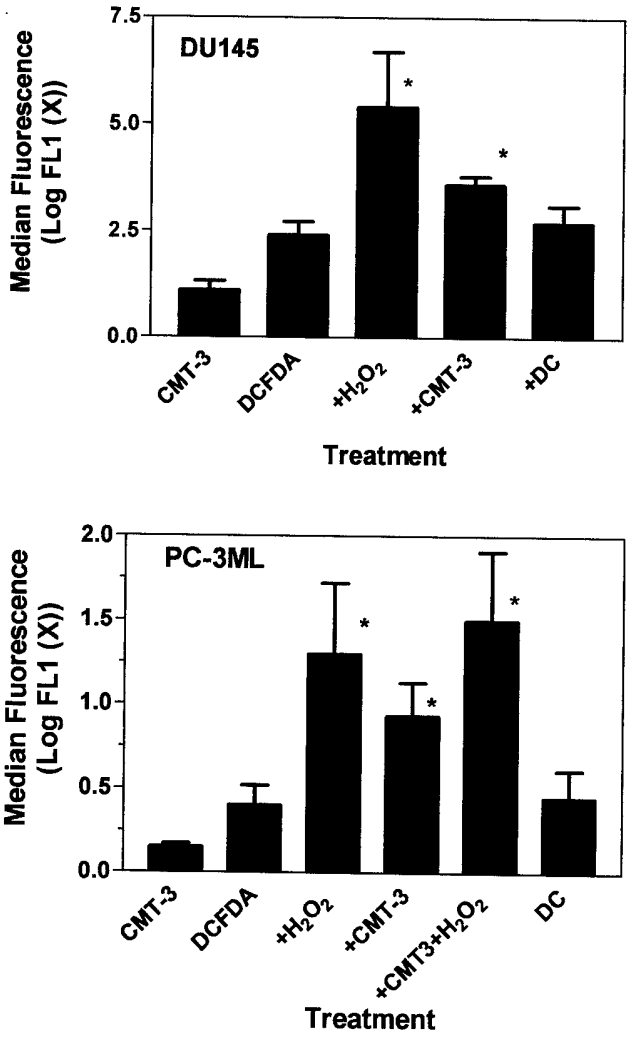

FIGURE 5 – Increase in free radical generation in CMT-3-treated cells. Fluorescence intensity of cells incubated with CMT-3 (5 μg/ml), DC (10 μg/ml) and CM-DCFDA (10 μg/ml), alone or together, was compared by flow cytometry. Cells incubated with CMT-3 and DC emitted weak green fluorescence. Histograms in the top panel represent the fluorescence intensity of DU 145 cells incubated with CMT-3 alone, DCFDA alone or DCFDA + H_2O_2, CMT-3 + DCFDA or DC + DCFDA. Cells incubated with DCFDA alone fluoresced significantly; this was further increased significantly when a free radical-generating agent (hydrogen peroxide) was added. Data are from 3 independent experiments. *, fluorescence levels significantly different from that of control ($p < 0.04$).

CaP cell lines.[10] We found a dose-dependent decrease in the levels of MMP-2 secreted by the CaP 139 cells treated with CMT-3 or DC. MMP-2 levels decreased by 51 and 74% at 20 μg/ml DC and 10 μg/ml CMT-3, respectively (Table V). Although traces of MMP-9 were detected by zymography, MMP-9 levels even in 10× concentrated medium were below the minimum detection levels of ELISA.

Levels of TIMP-1 and TIMP-2 in CMT-3- and DC-treated cultures

Since the MMP-dependent invasion process may also depend on the levels of their endogenous inhibitors, TIMP-1 and TIMP-2, we measured TIMP-1 and TIMP-2 levels in CMT-3- and DC-treated cells (Table V). We observed a decrease in the levels of TIMPs secreted by CaP 139 cells treated with CMT-3 or DC. The decreases in TIMP-1 levels were 33 and 38% at 10 and 20 μg/ml of CMT-3 or DC, respectively. These decreases are significantly less than those determined for MMP-2 ($p < 0.05$, *t*-test). Similarly, levels of TMP-2 were also decreased by 10.27 and 21.75% respectively, in CMT-3 (10 μg/ml) and DC (20 μg/ml) treated cultures. As noted above for TIMP-1, decreases in TIMP-2 levels were also significantly less than the decrease in MMP-2 levels under comparable treatment conditions ($p < 0.04$, t-test).

Effect of DC and CMT-3 on Dunning tumor growth and lung metastasis

Due to the multiple effects of CMT-3 and DC on prostate cancer cells, we next examined whether CMT-3 and DC show antitumor activity *in vivo*. Although CMT-2 was comparable in efficacy, it was not tested *in vivo* because of its poor bioavailability (Golub *et al.*, unpublished observation). In the first experiment, we started treating the animals with drugs on the same day as we implanted 1×10^6 tumor cells. Tumors were palpable (≥ 0.1 cm^3) after 6 days in more than 50% of the animals in all treatment groups. Tumors increased in volume rapidly, reaching >10 cm^3 at 15 days post implant. Regression analysis of tumor volumes showed no significant difference in the primary tumor growth between the control group and the DC- or CMT-3-treated groups (Fig. 8*a*).

The mean duration for the growth of a 1 cm^3 tumor was 10.3 ± 2.12 days in the control group and 12.0 ± 1.9 days in the CMT-3-treated group. Tumors in the control group, as well as from the drug-treated groups, developed a highly necrotic center as the tumors grew to a size of ≥10 cm^3.

Tumor foci were visible in lungs fixed in Bouin's fluid. Typically, they were ≤1 mm in diameter from all the treatment groups. As illustrated in Figure 8*b*, lungs in the control group had 59.5 ± 13.9 metastatic tumor foci (MTF)/rat (mean ± SD). The animals treated with DC had 39.7 ± 17.2 and 43.6 ± 18.8 MTF/rat in the low-dose (20 mg/kg) and high-dose (40 mg/kg) treatment groups, respectively. The most significant reduction in lung MTF (28.9 ± 15.4 MTF/rat) occurred in those rats treated with a high dose of CMT-3 (40 mg/kg): 51% reduction in MTF as compared with that of the control group ($p < 0.01$, Tukey-Kramer multiple comparison test). Histologic examination of the tumor foci did not reveal any apparent differences among the various treatment groups (data not shown).

In an attempt to increase the efficacy of therapy, a predosing drug regimen was next employed and combined with a reduction in the number of tumor cells implanted. Since recent studies indicate rapid extravasation of tumor cells from the capillaries,[5] we reasoned that a sustained serum level of the drugs should reduce the incidence of growth and metastasis of the injected tumor cells. In addition, we thought that reducing the tumor cell inoculum should increase the tumor latency. Therefore, 2 modifications to the first experiment were introduced: (*i*) rats were predosed with DC or CMT-3, both at 40 mg/kg, with daily gavage for 7 days; and (*ii*) the tumor cell inoculum was reduced to 2×10^5 cells/animal from the previous dose of 1×10^6 cells/site. Due to the decrease in the cell inocula, the tumor latency increased from 6 to 9 days in the control group. However, there was no change in the tumor growth rate in the control group, once the tumors became palpable (see below).

As shown in Figure 9*a*, tumor incidence was >90% in control and DC-treated groups in 3 independent experiments. Interestingly, the tumor incidence in CMT-3-treated rats varied from 28% (2/7) to 85% (6/7) in 4 separate experiments. This was significantly lower (55 ± 9%) than that for control or DC-treated groups. The rats with no primary tumor incidence remained tumor-free for up to 6 months, at which time they were euthanized. No histologically identifiable tumor focus was observed at the site of injection or in the lungs. In addition, among the rats in the CMT-3-treated group that developed measurable tumors (≤50%), tumor growth in the CMT-3-treated group was significantly slower, 20.2 ± 3.5 days (CMT-3-treated) *versus* 15.9 ± 2.0 days (control) *versus* 16.7 ±

TABLE III – CELL CYCLE PHASE COMPOSITION OF CMT-3- AND DC-TREATED CELLS[1]

Phase	Untreated (control)	CMT-3		DC		
		5 μg/ml	10 μg/ml	5 μg/ml	10 μg/ml	20 μg/ml
LNCaP cells						
G0–G1	51.8 ± 4.8	74.5 ± 4.1	78.0 ± 4.2	75.0 ± 4.1	75.2 ± 3.8	70.0 ± 5.6
S	32.7 ± 4.8	6.6 ± 4.1	5.1 ± 4.1	10.4 ± 4.2	5.0 ± 3.8	5.5 ± 5.6
G2–M	15.5 ± 4.8	18.9 ± 4.8	17.0 ± 4.1	14.8 ± 4.1	20.0 ± 5.0	24.6 ± 5.6
DU 145 cells						
G0–G1	47.5 ± 5.2	78.6 ± 5.4	85.3 ± 5.5	59.4 ± 5.2	79.2 ± 5.4	79.1 ± 5.4
S	34.9 ± 5.2	15.9 ± 5.6	5.1 ± 4.1	26.7 ± 5.7	5.8 ± 5.4	5.5 ± 5.6
G2–M	17.6 ± 1.8	5.6 ± 5.6	7.0 ± 5.5	13.9 ± 5.2	15.0 ± 5.4	12.8 ± 5.6

[1]Cell cycle phase composition of prostate cancer cells treated with either CMT-3 or DC was determined by flow cytometric analysis of propidium iodide (PI)-stained nuclei as described in Material and Methods. Data for each phase were extracted from analyzing histograms obtained from flow analysis of long-pass red filter PMT signals (PI staining) using ModFit LT software (Verity Software House). Percent of cells in a given phase shown is from 3 independent experiments for both cell lines.

1.9 days (DC, 40 mg/kg) to reach a tumor volume of 3 cm^3. Interestingly, in 2 separate experiments, we observed tumor regression in 20 and 30% of CMT-3-treated groups. In these animals tumors were palpable (volume $\leq$ 0.01 cm^3) at 8–10 days after tumor cell injection at the primary site but did not increase in volume and disappeared (impalpable) 4–7 days later. These animals remained tumor-free for up to 6 months. Although there was a significant reduction in tumor incidence and growth rate, predosing the animals with CMT-3 did not further enhance the inhibition of tumor metastasis to lungs. The MTF in CMT-3-treated animals was 46.3 ± 6.7 *versus* 74.2 ± 6.4 in control, a 37.6% reduction ($p \leq 0.01$).

Toxicity and adverse reactions to DC and CMT-3

None of the 142 animals used in our study demonstrated adverse effects of drug treatments, such as irritability, hypersensitivity to light, hair loss or diarrhea. As a gross measure of normal tissue injury, plausibly caused by DC or CMT-3, animals were weighed before, during and after treatment. In all experiments, we found no significant weight loss in any groups. The animals gained 1.5–3.2% of their weight during the 4-week treatment (with tumor measurement) and gained an additional 8–12% of their weight during the 6 months of posttreatment observation. These weight gains were similar to the weight gain in naive animals of matching age.

DISCUSSION

The results presented above show that DC and various CMTs inhibit multiple aggressive activities of tumor cells including cell proliferation, invasion, secretion of MMPs, primary tumor growth and metastasis. Possible mechanisms of these actions are discussed.

Tetracycline and cytotoxicity

In bacteria and protozoa the principal toxic action of tetracycline is disruption of 30S ribosomal protein synthesis,[47] a component that is absent in higher eukaryotes. We propose that tetracycline and CMTs may induce inhibition of cell proliferation of mammalian cells by at least 2 mechanisms, occurring in both cytoplasmic and mitochondrial compartments. Kroon and colleagues[16,20] hypothesized that tetracycline acts as a cytostatic drug since it inhibits protein synthesis in mitochondria and decreases the activity of cytochrome C oxidase. Results presented here show that tetracycline and its nonantibiotic derivatives (*e.g.*, CMT-3) are cytotoxic. Reduction of tumor cell viability (reduction in MTT reduction) and increase in apoptotic activity both support this conclusion. Decrease in conversion of MTT into insoluble formazan may be the result of DC and CMT-3 inhibiting mitochondrial function and reducing the cytoplasmic pyridine nucleotide pool, NADH and NADPH.[48,49] Furthermore, we have reported previously that CMTs and DC inhibit clonal survival of prostate tumor cells.[50] Cytotoxic activity shown by analogs of tetracycline, with or with-

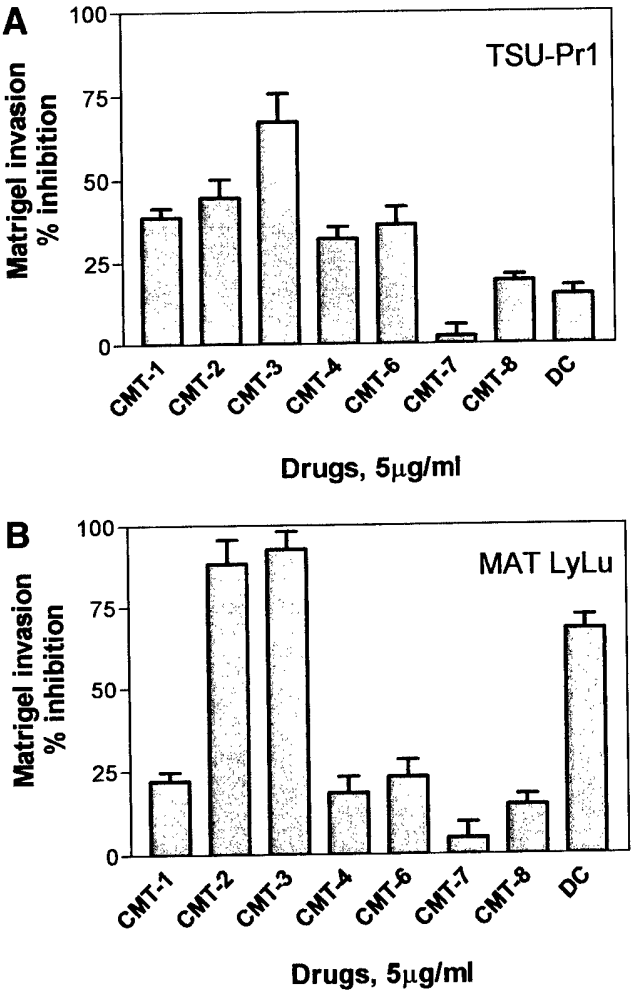

FIGURE 6 – Inhibition of invasive potential of tumor cells by DC and CMTs. Cells in the top chamber and those that had invaded the Matrigel-coated filter after 48 hr were quantitated (invasion index) using the MTT assay. The invasion index was defined as the ratio between the OD of the reduced MTT (formazan) in the bottom wells to the total OD (bottom plus the top wells). The invasion index of the control (0.1% DMSO) wells was 22 ± 8.3 % for TSU PR1 cells (*a*), and 17 ± 4.2 for MAT LyLu cells (*b*). DMSO (0.1%) had a negligible effect on the invasion index. Results presented are from 3 independent experiments.

TABLE IV – INHIBITION OF MMP ACTIVITY BY CMT-3 AND DC

Drug concentration (μM)	% Inhibition (mean ± SEM)[1]			
	CMT-3		Doxycycline	
	1 mM $CaCl_2$[2]	10 mM $CaCl_2$[3]	1 mM $CaCl_2$[2]	10 mM $CaCl_2$[3]
0.25	47.2 ± 3.6	20.7 ± 4.9	35.3 ± 2.7	0.0
0.50	64.4 ± 8.3	31.7 ± 6.5	44.8 ± 7.6	0.0
1.0	84.8 ± 10.1	37.8.1 ± 7.5	54.8 ± 0.6	0.0
2.0	96.7 ± 4.6	45.1 ± 2.9	77.4 ± 2.9	32.7 ± 11.4
10	97.2 ± 2.9	52.4 ± 3.5	87.5 ± 2.8	64.3 ± 19.5
20	100	69.9 ± 10.5	91.15 ± 3.3	75.5 ± 3.3
100.0	100	94.2 ± 3.3	100	93.2 ± 2.7

[1]Inhibition of gelatinase activity by CMT-3 or DC was calculated from the activity (expressed as ng of [^{3}H]gelatin solubilized/min/ml) of MAT LyLu conditioned medium. Culture-conditioned medium was incubated with [^{3}H]gelatin in the presence of various concentrations (0–100 μM) of either CMT-3 or DC and 1 or 10 mM $CaCl_2$, at 37°C for 4 hr. The assay was conducted as described in Material and Methods. Data presented are from 3 independent experiments. Inhibition of gelatinase activity was significantly different between 1 and 10 mM Ca^{2+} concentrations for both CMT-3 and DC at 0.25–20 μM concentrations, tested independently for both drugs ($p < 0.05$ for all groups, t-test).–[2]Total gelatinase activity in the presence of 1 mM $CaCl_2$, without inhibitors (control), was 18.95 ± 3.18 ng [^{3}H]gelatin digested/min/ml of the dialyzed culture-conditioned medium.–[3]Total gelatinase activity in the presence of 10 mM $CaCl_2$ without inhibitors (control) was 48.92 ± 2.7 ng/min/ml.

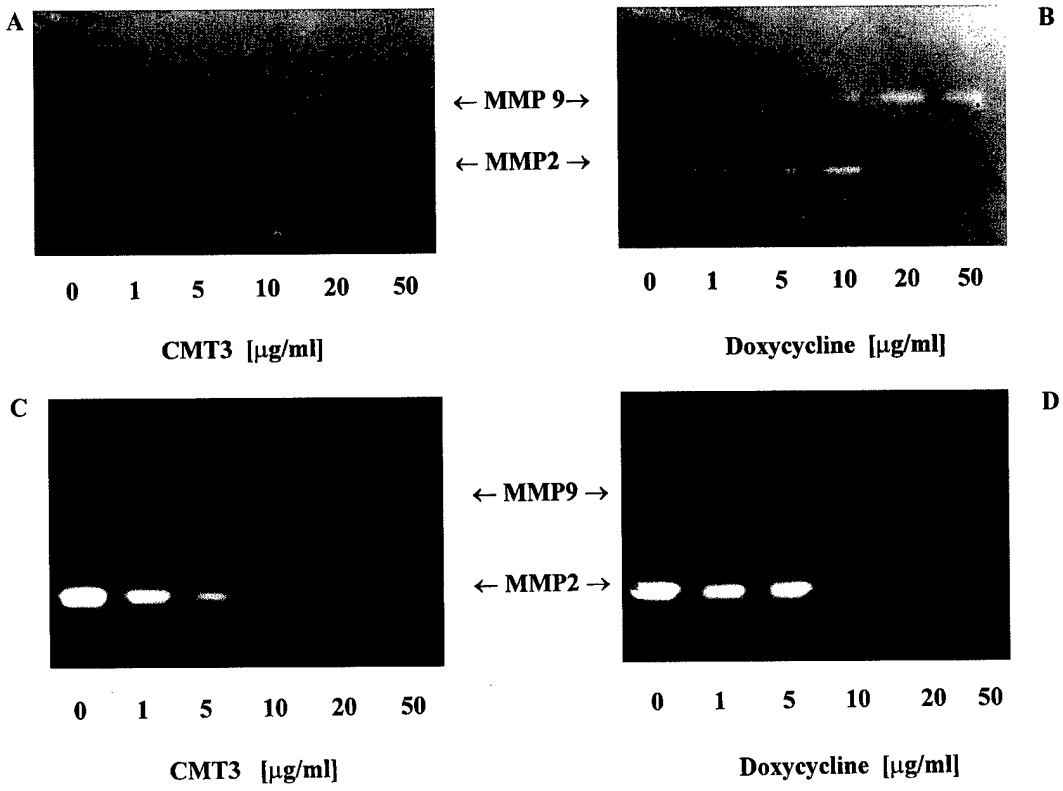

FIGURE 7 – Zymographic detection of gelatinases secreted into the conditioned media from cultures treated with CMT-3 or DC. Culture conditioned media (15 μl/lane, equivalent to 5×10^3 cells) from TSU PR1 (*a,b*) and MAT LyLu (*c,d*) cells were separated by SDS-PAGE (8% polyacrylamide) on a gelatin-embedded (1 mg/ml) gel and zymography. The positions of purified MMP-2 and MMP-9 are indicated. Note: the major fraction of MMP-2 from MAT LyLu (bottom) cell conditioned media was active (Mr ~ 64.000), whereas most of the TSU PR1 (top) MMP-2 was in the latent form (Mr 72,000).

out antimicrobial activity, shows that tumor cell cytotoxicity is independent of its antibiotic action.

We report here that CMT-3 is a specific and potent inducer of apoptotic cell death. Our selection of the assay system to measure apoptosis, the Cell Death ELISA-Plus kit, facilitated distinction between CMT-3- and DC-induced programmed cell death and necrosis. CMT-3 not only induced apoptosis but also caused necrotic cell death; the latter was increased at higher concentrations of the drug (≥10 μg/ml). Although necrotic cell death is often termed "nonspecific cell death," tetracycline-induced cell lysis may be attributable to the ability to complex and sequester divalent cations such as Ca^{2+}, Fe^{2+} and Zn^{2+}, thus leading to cell lysis.[51–53] The minimum incubation time required for CMT-3 to induce apoptosis was 4 hr (Table II), similar to the time interval for detectable depolarization of mitochondria (a decrease in $\Delta\Psi$, Fig. 4). This time range was also similar to the maximum increase observed in cellular [OH^-] (Fig. 5). The cellular [OH^-] levels in cells exposed to CMT-3 for 4 hr or more were comparable to those

TABLE V – INHIBITION OF MMP-2 AND TIMP-1 PRODUCTION BY CAP 139 CELLS BY CMT-3 AND DC[1]

Drug (μg/ml)	MMP-2	TIMP-1[2]	TIMP-2[3]
No drug, control	65.9 ± 2.2 (0)[4]	230 ± 11.2 (0)	18.2 ± 1.15
CMT-3			
1.0	43.1 ± 1.6 (35)	187.3 ± 5.5 (18.5)	17.64 ± 0.5 (3.13)
5.0	28.6 ± 1.1 (56.7)	170.0 ± 7.5 (26.3)*	16.53 ± 0.84 (9.22)
10.0	17.3 ± 8.3 (74.0)	144.3 ± 2.4 (37.9)*	16.59 ± 0.61 (10.27)
DC			
1.0	58.9 ± 1.64 (10.6)	193.3 ± 17.3 (16.0)	17.29 ± 0.38 (5.05)
5.0	63.8 ± 1.1 (3.0)	188.1 ± 2.8 (18.2)	16.1 ± 0.46 (11.87)
10.0	52.7 ± 0.8 (20.0)	169.0 ± 5.68 (26.5)	15.86 ± 1.64 (12.9)
20.0	32.3 ± 1.3 (50.9)	156.1 ± 3.8 (32.7)	14.25 ± 0.79 (21.75)

[1]MMP-2 and TIMPs in the drug-treated CaP 139 culture-conditioned medium were measured using ELISA kits (Oncogene Sciences). Amount of MMP-2 or TIMP proteins in the assay medium was calculated from a standard graph using assay standards provided in the kits. MMP-9 levels measured using a similar but separate assay kit was below the detection limit of the kit (<10 ng/ml conditioned medium). Data are ng/mg (mean ± SD), with percent reduction in parentheses.–[2]In data marked with an asterisk, the levels were significantly different from untreated cultures (control) ($p < 0.05$, *t*-test).–[3]TIMP-2 levels from drug-treated cultures did not significantly differ form control levels ($p > 0.05$, *t*-test).–[4]Data shown are from a single experiment with replicate (4) samples.

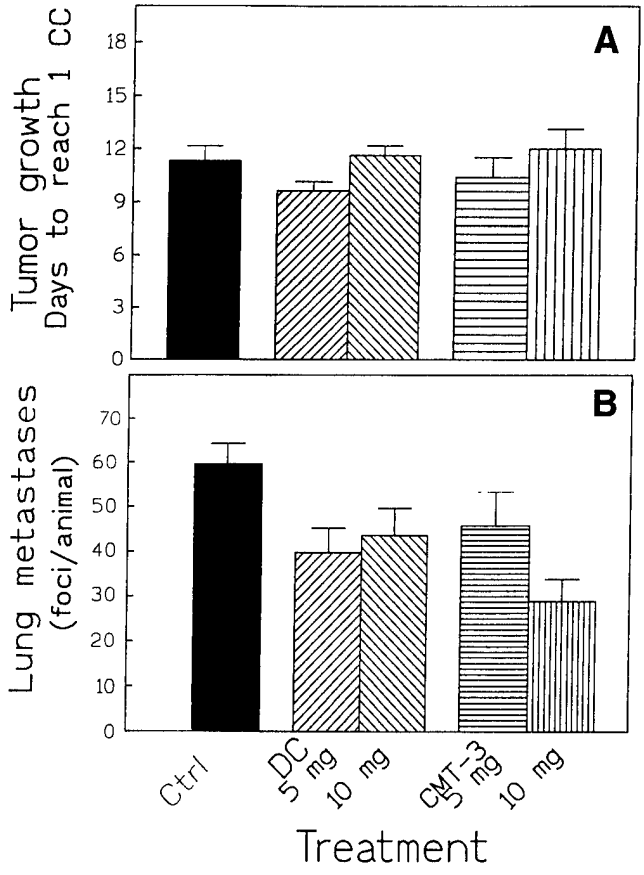

FIGURE 8 – Effect of DC and CMT-3 on growth and metastasis of Dunning tumor in rats. Tumor growth was initiated by injection of 1×10^6 tumor cells s.c. Drug dosing began the same day. (*a*) Growth profile of all tumors. (*b*) Number of metastatic tumor foci in lungs (mean ± SD). The doses of drugs, 5 and 10 mg/day, are actual amounts used in the gavage for a rat weighing ~250 g; this amounts to 20 and 40 mg/kg/day, respectively.

found in unexposed cells, indicating possible interaction of [OH^-] with their cellular targets and destruction of polarity of mitochondrial membrane. As shown in many types of cells including cardiac myocytes, release of reactive oxygen species is accompanied by mitochondrial depolarization.[54] Mitochondrial depolarization is frequently observed in cells undergoing apoptosis.[52] Therefore, it is compelling to conclude that CMT-3-induced apoptosis in CaP cells is associated with depolarization of mitochondria. Cytotoxic activity of CMTs by necrotic mechanisms may be irrelevant to their clinical application: in preclinical trials the peak plasma levels rarely reached ≤5 μg/ml without causing severe toxicity.[55] Therefore, induction of apoptosis is likely to be of major significance to potential application of CMTs in the clinic.

In addition to the induction of apoptosis, cell cycle progression was blocked in cultures exposed to CMT-3 and DC. A significant increase in accumulation at the G_0/G_1 phase and a decrease in S-phase fractions was observed in both androgen-sensitive LNCaP and androgen-insensitive DU145 cells. This G_1/S transition block is indicative of the inhibition of biochemical processes such as inhibition of cell cycle phase transition-related cyclin-dependent kinases.[56]

CMTs and antiinvasive activity

We have presented results showing evidence of the antiinvasive activity of DC and CMTs. DC and several CMTs inhibited invasive activity in both human and rat CaP cells. Since the invasion process is complex and involves both tumor cell motility and the ability to degrade basement membrane, our evidence suggests that the antiinvasive activity of CMT and DC is predominantly due to their anti-MMP activity. CMT-3 and DC not only inhibited the activity of secreted MMPs (MMP-2 and MMP-9) but also significantly decreased the production and/or secretion of these enzymes (Table V, Fig. 6). Interestingly, both TIMP-1 and TIMP-2 levels were much less inhibited by DC or CMT-3 than that for MMPs (Table V). This suggests that CMT-3 reduces invasive activity of tumor cells not only by binding to active MMPs and inhibiting their synthesis/secretion, but also by not affecting TIMP-1 and TIMP-2 levels significantly. Stearns *et al.*[57] have reported that the cytokine interleukin-10 (IL-10) decreases MMP levels in CaP cells, while simultaneously increasing TIMP-1 expression. Preliminary studies in our laboratory indicate that CMT-3 actually reduces the expression of IL-10 and other immunomodulatory cytokines such as IL-1β in some CaP cells such as the PC-3 cell line (unpublished observations). It is thus possible that the decrease in MMP-2 levels in CMT-3-treated cells is primarily due to the effect of CMT-3 and is not mediated by cytokine-mediated pathways.

We observed that CMT-3 and DC significantly reduced but did not abolish invasive activity (Fig. 7). This partial inhibition could be attributable to other proteinases secreted by tumor cells that are not inactivated by these drugs. An example is urokinase-like plasminogen activator (uPA), a serine proteinase that also facilitates invasion and metastasis.[40] It has been reported that DC or CMTs do not inhibit uPA secretion or activity.[58] However, they

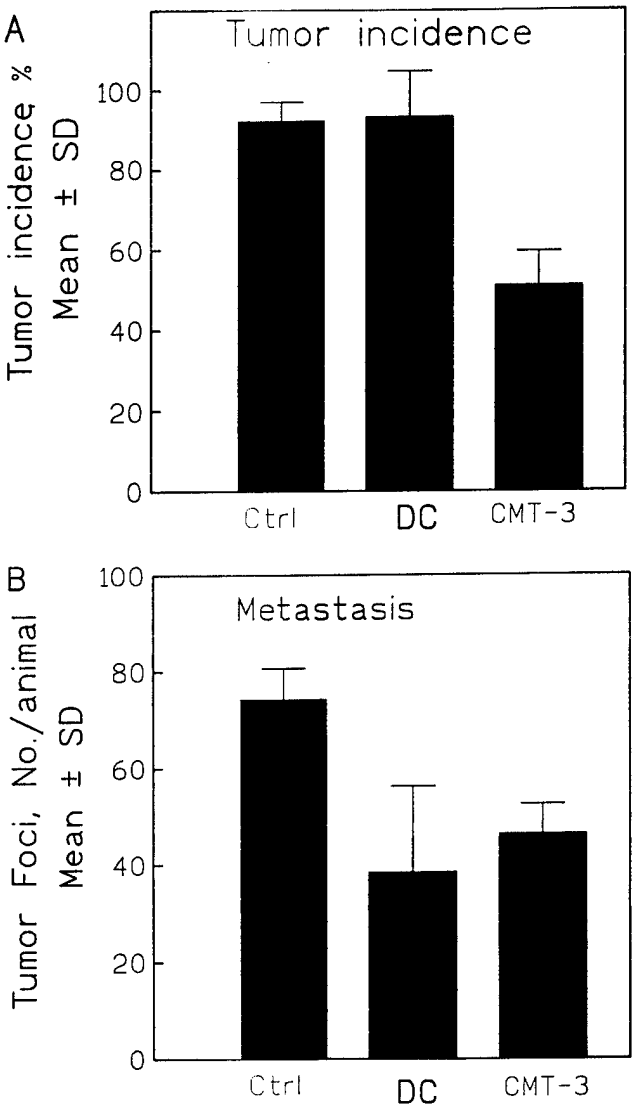

FIGURE 9 – Effect on tumor incidence and metastasis of predosing the rats with DC and CMT-3. (*a*) Tumor incidence. The experiments were conducted as described in Material and Methods. Animals without palpable tumors at the site of injection were considered tumor-free. The percentage of animals belonging to the CMT-3 treatment group in which tumors were resorbed varied between 20 and 30% in 3 separate experiments (2/10, 2/7 and 3/10, respectively). No tumor resorption was observed in other experimental groups. Data are mean ± SD. (*b*) Tumor foci in lungs. Data are mean ± SD from all animals in which the primary tumors grew to a size of ≥ 10 cm^3. Tumor foci were counted manually under a 2.5× magnifying lens. Specimens were selected randomly to avoid bias.

may inhibit other serine proteinases that activate MMPs, such as trypsinogen-2.[59] Recently, Meng *et al.*[60] showed that CMT-3 and CMT-8 also inhibit proliferation and invasive potential of MDAMB-468 human breast tumor cells. In addition, CMT-3 increased the expression of E-cadherin, catenins and BRACA1, a tumor suppressor. That report, along with ours, suggests a potential application of CMTs in early treatment for metastatic disease.

CMT and antitumor activity

A combined antitumor and antimetastatic efficacy of CMT-3, an orally administered tetracycline analog, is a significant finding of our study. Although administration of DC or CMT after tumor implant caused insignificant inhibition of primary tumor growth, inhibition of tumor metastasis was significant (Figs. 8,9). However, predosing rats with the drug caused a delay in primary tumor growth. It is unclear at present why primary tumor growth was unaffected if the dosing began after tumor implant. It is plausible that a significant fraction of tumor cells survive *in vivo* if drug dosing is initiated after tumor cells are implanted. However, predosing may result in significantly higher plasma levels of CMT-3 (>10 μg/ml), would be sufficient to kill most of the tumor cells. The selection of 7 days as the predosing period was arbitrary. Rodman *et al.*[55] (using the alternate term COL-3 for CMT-3) have shown that the absorption half-life is 5.4 hr and the elimination half-life is 7.1 hr after a single oral dose in rats. Furthermore, they reported that a significant amount remained in the circulation after 48 hr. In their studies, the plasma levels of CMT-3, after a single daily gavage of CMT-3 (30 mg/kg), exceeded the concentration required to inhibit the gelatinases by more than 2-fold. Taking our results together with these findings, it is possible to conclude that the enhanced efficacy of CMT-3 against tumor growth and metastasis over DC or other tetracyclines could be due to sustained high levels of CMT-3 in plasma.

Predosing the animals for 7 days with CMT-3 before implanting tumor cells also caused significant tumor remission and a remarkable reduction in tumor incidence. Tumor cell cytotoxicity combined with disruption of MMP-dependent early extravasations and angiogenesis[61,62] might have contributed to the reduced tumor incidence and tumor remission. It is possible that an immune-mediated response may a play part in abolishing tumor incidence in predosed animals. Although at present MAT LyLu cells are poorly immunogenic in syngenic Copenhagen (COP) rats, both bilateral tumors and regrowth of ablated tumors have been observed in this system, indicating a lack of tumor immunity in this model.[63] The absence of these effects by DC is not surprising. Although DC was able to inhibit invasive activity of MAT LyLu cells *in vitro* (present report) and is capable of inhibiting angiogenic activity,[64] its low tumor cell cytotoxicity and low peak plasma levels ($\leq$2.5 μg/ml[22]) may have limited its efficacy in this model. An enhanced efficacy of CMT-3 upon predosing shows that CMT-3 treatment can be effective if dosing is started even before clinical signs are apparent. This dosing schedule may be efficacious in situations such as immediately after surgical removal of malignant prostate or as adjuvant to radiation in the palliative treatment of metastatic disease.

The overall effectiveness of CMT-3 (demonstrated by a reduction in tumor incidence, tumor remission and decreased tumor metastasis in the Dunning MAT LyLu model) indicates that it also has a potential application in advanced hormone-refractory prostate cancer in men. Oral bioavailability, with minimal adverse reactions within a tolerable dose, suggests that it could be used as an adjuvant to hormone ablation or radiation therapy in prostate cancer. Similar studies in other models of cancer, notably melanoma and sarcoma, have also shown efficacy of CMTs as antitumor and antimetastatic drugs.[65,66] Fife *et al.*[67] have reported that DC induces apoptosis and antiinvasive activities in PC-3 and DU 145 cells *in vitro*, although the concentrations ($\geq$10 μg/ml) at which these activities were elicited were higher than those that could be achieved safely by oral administration.

While our work was in progress, a multicenter phase I clinical trial of CMT-3 (trade name COL-3, Metastat; CollaGenex Pharmaceuticals) was conducted by the Investigational Drug Branch, Cancer Therapy Evaluation Program (National Cancer Institute, Bethesda, MD). The study evaluated maximum tolerated dose (MTD) and dose-limiting toxicities in patients with refractory solid tumors. The study, initially involving 35 patients, demonstrated disease stabilization for up to 26 months in patients with nonepithelial types of malignancy. In addition, there was a significant reduction in plasma MMP-2 levels in the group that showed disease stabilization. The major dose-limiting toxicity was photosensitivity. Drug-induced photosensitivity to both UV-A and UV-B radiation was reported in 40–70% of patients receiving

COL-3 at a dose ≥70 mg/m^2/day. Based on the results of this study, the investigators have recommended a daily dose of 36 mg/m^2 for a phase II clinical trial.[68]

Several other inhibitors of MMPs also reduce experimental tumor growth and metastasis in animal tumor models.[14,69,70] For example, 2 synthetic inhibitors of MMPs, Batimastat and Marimastat (British Biotechnology and Pharmaceutical, Cowley, Oxford, UK) have shown significant antitumor activity in several experimental tumor models including ovarian,[14] colon,[71] breast[70] and melanoma models.[71] Recent experience, gained from clinical trials of various MMP inhibitors, however, shows a lack of significant efficacy in most of the human cancers and, furthermore, some are contraindicated.[72–75] A few, however, have been shown to be capable of stabilizing the disease.[75]

Some have argued that this was an anticipated result in the light of the stage and timing of therapeutic intervention. For example, Zucker *et al.*[76] have postulated that the therapeutic benefit of MMP inhibitors is seen in early-stage disease, in some tumors as soon as malignancy is detected. Furthermore, they have expressed concern that anti-MMP therapy at an advanced stage in any human cancer is likely to be ineffective, akin to "closing the barn door after the horses have escaped." Although this may be a valid concern for conventional anti-MMP agents in cancer therapy, CMTs may be potentially more effective if treatment begins at first diagnosis of cancer, even in situations in which clinical manifestations of the disease are not apparent. CMT-3 or its close derivatives have a potential advantage over other anti-MMP agents in that CMTs show antiproliferative activity against tumor cells. The antimetastatic and antiproliferative activity, as well as the oral bioavailability, makes this class of drug potentially more powerful than synthetic MMP inhibitors.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. A. Krishan for many suggestions and advice, Dr. N. Altman, Department of Pathology University of Miami, for help with histology and Mr. R. Ashley and Dr. B. Zerler of CollaGenex Pharmaceuticals (Newtown, PA) for a generous supply of CMTs. B.L.L. is grateful to Dr. V.B. Lokeshwar for encouragement, patience and understanding. BLL is grateful to NY Academy of Sciences for permission to reproduce Fig 2.

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