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β-Cyclodextrin tetradecasulfate/tetrahydrocortisol ± minocycline as modulators of cancer therapies in vitro and in vivo against primary and metastatic lewis lung carcinoma

Beverly A. Teicher, Enrique Alvarez Sotomayor, Zhen Dong Huang, Gulshan Ara, Sylvia Holden, Vrinda Khandekar, Ying-Nan Chen

Dana-Farber Cancer Institute and Joint Center for Radiation Therapy, 44 Binney Street, Boston, MA 02115, USA

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Abstract: Tetrahydrocortisol, β-cyclodextrin tetradecasulfate, and minocycline used alone or in combination are not very cytotoxic toward EMT-6 mouse mammary tumor cells growing in monolayer. Tetrahydrocortisol (100 μ*M*, 24 h) and β-cyclodextrin tetradecasulfate (100 μ*M*, 24 h) protected EMT-6 cells from the cytotoxicity of CDDP, melphalan, 4-hydroperoxycyclophosphamide, BCNU, and X-rays under various conditions of oxygenation and pH. Minocycline (100 μ*M*, 24 h) either had no effect upon or was additive with the antitumor alkylating agents or X-rays in cytotoxic activity toward the EMT-6 cells in culture. The combination of the three modulators either had no effect upon or was to a small degree protective against the cytotoxicity of the antitumor alkylating agents or X-rays. The Lewis lung carcinoma was chosen for primary tumor growth-delay studies and tumor lung-metastases studies. Tetrahydrocortisol and β-cyclodextrin tetradecasulfate were given in a 1:1 molar ratio by continuous infusion over 14 days, and minocycline was given i.p. over 14 days, from day 4 to day 18 post tumor implantation. The combination of tetrahydrocortisol/β-cyclodextrin tetradecasulfate diminished the tumor growth delay induced by CDDP and melphalan and produced modest increases in the tumor growth delay produced by cyclophosphamide and radiation. Minocycline co-treatment increased the tumor growth delay produced by CDDP, melphalan, radiation, bleomycin, and, especially cyclophosphamide, where 4 of 12 animals receiving minocycline (14 × 5 mg/kg, days 4–18) and cyclophosphamide (3 × 150 mg/kg, days 7, 9, 11) were long-term survivors. The 3 modulators given in combination produced further increases in tumor growth delay with all of the cytotoxic therapies, and 5 of 12 of the animals treated with the 3-modulator combination and cyclophosphamide were long-term survivors. Although neither tetrahydrocortisol/β-cyclodextrin tetradecasulfate, minocycline, nor the three modulator combination impacted the number of lung metastases, there was a decrease in the number of large lung metastases. Treatment with the cytotoxic therapies alone reduced the number of lung metastases. Addition of the modulators to treatment with the cytotoxic therapies resulted in a further reduction in the number of lung metastases. These results indicate that agents that inhibit the breakdown of the extracellular matrix can be useful additions to the treatment of solid tumors.

Introduction

To enlarge and invade locally or distally, a tumor must continue to cause the breakdown and restructuring of the extracellular matrix, including both basement membrane and interstitial matrices [11, 67–70]. It has been recognized for many years that the formation of a blood supply (angiogenesis) is critical for the growth of both normal and malignant tissues [10, 16, 53, 73]. For over 20 years cancer researchers have been searching for agents that could inhibit the processes of malignant cell invasion and growth in normal tissues. One focus of that search has used the inhibition of the formation of blood vessels, usually in the normal tissue model system of the CAM, to discover potential therapeutic agents that have been termed "angiostatic" or "antiangiogenic" [10, 11, 13, 16]. Evidence has been accumulating that indicates that the actions of some of the agents discovered through the angiogenesis assay occur at the level of extracellular matrix enzymes [12, 29, 31, 68, 69].

The angiostatic activity of several steroids was discovered some years ago; however, the mechanism by which these steroids inhibit vessel growth and/or produce

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Abbreviations: 14(SO_4)βCD, β-cyclodextrin tetradecasulfate; THC, tetrahydrocortisol; CDDP, *cis*-diamminedichloroplatinum(II); 4-HC, 4-hydroperoxycyclophosphamide; BCNU, *N,N-bis*(2-chloroethyl)-*N*-nitrosourea; CAM, chick embryo chorioallantoic membrane; IC_{50}, concentration of a drug required to kill 50% of the cells

Correspondence to: Dr. Beverly A. Teicher, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA

regression of growing vessels is only now being elucidated [12, 13, 29, 31]. Angiostatic steroids appear to induce basement membrane dissolution as part of their antiangiogenic action [12, 29, 31]. In fact, in the CAM assay, angiostatic steroids had a direct effect on extracellular matrix turnover, resulting in a decrease in collagen accumulation when applied in combination with heparin. The steroids had a modest effect on collagen accumulation and heparin had no effect on collagen accumulation when used alone [31]. Therefore, a direct effect on extracellular matrix metabolism resulted in inhibition of new capillary growth. Of the naturally occurring angiostatic steroids, tetrahydrocortisol was identified as the most potent [12].

In 1983, Folkman et al. [14] reported that heparin or a heparin fragment used in combination with cortisone prevented angiogenesis in the CAM assay and inhibited the growth of several solid murine tumors. Inhibition of angiogenesis or tumor response has been reported in other model systems after treatment with cortisone or cortisone acetate; however, in most studies, heparin did not increase the effect obtained with the steroid [37, 45]. More recently, Folkman et al. [17] reported that β-cyclodextrin tetradecasulfate in combination with hydrocortisone was 100–1000 times more effective than heparin in combination with hydrocortisone in inhibiting capillary formation in the CAM assay and in preventing neovascularization induced by endotoxin in the rabbit cornea [17].

Several enzymes are involved in the degradation of the extracellular matrix during malignant tumor growth and invasion. Serine proteases and metalloproteases are the most prominent of the enzymes [1, 40, 67–69]. Type IV collagen is the main component of the tight structure of the basement membrane [67–69]. At least two specific type IV collagenases have been isolated and characterized [6, 47, 55, 66]. The activity of type IV collagenase has been associated with the metastatic phenotype. Relatively high concentrations of cortisol have been shown to inhibit type IV collagenase activity in human skin fibroblast cultures [55]. The interstitial collagen types (I, II, and III) are remarkably resistant to the attack of proteinases, but they can be degraded by highly specific metalloproteinases called interstitial collagenases that have been isolated from a variety of mammalian cells and tissues, including rabbit synovial fibroblasts, rheumatoid synovium, rabbit VX2-carcinoma, human skin, and pig synovium [4, 26, 27, 68]. It has been recognized for some time that the tetracyclines can inhibit tissue collagenase activity, and tetracycline administration has been used in the treatment of peridontal disease [18] and of gingival collagenolytic activity in diabetes [18, 19] and to inhibit joint deterioration in patients with rheumatoid arthritis [22, 74]. This inhibitory activity has been associated with both type IV collagenase and interstitial collagenase [21]. Recently, Tamargo et al. [61] reported that minocycline, a semisynthetic tetracycline with a relatively long circulating half-life, inhibited neovascularization in the rabbit cornea implanted with the VX2 carcinoma.

In the current study, the effect of the exposure of EMT-6 mouse mammary carcinoma cells to tetrahydrocortisol, β-cyclodextrin tetradecasulfate, or minocycline alone or in combination with antitumor alkylating agents or X-rays was examined. In vivo, the effect of tetrahydrocortisol/β-cyclodextrin tetradecasulfate delivered via osmotic pump, minocycline, and tetrahydrocortisol/β-cyclodextrin tetradecasulfate/minocycline on the primary tumor growth delay and lung metastasis formation in the Lewis lung carcinoma following their administration alone and in combination with cytotoxic therapies was explored.

Materials and methods

Drugs. Tetrahydrocortisol, minocycline, melphalan, cyclophosphamide, and Adriamycin were purchased from Sigma Chemical Co. (St. Louis, Mo.). *cis*-Diamminedichloroplatinum(II) (CDDP) was a gift from Dr. Alfred Crosswell, Bristol-Myers-Squibb Co. (Wallingford, Conn.). 4-Hydroperoxycyclophosphamide (4-HC) was a gift from Drs. P. Hilgard and J. Pohl, Asta Pharma (Bielefeld, Germany).

β-Cyclodextrin tetradecasulfate was prepared in our laboratory by an improved method as compared with those previously published [2, 17, 39]. Briefly, a solution of sulfur trioxide pyridine complex (6.82 g, 0.042 mol.; Aldrich Chemical Co., Milwaukee, Wis.) in anhydrous pyridine (12 ml) was heated on an oil bath in a three-necked flask equipped with a condensor topped by a drying tube (calcium chloride) and a thermometer. Magnetic stirring was maintained at 80° – 85° C for 20 min to produce a pale yellow liquid.

To this solution, β-cyclodextrin (1.135 g, 0.001 mol.; Sigma Chemical Co., St. Louis, Mo.) was added rapidly with constant stirring. The reaction mixture was stirred at 80° – 85° C for 6 h and then allowed to stand at room temperature for 36–40 h to form a dark brown semisolid, which was treated with methanol (500 ml) and stirred for 1 h to precipitate out crude β-cyclodextrin tetradecasulfate-pyridine salt. The crude material was collected by filtration through a sintered glass funnel under suction. After being washed several times with methanol, it was air-dried and redissolved in a 30% sodium acetate solution (1.933 g of sodium acetate · $3H_2O$ in 6.5 ml of water) and water (7 ml) to convert the pyridium salt into the sodium salt. This solution was gradually added into 100 ml of absolute ethanol and stirred for 1 h to allow precipitation of the β-cyclodextrin tetradecasulfate sodium salt, which was collected by filtration, washed with alcohol, and then air-dried. The crude sodium salt was redissolved in a 30% sodium solution (4 ml) and water (7 ml) and reprecipitated from alcohol (120 ml) three times as described above. The purified material was dried under vacuum over phosphorus pentoxide at 78° C/100 torr in a drying pistol for 4 h to give a white amorphous powder (2.35 g, 83.6% yield). An elemental analysis for $C_{42}H_{56}O_{77}S_{14}Na_{14} \cdot 14H_2O$ (mol.wt., 2815.7761) revealed calculated values of C, 17.91; H, 3.00; and S, 15.94; the measured values were C, 18.12; H, 2.94; and S, 15.95.

All other drugs were purchased from the Dana-Farber Cancer Institute pharmacy.

Cell culture. EMT-6 mouse mammary tumor cells have been widely used for the study of hypoxia [49 – 51]. Cultures were maintained in exponential growth in Waymouth's medium (I.S.I. Corp., Chicago, Ill.) supplemented with 15% newborn calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml; Grand Island Biological Co., Grand Island, N.Y.). The doubling time of these cultures, growing at 37° C in a humidified atmosphere containing 5% CO_2/95% air, was 16–19 h [52]. The in vitro plating efficiencies of control cultures ranged from 65% to 80%.

The pH of the medium was adjusted using a sodium bicarbonate/5% CO_2 buffer system [28]. To produce hypoxia, the plastic flasks containing exponentially growing monolayers in complete medium plus serum were fitted with sterile rubber septa and exposed to a continuously flowing 95% N_2/5% CO_2 humidified atmosphere for 4 h at 37° C as previously reported [64, 65]. Parallel flasks were maintained in 95% air/5% CO_2. At the end of 4 h, the drug or vehicle was added to the flasks by injection through the rubber septum without disturbing the hypoxia. EMT-6 cells were exposed to various concentrations (5, 10, 50, 100, 250, or 500 μ*M*) of THC, 14(SO_4)βCD, or minocycline under normally oxygenated conditions for 24 h or under hypoxic conditions for 5 h followed

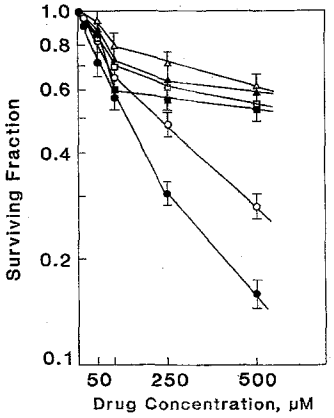

Fig. 1. Survival curve of exponentially growing normally oxygenated (▲,■,●) and hypoxic (△,□,○) EMT-6 cells exposed to various concentrations of tetrahydrocortisol (▲,△), β-cyclodextrin tetradecasulfate (■,□), or minocycline (●,○) for 24 h Hypoxia was maintained for the first 5 h of drug exposure. *Points*, Mean values for 3 independent determinations; *bars*, SEM

by normally oxygenated conditions for 19 h. In combination-treatment experiments, EMT-6 cells were exposed to 100 μ*M* of THC, 14(SO4)βCD, or minocycline alone or in combination for 4 h prior to, during 1 h exposure to melphalan, 4-hydroperoxycyclophosphamide, CDDP, or BCNU or during radiation delivery, and for an additional 19 h. In the combination studies, hypoxia was maintained for the first 5 h of drug exposure.

Cell viability was measured by the ability of single cells to form colonies in vitro as described previously [64, 65]. Each experiment was repeated three to five times, and each data point per experiment represents the results obtained for three different dilutions of cells plated in triplicate.

Tumors. The Lewis lung tumor [57–59] was carried in male C57BL mice (Taconic Laboratories, Germantown, N.Y.). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted s.c. into the legs of 8- to 10-week-old male mice.

Tumor growth-delay experiments. By day 4 post tumor cell implantation, Lewis lung tumors have begun neovascularization. Animals bearing Lewis lung tumors were implanted s.c. with 14-day mini-osmotic pumps (Alzet pumps, model 2002; Alza Corp., Palo Alto, Calif.) containing 14(SO4)βCD (1 g/kg) and THC (125 mg/kg) in a 1:1 molar ratio and/or were treated with minocycline (5 mg/kg) i.p. daily for days 4–18 post tumor implantation. When the Lewis lung tumors were approximately 100 mm^3 in volume, at day 7 post tumor cell implantation, cytotoxic therapy was initiated. CDDP (10 mg/kg), melphalan (10 mg/kg), and cyclophosphamide (150 mg/kg) were given i.p. on day 7. Cyclophosphamide (150 mg/kg) was given on days 7, 9, and 11 post tumor implantation. Radiation was delivered locally to the tumor-bearing limb as 20 Gy on day 7 or 3 Gy daily on days 7–11. Adriamycin (1.75 mg/kg) was injected i.p. daily on days 7–11, and bleomycin (10 mg/kg) was given i.p. on days 6, 10, 13, and 16.

The progress of each tumor was measured thrice weekly until it reached a volume of 500 mm^3. Tumor growth delay was calculated as the number of days taken by each individual tumor to reach a volume of 500 mm^3 as compared with the untreated controls. Each treatment group had six animals, and the experiment was repeated three times. The days of tumor growth delay are the mean values ± SE for the treatment group as compared with the control.

Lung metastases. The number of external lung metastases from animals treated as described above on day 20 post tumor implantation were counted manually and scored as ≥3 mm^3 in diameter. The data are shown as the means values obtained for 6–12 pairs of lungs. Parentheses indicate the number of large (vascularized) metastases and the percentage of the total number of metastases that were large.

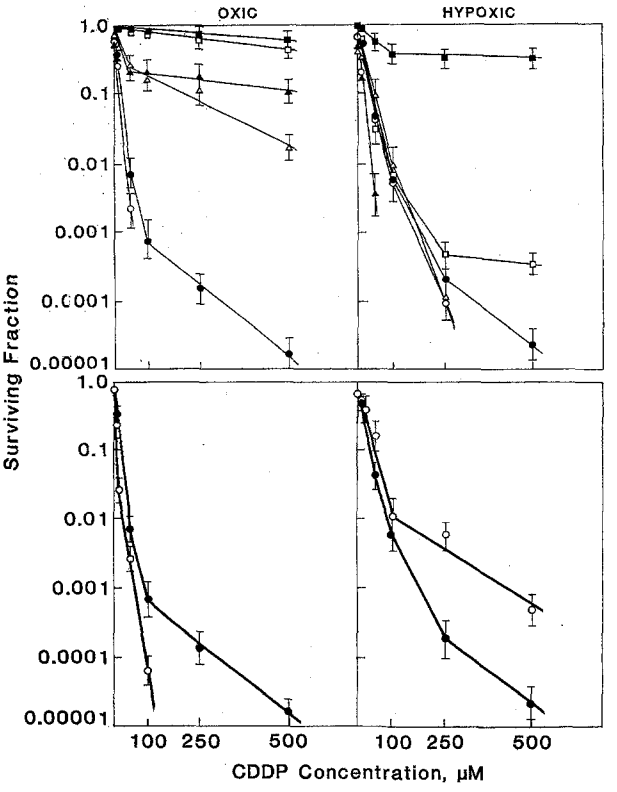

Fig. 2. Survival curve of exponentially growing normally oxygenated and hypoxic EMT-6 cells exposed to various concentrations of CDDP alone (●) or *upper panels:* in combinations with tetrahydrocortisol (100 μ*M*, 24 h) at pH 7.40 (□) or pH 6.45 (△), β-cyclodextrin tetradecasulfate (100 μM, 24 h) at pH 7.40 (■) or pH 6.45 (▲), or minocycline (100 μ*M*, 24 h) at pH 7.40 (○); or *lower panels:* in combination with the three modulators at pH 7.40 as described above (○) under normally oxygenated or hypoxic conditions during the 5th h of modulator exposure. *Points*, Mean values for 3 independent determinations; *bars*, SEM

Results

Neither THC nor 14(SO4)βCD was very cytotoxic toward normally oxygenated or hypoxic (5 h of hypoxia + 19 h of normal oxygenation) EMT-6 cells upon 24 h of exposure to the modulators (Fig. 1). Even with exposure to 500 μ*M* of THC or 14(SO4)βCD, IC_{50} values were not reached. Minocycline was more cytotoxic toward normally oxygenated cells than toward hypoxic cells. The IC_{50} value for minocycline in normally oxygenated EMT-6 cells was 132 μ*M*, and that in hypoxic EMT-6 cells was 220 μ*M*. There was no difference in the cytotoxicity of CDDP in EMT-6 cells in relation to the oxygenation level of the cells (Fig. 2). Exposure of the cells to 100 μ*M* of THC or 14(SO4)βCD prior to, during, and after exposure to CDDP nearly ablated the effect of the alkylating agent in normally oxygenated EMT-6 cells. When the extracellular pH was lowered to 6.45 for the first 5 h of drug exposure, the inhibitory effects of THC and 14(SO4)βCD were somewhat reduced. When the first 5 h of drug exposure occurred under hypoxic conditions, the inhibitory effect of 14(SO4)βCD was maintained, whereas THC at pH 7.40 or pH 6.45 and 14(SO4)βCD at pH 6.45 resulted in survival values that were not different those observed for CDDP. Exposure of the cells to minocycline (100 μ*M*, 24 h) with

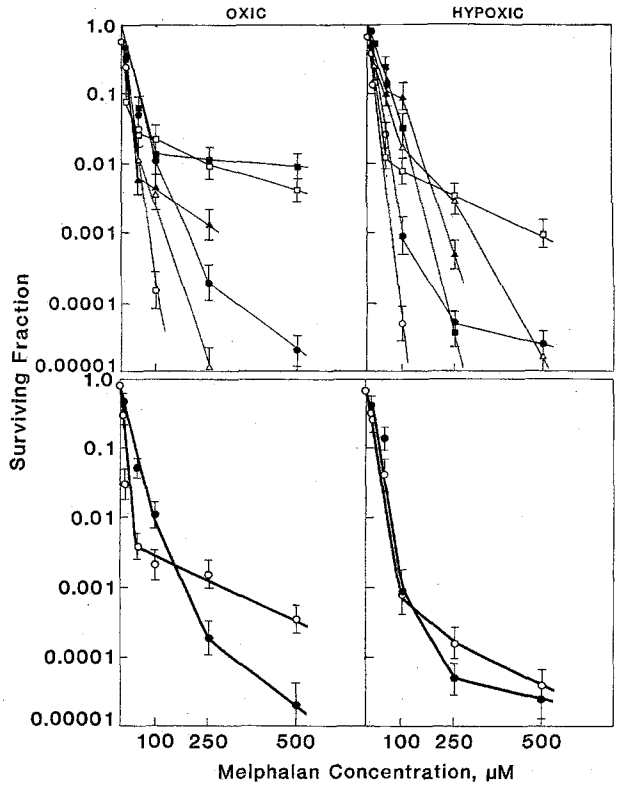

Fig. 3. Survival curve of exponentially growing normally oxygenated and hypoxic EMT-6 cells exposed to various concentrations of melphalan alone (●) or *upper panels:* in combinations with tetrahydrocortisol (100 μ*M*, 24 h) at pH 7.40 (□) or pH 6.45 (△), β-cyclodextrin tetradecasulfate (100 μ*M*, 24 h) at pH 7.40 (■) or pH 6.45 (▲), or minocycline (100 μM, 24 h) at pH 7.40 (○); or *lower panels:* in combination with the three modulators at pH 7.40 as described above (○) under normally oxygenated or hypoxic conditions during the 5th h of modulator exposure. *Points,* Mean values for 3 independent determinations; *bars,* SEM

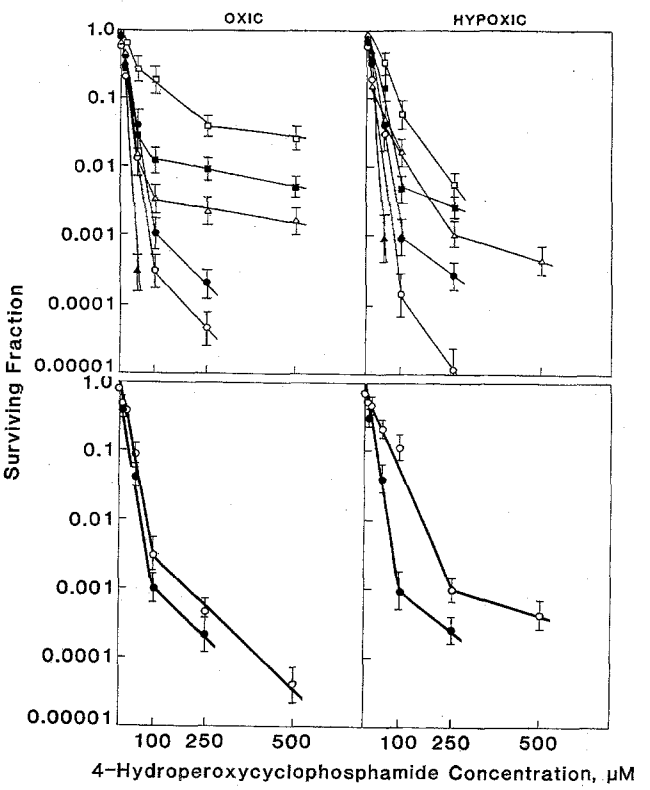

Fig. 4. Survival curve of exponentially growing normally oxygenated and hypoxic EMT-6 cells exposed to various concentrations of 4-hydroperoxycyclophosphamide alone (●) or *upper panels:* in combination with tetrahydrocortisol (100 μ*M*, 24 h) at pH 7.40 (□) or pH 6.45 (△), β-cyclodextrin tetradecasulfate (100 μ*M*, 24 h) at pH 7.40 (■) or pH 6.45 (▲), or minocycline (100 μ*M*, 24 h) at pH 7.40 (○); or *lower panels:* in combination with the three modulators at pH 7.40 as described above (○) under normally oxygenated or hypoxic conditions during the 5th h of modulator exposure. *Points,* Mean values for 3 independent determinations; *bars,* SEM

CDDP treatment during the 5th h of minocycline exposure did not significantly alter the cytotoxicity of CDDP. The three modulators in combination did not significantly alter the cytotoxicity of CDDP toward normally oxygenated cells but protected hypoxic cells from high concentrations of CDDP.

The survival of EMT-6 cells exposed for 1 h to various concentrations of melphalan under normally oxygenated or hypoxic conditions was the same (Fig. 3). Exposure to 100 μ*M* of THC or 14(SO_4)βCD prior to, during, and after exposure to melphalan protected normally oxygenated EMT-6 cells from the cytotoxicity of high concentrations of the drug. When drug exposure was performed at pH 6.45 (4 h prior to and during exposure to melphalan), the cytotoxicity achieved was not different from that produced by the alkylating agent alone. When drug exposure was performed under hypoxic conditions, THC at pH 7.40 or pH 6.45 was somewhat protective from melphalan cytotoxicity, whereas 14(SO_4)βCD resulted in cell killing that was not different from that observed for melphalan alone. Treatment with minocycline with melphalan exposure during the 5th h of minocycline treatment resulted in additive cytotoxicity of the two agents. The three modulators in combination protected normally oxygenated cells from high concentrations of melphalan but did not alter the cytotoxicity of melphalan toward hypoxic cells.

4-Hydroperoxycyclophosphamide was equally cytotoxic toward normally oxygenated and hypoxic EMT-6 cells (Fig. 4). Exposure to 100 μ*M* of THC or 14(SO_4)βCD prior to, during, and after exposure to 4-HC protected normally oxygenated and hypoxic EMT-6 cells from the cytotoxicity of 4-HC. However, acidic pH conditions along with 14(SO_4)βCD treatment prior to and during exposure to 4-HC resulted in additive cytotoxicity of the drug combination toward both normally oxygenated and hypoxic EMT-6 cells. Exposure of EMT-6 cells under either oxygenation condition to minocycline with the addition of 4-hydroperoxycyclophosphamide during the 5th h of minocycline exposure resulted in cytotoxicity that appeared to be additive. The combination of the three modulators had no effect on the cytotoxicity of 4-HC toward normally oxygenated cells but protected hypoxic cells from the cytotoxic actions of the drug.

Similarly, BCNU was equally cytotoxic toward EMT-6 cells under normally oxygenated and hypoxic conditions (Fig. 5). Exposure to THC or 14(SO_4)βCD for 4 h prior to and during as well as for 19 h after exposure to BCNU resulted in a marked diminution in the cytotoxicity of the

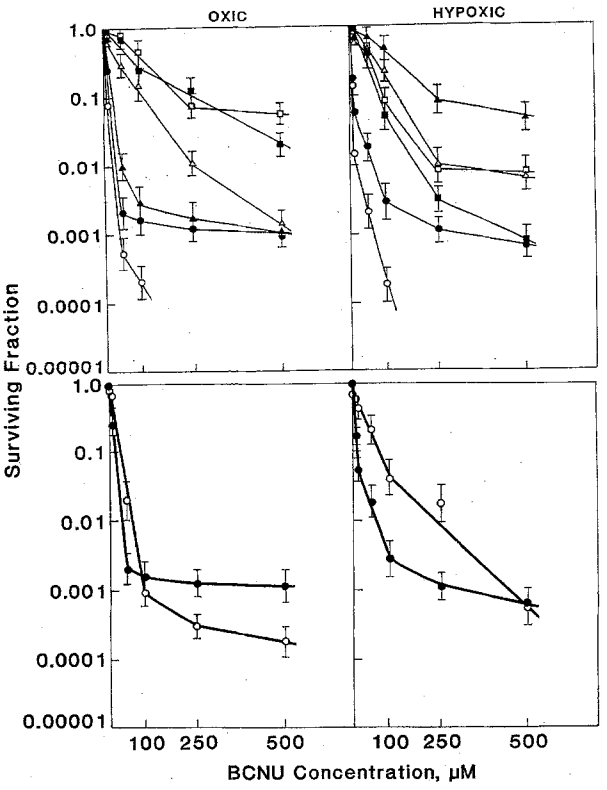

Fig. 5. Survival curve of exponentially growing normally oxygenated and hypoxic EMT-6 cells exposed to various concentrations of BCNU alone (●) or *upper panels:* in combination with tetrahydrocortisol (100 μ*M*, 24 h) at pH 7.40 (□) or pH 6.45 (△), β-cyclodextrin tetradecasulfate (100 μ*M*, 24 h) at pH 7.40 (■) or pH 6.45 (▲), or minocycline (100 μ*M*, 24 h) at pH 7.40 (○); or *lower panels:* in combination with the three modulators at pH 7.40 as described above (○) under normally oxygenated or hypoxic conditions during the 5th h of modulator exposure. *Points*, Mean values for 3 independent determinations; *bars*, SEM

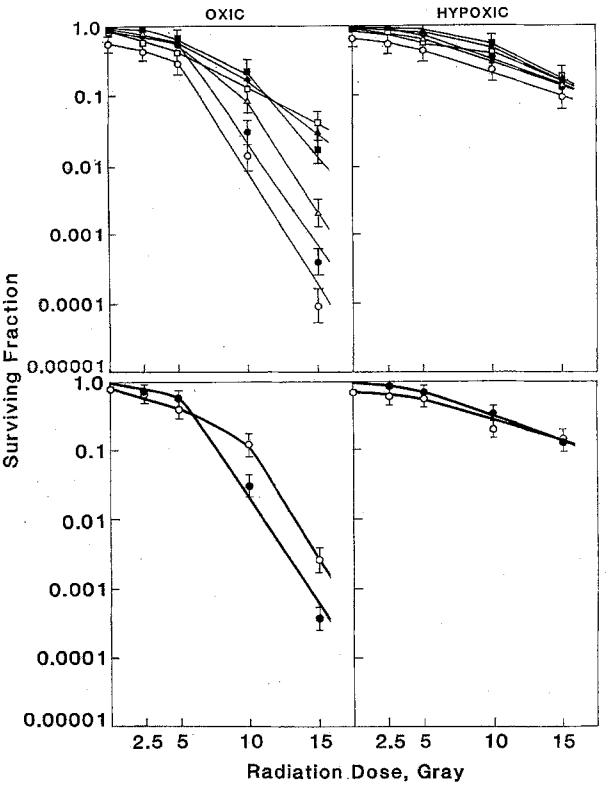

Fig. 6. Survival curve of exponentially growing normally oxygenated and hypoxic EMT-6 cells exposed to various concentrations of X-rays alone (●) or *upper panels:* in combination with tetrahydrocortisol (100 μ*M*, 24 h) at pH 7.40 (□) or pH 6.45 (△), β-cyclodextrin tetradecasulfate (100 μ*M*, 24 h) at pH 7.40 (■) or pH 6.45 (▲), or minocycline (100 μ*M*, 24 h) at pH 7.40 (○); or *lower panels:* in combination with the three modulators at pH 7.40 as described above (○) under normally oxygenated or hypoxic conditions during the 5th h of modulator exposure. *Points*, Mean values for 3 independent determinations; *bars*, SEM

nitrosourea toward normally oxygenated EMT-6 cells and, to a lesser extent, toward hypoxic EMT-6 cells. Although reducing the extracellular pH to 6.45 lessened the protective effect of both THC and 14(SO4)βCD, the overall cytotoxicity of the combinations was nonetheless lower than that of BCNU alone. Treatment with minocycline with BCNU exposure during the 5th h of minocycline treatment resulted in additive cytotoxicity of the two agents toward both normally oxygenated and hypoxic EMT-6 cells. The three-modulator combination resulted in a 4- to 5-fold increase in the cytotoxicity of BCNU toward normally oxygenated cells but protected hypoxic cells from the cytotoxicity of moderate concentrations of BCNU.

Radiation was more cytotoxic toward normally oxygenated EMT-6 cells than toward hypoxic EMT-6 cells (Fig. 6). Exposure to THC or 14(SO4)βCD under normal (pH 7.40) or acidic (pH 6.45) conditions protected normally oxygenated EMT-6 cells from the cytotoxic effects of radiation. The survival of EMT-6 cells after exposure to radiation under hypoxic conditions was not altered when the cells were exposed to THC or 14(SO4)βCD prior to, during, and after radiation delivery under normal or acidic conditions. When EMT-6 cells under either oxygenation condition were exposed to minocycline for 24 h with radiation delivery during the 5th h of minocycline exposure, there was no enhancement in the cytotoxicity of the radiation. The three-modulator combination protected EMT-6 cells from the cytotoxicity of relatively high doses of radiation but did not alter the radiation response of hypoxic cells.

The Lewis lung carcinoma growing in C57BL mice was chosen for tumor growth-delay studies because this tumor is relatively resistant to many cancer therapies and because this tumor metastasizes avidly to the lungs from s.c. implants. For tumor growth-delay studies, THC (125 mg/kg) and 14(SO4)βCD (1000 mg/kg) were prepared in a 14-day osmotic pump and implanted s.c. in the animals on day 4 post tumor cell implantation, by which time neovascularization of the tumors has begun [24, 25]. Administration of minocycline (5 mg/kg) i.p. daily was also initiated on day 4 post tumor cell implantation and continued until day 18. Neither the 14-day continuous infusion of THC/14(SO4)βCD nor the daily i.p. injection of minocycline for 2 weeks altered the growth of the Lewis lung carcinoma (Table 1). The three modulators given together [THC/14(SO4)βCD/minocycline] produced a modest tumor growth delay of 1.2 days in the Lewis lung carcinoma. Single-agent chemotherapy or radiation therapy was

Table 1. Growth delay of the Lewis lung tumor produced by various anticancer treatments given alone or in combination with β-cyclodextrin tetradecasulfate/tetrahydrocortisol, minocycline, or the combination of modulators

Treatment group	Dose[a]	Tumor growth delay, days[b]			
		Alone	+14(SO_4)βCD, THC	+ Minocycline	+14(SO_4)βCD, THC, Mino
14(SO_4)βCD, THC	1000 mg/kg, 125 mg/kg over 14 days		0.6±0.3		
Minocycline	14× 5 mg/kg			0.6±0.3	
14(SO_4)βCD, THC, Mino	As above				1.2±0.4
CDDP	1× 10 mg/kg	4.5±0.3	2.2±0.3	5.0±0.3	26.2±2.5
Melphalan	1× 10 mg/kg	2.7±0.3	1.1±0.3	4.3±0.3	10.5±0.9
Cyclophosphamide	1×150 mg/kg	7.2±0.4	16.2±1.2	24.7±2.7	27.6±2.8
	3×150 mg/kg	21.5±1.7	36.8±3.4	45.2±2.9[c]	48.8±3.3[d]
Radiation	1× 20 Gy	6.2±0.5	8.3±0.5	11.9±1.4	13.8±1.3
	5× 3 Gy	4.4±0.3	7.1±0.7	7.8±0.6	12.6±1.2
Adriamycin	5× 1.75 mg/kg	7.0±0.6	–	9.8±0.8	11.7±1.2
Bleomycin	4× 10 mg/kg	8.5±0.6	–	12.0±1.2	12.9±1.3

[a] β-Cyclodextrin tetradecasulfate (1000 mg/kg) and tetrahydrocortisol (125 mg/kg) were given in a 1 : 1 molar ratio by continuous infusion over 14 days in an Alzet osmotic pump on days 4–18 post tumor implantation. Minocycline (5 mg/kg) was given i.p. on days 4–18 after tumor implantation. CDDP (10 mg/kg), melphalan (10 mg/kg), and cyclophosphamide (150 mg/kg) were given i.p. on day 7 post tumor implantation. Cyclophosphamide (150 mg/kg) was also given on days 7, 9, and 11 after tumor implantation. Radiation was delivered locally to the tumor-bearing limb as 20 Gy on day 7 or 3 Gy daily on days 7–11. Adriamycin (1.75 mg/kg) was given i.p. daily on days 7–11. Bleomycin (10 mg/kg) was given i.p. on days 6, 10, 13, and 16

[b] Tumor growth delay is the difference in the number of days required for treated tumors to reach a volume of 500 mm^3 as compared with untreated control tumors. Untreated control tumors reach a volume of 500 mm^3 in about 14 days. Data represent mean values ± SE for 15 animals

[c] 4 of 12 animals were long-term survivors (>120 days)

[d] 5 of 12 animals were long-term survivors (>120 days)

delivered to the tumor-bearing animals beginning on day 7, when the volume of the tumors was about 100 mm^3. Each treatment agent was given at a standard dose and schedule.

Consistent with the cell-culture studies, the tumor growth delays produced by CDDP (10 mg/kg) and melphalan (10 mg/kg) were decreased about 2-fold in animals receiving THC/14(SO_4)βCD as compared with those treated only with the antitumor alkylating agents. In contrast to the cell-culture results obtained with 4-HC, the tumor growth delays produced by CDDP (10 mg/kg) and melphalan (10 mg/kg) were decreased about 2-fold in animals receiving THC/14(SO_4)βCD as compared with those treated only with the antitumor alkylating agents. In contrast to the cell-culture results obtained with 4-HC, the tumor growth delay produced by cyclophosphamide was increased about 2.2-fold with a single dose of cyclophosphamide and about 1.7-fold with three doses of cyclophosphamide. The growth delay of tumors treated with single-dose (20 Gy) or multiple-dose (5 × 3 Gy) radiation in the presence of THC/14(SO_4)βCD was enhanced about 1.3-fold or about 1.6-fold, respectively, as compared with that observed for the radiation regimens only.

The addition of minocycline to treatment with CDDP did not alter the tumor growth delay produced by that drug (Table 1). The growth delay produced by melphalan was enhanced by about 1.6-fold in animals treated with minocycline and melphalan as compared with those receiving melphalan alone. A marked enhancement in tumor growth delay was observed with the combination of minocycline and cyclophosphamide. The addition of minocycline to treatment with cyclophosphamide resulted in a 3.4-fold and a 2.1-fold increase in tumor growth delay with the single-dose and multiple-dose regimens of cyclophosphamide, respectively. In the multiple-dose cyclophosphamide regimen plus minocycline group, 4 of 12 animals were long-term survivors (> 120 days). There was nearly a 2-fold increase in tumor growth delay when minocycline administration was added to treatment with the single-dose or fractionated radiation. The tumor growth delays produced by standard regimens of Adriamycin and bleomycin were increased about 1.4-fold with the addition of minocycline to treatment with those drugs.

The combination of the three modulators [THC/14(SO_4)βCD and minocycline] was most effective at increasing the response of the Lewis lung tumor to these cytotoxic therapies (Table 1). The tumor growth delay produced by the antitumor alkylating agents were increased by about 5.8-fold, 3.9-fold, 3.8-fold, and 2.3-fold for CDDP, melphalan, and single and multiple doses of cyclophosphamide, respectively. In all, 5 of 12 of the animals treated with the three-modulator combination and multiple doses of cyclophosphamide were long-term survivors. The tumor growth delays produced by single-dose and multiple-dose radiation therapy were increased about 2.2- and 2.8-fold, respectively, in the presence of the three-modulator combination. The increases in tumor growth delay observed with Adriamycin and bleomycin with the addition of the three modulators to the regimen were about 1.7-fold and 1.5-fold, respectively, as compared with that produced by the antitumor agents alone.

Untreated control animals bearing the Lewis lung tumors survived for 21–25 days post tumor implantation

Table 2. Numbers of lung metastases from s. c. Lewis lung tumors detected on day 20 after various anticancer treatments given alone or in combination with β-cyclodextrin tetradecasulfate/tetrahydrocortisol, minocycline, or the combination of modulators

Treatment group	Dose[b]	Mean number of lung metastases (number and % of vascularized metastases)[a]			
		Alone	+14(SO4)βCD, THC	+ Minocycline	+14(SO4)βCD, THC, Mino
Untreated controls		15 (10; 66%)			
14(SO4)βCD, THC	1000 mg/kg, 125 mg/kg over 14 days		14.5 (10; 69%)		
Minocycline	14× 5 mg/kg			12 (5; 43%)	
14(SO4)βCD, THC, Mino	As above				13 (6; 46%)
CDDP	1× 10 mg/kg	12	15 (10; 67%)	10.5 (5; 48%)	6 (2.5; 39%)
Melphalan	1× 10 mg/kg	8	8 (4; 48%)	6 (3; 50%)	5 (2.5; 47%)
Cyclophosphamide	1×150 mg/kg	6.5	6 (2; 32%)	3 (1; 33%)	4 (2; 44%)
	3×150 mg/kg	3.5	3 (0.5; 16 %)	0.5 (0; 18%)	0.5 (0; 20%)
Radiation	1× 20 Gy	8	7 (3; 40%)	8 (1; 25%)	7 (3; 46%)
	5× 3 Gy	7	7 (2.5; 35%)	6.5 (2; 30%)	7 (3; 47%)
Adriamycin	5× 1.75 mg/kg	8	–	7.5 (5; 63%)	5 (3; 57%)
Bleomycin	4× 10 mg/kg	7	–	7 (4.5; 64%)	4 (2; 45%)

[a] The number of external lung metastases on day 20 post tumor implantation were counted manually and scored as ≥ 3 mm^3 in diameter. Data represent mean values for 6–12 pairs of lungs. Parentheses indicate the number of large (vascularized) metastases and the percentage of the total number of metastases that were large

[b] The schedules of drug administration were as shown under Table 1

and succumbed to disease metastatic to the lungs. The number and size of lung metastases detected in untreated and treated animals were scored on day 20 post tumor implantation (Table 2). Treatment with THC/14(SO4)βCD by continuous infusion from day 4 through day 18 post tumor implantation did not alter the number of lung metastases or the percentage of large metastases observed in these animals. Treatment with minocycline or with the three-modulator combination [THC/14(SO4)βCD and minocycline] over the same period had little effect on the total number of metastases; however, only 40%–50% of the metastases were large as compared with about 70% in the untreated control animals. The cytotoxic antitumor treatments reduced the number of lung metastases in many cases to about one-half the number observed in the untreated control animals. Treatment with THC/14(SO4)βCD did not alter the lung metastases produced in animals treated with CDDP, melphalan, cyclophosphamide, or radiation therapy. The combination of minocycline with these cytotoxic therapies did not alter the number of lung metastases produced in animals treated with CDDP, melphalan, Adriamycin, bleomycin, or radiation therapy; however, in animals treated with cyclophosphamide and minocycline, the number of lung metastases was reduced to 50% and 14% of the number observed in those treated with single-dose or multiple-dose cyclophosphamide alone. The three-modulator combination [THC/14(SO4)βCD and minocycline] along with the cytotoxic therapies was more effective against metastastic disease except when the cytotoxic therapy was radiation delivered to the primary tumor. In most cases, the number of lung metastases was reduced to about 50% of the number observed with the cytotoxic therapy alone and the number of large metastases was 40%–50% of those. The lowest number of large metastases were found in animals treated with cyclophosphamide and minocycline or the three-modulator combinations; in fact, with multiple doses of cyclophosphamide in combination with minocycline, there was no large lung metastasis present on day 20.

Discussion

Although cytotoxic therapies often make some impact in the treatment of solid tumors, a cure is rarely attained. We have been searching for modulators to add to standard therapies, which, by virtue of their effects on the physiological, biological, or biochemical properties of the tumor, would increase the susceptibility of the tumor to cytotoxic treatment without increasing the toxicity to the host. Agents that inhibit processes involved in extracellular matrix restructuring could be envisioned to act as modulators of other therapies by inhibiting further growth or regrowth of both primary and metastatic disease and inhibiting further metastatic invasion. In the ideal case, an angiostatic condition might lead to the death of those tumor cells most distal from the established tumor vasculature and could thereby reduce the tumor burden of the host and result in a tumor mass more easily permeated by chemotherapy agents and treated by radiation therapy. The search for such substances has led primarily to the discovery of proteins and small molecules that inhibit various steps in the breakdown of the basement membrane [67, 68]. These include naturally occurring proteins such as protamine [63], interferon-α [23, 72], platelet factor [63], tissue inhibitors of metalloproteinases (TIMPs) [60, 71]; peptides derived from cartilages [36, 41], vitreous humor [62], smooth muscle [8], and aorta [9]; as well as synthetic

peptides such as synthetic laminin peptide (CDPG) YIGSR-NH_2 [54] and somatostatin analogs such as somatuline [3]. Active small molecules include naturally occurring heparins [17]; a variety of steroids [7, 13, 15, 38]; several retinoids [29, 35, 43, 56]; warfarin [5], fumagillin [30, 34]; as well as synthetic agents such as sulfated chitin derivatives [42], sulfated cyclodextrins [17], SC44463 [48], SC39026 [32], derivatives of fumagillin [42], and minocycline [61]. Radiation also inhibits blood vessel growth [32, 33, 44, 46].

In the development of new drug combinations the potential for both positive and negative interactions exists. Cell-culture studies with exponentially growing EMT-6 cells under normally oxygenated and hypoxic conditions indicated that whereas both THC and 14(SO_4)βCD were minimally cytotoxic, exposure to THC or 14(SO_4)βCD during treatment with several antitumor alkylating agents or radiation could markedly reduce the cytotoxic effects of those treatments. These in vitro studies also indicated that minocycline was minimally cytotoxic toward EMT-6 cells upon 24 h of exposure, but only at very high concentrations. Combinations of minocycline and radiation or chemotherapy showed only additive cytotoxicities of the treatments, indicating no direct interaction between minocycline and the other treatments. The protective effects of THC/14(SO_4)βCD were evidenced in vivo in the reduced tumor growth delays observed with CDDP and melphalan in animals being infused with those modulators.

The ability of tetracyclines to inhibit tissue collagenase activity was first reported by Golub et al. [18, 20] in the early 1980s. At about the same time, Folkman et al. [14] reported that heparin or a heparin fragment in the presence of cortisone caused angiogenesis inhibition and tumor regression of several established murine solid tumors. Because of the side effects of the cortisone treatment, Folkman et al. [14] also treated their tumor-bearing mice with tetracycline and bactrim in the drinking water throughout the heparin/cortisone treatment period and for 2 additional weeks. In a subsequent study, Penhaligon and Camplejohn [45] examined the effect of five different heparin preparations and cortisone on two transplantable mouse tumors. These investigators treated the tumor-bearing animals with oxytetracycline and bactrim beginning on the day of tumor implantation. Lee et al. [37] examined the potential of cortisone acetate to inhibit tumor angiogenesis in C3H mice bearing MBT-2 tumors. Lee et al. [37] also included tetracycline and sulfatrim in the drinking water of the animals throughout the course of treatment with cortisone acetate. In each case, the animals in these three studies probably received a tetracycline dose greater than or comparable with the dose of minocycline used in the current study. It is therefore quite likely that the tetracycline treatment was an active component in the antiangiogeneic effects observed in these studies. Whereas tetrahydrocortisol and β-cyclodextrin tetradecasulfate are experimental agents, minocycline is clinically available, and the minocycline dose used in the in vivo studies reported herein is readily achievable in humans.

The pathways available for extracellular matrix breakdown and restructuring in vivo are highly redundant. It is unlikely that 100% blockade of any given enzyme can be achieved in vivo; however, partial inhibition of multiple pathways may achieve a significant biological effect. When the three modulators examined in this study were given together, marked improvements in the response of primary and metastatic disease to several of the cytotoxic therapies occurred. The antiangiogenic modulators did not increase the toxicity of the cytotoxic therapies to the animals as evidenced by weight loss or early deaths. Overall, therefore, these modulators, which may be acting primarily on a critical normal process within the tumor mass, provided a positive effect on the treatment outcome of several chemotherapeutic agents and radiation therapy in vivo.

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