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Effects of Mithramycin, Mitomycin, Daunorubicin, and Bleomycin on Human Subconjunctival Fibroblast Attachment and Proliferation

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Scar formation and fibrosis are the most common causes of ultimate glaucoma filtration surgery failure. The authors used Coulter counter, hexosaminidase, and ^{3}H-thymidine assays to evaluate the effects of mithramycin, mitomycin, daunorubicin, and bleomycin on human subconjunctival fibroblast growth in tissue culture. Fifty percent inhibitory doses (ID_{50}) for each drug on different days of incubation with fibroblasts were calculated with these three assays. Among these four drugs only daunorubicin exerted a significant effect on cellular attachment, showing cytotoxicity at 4×10^{-6} M. However, all drugs had antiproliferative effects. Daunorubicin was the most potent drug in the concentration range studied. Mithramycin had a narrow therapeutic range in tissue culture. Cellular antiproliferative responses to mitomycin and bleomycin were more gradual. These drugs have potential applications in the prevention of glaucoma filtration surgery failure. Invest Ophthalmol Vis Sci 31:2136–2144, 1990

Glaucoma filtration surgery for patients refractory to medical treatment creates a passage of aqueous humor flow from the anterior chamber to the subconjunctival space. This procedure fails usually as a result of fibroblast proliferation and subconjunctival and bleb fibrosis.[1] To inhibit the fibroblast proliferative response, many antineoplastic antibiotics have been examined in experimental settings.[2-6] Although these investigations found some antineoplastic agents successful in suppressing cell growth, tissue culture data investigating the effectiveness of these drugs in inhibiting the proliferation of human Tenon's fibroblasts is still limited.

To evaluate the efficacy of several antineoplastic agents in preventing subconjunctival scar formation after glaucoma filtration surgery, we focused on four antineoplastic antibiotics produced by various strains of the soil fungus streptomyces: mithramycin, mitomycin, daunorubicin, and bleomycin. We assessed the effectiveness of these four agents on suppressing human Tenon's fibroblast attachment and growth in vitro with the Coulter counter and the hexosaminidase and ^{3}H-thymidine assays. Concentrations of each antibiotic that resulted in 50% inhibition of the fibroblast proliferation (ID_{50}) at various days after drug exposure were determined by these three assay methods.

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Materials and Methods

Cell Culture

Cell cultures of human Tenon's capsule fibroblasts were established from specimens of muscle, cataract, and glaucoma filtration procedures with the approval of the UCLA Human Subject Protection Committee. The tissue was minced, placed under a coverslip in a 30-mm tissue culture Petri dish, placed in Eagle's modified minimal essential media (MEM; Flow, McLean, VA), supplemented with glutamine, 15% fetal bovine serum (FBS), 100 IU/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B (an antibiotic combination previously shown to have no net effect on cell growth in tissue culture),[7] and incubated at 37°C with 5% CO_2. By 3–4 weeks, fibroblasts proliferated to reach a confluent monolayer in the Petri dish. The medium was discarded, and the cells were incubated in 0.05% trypsin at 37°C for 10 min, centrifuged, and washed with Dulbecco's calcium- and magnesium-free phosphate-buffered saline (PBS), transferred to a 75-cm^2 flask, and allowed to grow in the medium consisting of MEM, 10% FBS, penicillin, streptomycin, and amphotericin B. When a monolayer formed, the medium was discarded, cells were incubated in 0.05%

trypsin at 37°C for 10 min, washed with PBS, centrifuged at 1000 rpm for 10 min, rewashed, recentrifuged, and transferred to three 75-cm^2 flasks. Once all three flasks had reached confluency, cells were passaged again and transferred to three 150-cm^2 flasks as just described. A monolayer in all three 150-cm^2 flasks provided an adequate cell number for one drug experiment. No cell culture was used beyond 12 passages, and more than three different cell lines were used for each drug.

Drugs

All drugs were dissolved and diluted serially from original vials in MEM. Daunorubicin was donated by Wyeth, Philadelphia, PA. Bleomycin, mithramycin, and mitomycin were purchased from Sigma, St. Louis, MO. Daunorubicin and mithramycin were sterilized by passage through a filter unit before they were added to the culture plates. The concentrations were chosen around a known toxicity concentration to establish a dose-dependent survival curve for future experimentation.

Assays

All three different assays were done in quadruplicate in each experiment in 96-well flat-bottom microtiter plates, and every experiment was repeated three times with different patient cell lines and passages. The live cell number per milliliter was counted on a hemocytometer with the trypan blue exclusion method. Approximately 1000 fibroblasts in 100 μl of medium were aliquoted to each well.

Attachment: To assess the effects on cell attachment, drugs were added immediately after plating the cells. One hundred microliters of different concentrations of a drug were added to each well, resulting in a final volume of 200 μl per well. Control wells received 100 μl of MEM without drugs. In addition, 16 wells located peripherally in each plate were reserved as "mote" where only 200 μl of MEM was aliquoted to minimize evaporative losses. Twenty-four hours after plating cells and adding the drugs, plates were washed with PBS three times so that unattached cells were removed from the plates. The number of attached cells was assessed by Coulter counter and hexosaminidase methods.

Proliferation: Cells were allowed to attach to the bottom of the plates for 22 hr at 37°C. To assess the effects on cell proliferation, drugs were added 22 hr after plating the cells as already described. Proliferative plates were analyzed 1, 2, 3–4, 7–8, and 10–11 days after adding the drugs. The number of cells was assessed by Coulter counter and hexosaminidase methods. The effect on DNA synthesis was assessed by ^{3}H-thymidine uptake.

Coulter counter: The culture medium was discarded, and the wells were washed with PBS and incubated at 37°C for 10 min in 50 μl of 0.05% trypsin. The trypsin enzymatic reaction was stopped with 100 μl of 10% FBS containing medium after incubation. Cell suspensions were immediately pipetted into vials containing 10 ml of Isotone (Fisher Scientific, Springfield, NJ). Each vial was counted four times consecutively with the Coulter counter model ZM (Hialeah, FL). The four counts for each well were averaged for the cell number value of that well.

Hexosaminidase: This colorimetric assay was described by Landegren.[8] After all the medium in each plate was discarded, cells were washed with PBS and incubated for 1–2 hr in 50 μl of p-nitrophenol substrate solution with 0.1 M citrate buffer at pH 5.0 and 0.25% Triton X-100. The latter was used to disrupt cell membranes so that the substrate could be exposed to the hexosaminidase enzyme inside of the cells. This reaction was terminated by adding 25 μl of 6.5 mM ethylenediaminetetraacetic acid buffered with 75 μl of 0.2 M glycine buffer at pH 10.4 to each well. Optical density in each well was measured by a Titertek Multiskan MCC ELISA reader (Flow) at 405 nm.

^{3}H-Thymidine: Cultured cells were incubated with 1 μCi of ^{3}H-thymidine per well (specific activity 15.0 Ci/mmol; New England Nuclear, DuPont Co, Wilmington, DE) for 22 hr before harvesting. On the harvesting day, supernatant from each well was removed with a semiautomatic cell harvester (Otto Hiller, Madison, WI). The remaining attached cells were washed three times with PBS, incubated in 50 μl of 0.05% trypsin for 10 min at 37°C, and then harvested on a fiberglass filter paper (Whittaker, Walkersville, MA). Cells were sequentially washed at least three times with PBS, 5% cold trichloroacetic acid, and absolute alcohol. Filter papers were then sectioned into pieces, transferred to scintillation counting vials (Packard, Downers Grove, IL), air dried for 10 min, and immersed in 100 μl of 0.01 M sodium hydroxide for 20 min. Five ml of Protein Ready+ scintillation liquid (Beckman, Fullerton, CA) was added to each vial. The vials were then vortexed, and disintegrations per minute was measured by counting 1 min per vial with a 1900 CA TRICARB liquid scintillation analyzer (Packard).

Statistical Analysis

Average response values obtained by these three assays were standardized by dividing the response mean at each drug concentration by the control (no drug) response to yield percent control values. The ID_{50} for each experiment was calculated empirically using a method suggested by Finney,[9] which uses log

scale linear interpolation to determine the ID_{50} estimate and confidence interval. Adjustments were made for nonzero background response at high dose before the ID_{50} values was computed.

Results

Of the four drugs studied at the indicated concentration range, only daunorubicin directly affected fibroblast attachment. Marked toxicity was apparent at the concentration of 4×10^{-6} M daunorubicin showing more than 80% cell death with respect to control from both Coulter counter and hexosaminidase assays (Fig. 1). The other three drugs had no effect on fibroblast attachment (Figs. 2–4). The representative dose-response curves at different time intervals with different patients' cell lines and passage numbers from three types of assays are presented in Figures 1–4. All four drugs appeared to inhibit proliferation in a dose-dependent manner especially at the highest concentration tested after 10 days' exposure. As can be seen in Figure 1, daunorubicin had the most potent growth inhibitory effects and a more gradual slope in the dose response curve. At 4×10^{-10} M and a higher concentration, more than 90% of fibroblasts stopped their DNA synthesis activity as measured by ^{3}H-thymidine assays on days 2 and 4. With concentrations diluted up to 4×10^{-17} M (not shown), we still observed fibroblast DNA synthesis activity to be lower than 60% of the control on days 2 and 4.

The dose-response curve of mithramycin (Fig. 2) showed a narrow therapeutic range. By day 10, concentrations higher than 4×10^{-8} caused more than 90% cell death, while concentrations lower than 4×10^{-9} M showed no significant difference compared with the control by both Coulter counter and hexosaminidase assays. Similarly, DNA synthesis activity with mithramycin decreased more than 90% with concentrations higher than 4×10^{-8} M compared with the control by ^{3}H-thymidine assay on day 4.

Bleomycin inhibited DNA synthesis completely at 1.9×10^{-7} M on day 4, but at least 1000-fold higher concentrations were required to inhibit cell numbers (Fig. 3). The dose-response curve of bleomycin showed a steep slope in the concentration range of 10^{-3}–10^{-5} M by both Coulter counter and hexosaminidase assays. At days 10–11 with the concentration at 1.9×10^{-4} M, more than 10% of fibroblasts were still present by both Coulter counter and hexosaminidase assays. When the concentration was dou-

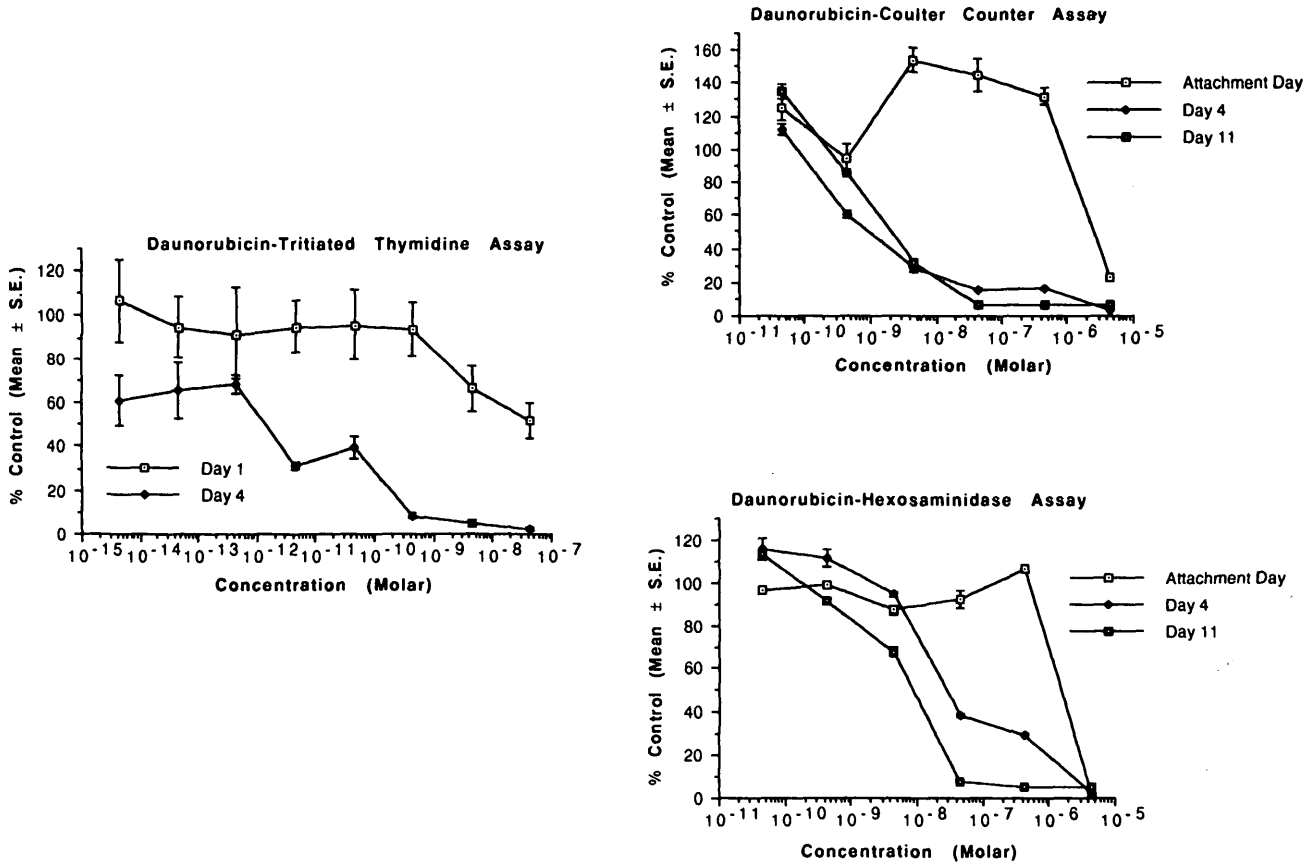

Fig. 1. Semilogarithmic plot of Coulter counter and hexosaminidase assays of human Tenon's fibroblasts proliferation at attachment day, day 4, and day 11 and ^{3}H-thymidine assay at day 1 and day 4 following incubation with media and daunorubicin at indicated concentrations.

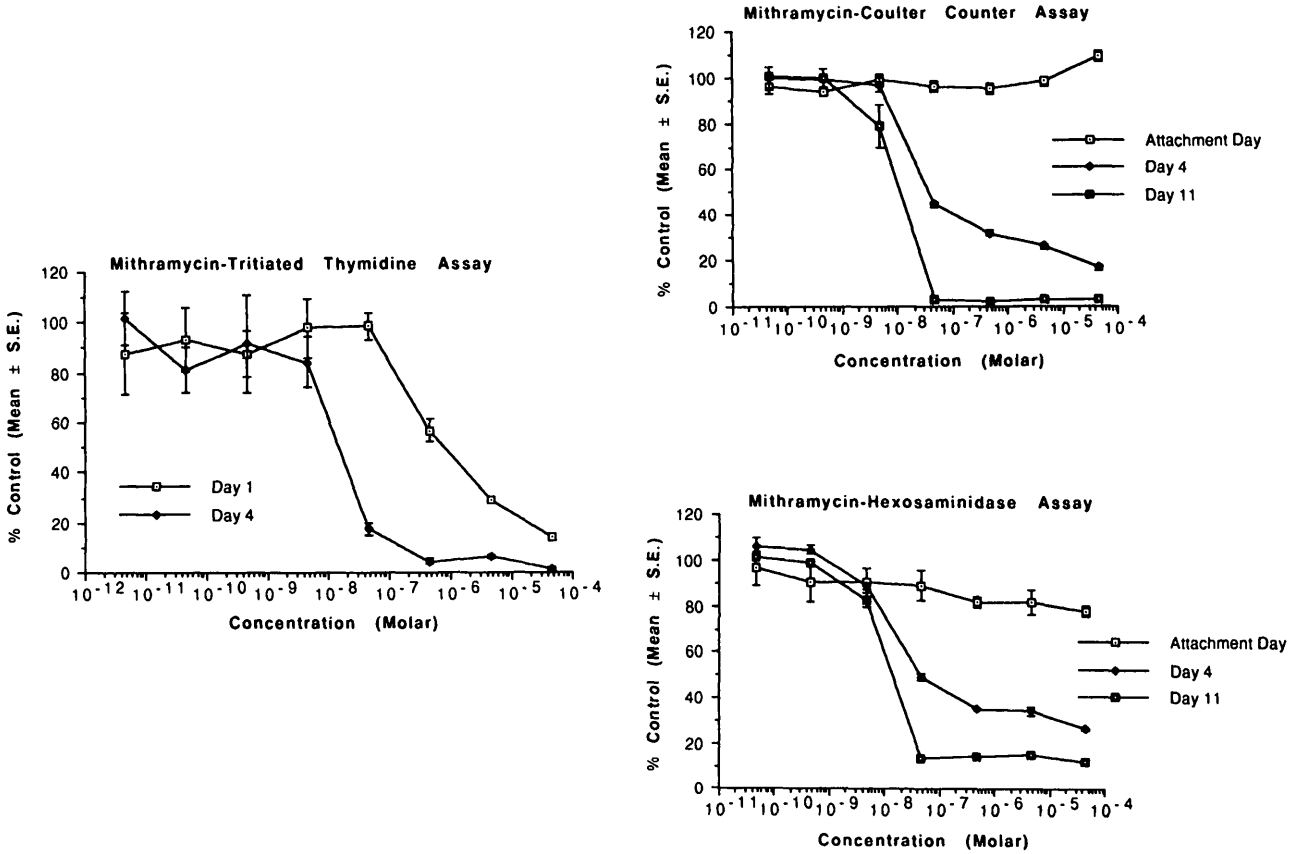

Fig. 2. Semilogarithmic plot of Coulter counter and hexosaminidase assays of human Tenon's fibroblast proliferation at day 4, and day 11 and ^{3}H-thymidine assay at day 1 and day 4 following incubation with media and mithramycin at indicated concentrations.

bled to a maximum of 3.8×10^{-4} M, less than 10% of the cells were viable by Coulter counter and hexosaminidase assays on days 7–8 and beyond. This observation was confirmed by the trypan blue exclusion test which showed cell viability to be less than 2% on days 7–8.

Mitomycin inhibited the fibroblast proliferation at 2×10^{-6} M by all three assays (Fig. 4). DNA synthesis was inhibited gradually over a wide range. The hexosaminidase assay demonstrated a steep dose-response curve at a concentration range of 10^{-6} M.

The results of average ID_{50} from three independent experiments, presented in Figure 5, compare the effectiveness of mithramycin, mitomycin, daunorubicin, and bleomycin in inhibiting Tenon's fibroblast proliferation on days 3–4. Daunorubicin is the most effective drug in its ability to reduce cell viability and inhibit DNA synthesis as evaluated by Coulter counter and ^{3}H-thymidine assays in the concentrations chosen. On the other hand, bleomycin is least effective in reducing cell numbers as measured by hexosaminidase assay.

The mean ID_{50} and its 95% confidence interval at various days of exposure to the drug are shown in Tables 1–4. The three assays showed a decrease of ID_{50} with time in all drugs except for bleomycin. Cell cultures from different patients did not show identical responses to the drugs, a common clinical finding with most drugs. Cells in early passage, which better characterized fibroblasts in early stages of wound healing, had a heightened proliferative drive and enhanced drug sensitivity. All of the data in Tables 1–4 were taken from early (less than 12) passage cells. No definitive conclusion can be obtained on the effect of patient ages and cases (cataract, glaucoma, or muscle case) from these tables. Although there was a tendency of older patient cell lines to be less responsive to drugs and therefore to have higher ID_{50}s. There was no statistically significant correlation between patient age and ID_{50} for any of the drugs tested by the Spearman (rank) correlation test. In looking at the simple correlation between ID_{50} and age, the effect of day on ID_{50} was ignored.

Discussion

Failure of glaucoma filtration surgery due to postoperative subconjunctival fibroblast proliferation and scar formation continues to be a dreaded complication. Using a tissue culture model of human subconjunctival fibroblasts, we attempted to show that antineoplastic agents could inhibit this process.

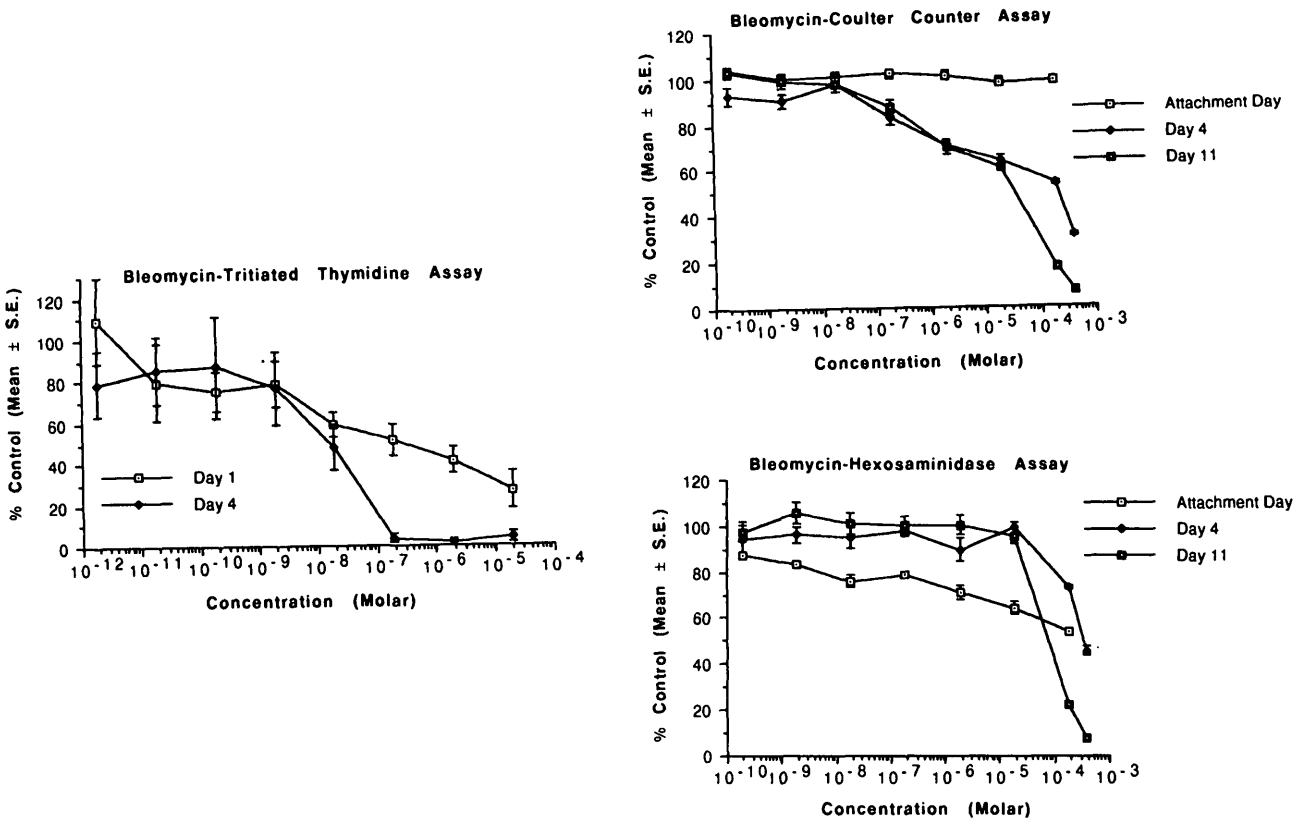

Fig. 3. Semilogarithmic plot of Coulter counter and hexosaminidase assays of human Tenon's fibroblast proliferation at day 4 and day 11 and ^{3}H-thymidine assay at day 1 and day 4 following incubation with media and bleomycin at indicated concentrations.

Although daunorubicin is not cell-cycle specific, it is most toxic to cells entering S-phase.[10] It has been shown to be one of the most active ocular antiproliferative agents in the treatment of proliferative vitreoretinopathy.[11] Although daunorubicin inhibits fibroblast proliferation and migration, it has no effect on contraction.[3] In fact, cells surviving daunorubicin action continue to produce collagen, suggesting that collagen synthesis and transport remain functional.[4] In our studies, daunorubicin was toxic at the highest concentration before attachment, possibly due to a direct disruption of cellular membrane integrity with loss of ion transport and selective permeability mechanisms.[10] Furthermore, by comparing results obtained from Coulter counter and ^{3}H-thymidine assay, we showed that daunorubicin at noncytotoxic concentrations produced more than 50% inhibition of cellular DNA synthesis. This phenomenon can be explained by the fact that daunorubicin can condense chromatin and render it resistant to micrococcal nuclease digestion, thereby preventing thymidine from binding to DNA.[12] Therefore, inhibition of DNA synthesis does not necessarily lead to cellular death, particularly at lower concentrations. McDonnell and associates[13] reported that lens epithelial cell proliferation was inhibited by 50% with a single dose of 5 ng/ml on day 4 after the initial 24-hr exposure to the drug. Their result agreed closely with our Coulter counter ID_{50}. In addition, our studies showed that the inhibitory effect of daunorubicin became stronger as the time incubation increased. This time-dependent dose-response effect can be caused by microsomal enzyme P-450 reductase-dependent formation of free radicals during the metabolism of daunorubicin,[14] and these radicals can precipitate cell damage. Further work, perhaps involving a single "pulse" treatment to cells and long-term treatment at very low concentrations, is needed.

Mithramycin binds preferentially to guanine–cytosine base pairs, inhibiting transcription of poly(dG)-poly(dC).[15] At higher concentrations (10^{-4} M) mithramycin not only inhibits DNA-dependent RNA synthesis but causes single-strand breaks in the DNA.[15] Moreover, rate and extent of mithramycin intercalation into DNA base pairs is dependent on the Mg^{2+} concentration.[16] With exposure times of 24 hr, in vitro studies on mithramycin using HeLa cells have shown the growth rate of cells decreased to approximately one half the control value at a concentration of 10^{-8} M[15]. This value is about 10–100 times lower than the one shown in our experiment. On the other hand, Gupta[17] demonstrated a 1% colony sur-

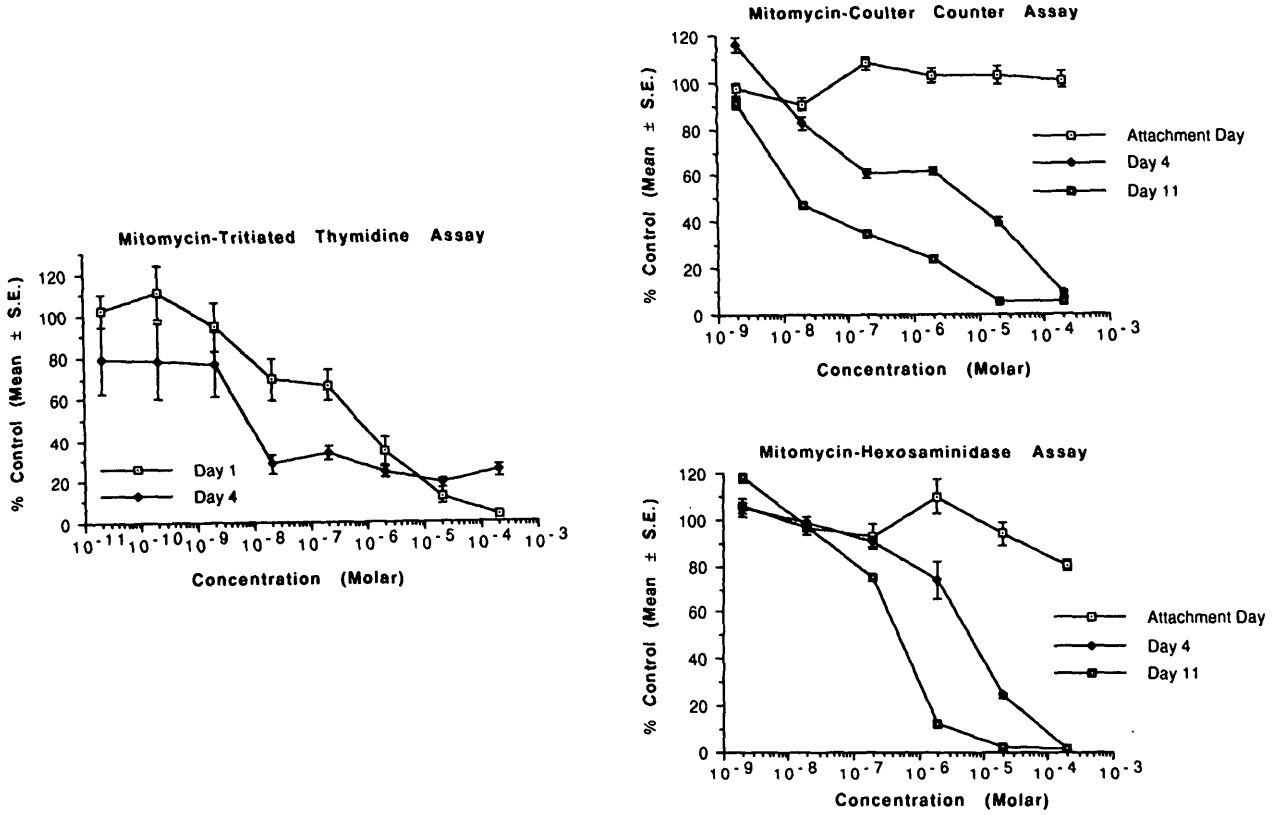

Fig. 4. Semilogarithmic plot of Coulter counter and hexosaminidase assays of human Tenon's fibroblast proliferation at day 4 and day 11 and ^{3}H-thymidine assay at day 1 and day 4 following incubation with media and mitomycin at indicated concentrations.

vival of both HeLa and HSC 172 human fetal fibroblast cell lines when incubated with 0.02 μg/ml mithramycin for 7–10 days. This concurred with our results of near total cell death after 11-day exposure to concentrations higher than 4×10^{-8} M. In our tissue culture experiments, mithramycin had a narrow therapeutic range. It has been reported that mithramycin can cause severe hemorrhagic diathesis

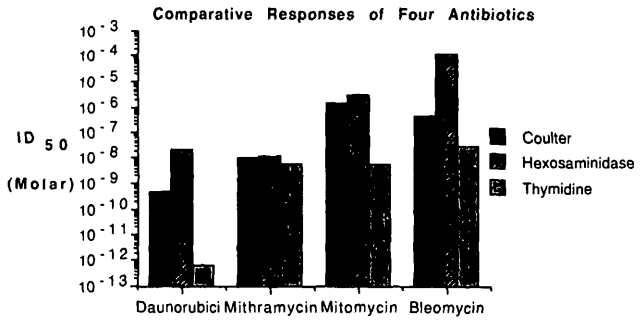

Fig. 5. Effects of mithramycin, mitomycin, daunorubicin, and bleomycin on causing 50% reduction in proliferation of human Tenon's fibroblasts, ID_{50}, via Coulter counter, hexosaminidase, and ^{3}H-thymidine assays. Data represent the means of ID_{50}s obtained from three experiments performed separately with different cell lines and passage numbers at day 3–4 of incubation with the agents.

at doses up to 30 μg/kg/day.[15] Clinical ophthalmic application of this drug would require careful patient monitoring, especially for possible subconjunctival or retinal hemorrhage. Further animal toxicity studies are required.

Mitomycin is most effective in treating hypoxic tumors[10] since it requires metabolic activation through a cytochrome P-450 reductase-mediated reduction. When applied to human foreskin fibroblasts, mitomycin C at concentrations of 0.19–0.66 μg/ml showed no effect on cell survival.[18] By using neutral red dye uptake method, Isomura and associates[19] showed the ID_{50} of mitomycin incubated with HeLa cells to be 0.11 μg/ml, which concurred closely with the ID_{50} we obtained by Coulter counter method on day 7. In our experiment mitomycin did not show any effects on cellular attachment, and it had a more gradual response than mithramycin over the different concentrations and time. This time-dependent effect may be due to the reduction of the drug needed for its activation. The fact that ^{3}H-thymidine assay did not show this time-dependent response suggests that mitomycin may cause a direct inhibition of DNA synthesis in addition to its alkylation and scission of DNA produced by its activation. Since its activity is heightened under hypoxic conditions, mitomycin

Table 1. ID_{50} (with 95% confidence interval) of mithramycin on human ocular fibroblast proliferation as determined by Coulter counting, hexosaminidase, and tritiated thymidine assays

	Coulter counting				*Hexosaminidase*				*Tritiated thymidine*			
Day	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*
0	ND		M	8	ND		M	8		—		
0	ND		M	19	ND		M	19		—		
0	ND		M	12	ND		M	12		—		
1	2.04×10^{-8}	$1.25\text{–}3.31 \times 10^{-8}$	M	8	1.91×10^{-6}	$0.05\text{–}70.4 \times 10^{-6}$	M	8	4.00×10^{-6}	$1.04\text{–}15.4 \times 10^{-6}$	M	8
1	5.87×10^{-7}	$4.42\text{–}7.80 \times 10^{-7}$	M	19	4.80×10^{-6}	$2.51\text{–}9.17 \times 10^{-6}$	M	19	8.34×10^{-7}	$0.80\text{–}88.5 \times 10^{-7}$	G	66
1	2.49×10^{-8}	$2.08\text{–}2.99 \times 10^{-8}$	M	12	5.45×10^{-6}	$3.27\text{–}9.10 \times 10^{-6}$	M	12	4.51×10^{-7}	$1.82\text{–}11.2 \times 10^{-7}$	C	38
2	1.18×10^{-8}	$1.01\text{–}1.37 \times 10^{-8}$	M	8	9.46×10^{-8}	$6.46\text{–}13.8 \times 10^{-8}$	M	8	8.74×10^{-8}	$3.02\text{–}25.3 \times 10^{-8}$	M	8
2	6.13×10^{-9}	$5.06\text{–}7.43 \times 10^{-9}$	M	19	1.40×10^{-8}	$1.13\text{–}1.74 \times 10^{-8}$	M	19	2.6×10^{-10}	$0.08\text{–}92.7 \times 10^{-10}$	G	67
2	9.01×10^{-8}	$8.15\text{–}9.96 \times 10^{-8}$	M	12	1.49×10^{-7}	$1.06\text{–}2.11 \times 10^{-7}$	M	12	1.30×10^{-8}	$0.95\text{–}1.77 \times 10^{-8}$	C	27
3–4	5.92×10^{-9}	$4.92\text{–}7.13 \times 10^{-9}$	M	8	2.06×10^{-8}	$1.94\text{–}2.20 \times 10^{-8}$	M	8	2.17×10^{-9}	$1.29\text{–}3.66 \times 10^{-9}$	G	67
3–4	4.1×10^{-10}	$3.90\text{–}4.3 \times 10^{-10}$	M	19	1.33×10^{-9}	$1.33\text{–}1.53 \times 10^{-9}$	M	19	1.47×10^{-8}	$1.03\text{–}2.10 \times 10^{-8}$	M	8
3–4	2.45×10^{-8}	$2.25\text{–}2.67 \times 10^{-8}$	M	12	1.74×10^{-8}	$1.37\text{–}2.21 \times 10^{-8}$	M	12	2.9×10^{-10}	$0.6\text{–}13.8 \times 10^{-10}$	C	27
7–8	6.87×10^{-9}	$6.54\text{–}7.23 \times 10^{-9}$	M	8	1.29×10^{-8}	$1.22\text{–}1.37 \times 10^{-8}$	M	8		—		
7–8	2.1×10^{-10}	$2.00\text{–}2.2 \times 10^{-10}$	M	19	2.10×10^{-10}	$1.90\text{–}2.30 \times 10^{-10}$	M	19		—		
7–8	1.01×10^{-8}	$0.96\text{–}1.06 \times 10^{-8}$	M	12	1.38×10^{-8}	$1.19\text{–}1.59 \times 10^{-8}$	M	12		—		
10–11	7.13×10^{-9}	$6.53\text{–}7.79 \times 10^{-9}$	M	8	9.83×10^{-9}	$9.03\text{–}10.7 \times 10^{-9}$	M	8		—		
10–11	1.9×10^{-10}	$1.80\text{–}1.90 \times 10^{-10}$	M	19	1.50×10^{-10}	$1.30\text{–}1.7 \times 10^{-10}$	M	19		—		
10–11	1.07×10^{-8}	$1.05\text{–}1.09 \times 10^{-8}$	M	12	1.12×10^{-8}	$0.99\text{–}1.27 \times 10^{-8}$	M	12		—		

C = a cataract case; G = a glaucoma case; M = a muscle case; ND = not detectable; — = not done.

may be more effective in preventing bleb fibrosis when administered before the vascularization stage of wound healing.

Bleomycins are a glycopeptide antibiotic group which induces DNA cleavage. When systemically applied, there is a dose-related toxicity after bleomycin therapy that causes pulmonary interstitial fibrosis.[10] Unexpectedly, fibroblast proliferation increased when a concentration of 10^{-8} U/ml of bleomycin was added to fibroblast growth factors.[5] The effect of bleomycin on human Tenon's capsule fibroblast was studied by Litin and associates.[5] They found that bleomycin at 200 ng/ml reduced ^{3}H-thymidine uptake to one third of control in human Tenon's capsule cells. Doubling the bleomycin concentration further decreased the percentage of labeled cells to one

Table 2. ID_{50} (with 95% confidence interval) of mitomycin on human ocular fibroblast proliferation as determined by Coulter counting, hexosaminidase, and tritiated thymidine assays

	Coulter counting				*Hexosaminidase*				*Tritiated thymidine*			
Day	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*
0	ND		G	70	ND		G	70		—		
0	ND		C	64	ND		C	64		—		
0	ND		C	58	ND		C	58		—		
1	7.38×10^{-5}	$6.54\text{–}8.34 \times 10^{-5}$	G	70	5.60×10^{-5}	$4.13\text{–}7.59 \times 10^{-5}$	G	70	3.80×10^{-8}	$1.15\text{–}12.5 \times 10^{-8}$	G	67
1	1.01×10^{-4}	$0.88\text{–}1.16 \times 10^{-4}$	C	64	4.36×10^{-5}	$2.18\text{–}8.72 \times 10^{-5}$	C	64	5.62×10^{-6}	$2.27\text{–}13.9 \times 10^{-6}$	M	8
1	3.43×10^{-6}	$2.03\text{–}5.78 \times 10^{-6}$	C	58	4.85×10^{-5}	$3.68\text{–}6.40 \times 10^{-5}$	C	58	5.82×10^{-7}	$1.15\text{–}29.3 \times 10^{-7}$	G	66
2	3.95×10^{-5}	$3.48\text{–}4.50 \times 10^{-5}$	G	70	2.73×10^{-5}	$2.31\text{–}3.24 \times 10^{-5}$	G	70	1.22×10^{-8}	$0.16\text{–}9.05 \times 10^{-8}$	G	67
2	7.06×10^{-6}	$5.69\text{–}8.76 \times 10^{-6}$	C	64	5.47×10^{-6}	$3.56\text{–}8.40 \times 10^{-6}$	C	64	1.18×10^{-8}	$0.09\text{–}14.4 \times 10^{-8}$	M	8
2	1.49×10^{-7}	$1.33\text{–}1.68 \times 10^{-7}$	C	58	2.80×10^{-6}	$2.35\text{–}3.33 \times 10^{-6}$	C	58	4.71×10^{-8}	$0.75\text{–}29.5 \times 10^{-8}$	G	66
3–4	4.42×10^{-6}	$3.62\text{–}5.39 \times 10^{-6}$	G	70	5.85×10^{-6}	$5.00\text{–}6.86 \times 10^{-6}$	G	70	1.00×10^{-9}	$0.02\text{–}68.8 \times 10^{-9}$	G	67
3–4	8.18×10^{-8}	$2.66\text{–}25.1 \times 10^{-8}$	C	64	1.37×10^{-6}	$1.15\text{–}1.68 \times 10^{-6}$	C	64	1.41×10^{-8}	$0.63\text{–}3.18 \times 10^{-8}$	M	8
3–4	1.18×10^{-7}	$1.02\text{–}1.35 \times 10^{-7}$	C	58	1.53×10^{-6}	$1.33\text{–}1.75 \times 10^{-6}$	C	58	4.00×10^{-9}	$0.40\text{–}35.7 \times 10^{-9}$	G	66
7–8	6.73×10^{-8}	$4.99\text{–}9.07 \times 10^{-8}$	G	70	5.72×10^{-7}	$5.24\text{–}6.25 \times 10^{-7}$	G	70		—		
7–8	9.37×10^{-8}	$4.17\text{–}21.0 \times 10^{-8}$	C	64	5.02×10^{-7}	$4.68\text{–}5.39 \times 10^{-7}$	C	64		—		
7–8	1.66×10^{-7}	$1.50\text{–}1.84 \times 10^{-7}$	C	58	1.80×10^{-6}	$1.57\text{–}2.06 \times 10^{-6}$	C	58		—		
10–11	1.45×10^{-8}	$1.23\text{–}1.72 \times 10^{-8}$	G	70	4.89×10^{-7}	$4.60\text{–}5.20 \times 10^{-7}$	G	70		—		
10–11	1.93×10^{-8}	$1.67\text{–}2.23 \times 10^{-8}$	C	64	4.04×10^{-7}	$3.83\text{–}4.27 \times 10^{-7}$	C	64		—		
10–11	1.04×10^{-7}	$0.92\text{–}1.18 \times 10^{-7}$	C	58	1.03×10^{-6}	$0.92\text{–}1.15 \times 10^{-6}$	C	58		—		

C = a cataract case; G = a glaucoma case; M = a muscle case; ND = not detectable; — = not done.

Table 3. ID_{50} (with 95% confidence interval) of daunorubicin on human ocular fibroblast proliferation as determined by Coulter counting, hexosaminidase, and tritiated thymidine assays

	Coulter counting				*Hexosaminidase*				*Tritiated thymidine*			
Day	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*
0	2.24×10^{-8}	$1.85\text{–}2.70 \times 10^{-8}$	G	62	1.58×10^{-6}	$1.43\text{–}1.74 \times 10^{-6}$	G	62		—		
0	2.89×10^{-6}	$2.61\text{–}3.21 \times 10^{-6}$	M	12	1.51×10^{-6}	$1.42\text{–}1.59 \times 10^{-6}$	M	12		—		
0	1.98×10^{-6}	$1.62\text{–}2.42 \times 10^{-6}$	C	64	1.42×10^{-6}	$1.30\text{–}1.57 \times 10^{-6}$	C	64		—		
1	6.59×10^{-7}	$4.97\text{–}8.73 \times 10^{-7}$	G	62	1.27×10^{-6}	$0.99\text{–}1.65 \times 10^{-6}$	G	62	1.97×10^{-9}	$0.16\text{–}23.8 \times 10^{-9}$	M	8
1	1.61×10^{-6}	$1.31\text{–}1.97 \times 10^{-6}$	M	12	1.35×10^{-6}	$1.20\text{–}1.51 \times 10^{-6}$	M	12	6.99×10^{-9}	$1.40\text{–}35.0 \times 10^{-9}$	C	38
1	1.79×10^{-6}	$1.95\text{–}2.11 \times 10^{-6}$	C	64	1.29×10^{-6}	$1.13\text{–}1.48 \times 10^{-6}$	C	64	1.91×10^{-11}	$0.003\text{–}1270 \times 10^{-11}$	G	66
2	5.20×10^{-11}	$2.40\text{–}10.9 \times 10^{-5}$	G	62	1.28×10^{-7}	$0.25\text{–}6.44 \times 10^{-7}$	G	62	2.00×10^{-13}	$0.05\text{–}82.0 \times 10^{-13}$	M	8
2	1.16×10^{-6}	$0.009\text{–}1.50 \times 10^{-6}$	M	12	4.19×10^{-7}	$1.48\text{–}11.8 \times 10^{-7}$	M	12	6.00×10^{-14}	$0.20\text{–}232 \times 10^{-14}$	C	27
2	2.76×10^{-8}	$1.99\text{–}3.81 \times 10^{-8}$	C	64	7.05×10^{-7}	$6.60\text{–}7.53 \times 10^{-7}$	C	64	2.75×10^{-15}	$0.81\text{–}9.35 \times 10^{-15}$	M	15
3–4	6.20×10^{-11}	$3.60\text{–}10.9 \times 10^{-11}$	G	62	7.18×10^{-9}	$6.73\text{–}7.66 \times 10^{-9}$	G	62	1.30×10^{-12}	$0.15\text{–}11.1 \times 10^{-12}$	M	8
3–4	8.90×10^{-10}	$6.60\text{–}12.0 \times 10^{-10}$	M	12	2.62×10^{-8}	$2.42\text{–}2.83 \times 10^{-8}$	M	12	4.00×10^{-15}	$1.00\text{–}10.0 \times 10^{-15}$	C	38
3–4	3.80×10^{-10}	$2.60\text{–}5.50 \times 10^{-10}$	C	64	3.30×10^{-8}	$2.55\text{–}4.26 \times 10^{-8}$	C	64	2.95×10^{-15}	$2.85\text{–}3.05 \times 10^{-15}$	M	15
7–8	5.00×10^{-10}	$0.70\text{–}38.1 \times 10^{-10}$	G	62	2.04×10^{-9}	$1.39\text{–}3.01 \times 10^{-9}$	G	62		—		
7–8	2.63×10^{-9}	$1.93\text{–}3.58 \times 10^{-9}$	M	12	1.29×10^{-8}	$1.22\text{–}1.36 \times 10^{-8}$	M	12		—		
7–8	1.23×10^{-9}	$0.80\text{–}1.90 \times 10^{-9}$	C	64	6.98×10^{-9}	$6.58\text{–}7.40 \times 10^{-9}$	C	64		—		
10–11	9.83×10^{-9}	$4.78\text{–}20.2 \times 10^{-9}$	G	62	9.63×10^{-11}	$3.02\text{–}30.7 \times 10^{-11}$	G	62		—		
10–11	1.72×10^{-9}	$1.58\text{–}1.88 \times 10^{-9}$	M	12	7.88×10^{-9}	$7.53\text{–}8.25 \times 10^{-9}$	M	12		—		
10–11	6.8×10^{-11}	$3.3\text{–}14.0 \times 10^{-11}$	C	64	7.29×10^{-9}	$6.54\text{–}8.12 \times 10^{-9}$	C	64		—		

C = a cataract case; G = a glaucoma case; M = a muscle case; ND = not detectable; — = not done.

fifth of the control values.[5] Our data supported such toxicity at these low levels. Our Coulter counter data also concurred with data by Fiscella and associates[6] where rabbit corneal fibroblasts were inhibited by 58% with 10 μg/ml. Kay and associates,[20] using a collagen sponge delivery system impregnated with 1.35 mg of bleomycin, were able to increase functional bleb duration in the rabbit up to 50 days subsequent to filtration surgery. Paradoxically, Smith[21] showed that while bleomycin-induced DNA strand breaks in fibroblasts increased with a ferrous iron supply, survival capacity of drug-treated cells also rose. Similar to mitomycin, bleomycin showed no effect on fibroblast cellular attachment and a more gradual dose-related cellular effect over the concentrations tested.

Table 4. ID_{50} (with 95% confidence interval) of bleomycin on human ocular fibroblast proliferation as determined by Coulter counting, hexosaminidase, and tritiated thymidine assays

	Coulter counting				*Hexosaminidase*				*Tritiated thymidine*			
Day	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*	*ID_{50} (molar)*	*95% CI*	*Case*	*Age (years)*
0		ND	M	19		ND	M	19		—		
0		ND	M	13		ND	M	13		—		
0		ND	G	22		ND	G	22		—		
1	8.66×10^{-5}	$5.94\text{–}12.6 \times 10^{-5}$	M	15	2.02×10^{-5}	$0.99\text{–}4.16 \times 10^{-5}$	M	19	5.69×10^{-8} M	$0.08\text{–}426 \times 10^{-8}$	G	66
1	4.03×10^{-6}	$1.58\text{–}10.2 \times 10^{-6}$	M	13	1.13×10^{-4}	$0.79\text{–}1.63 \times 10^{-4}$	M	15	1.12×10^{-8} M	$0.007\text{–}183 \times 10^{-8}$	G	66
1	1.50×10^{-4}	$0.44\text{–}5.60 \times 10^{-4}$	G	22	2.30×10^{-4}	$2.10\text{–}2.60 \times 10^{-4}$	G	22	9.97×10^{-7} M	$0.40\text{–}251 \times 10^{-7}$	C	38
2	9.27×10^{-8}	$7.21\text{–}11.9 \times 10^{-8}$	M	19	8.77×10^{-6}	$5.56\text{–}13.7 \times 10^{-6}$	M	19	5.35×10^{-8} M	$1.18\text{–}24.3 \times 10^{-8}$	M	8
2	5.10×10^{-8}	$1.82\text{–}14.3 \times 10^{-8}$	G	67	1.60×10^{-4}	$1.02\text{–}2.51 \times 10^{-4}$	G	77	5.60×10^{-9} M	$1.00\text{–}30.9 \times 10^{-9}$	G	66
2	8.79×10^{-8}	$0.63\text{–}124 \times 10^{-8}$	M	8	4.28×10^{-5}	$1.25\text{–}14.7 \times 10^{-5}$	M	8	3.90×10^{-9} M	$0.20\text{–}87.6 \times 10^{-9}$	C	27
3–4	1.12×10^{-7}	$0.98\text{–}1.27 \times 10^{-7}$	M	19	2.53×10^{-5}	$1.99\text{–}3.20 \times 10^{-5}$	M	19	7.31×10^{-8} M	$1.25\text{–}42.9 \times 10^{-8}$	M	8
3–4	9.46×10^{-8}	$4.12\text{–}21.8 \times 10^{-8}$	G	67	2.16×10^{-4}	$1.83\text{–}2.54 \times 10^{-4}$	G	77	2.50×10^{-9} M	$0.30\text{–}20.9 \times 10^{-9}$	G	66
3–4	1.14×10^{-6}	$0.02\text{–}62.0 \times 10^{-6}$	M	8	1.31×10^{-4}	$0.65\text{–}2.65 \times 10^{-4}$	M	8	1.33×10^{-8} M	$0.03\text{–}67.4 \times 10^{-8}$	C	27
7–8	2.44×10^{-7}	$2.07\text{–}2.87 \times 10^{-7}$	M	19	5.14×10^{-5}	$4.85\text{–}5.46 \times 10^{-5}$	M	19		—		
7–8	2.25×10^{-5}	$1.44\text{–}3.53 \times 10^{-5}$	G	67	2.51×10^{-5}	$2.12\text{–}2.98 \times 10^{-5}$	G	66		—		
7–8	3.06×10^{-5}	$2.04\text{–}4.60 \times 10^{-5}$	M	8	4.01×10^{-5}	$2.85\text{–}5.65 \times 10^{-5}$	M	8		—		
10–11	1.55×10^{-7}	$1.40\text{–}1.71 \times 10^{-7}$	M	19	2.79×10^{-5}	$2.40\text{–}3.23 \times 10^{-5}$	M	19		—		
10–11	5.18×10^{-7}	$0.20\text{–}134 \times 10^{-7}$	G	67	7.19×10^{-5}	$5.88\text{–}8.79 \times 10^{-5}$	G	77		—		
10–11	2.71×10^{-5}	$1.51\text{–}4.86 \times 10^{-5}$	M	8	7.05×10^{-5}	$5.17\text{–}9.61 \times 10^{-5}$	M	8		—		

C = a cataract case; G = a glaucoma case; M = a muscle case; ND = not detectable; — = not done.

The Coulter counter and hexosaminidase assays consistently presented higher ID_{50} values compared with the ^{3}H-thymidine assay. The Coulter counter assay is a direct reflection of actual cell numbers, while the hexosaminidase assay reflects the amount of the intracellular enzyme hexosaminidase present in the treated cell population. Therefore, the hexosaminidase assay is an indicator of cell numbers provided that the enzyme activity is unaltered by experimental drugs and is equally distributed throughout the entire cell population. Since DNA synthesis measured by the ^{3}H-thymidine assay precedes protein synthesis and cell division, we would expect that less time is required to observe inhibition of DNA synthesis than for protein synthesis and cellular division. In this experiment, we employed the Coulter counter as an indirect measure of cytocidal effect and ^{3}H-thymidine as an indirect measure of cytostatic and cytocidal effects of drugs. Although the toxicity of daunorubicin is the highest among the drugs we tested, it probably is the most effective drug in its ability to achieve the inhibition of cell proliferation. There was a large discrepancy between ID_{50} obtained from Coulter counter and ^{3}H-thymidine assays which may indicate a high cytostatic effect.

Wound healing is a complex multifactorial process which cannot be completely and accurately reproduced in an in vitro environment. However, these experiments provide important data on the effects of these antineoplastic agents on human subconjunctival fibroblasts under tissue culture conditions. An "ideal" drug would inhibit fibroblast proliferation and not be toxic to other ocular tissue. The results provide a starting point for future experimentation with these drugs and their potential clinical utility.

Key words: daunorubicin, bleomycin, mithramycin, mitomycin, fibroblasts

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