Ocular Cicatricial Disease

Drug Effects in Vitro on Cell Proliferation, Contraction, and Viability

T. D. Heath,* C. S. Brown,* and W. H. Stern†

Fluoroorotate, tunicamycin, and actinomycin D exhibit optimal effects on cell contractility if cells are exposed to the drug for 72 hr, followed by 24 hr of exposure during the contractility assay. Under these conditions, fluoroorotate and tunicamycin inhibit contractility at concentrations very similar to those required to inhibit proliferation, and at higher concentrations affect cell viability. In contrast, the concentrations at which actinomycin D inhibits cell contractility and viability are very similar, and the concentration at which it inhibits cell proliferation is much lower. These results suggest that fluoroorotate and tunicamycin inhibit cell contractility by inhibiting membrane protein glycosylation. Actinomycin D, which inhibits RNA synthesis, appears to block cell contractility only by blocking cell viability, and its most potent effect inhibits only cell proliferation. Daunomycin exhibits very similar effects on cell contractility if cells are exposed to drug for 48 or 72 hr prior to the assessment of contractility, and its effects are not appreciably increased by the further inclusion of the drug for 24 hr during the contractility assay. Daunomycin also has appreciable effects on contractility if cells are exposed to the drug only for the 24 hr of the contractility assay. Similar to actinomycin D, daunomycin inhibits cell contractility and viability at similar concentrations, and inhibits cell proliferation at much lower concentrations. Moreover, daunomycin can appreciably inhibit cell viability in 24 hr of exposure. Therefore, daunomycin appears only to block cell contractility by blocking cell viability, and its most potent effect inhibits only cell proliferation. Invest Ophthalmol Vis Sci 31:1245-1251, 1990

There is considerable interest in the use of chemotherapy as an adjunct to the surgical treatment of proliferative vitreoretinopathy (PVR) and glaucoma. Clinical studies of retinal detachment in patients with PVR have indicated the desirability of ocular chemotherapy to control the proliferation and contraction of cells on tissue surfaces within the eye. Clinical studies of patients who have undergone glaucoma filtration surgery have shown that ocular chemotherapy can enhance the success of filtration surgery.

Experimental studies have demonstrated the ability of cultured cells to cause traction retinal detachment after injection into the vitreous or onto the retina, and thereby have established animal models for PVR. These model systems have been used to screen potential inhibitors of traction retinal detachment. The time required for in vivo evaluation has prompted investigators to use more rapid in vitro assays to screen potential agents. These assays attempt to establish whether a potential therapeutic agent can inhibit certain cell functions. The cell functions that have been considered relevant by investigators include cell proliferation, cell contractility, and collagen synthesis.

Previously, we have demonstrated that the RNA-directed fluoropyrimidines inhibit both cell contraction and proliferation. In the current study, we explore the relationship between inhibition of cell proliferation and contractility more fully, and examine how the effects of drugs on cell viability may also play a role in their efficacy.

Materials and Methods

Fluoroorotate was obtained from Pharmacia (Piscataway, NJ). Tunicamycin, actinomycin D, and daunomycin were obtained from Sigma (St. Louis, MO). Collagen from rat tail ligaments was purchased from Collaborative Research (Boston, MA) as a 2.5-4.0-mg/ml solution in 0.02 M acetic acid, and was used as supplied. Medium, fetal calf serum, antibiotics, and trypsin for cell culture were purchased from Gibco (Grand Island, NY). All other reagents...
were of analytic grade, and were obtained from standard suppliers.

Rabbit dermal cells were obtained as described previously. They were grown in RPMI 1640 medium supplemented with 20% fetal calf serum, 100 units/ml penicillin/streptomycin. The cells were used for no more than five passages before being discarded. When large numbers of cells were available, samples were frozen at passage 2 or 3, and were thawed and grown as needed. For measurement of growth inhibition, cells were plated at 2 × 10^4 per well in 24-well plates, with 1 ml medium per well. After overnight incubation, triplicate wells were treated with drug at the concentrations shown in Results. After 72 hr of growth, cells were washed, trypsinized, and counted in a Coulter counter (model ZM; Coulter, Hialeah, FL). Percentage growth was determined and plotted against the log_{10} of the drug concentration.

Cell contractility was determined by the method of van Bockxmeer and Martin with some modifications. Cells were plated at 1.5 × 10^5 per ml, 2 ml per well in 6-well plates, and were allowed to grow for 24 hr before treatment. Single wells were treated with the specified drug concentration for 24–72 hr. The medium, which contained any cells that had become detached from the plate, was aspirated from the well and transferred to a 15-ml centrifuge tube. The cells were centrifuged to eliminate the drug-containing medium, and resuspended in 2 ml growth medium. The adherent cells then were trypsinized and added to the contents of the centrifuge tube. The combined cell population was counted in a Coulter counter, and a portion of the cell suspension containing 1.34 × 10^5 cells was transferred to a fresh 15-ml centrifuge tube. The cells were pelleted, the supernatant was aspirated, and the cells were resuspended in 0.5 ml collagen suspension. Portions of 150 μl (six replicates per drug concentration) were added to the center wells of an ice-chilled 96-well plate that was not treated for cell culture (Flow Labs, McLean, VA). Controls included samples that contained untreated cells (100% contraction) and samples that did not contain cells (0% contraction). After all samples had been added, the plate was allowed to gel at 37°C for 15 min. A 100-μl volume of standard growth medium was layered over the gels. The plate was then incubated for 18 hr at 37°C, and the gels were released from the edge of the wells with a 22-gauge needle. After an additional 6 hr of incubation at 37°C, the gels were removed from the plate and weighed. The percentage of contraction inhibition was determined from the weights of the sample gels, contracted control gels, and uncontracted control gels as described previously. The means were plotted against the log_{10} of the drug concentration. For all drugs except tunicamycin, concentration was expressed in moles per liter. For tunicamycin, the concentration was expressed as micrograms per milliliter, because tunicamycin is a complex mixture of different components whose molecular weights vary between 300 and 700 D.

For viability determination, 0.1–0.2-ml portions of cells were mixed with an equal volume of 0.2% trypan blue and allowed to stand for 10 min at room temperature. The cells were then placed in a modified Neubauer counting chamber, and viable and nonviable cells were determined at 100× magnification under a phase-contrast microscope (Nikon Labophot, Garden City, NJ).

Results

In previous studies, we have exposed cells to drug for up to 72 hr prior to measuring the contractility of the cells in the absence of the drug. This protocol may allow the cells to recover partly from the effects of the drug while contractility is measured. Figure 1 shows the inhibitory effects of fluoroorotic acid on cell contraction with 72 hr of exposure, either with or without drug added back during the 24-hr contractility measurement. The inhibition that occurred after 72 hr of exposure was very similar to what we saw...
previously.\textsuperscript{14} Partial inhibition of contractility occurred at concentrations as low as 3 $\mu$M, and complete inhibition was seen at 100 $\mu$M. Exposure for the additional 24 hr of the contraction assay had no effect on the minimum concentration at which contraction inhibition occurred, and reduced the concentration at which complete inhibition of contraction occurred by 10-fold. This suggests that a partial recovery can occur if fluoroorotic acid is removed prior to measuring the contractility of the cells.

Figure 2 shows the inhibition of cell growth, contractility, and viability by fluoroorotic acid. Inhibition of growth and contraction both occur at drug concentrations between 0.3 and 10 $\mu$M. The midpoints of the curves occur at 0.9 $\mu$M for growth, and 2.3 $\mu$M for contraction. In contrast, cell viability is only minimally inhibited by fluoroorotic acid after either 72 or 96 hr of exposure. This suggests that the inhibition of growth and contraction by fluoroorotic acid occurs primarily through metabolic effects that do not inhibit cell viability.

Figure 3 shows the effects of tunicamycin on cell contractility. Cells, whose contractility was assessed in the absence of drug after exposure to tunicamycin for 24–72 hr, were partly inhibited at concentrations between 0.3 and 3 $\mu$g/ml tunicamycin. The potency of tunicamycin was marginally increased as exposure length was increased from 24–72 hr, but not greatly so. In contrast, the potency of tunicamycin was increased 10-fold by its presence during the measurement of contractility after 72 hr of prior exposure. This suggests that tunicamycin, like fluoroorotic acid, reversibly inhibits cell contractility, and that the cells partly recover if the drug is not present during the contractility assay. Exposure of cells to tunicamycin for 24 hr prior to contractility measurement and the presence of tunicamycin during the measurement of cell contractility created an effect that is 2.5 times greater than the effect obtained by exposing the cells to tunicamycin for 48 hr prior to contractility measurement. This rules out the possibility that the increased inhibition created by adding the drug back is caused simply by the increase in exposure length. If tunicamycin was present only during the contractility assay, inhibition of cell contractility required concentrations 10-fold higher than those required if the cells were treated with the drug for 24 hr prior to contractility measurement. This suggests that prior exposure is essential for full expression of tunicamycin-mediated inhibition of cell contractility.

Figure 4 shows the inhibition of cell growth, contractility, and viability by tunicamycin. Growth and contractility were affected very similarly by tunicamycin. The midpoint of the curves is 0.14 $\mu$g/ml for growth and 0.16 $\mu$g/ml for contractility. Cell viability is affected by tunicamycin at higher concentrations, and the extent of the effects varies considerably between 72 and 96 hr of exposure. The midpoint of the viability curves occurs at a concentration that is between three and ten times higher than the concentration required to half-inhibit growth or contractility. This suggests that the inhibition of growth and contraction by tunicamycin occurs primarily through metabolic effects that do not inhibit cell viability.

Figure 5 shows the inhibition of cell contractility by actinomycin D. Cells, whose contractility was assessed in the absence of drug after exposure to acti-
Drug Concentration (μg/ml)

![Graph showing drug concentration vs. growth, contraction, or viability.]

**Fig. 4.** The effects of tunicamycin on cell proliferation, contraction, and viability. Open circles, proliferation during 72 hr of exposure; filled circles, contraction after 72 hr of exposure, with drug present during contractility measurement; viability after 72 hr (filled triangles) or 96 hr (open triangles) of exposure.

Actinomycin D for 24–72 hr, showed a partial inhibition of their contractility at concentrations between 0.01 and 0.03 μM actinomycin D. The potency of actinomycin D was the same for exposure lengths between 24 and 72 hr. In contrast, the potency of actinomycin D was increased 10-fold by its presence during the measurement of contractility. This suggests that actinomycin D, like fluoroorotic acid and tunicamycin, reversibly inhibits contractility, and that the cells partly recover if the drug is not present during the contractility assay. Exposing the cells to actinomycin D for 24 hr prior to contractility measurement and the presence of actinomycin D during the measurement of cell contractility inhibit contractility more than does the exposure of the cells to actinomycin D for 48 hr prior to contractility measurement. This rules out the possibility that the increased inhibition created by adding the drug back is caused simply by the increase in exposure length. If actinomycin D is present only during the contractility assay, inhibition of cell contractility requires concentrations that are 10-fold higher than those required if the cells are treated with the drug for 24 hr prior to contractility measurement. This suggests that prior exposure is essential for full expression of actinomycin D effects.

Figure 6 shows the effects of actinomycin D on cell growth, contraction, and viability. Growth inhibition occurred at concentrations of between 0.0001 and 0.0003 μM actinomycin D. This drug is a very potent growth inhibitor, and the midpoint of the growth curve occurred at 0.00018 μM. In contrast, actinomycin D inhibited cell contractility and viability at much higher concentrations. The extent of actinomycin D inhibition of cell viability was much less after 72 hr of exposure than after 96 hr of exposure. Consequently, the contractility and 96-hr viability curves are virtually identical, whereas the inhibition of viability in 72 hr of exposure required concentrations that are 10-fold higher. These results suggest that actinomycin D inhibition of cell growth occurs primarily through metabolic effects that do not inhibit cell viability or contraction. The inhibition of contraction may be caused by the same metabolic effects that ultimately lead to cell death, or may possibly be caused by the loss of cell viability itself.

Figure 7 shows the inhibition of cell contractility by daunomycin. Cells, whose contractility was assessed in the absence of drug after exposure to daunomycin for 24–72 hr, were partly inhibited at concentrations of between 0.3 and 3 μM daunomycin. The potency of daunomycin increased by nearly 3-fold as the exposure length was increased from 24 to 72 hr. The potency of daunomycin was not increased by its presence during the measurement of contractility. This suggests that the inhibition of contractility by daunomycin is not reversed if the drug is removed during the contractility assay. When daunomycin was present only during the contractility assay, contraction was inhibited almost as much as it was when cells were exposed for 24 hr prior to measurement of contractility. This suggests that expression of daunomycin inhibition of contractility can be very rapid, occurring within hours of incubation with cells.

Figure 8 shows the effects of daunomycin on cell growth, contraction, and viability. Growth inhibition occurred at concentrations of between 0.003 and 0.03 μM daunomycin. These results suggest that the expression of daunomycin inhibition of contractility is rapid, occurring within hours of incubation with cells.
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Fig. 6. The effects of actinomycin D on cell proliferation, contraction, and viability. Open triangles, proliferation during 72 hr of exposure; filled triangles, contraction after 72 hr of exposure, with drug present during contractility measurement; viability after 72 hr (open squares), or 96 hr (filled squares) of exposure.

μM daunomycin, with the midpoint of the growth curve occurring at 0.014 μM. Like actinomycin D, daunomycin inhibited cell contractility and viability at much higher concentrations. The extent of daunomycin inhibition of cell viability was less after 72 hr of exposure than it was after 96 hr of exposure. Unlike actinomycin D, the contractility curve lay between the 72-hr and the 96-hr viability curves. These results suggest that daunomycin inhibition of cell growth occurs primarily through metabolic effects that do not inhibit cell viability or contraction. The inhibition of contraction may be caused by the same metabolic effects that ultimately lead to cell death, or may possibly be caused by the loss of cell viability itself. The very rapid expression of daunomycin effects on contractility prompted us to examine the effects of daunomycin on cell viability in 24 hr of exposure (Fig. 8). Viability was affected in a manner that was very similar to the effects that were seen on cell contractility. This suggests that very rapid inhibition of cell contractility by daunomycin is also associated with daunomycin effects on cell viability.

Discussion

The RNA-directed fluoropyrimidines, fluoroorotic acid, fluorouracil, and fluorouridine all have been shown to have effects on cell contractility, whereas fluorodeoxyuridine, a fluoropyrimidine that is incorporated into the deoxynucleotide pools, does not inhibit cell contractility.13 The RNA-directed fluoropyrimidines are also known to be effective postsurgical adjuncts for experimental PVR. RNA-directed fluoropyrimidines all are known to be incorporated into cells by metabolism to fluorouridine monophosphate, the form in which they are subsequently incorporated into RNA. RNA incorporation of fluoropyrimidines alters the function of ribosomal and messenger RNA species.19,20 Fluorouridine monophosphate is also converted to fluoruridine diphosphate (FUDP) glucose, which interferes with the synthesis of membrane glycoproteins.21 Therefore, there are at least two possible mechanisms by which fluoropyrimidines may inhibit cell contractility. In order to determine if either of these mechanisms can affect cell contractility, and to distinguish which of these two mechanisms is more likely, we have compared fluoroorotic acid to tunicamycin and actinomycin D. Tunicamycin is known to inhibit protein glycosylation, and actinomycin D is known to inhibit RNA synthesis.

The comparison of these three drugs suggests that fluoroorotic acid is more similar to tunicamycin than to actinomycin D. Tunicamycin inhibits cell growth and contractility at very similar concentrations, which are lower than the concentrations required for inhibition of cell viability. This suggests that the most potent metabolic effect of tunicamycin, the inhibition of membrane protein glycosylation, inhibits both cell proliferation and contraction. In contrast, actinomycin D inhibits cell growth at a much lower concentration than is required to inhibit either cell contraction or viability. This suggests that the most potent metabolic effect of actinomycin D, the inhibition of RNA synthesis, does not inhibit cell contraction. Moreover, inhibition of cell viability may be the mechanism by which this drug inhibits cell contraction. Fluoroorotic acid inhibits both proliferation and ...
contractility at concentrations that do not have appreciable effects on cell viability. This suggests that the most potent metabolic effect of fluoroorotic acid does not inhibit cell viability. Growth inhibition occurs at concentrations that are slightly lower than those required for contractility inhibition. Therefore, it seems likely that RNA effects may occur at slightly lower concentrations, whereas membrane protein glycosylation effects occur at slightly higher concentrations. We conclude that fluoroorotate inhibits cell contractility by inhibition of membrane protein glycosylation.

A number of investigators have attempted to correlate the in vitro inhibition of cell contractility with the possible efficacy of drugs for treatment of ocular cicatricial diseases. Fluorouracil, fluorouridine, and fluoroorotate have all demonstrated the ability to inhibit cell contractility, and are to some extent effective for treatment of experimental PVR. The experiments described here show that the effects that fluoroorotate has on cell contractility occur at similar concentrations to those that affect growth, and that cell viability is affected only to a limited extent by these concentrations. In treating ocular cicatricial diseases, this limited effect may allow the inhibition of retinal detachment without causing major ocular toxicity. However, it may also mean that the effects of the drug are short-lived if the cells recover their full contractile potency after the drug has been eliminated from the eye.

Daunomycin has been studied extensively as a potential treatment for PVR, although its ability to inhibit cell contractility has not been explored before now. We have demonstrated here that daunomycin, like most other agents used for PVR treatment, inhibits the contractility of cells. There are a number of features of daunomycin that should be noted. First, it has the capability of very rapid inhibition of cell contractility, which is a useful property for PVR treatment. Intravitreally injected drugs have very short residence times in the eye, and must act rapidly to be effective. Wiedemann et al have noted that daunomycin very rapidly inhibits cell growth, and have devised clinical protocols that expose the eye to daunomycin for only short periods of time. Second, daunomycin kills cells at the concentrations required for inhibition of contractility. This is true even with short exposure times, and is potentially advantageous, because the killing of the cells that cause retinal detachment will inhibit the disease irreversibly. However, it is also potentially disadvantageous, because a concentration that kills cells that cause retinal detachment may also kill sensitive tissues in the eye. Issues such as these can only be resolved using in vivo experiments, because it is difficult to predict the sensitivity of the different ocular tissues, many of which do not divide, using in vitro cell proliferation studies.

Actinomycin D has been examined as a treatment for PVR, although not outside of experimental studies, since it is toxic at concentrations lower than those required for inhibition of retinal detachment. The observations that we have made here may demonstrate why this drug is not suitable for the treatment of PVR. The inhibition of cell contractility by this drug requires a drug concentration that kills cells. Moreover, the manifestation of the anticontractile effects requires the exposure of the cells to actinomycin D for several days. Although daunomycin also kills cells at the concentrations required for inhibition of contractility, it inhibits cell contractility very rapidly.

It is clearly very difficult to predict from in vitro studies what toxic effects will occur in vivo. Nonetheless, these in vitro studies do highlight some of the major features that affect the possible use of these drugs for treatment of the disease, and may be useful in predicting which compounds will be effective, what kind of possible toxicity problems may arise from their use, and how these problems may be overcome.

Key words: Proliferative vitreoretinopathy, fluoropyrimidine, daunomycin, actinomycin D, tunicamycin

References


