Evaluation of Antiproliferative Agents
Using a Cell-Culture Model


Chinese hamster ovarian (CHO) cells in culture were used to evaluate the relative antiproliferative potential of drugs. These agents have been used to improve the clinical response after glaucoma filtering surgery. The following drugs were evaluated: S-fluorouracil (5-FU) as the benchmark, 5-fluorouridine (FUR), 5-fluorodeoxyuridine (FUDR), 5'-deoxy-5-fluorouridine (DFUR), bleomycin, and cytarabine (ARA-C). In addition to cell counting, a colorimetric assay based on the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to follow growth response. The MTT assay was found to be extremely convenient and an indirect measure of cell activity, offering an alternate or addition to a measure of cell number. All agents tested were shown to inhibit cellular proliferation. Dose-response curves for each agent indicate the following absolute potency: FUDR > FUR > ARA-C > 5-FU = bleomycin > DFUR. Besides absolute potency, an evaluation of the effects of equivalent inhibitory concentrations of each drug on growth rate was assessed. Several agents affected the proliferation rate patterns differently. Based on these studies, it is suggested that the in vitro model can identify potential agents through an assessment of their overall activity profile in CHO cells, which includes not only their potency based on dose response, but their onset of activity, duration of effect, and potential for toxicity. Invest Ophthalmol Vis Sci 31:2572-2578, 1990

Several different approaches have been used to improve the success rates in glaucoma filtering surgery (GFS) in patients with a poor prognosis. Perhaps the most promising area of research involves pharmacologic alteration of the cellular response in scar formation. Although investigation of the effectiveness of corticosteroids, D-penicillamine, and β-amino-propionitrile as adjuncts to GFS has been reported, most research at present is focused on the evaluation of antiproliferative agents. The agent most closely studied is 5-fluorouracil (5-FU), which acts primarily as an S-phase specific agent. Recent studies show that subconjunctival injections of 5-FU promote bleb longevity in animals and improve the success of filtering operations in patients with poor surgical prognoses. The proposed mechanism of action responsible for the increased success rates is inhibition of fibroblast proliferation and consequent attenuation of postoperative scar formation. Despite encouraging results, subconjunctival injections of 5-FU are associated with numerous disadvantages. Under current treatment recommendations, patients receive once or twice daily subconjunctival injections over 2 weeks. Not only is this frequency of administration inconvenient, but the patient also is uncomfortable with each injection. In addition, the injections are associated with corneal epithelial defects in approximately 45% of cases and wound or needle tract leaks in approximately 41% of cases.

Approaches to improve the clinical response to treatment with antiproliferative agents include more effective agents and better ways to deliver them in terms of dosing schedules and novel drug-delivery systems. In exploring these possibilities for application to GFS, an in vitro model was sought to screen drug candidates with respect to physiologic properties to provide important information in identifying more efficient compounds and appropriate protocols to be used in their clinical evaluation. Also, since the design of a drug-delivery system is dependent on its biologic profile, knowledge of a drug's activity in terms of onset of activity, duration of action, and potential for toxicity is critical.

We focused on the development of an in vitro model, based on Chinese hamster ovarian (CHO) cells grown in culture to assess the cellular response to antiproliferative agents. These cells were chosen because they are easy to grow in a monolayer and represent a homogeneous, rapidly proliferating, continuous cell line which is commercially available. Their use thus could lead to a highly reproducible protocol, which is essential in comparative drug screening. Our purpose was not to simulate in vivo fibroblast overproliferation, but to develop an in vitro model which identifies compounds as having antiproliferative properties and provides additional information with which to compare several agents. The antiprolifera-

From the Department of Ophthalmology and College of Pharmacy, Ohio State University, Columbus, Ohio.
Supported by the Ohio Lions Eye Research Foundation.
Submitted for publication: November 9, 1989; accepted May 15, 1990.
Reprint requests: P. A. Weber, Department of Ophthalmology, The Ohio State University Hospitals, 5156 Hospital Clinics, 456 West 10th Avenue, Columbus, OH 43210.
ative compounds examined included: 5-FU as the benchmark for comparison, 5-fluorouridine (FUR), 5-fluorodeoxyuridine (FUDR), 5'-deoxy-5-fluorouridine (DFUR), bleomycin sulfate, and cytarabine (ARA-C). The compounds are believed to have different modes of action which offered the possibility for differentiation using the method described: 5-FU and its metabolites inhibit DNA synthesis through inhibition of thymidylate synthetase, bleomycin sulfate causes scission of single- and double-stranded DNA, and ARA-C is converted to ARA-C triphosphate, a potent inhibitor of DNA polymerase.

Materials and Methods

The Ohio State University Cell Culture Service provided CHO, Eagle's minimal essential medium, bovine calf serum, penicillin, streptomycin, amphotericin B, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Isotone (Coulter Electronics Inc., Hialeah, FL) and spectrophotometric-grade dimethylsulfoxide (DMSO). The 5-FU was purchased from Sigma (St. Louis, MO), ARA-C from Quad Pharmaceuticals (Indianapolis, IN), and bleomycin sulfate from Mead Johnson Oncology Products (Bethel Park, PA). The 5-FU, FUDR, and DUFR were provided by Hoffman-La-Roche (Nutley, NJ). The analytic equipment consisted of a Coulter counter (Model ZBI, Coulter) and a multiwell scanning spectrophotometer (Model MR700; Dynatech Laboratories, Chantilly, VA).

The CHO cells were maintained by incubation at 37°C in 90% humidity and 5% CO₂. The cells were grown in a modified Eagle's minimal essential medium that was supplemented with 10% bovine calf serum. Penicillin, streptomycin, and amphotericin B were added to protect against microbial contamination.

Correlation of Assay Techniques

Cell response was determined with a Coulter counter and by colorimetric assay using MTT.

The following procedures were used. The CHO cells were removed enzymatically from T25 flasks using a 15-min incubation with 0.01% trypsin. Next they were diluted to appropriate concentrations using a Coulter counter and then plated into 24-well tissue-culture plates. Growth curves were generated to establish appropriate inoculation densities to achieve cell counts ranging from 10,000–300,000 cells/well after a 24-hr incubation. The experimental design consisted of using eight wells per inoculation concentration: four wells were analyzed with the Coulter counter and four wells by the MTT assay. The Coulter counter assay involved: (1) rinsing the cell wells with phosphate-buffered saline (PBS), (2) disso-
every 24 hr. Four control wells and four test wells were assayed by both the MTT assay and the Coulter counter assay every 12–24 hr depending on the growth rate. Total incubation periods ranged from 84 (control wells)–288 hr (FUR wells) depending on the antiproliferative agent. The six agents listed previously were used in both experimental designs. Onset of activity was assessed by estimating the time at which the curve for the drug-treated system deviated from the control curve. The duration of effect was estimated by noting the time at which the slope of the proliferation curve increased after removal of the drug. A potential for toxicity was judged through a determination of the point at which the slope of the proliferation curve decreased after continuous drug treatment.

Results

Correlation of Assay Techniques

Figure 1 summarizes the results that compare the Coulter counter (x-axis) and MTT (y-axis) methods of assessing cell population. Each point represents one of 12 different initial CHO cell inoculations that resulted in cell counts ranging from 10,000–300,000 cells/well after 24 hr. At each population density or data point represented, eight wells were analyzed: four by Coulter counting and four by the MTT procedure. The length of the bars around the average in the x- and y-directions represents the error in the respective methods.

Dose-Response Studies

Cell count or MTT absorbance in the presence of varying amounts of the six drugs studied relative to controls without drug was determined and plotted as a function of the logarithm of the drug concentration added. Figure 2 gives the results. Solid lines are the results from the Coulter counter and broken lines those from the MTT assay. The error bars represent the error about the mean percent of control for four replicates. Table 1 lists the average IC50 values for the three individual runs and the standard error found.

Drug Effect Over Time

Our results found in the studies assessing drug effects over time are shown in Figure 3 in which MTT optical densities of two experimental groups (continuous drug and drug flushed at 72 hr) is compared with the control group (no drug). In each panel (representing a different drug), the upper curve represents the control system where no drug was added, the middle curve represents the system where drug was added to the culture at its IC50 concentration but then flushed and replaced with drug-free medium, and the lower curve represents systems where the cells are exposed to drug at its IC90 concentration for the entire time. These plots were compared in a semiquantitative way to identify trends in the time-action profiles and used to differentiate the agents. Significant differences were not observed in onset of activity between the agents, and all agents were active within 24 hr of drug treatment. The duration of effect for FUR, bleomycin sulfate, ARA-C, 5-FU, and FUDR was 192, 144, 96, 72, and 72 hr, respectively, as reflected by the time when cell response began to increase after drug removal. The estimations of potential for toxicity were 120, 120, 144, 144, and 168 hr for FUR, ARA-C, bleomycin sulfate, FUDR, and 5-FU, respectively, determined as the point at which the slope of the curve decreased with continuous treatment. No decrease in slope was observed for DFUR after continuous drug treatment.

Discussion

Correlation of Assay Techniques

Until reduced by dehydrogenase enzymes in the mitochondria of living cells, MTT is a yellow substrate. Once reduced, a purple compound (MTT formazan) is formed which is insoluble in media but can be dissolved in DMSO and quantified spectrophotometrically. This method for assaying viable cells has been shown to be much more rapid and equally as accurate as other methods such as the hemocytometer, the Coulter counter, incorporation of radioactive nucleotides, and 51Cr labeling of cell proteins. The amount of MTT formazan produced (as the reduced form) is directly proportional to the number of living cells, with many cell lines.
activity (as determined by the MTT method) was found for CHO cells in the range studied (Fig. 1). With cell numbers greater than 300,000 (resulting in optical densities greater than 1.2), the results were erratic, and the absorbance was no longer proportional to the number of cells. The error associated with each method was similar as evidenced through the error bars (Fig. 1) and standard deviation (Table 1). The linearly correlated counting and activity measures may have been fortuitous. The MTT method quantitates cell number by the measured amount of enzyme contained in the cell spectrophotometrically, which may give an indirect measure of metabolism and cell viability and may be a more significant assessment of potency than cell number for a group of compounds like the antiproliferatives.
Dose-Response Studies

Tukey's studentized range test was used to verify the differences in relative potency based on the IC₅₀ determinations. Bleomycin sulfate and 5-FU were found to be equipotent; all other agents tested had different potencies at a level of significance of 0.05. The rank order found was: FUDR > FUR > ARA-C > 5-FU = bleomycin > DFUR. The statistical results were identical for IC₅₀ determinations using both the MTT and Coulter counter assay techniques.

Drug Effect Over Time

Although dose-response curves for numerous antiproliferative agents have been generated in our laboratories and those of others, there are no published reports to our knowledge that use cell-culture techniques to examine the effects of antiproliferative agents over time. This type of analysis can reveal relative differences in onset of activity, duration of effect (reversibility), and potential for toxicity. Each of the six agents studied had its own unique set of cell response versus time curves after treatment with equivalent doses (IC₅₀). Although differences in onset of action were not found, analysis of the results revealed differences in activity in terms of potential reversibility of cell growth inhibition after drug removal and the propensity to apparent toxicity after continuous cell treatment. Based on the estimates of the duration of effect, FUR and bleomycin sulfate produce a cellular inhibition that is twice as long as 5-FU and FUDR, whereas 5-FU and FUDR produce the same degree of inhibition. Although decreasing cell numbers with time after continuous drug treatment may have been due to secondary factors such as nutritional deficiency in the media or conversion of the drug to a more potent metabolite, it seemed reasonable to assume that this represented toxicity and that the relative toxicity may be assessed by noting how soon the growth curve slope became negative. Judging toxicity potential in this way, DFUR was the only agent tested that might be relatively free of toxicity. The other agents showed a potential for toxicity in the order listed (low to high): 5-FU > FUDR > bleomycin sulfate > ARA-C > FUR. These findings are semiquantitative and subjective, but they indicate that equivalent doses of antiproliferative agents do not produce equivalent responses in terms of their activity over time.

Summary

Our procedures represent a simple, reproducible method to evaluate drug activity with rapidly proliferating cells. The reproducibility of the model allows experiment-to-experiment and interlaboratory comparisons of activity. This protocol can be used to screen a series of compounds with respect to differences in their relative activity. The method shows that the overall activity profile of an antiproliferative agent should include not only its potency based on dose response, but its onset of activity, duration of effect, and potential for toxicity determined by time-course studies. The differences noted can be applied to the design of clinical studies by providing equivalent doses and dosing schedules. The studies emphasize that antiproliferative potential based on equivalent doses derived from absolute potency does not necessarily confer therapeutic equivalence. The regimen for each drug also should be optimized before comparisons of clinical response can be made. When considering a controlled drug-delivery system, the significance of these differing properties is even greater, since ultimately it is the absolute potency, duration of effect, and potential for toxicity of the active agent which determines the required delivery rate.
Fig. 3. The relationship between absorbance (cell response) and time under different experimental conditions. In each panel the upper curve represents the control where no drug is added, the middle curve represents the system where drug is added to the culture at its IC90 concentration but then flushed and replaced with drug-free medium, and the lower curve refers to systems where the cells are exposed to drug at its IC90 concentration over the entire time course. Each panel is a different drug: A, 5-FU; B, FUR; C, FUDR; D, bleomycin; E, ARA-C; F, DFUR.

Key words: antiproliferatives, in vitro model, cell culture, CHO cells, potency, growth rate

References
5. Gressel MG, Parrish RK, and Folberg R: 5-Fluorouracil and