Antiviral Research, 9 (1988) 263–272
Elsevier

AVR 00291

An in vitro study of ophthalmic antiviral agent toxicity on rabbit corneal epithelium

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(Received 19 December 1987; accepted 29 March 1988)

Summary

Using an in vitro system we measured the corneal epithelial cytotoxicity and the antiviral activity of the antiviral agents idoxuridine (IDU), trifluridine (TFT), ethyldeoxyuridine (EDU), and (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU). Confluent rabbit corneal epithelial cell cultures were established, and the antiviral agents were added for 5, 30, or 60 min at a range of concentrations including that used clinically (IDU 0.1%, TFT 1.0%, BVDU 0.1%, EDU 2.0%). Twelve hour [^{3}H]thymidine incorporation then was measured and expressed as % inhibition of control cultures. In separate experiments confluent corneal epithelial cell monolayers were inoculated with 10^4 plaque forming units (PFU) of HSV type 1 (McKrae strain) for 1 h, and IDU 0.1%, TFT 1.0%, and BVDU 0.1% were added to the culture for determination of PFU inhibition. Significant dose-, but not time-dependent, toxicity was observed at the clinical concentrations of IDU, TFT, and EDU. Toxicity was absent for BVDU. TFT and IDU were the most toxic, and EDU was of intermediate toxicity. IDU, TFT, and BVDU showed significant antiviral activity in this corneal epithelial cell culture system (TFT$^>$ BVDU$^>$IDU). The results of this in vitro study paralleled the findings of previous in vivo corneal epithelial toxicity studies of IDU, TFT, and BVDU. Our data, however, suggest that EDU has a potential for clinical toxicity and further studies are recommended. Our model may be useful in the future toxicologic study of new antiviral agents.

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Cytotoxicity, in vitro; Corneal epithelium; Antiviral agent

Introduction

More effective, topical and systemic antiviral agents are needed because of the large number of viruses that cause significant ocular disease (Vastine, 1987) and the inability of topical agents to treat deep corneal and uveal infections (Meyer and Kaufman, 1978; Pavan-Langston, 1987). The agents available today, including idoxuridine (IDU), Ara-A, and trifluridine (TFT), have a limited spectrum of disease activity (Meyer and Kaufman, 1978; Pavan-Langston, 1987). In addition, they are associated with considerable toxicity to the normal ocular surface, including punctate keratitis, punctal occlusion, follicular conjunctivitis, and conjunctival cicatrization (Lass et al., 1983; Pavan-Langston and Foster, 1977; Wilson, 1979), due to their non-selective activation in uninfected, host cells (Pavan-Langston, 1987). Topical antiviral agents have been developed more recently which are selectively activated by viral-induced thymidine kinase, sparing the uninfected host cells; these agents include acyclovir, (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU), and ethyldeoxyuridine (EDU) (De Clercq et al., 1979; Fischer and Prusoff, 1983; Gauri and Walter, 1973). Both in experimental or clinical studies these newer agents possess at least equal potency with less or absent toxicity when compared to the other agents (Elze, 1979; Lass et al., 1979; Maudgal et al., 1981; Pavan-Langston, 1987).

Few comparative in vivo or in vitro studies are available for the cytotoxic effects of antiviral agents on the corneal epithelium (Foster and Pavan-Langston, 1977; Hanna, 1966; Langston et al., 1974; Lass et al., 1979, 1984; Pavan-Langston unpublished data 1986; Vastine, 1987). Most pre-clinical studies have utilized animals treated with topical antiviral agent for days to weeks, and the toxic effects and/or delay in experimental wound closure were then assessed in a semi-quantitative manner, using photographic and histologic methods. Although an important part of pre-clinical evaluation, these studies have been qualitative, subjective, and lack standardization.

We report a comparative in vitro analysis of the corneal epithelial cytotoxicity of currently available and investigational topical antiviral solutions on the corneal epithelium. To confirm the antiviral activity of these agents in vitro on corneal epithelial cells and to assure the validity of the cytotoxicity assay, concurrent herpes simplex virus (HSV) reduction inhibition studies also were performed.

Materials and Methods

Cytotoxicity assay

Confluent cultures of rabbit corneal epithelium were prepared from adult New Zealand albino rabbits (2–3 kg) (Botti et al., 1987; Gipson and Grill, 1982; Im-

peria et al., 1986). After an overdose of intravenous pentobarbital, corneas, excluding sclera, were excised near the limbus. The posterior stroma was mechanically removed using forceps. The anterior half, containing the epithelium, was incubated for 1 h at 37°C in Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (Grand Island Biologic Co, Grand Island, NY) containing (final concentration) 1 unit/ml Dispase II (Boehringer Mannheim, Indianapolis, IN) (Botti et al., 1987; Gipson and Grill, 1982; Imperia et al., 1986). The corneal epithelium then was teased off the stroma and dispersed into pieces on 60 cm^2 culture plates (Falcon, Oxnard, CA).

Primary cultures were established and were grown to confluence at 37°C in 5% CO_2, 95% air in complete medium composed of minimal essential medium with d-valine (Gibco) with (final concentration) 10% heat-inactivated calf serum (Sterile Systems, Logan, UT), penicillin 100 units/ml and streptomycin 100 μg/ml (Gibco), 10 units crystalline insulin (Eli Lilly, Indianapolis, IN), epidermal growth factor (Collaborative Research, MA) 20 ng/ml, and cholera toxin (Sigma Chemicals, St. Louis, MO) 0.1 ng/ml (total medium) (Gilbert and Migeon, 1975; Jumblatt and Neufeld, 1983). The latter agent was used to stimulate epithelial cell growth (Jumblatt and Neufeld, 1983). Medium was changed every 48 h. After 10–14 days confluent cultures were incubated at 37°C in DPBS without calcium and magnesium, containing 1 unit/ml Dispase II for 15 min. The Dispase-containing medium was removed and replaced with complete medium. The cells were mechanically removed from the culture plate surface and clumps were dispersed using a flame-polished Pasteur pipette.

Cells were reseeded from the master primary culture plate into a 24-well Primaria tissue culture plate (Falcon) at 0.5–1.5 × 10^4 cells in 0.5 ml complete medium per well. After 36 h in culture, either ophthalmic antiviral solution or complete medium (control) were added. Stock solutions of IDU (Allergan Corporation, Irvine, CA), TFT (Burroughs Wellcome Co., Research Triangle Park, NC), EDU (Ortho Pharmaceutical Corporation, Raritan, NJ), and BVDU (provided by Dr. Erik De Clerq) were prepared immediately prior to use by dissolving each agent in complete medium at initial concentrations of 10% (w/v) for TFT and EDU, 0.2% for IDU, and 0.15% for BVDU prior to further dilution in complete medium. Antiviral or control were added at final culture concentrations of IDU 0.005–0.2%, TFT 0.1 – 1.0%, EDU 0.5 – 2.0%, and BVDU 0.01–0.15%. After 5, 30, or 60 min the medium containing antiviral drug was removed, culture plates were twice washed with DPBS, and 0.5 ml of complete medium, containing 1 microcurie of sterile [^{3}H]thymidine 6.7 mCi/mmol (New England Nuclear, Boston, MA), was added to each well. After 12 h of incubation, DNA was extracted (Martin et al., 1981). Samples were solubilized in Aquasol (New England Nuclear) and counted in a liquid scintillation counter (Hewlett Packard, Corvallis, OR).

Antiviral efficacy testing

Confluent corneal epithelial cell monolayers (as described above) were inoculated with 10^4 plaque forming units McKrae Strain HSV-1 by adsorption for 1 h at 37°C in 5% CO_2, and 95% air. Then, the inoculum was aspirated and com-

plete media containing each antiviral agent (IDU 0.1%, TFT 1.0%, and BVDU 0.1%) were added. The most (IDU and TFT) and least toxic (BVDU) antiviral agents in our cytotoxicity assay were chosen for examination of antiviral activity in this same system. Cultures were incubated for 48 h at 37°C in 5% CO_2 and 95% air in medium containing the antiviral agent. Cultures were monitored each day and at the end of 48 h, cultures were frozen at −20°C. The monolayer cells were disrupted by freeze-thawing (3 cycles) and the cells were centrifuged at 1500 × *g* to pellet cell debris. Serial dilutions (10^0–10^{-6}) of each antiviral-inhibited culture were plated onto confluent Vero cell monolayers (100 μl/35mm plate) and the viral adsorbed onto the monolayers for 1 h at 37°C. The virus inoculum was then aspirated and fresh medium was added to the culture plates. Cultures were incubated at 37°C in 5% CO_2, 95% air for 24 h. Virus titers in the antiviral-inhibited cell cultures were determined by counting virus plaques after staining the Vero cell monolayers with 1% methylene blue in 70% alcohol. All corneal epithelial cell culture antiviral inhibition studies and virus plaque reduction assays were repeated in quadruplicate and the data represents the mean.

Statistical analysis

Data were expressed as the percent inhibition of titrated thymidine incorporation compared to control (i.e. 0%) versus antiviral concentration at 5, 30 and 60 min exposure. Each point represents the mean of three values ± SE. Statistical significance was determined using the Student's *t* test.

Results

Figs. 1–4 show the inhibition of [^{3}H]thymidine incorporation as a function of antiviral concentration and duration of exposure. Absolute counts per minute in con-

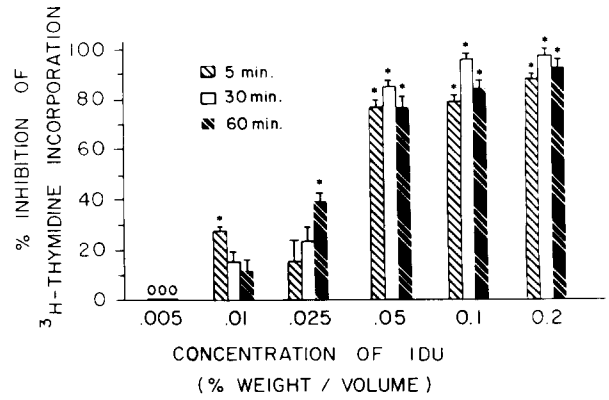

Fig. 1. Percent inhibition of [^{3}H]thymidine incorporation by treatment of corneal epithelial cells with idoxuridine (IDU). The height of each bar represents the mean of three values and the symbol (T) represents the SE. The symbol (*) denotes statistical significance at the 0.05 level.

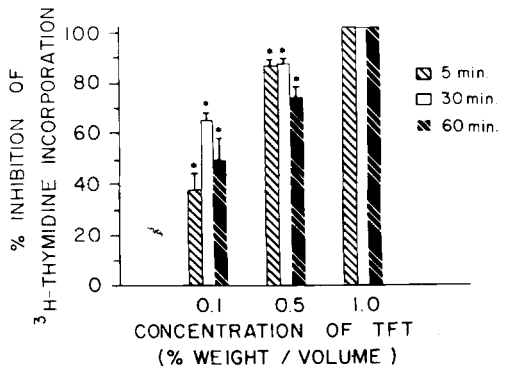

Fig. 2. Percent inhibition of [^{3}H]thymidine incorporation by treatment of corneal epithelial cells with trifluridine (TFT). The height of each bar represents the mean of three values and the symbol (T) represents the SE. The symbol (*) denotes statistical significance at the 0.05 level.

trol cultures varied in different experiments with a range of 3930 to 4818 cpm. For all antiviral agents, except BVDU, toxicity was dose-dependent, but not time-dependent (Figs. 1–4). For example, a progressive impairment in [^{3}H]thymidine incorporation was noted for IDU throughout the range of concentrations tested in vitro (0.005–0.2%); however, increasing the exposure duration from 5 to 60 min was not associated with significantly greater impairment of [^{3}H]thymidine incorporation at each concentration tested (Fig. 1).

With the exception of BVDU, all antiviral agents tested were associated with significant impairment of [^{3}H]thymidine incorporation, compared to control throughout the range of concentrations tested (Figs. 1–4). The concentration of drug which was associated with significant impairment of [^{3}H]thymidine incorporation at all three durations of exposures (5, 30, and 60 min) was: IDU 0.05% (Fig.

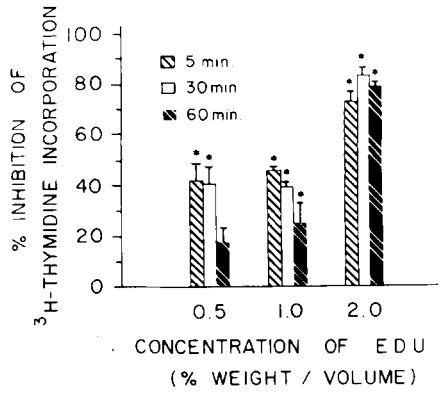

Fig. 3. Percent inhibition of [^{3}H]thymidine incorporation by treatment of corneal epithelial cells with ethyldeoxyuridine (EDU). The height of each bar represents the mean of three values and the symbol (T) represents the SE. The symbol (*) denotes statistical significance at the 0.05 level.

TABLE 1

Relative comparative antiviral cytotoxicity

Antiviral	Clinical concentration (%)	Minimum Toxic Conc. (%)[a] In vitro exposure time		
		5 min.	30 min.	60 min.
IDU	0.1	0.01	0.05	0.025
TFT	1.0	0.01	0.05	0.025
EDU	2.0	0.1	0.1	0.1
BVDU	0.1	NS	NS	NS

[a]Minimum concentration (and exposure time) for each agent which was associated with significant impairment of [^{3}H]thymidine incorporation ($P < 0.05$).
NS= No significant toxicity noted.

1), TFT 0.1% (Fig. 2), and EDU 1.0% (Fig. 3).

Significant inhibition ($P<0.05$) occurred at the concentrations employed clinically, i.e. IDU 0.1%, TFT 1.0%, EDU 2% (Table 1). BVDU at all concentrations, including that clinically employed (0.1%), showed no significant toxicity. When statistically compared at their clinically employed concentrations, a significant ($P < 0.05$) difference in toxicity to corneal epithelial cells among the four agents was noted. BVDU was the least toxic, EDU of intermediate toxicity, while IDU and TFT were the most toxic. This applied for all time exposures tested.

In our culture system IDU 0.1%, TFT 1.0% and BVDU 0.1% showed significant reduction in HSV plaque forming units reflecting antiviral activity when compared to control (Table 2). TFT showed the highest antiviral activity followed by BVDU and IDU, respectively.

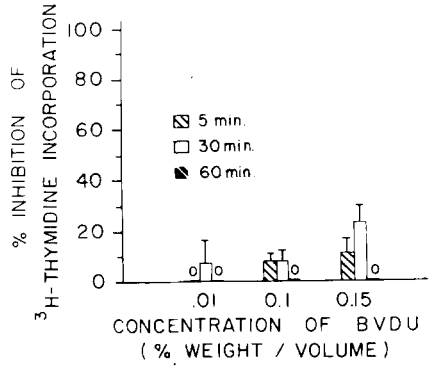

Fig. 4. Percent inhibition of [^{3}H]thymidine incorporation by treatment of corneal epithelial cells with (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU). The height of each bar represents the mean of three values and the symbol (T) represents the SE. The symbol (*) denotes statistical significance at the 0.05 level.

TABLE 2

Antiviral efficacy in corneal epithelium (PFU/ml)[a]

Antiviral agent	Dilution[b]		
	10^{0}	10^{-1}	10^{-2}
Placebo	1.2×10^{3}	4.0×10^{2}	2.0×10^{1}
IDU 0.1%	3.2×10^{2}	6.0×10^{1}	0
TFT 1.0%	8.0×10^{1}	1.0×10^{1}	0
BVDU 0.1%	2.0×10^{2}	8.0×10^{1}	0

[a]Plaque forming units per ml (PFU/ml).

[b]Confluent rabbit corneal epithelial cell cultures were inoculated with HSV type 1 (McKrae strain; 10^{4} PFU) for one hour. Cultures were harvested after 24-h incubation in medium containing the appropriate antiviral agent. The virus titer was determined by plaque reduction assay on Vero cell monolayers.

Discussion

We have shown our model to be useful for the evaluation of corneal epithelial cytotoxicity of antiviral agents by the good correlation of in vitro data with observations previously reported in vivo at the same drug concentrations. For IDU, TFT, and EDU, we noted an increase in inhibition of [^{3}H]thymidine incorporation compared to controls in the range of concentrations used clinically. BVDU was the least toxic, EDU of intermediate toxicity, while IDU and TFT were the most toxic. Although the minimum toxic concentrations for IDU and TFT were identical, IDU can be considered the most toxic of the two antiviral agents, since its clinical concentration is 10-fold less than TFT (Table 1). These dose-response effects observed in our in vitro system parallel the situation in clinical use or in the intact lab animal. In addition, our system parallels the clinical situation in terms of antiviral efficacy (Pavan-Langston, 1987). Our HSV reduction inhibition studies, using rabbit corneal epithelium, show that TFT and BVDU were the most effective and IDU was the least effective in HSV eradication. Thus, the degree of cytotoxicity observed with current antiviral agents can by used as a comparative standard to judge the potential toxicity of new agents.

Of the four agents examined, only BVDU did not appear to be toxic to corneal epithelial cells in culture. Significant in vitro cytotoxicity, however, was observed with IDU and TFT, antiviral agents with well recognized ocular surface toxicity (Foster and Pavan-Langston, 1977; Langston et al., 1974; Lass et al., 1983, 1984; Meyer and Kaufman, 1978; Pavan-Langston, 1987; Pavan-Langston and Foster, 1977; Vastine, 1987; Wilson, 1979). EDU, another agent which is purported to have a high therapeutic index in vivo due to its selective viral-induced thymidine kinase activation in a manner similar to BVDU (Elze, 1979; Gauri and Walter, 1973), did show significant toxicity in our model. The concentration of EDU used clinically is 2%; however, we observed 40% inhibition of [^{3}H]thymidine incorporation with concentrations as low as 0.5%.

Clinical trials with EDU remain limited (Elze, 1979). A recent phase I toxicity trial in 24 normal volunteers found no ocular side effects and mild transient ocular irritation when EDU was given as 2 or 4% drops 8 times daily for 14 days (Jackson, 1986, unpublished data). Phase II clinical trials in the United States in the treatment of active HSV keratitis, however, were abandoned in the last year because of lack of healing and an increased incidence of trophic ulceration (Pavan-Langston, 1986, unpublished data). These clinical results are not surprising based on our in vitro data, indicating EDU's apparent non-selective toxic effects on uninfected normal cells. Predictions using in vitro results, such as these, could become very important with the introduction of new agents in pre-clinical studies and circumvent potential toxicity of future agents.

We have demonstrated several advantages to our in vitro system when compared to existing in vivo methodologies. It is objective, quantitative, and standardized. Observer variation in bias, animal variability and host response variables are eliminated. Multiple dose and time variables can be easily assessed. Data can be gathered over considerably less time and at less expense since this technique requires 50-fold fewer animals (one cornea yields 50 cultures). Different drugs can be comparatively assessed and results subjected to statistical analysis. We have previously shown that for chemotherapeutic drugs and preservatives, this system correlates closely with clinical data (Botti et al., 1987; Imperia et al., 1986). In those previous studies, increases in duration of drug exposure, as well as drug concentration, were associated with increasing toxicity to corneal epithelial cells. The fact that duration of exposure did not correlate with [^{3}H]thymidine incorporation suggested that antiviral agents may have a different mechanism of action for toxicity as compared to other toxins such as the chemotherapeutic drugs or preservatives.

We studied antiviral agent cytotoxicity as well as antiviral efficacy in corneal epithelium. We believe this approach to be more rational than indirectly assessing cytotoxicity and efficacy using in vitro assays of non-ocular tissues. For example, in mouse melanoma cells EDU produces 100% irreversible inhibition of cell growth at a 0.26% concentration (Silagi et al., 1977). This concentration is well below the concentrations used clinically and those which we tested in vitro that were associated with a partial inhibition of corneal epithelial cell growth. Similar disparity in toxicity between non-ocular tissues was noted in a study showing a 50% inhibition of leucine incorporation with EDU, 0.04–0.1%, in leukemic cells, while a similar inhibition in PHA-stimulated lymphocytes was noted with an approximately 100-fold increase in EDU concentration (Tuominen et al., 1985). Thus, the use of corneal epithelium for in vitro studies more closely approximates the in vivo situation.

New antiviral agents with presumably less toxicity and/or more efficacy in treating latent or deep herpetic corneal or uveal infections will continue to be introduced. These agents will require toxicologic evaluation and comparisons to current antiviral agents. We believe our model can be an important adjunct in accomplishing this goal in a more expeditious and timely manner, and at less cost.

Acknowledgements

Supported in part by grants from BioSpecific Technologies Inc. (HML), the Ohio Lions Eye Research Fund (JHL), PHS P30CA43703 (Ireland Cancer Center), EY06584-01 (ECD), EY 5966-03 (DPL) and the Prince Charitable Trust, Chicago, Illinois (DPL, ECD). The antiviral agents were generously provided by Allergan Corporation (IDU), Burroughs Wellcome Company (TFT), Ortho Pharmaceutical Corporation (EDU) and Dr. Erik De Clercq (BVDU).

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