The corneal penetration of trifluorothymidine, adenine arabinoside, and idoxuridine: a comparative study

W. J. O'Brien and H. F. Edelhauser

Trifluorothymidine (F₃TdR) and idoxuridine (IDU) were observed to penetrate through the cornea from the epithelial side at a greater rate than adenine arabinoside (ARA-A) during in vitro corneal perfusions. Removal of the epithelium increased the rate of penetration of F₃TdR and IDU by about twofold and the rate of ARA-A penetration by fivefold. The kinetics of antiviral penetration did not display saturation points at high antiviral concentrations, thus indicating that these three antiviral drugs penetrate the cornea by nonfacilitated diffusion. The sole breakdown product detected following F₃TdR penetration in vitro, in situ, and in controls was 5-carboxy-2'-deoxyuridine (5-COOH-2'-dUd). The sole breakdown product isolated during ARA-A penetration experiments was hypoxanthine arabinoside (ARA-HX), and control experiments indicated that ARA-A was stable at pH 7.6. IDU was degraded to 2'-deoxyuridine (dUd) in control experiments, but during corneal penetration experiments IDU was degraded to a mixture of dUd and iodouracil (IU).

Key words: trifluorothymidine, 5-carboxy-2'-deoxyuridine, adenine arabinoside (Vidarabine), hypoxanthine arabinoside, idoxuridine, iodouracil, 2'-deoxyuridine, corneal penetration.

Topical trifluorothymidine (F₃TdR) has been reported to be effective in the treatment of herpetic iritis and uveitis as well as epithelial herpetic disease.¹ ² These observations suggest that F₃TdR or derivat...

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Fig. 1. Scans of thin layer chromatograms containing tritium-labeled F$_3$TdR. Crude $^3$H-F$_3$TdR was chromatographed in lane 1 of a silica gel G TLC plate developed in CICtLCH.OH (4:1) (Fig. 1, A). The area of thin layer containing the tritium-labeled F$_3$TdR was visualized under short-wave ultraviolet (UV) light and scraped from the TLC plate. The radioactive compound was eluted from the scrapings in 0.01N HCl. The eluate was filtered through a 0.2 μm filter to remove the particles and to sterilize the solution. The volume of the eluate was reduced by rotary evaporation at 60°C. Rotary evaporation was continued until all tritiums that were exchangeable with water were removed and a final product with constant specific activity was obtained. This final product possessed the UV spectral properties of purified F$_3$TdR$^+$ (specific activity: 22.5 μCi/μmol) and migrated as a single UV-absorbing band possessing greater than 99 percent of the radioactivity when subjected to analytical TLC (Fig. 1, B).

Adenine β-D-arabinofuranoside (adenine-2-$^3$H(N)) (specific activity: 11.0 μCi/μmol) was obtained from New England Nuclear and 5-ido-2’-deoxy (6-$^3$H) uridine (specific activity: 1.0 μCi/μmol) was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). The commercially available isotopes were subjected to TLC analysis in two systems and were found to be greater than 98 percent pure. These systems are shown in Table I.

Measurement of corneal penetration of antiviral drugs. Albino rabbits (2 to 3 kg.) were sacrificed, and the globes were enucleated with their conjunctival sac and lids attached. The isolated corneas were then mounted in a Lucite-block perfusion system$^6$ with a corneal holder that had been modified according to the method of Dikstein and Maurice.$^8$ This method prevents trauma to the corneal epithelium and distortion of the corneal curvature during clamping. The exposed surface area of the cornea was approximately 1 cm.$^2$ in all experiments. Six milliliters of bicarbonate-Ringer’s solution (BR) containing reduced glutathione, which has been shown to maintain endothelial function for up to 6 hr., was added to the reservoirs bathing either side of the cornea (NaCl, 6.2 gm./L; KCl, 0.358 gm./L; CaCl$_2$, 0.115 gm./L; MgCl$_2$, 0.159 gm./L; NaH$_2$PO$_4$, 0.103 gm./L; NaHCO$_3$, 2.454 gm./L; glucose, 0.90 gm./L; and reduced glutathione, 0.92 gm./L.).

Constant mixing of the reservoir solution was achieved with an airlift siphon gassed with 95 percent air–5 percent CO$_2$ to maintain a pH at 7.6. The block system and the bathing solutions were maintained at a constant temperature of 34°C throughout the experiment by a circulating water bath. In each experiment paired rabbit
Fig. 2. Rate of appearance of radioactive molecules on the endothelial side of excised rabbit corneas when \(^{3}H\)-F\(_3\)TdT is used to initiate the experiment. The initial antiviral drug concentrations used in these experiments were (●) 12.4 μM, (■) 24.8 μM, and media (▲) 49.6 μM. The points were fitted by regression analysis with the correlation coefficients \(r^2\). The slope, \(m \pm S.E.\) is listed for each concentration. Each point represents an N of 3. A, Epithelium present. B, Epithelium absent.

Table I. Systems of TLC analysis

<table>
<thead>
<tr>
<th>Antiviral drug</th>
<th>Solid support</th>
<th>Developing solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>F(_3)TdT</td>
<td>Mn Cellulose</td>
<td>n-Butanol:acetic acid:water (2:1:1)*</td>
</tr>
<tr>
<td>ARA-A</td>
<td>Mn Cellulose, PEI Cellulose</td>
<td>n-Butanol:acetic acid:water (2:1:1), n-Butanol:acetone:acetic acid:5% ammonium hydroxide: (H_2O) (45:25:10:10)</td>
</tr>
<tr>
<td>IDU</td>
<td>PEI Cellulose</td>
<td>n-Butanol:acetone:acetic acid:5% ammonium hydroxide: (H_2O) (45:25:10:10)</td>
</tr>
<tr>
<td></td>
<td>Silica Gel GF</td>
<td>70% ethanol, 1st solvent; chloroform:isopropanol (3:1), 2nd solvent</td>
</tr>
</tbody>
</table>

*Parts by volume.
†PEI = polyethyleneimine.

Corneas were utilized, one with the epithelium intact and the other cornea with the epithelium removed by scraping with a Gill knife. All experiments were initiated by the addition of radioactive antiviral drugs (0.12 to 0.25 μCi) to the media on the epithelial side of the corneas.

Serial samples of 100 μl were removed from each reservoir at various time intervals. Samples were placed in 15 ml of scintillation counting solution and were counted in a liquid scintillation counter (Unilux II; Nuclear Chicago, Des Plaines, Ill.) at 1 percent error. The scintillation solution contained 80 gm. of naphthalene, 5 gm. of 2, 5 diphenyloxazole (PPO) and 50 mg of 1, 4 bis-(2,5-phenyloxazolyl) benzene (POP) and made up to 1 L with dioxane. Counts were corrected for background, and quenching was determined with the use of an external standard. The rate of corneal penetration of the radioactive molecules was calculated by plotting the disintegrations per minute per 100 μl aliquot vs. time. These data were subjected to regression
Fig. 3. Velocity of F3Tdr corneal penetration as a function of the initial molar F3Tdr concentration. Velocity (V) of penetration (pmol/min./cm.²) is expressed as the penetration rate of radioactive molecules into the endothelial medium as measured with the epithelium present. The reciprocal of the millimolar concentration of F3Tdr is plotted on the abscissa, and r² is the correlation coefficient which describes the fit of points to the straight line drawn from regression analysis.

Results

**Kinetics of corneal penetration.** The rate of penetration of F3Tdr, as measured by the appearance of radioactive molecules on the endothelial side of the cornea was a linear function with respect to time, in both the presence and absence of the corneal epithelium. The rate of penetration was increased about twofold by removal of the epithelium (Fig. 2, A and B). The rate of penetration was also a linear function of the initial F3Tdr concentration over a 2,000-fold range from 1.24 to 25.5 mM (Fig. 3).

The rate of penetration of radioactive molecules as a function of time was linear when the experiments were initiated with 3H-ARA-A. The rates were observed to be linear in both the presence and absence of the corneal epithelium. The rate of penetration by radioactive molecules was increased more than twofold upon removal of the epithelium (Fig. 4, A and B). The low solubility of ARA-A restricted the concentration range over which penetration rates could be measured; however, the rate of appearance of radioactive molecules on the endothelial side of the cornea was a linear function at initial ARA-A concentrations between 16 and 66 μM.

The kinetics of penetration of radioactive molecules were also linear in both the presence and absence of the corneal epithelium when experiments were initiated...
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with ³H-IDU, but the rate of penetration did not increase upon removal of the epithelium (Fig. 5, A and B). The rate of penetration of radioactive molecules to the endothelial side of the corneal was measured at initial IDU concentrations between 5 and 44 µM.

**Chemical breakdown of F₂TdT, ARA-A, and IDU in experimental controls.** Experimental controls indicate that ARA-A was stable in BR at pH 7.6 and 34°C, but that F₂TdT and IDU were not (Table II). F₂TdT was found to be degraded to 5-carboxy-2'-deoxyuridine (5-COOH-2'-dUd), indicating that the fluorine atoms had come off the CF₃ group attached to the pyrimidine ring. Trifluorothymine (F₃T) and 5-carboxyuracil (5-COOH-U) were not found in these samples.

Identification of 5-COOH-2'-dUd was made by scanning difference spectrophotometry. After chromatography of the concentrated perfusion media, the UV-absorbing compounds with Rf's equal to those of 5-COOH-2'-dUd and 5-COOH-U were eluted from the thin layer in 0.01N HCl. One aliquot of the eluate was diluted to 1.0 ml in 0.01N HCl, and an equal aliquot was adjusted to a volume of 1.0 ml with 10N KOH so that the final KOH concentration was about 3N. Reference samples were prepared in the same manner by scraping and eluting an area of thin layer containing no UV-absorbing compound. Fig. 6 shows the difference spectra of (A) 5-COOH-2'-dUd and (B) 5-COOH-U. The bathachromic shift of the maximum absorption peak from 273 nm in acid solution to 290 nm in basic solution is characteristic of 5-COOH-U and

**Table II. Chemical breakdown of antiviral drugs in BR at pH 7.6 and 34°C.**

<table>
<thead>
<tr>
<th>Antiviral drug</th>
<th>Breakdown products</th>
<th>% breakdown after 3 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₂TdT</td>
<td>5-COOH-2'-dUd</td>
<td>18-25</td>
</tr>
<tr>
<td>ARA-A</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>IDU</td>
<td>dUd</td>
<td>16-21</td>
</tr>
<tr>
<td></td>
<td>X*</td>
<td>2</td>
</tr>
</tbody>
</table>

*Unidentified compound.
Fig. 5. Rate of penetration of radioactive molecules to the endothelial side of the cornea when H-IDU was used to initiate the experiment. Initial drug concentrations were (●) 11 μM, (■) 22 μM, and (▲) 44 μM. The slopes of the line, M, ± the standard error of the slope is reported. Each point is a mean of three determinations, and r² is the correlation coefficient. A, Epithelium present, B, Epithelium absent.

Fig. 6. Spectrophotometric scans of 5-COOH-U and 5-COOH-2'-dUd. Scans were run from 340 to 200 nm. A, Spectrum of 5-COOH-2'-dUd in acid (---) (λmax 277 nm.) and in base (----) (λmax 272 nm.). B, Spectrum of 5-COOH-U in acid (---) (λmax 276 nm.) and in base (----) (λmax 289 nm.).
clearly distinguishes 5-COOH-2′-dUd from 5-COOH-U.

IDU was degraded under the same condition of ionic strength, temperature, and pH as stated for the other control mixtures (Table II). The radioactive compounds were eluted from TLC plates and subjected to scanning spectrophotometric analysis. The data indicate that the sole degradation product which absorbed UV light was 2′-deoxyuridine (dUd) (λmax, acid, 261 nm.; λmax, base, 263 nm.). About 2 percent of the radioactive material recovered from the thin layer no longer possessed UV-absorption properties. The product appeared to be the result of pyrimidine ring cleavage at a mildly alkaline pH.

Chemical breakdown and metabolism during corneal penetration experiments. The breakdown products isolated from the epithelial and endothelial perfusion media were the same as those observed in the control media when F₃TdR was used to initiate penetration experiments. This observation suggests that pH and ionic strength were the main factors contributing to the breakdown.

F₃TdR was found to be broken down in such a way that both the epithelial and endothelial perfusion media contained between 19 and 29 percent 5-COOH-2′-dUd (Fig. 7, A). The presence or absence of the corneal epithelium did not appear to make any significant difference in the percentage of 5-COOH-2′-dUd formed or in the relative amount of this breakdown product found on either side of the cornea. In experiments in which 4 percent ophthalmic solution of F₃TdR was either dropped onto the corneal surface or injected directly into the anterior chamber, F₃TdR and 5-COOH-2′-dUd were recovered from the aqueous humor in approximately the same proportion as shown for the in vitro corneal perfusion.

During the course of the perfusion experiments ARA-A was metabolized by adenosine deaminase to ARA-HX. When the epithelium was present, essentially all the radioactive molecules remaining on the epithelial side of the cornea were isolated and identified as ARA-A (Fig. 7, B). However, about 70 percent of the radioactive molecules which had penetrated the cornea were identified as ARA-HX. Removal of the corneal epithelium resulted in the isolation of a small amount of ARA-HX from the epithelial side media (Fig.
Table III. Rates of corneal penetration of antiviral drugs

<table>
<thead>
<tr>
<th>Antiviral drug*</th>
<th>Rate of penetration (pmol/min/cm.²)</th>
<th>Epithelium present</th>
<th>Epithelium absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₃TdR</td>
<td></td>
<td>14.2</td>
<td>28.5</td>
</tr>
<tr>
<td>IDU</td>
<td></td>
<td>11.6</td>
<td>25.73</td>
</tr>
<tr>
<td>ARA-A</td>
<td></td>
<td>2.6</td>
<td>13.4</td>
</tr>
<tr>
<td>Σ ARA-A + ARA-Hx</td>
<td></td>
<td>8.5</td>
<td>23.9</td>
</tr>
<tr>
<td>ARA-A†</td>
<td></td>
<td>7.4</td>
<td></td>
</tr>
</tbody>
</table>

*aInitial antiviral concentration of 25 μM.  
†Covidarabine, 0.04 μg/0.6 ml of reservoir solution.

7, B). In addition, the relative percentage of ARA-A isolated from the endothelial media was increased. In experiments in which 0.4 μg of covidarabine, an inhibitor of adenosine deaminase, was added to both the epithelial and endothelial perfusion media prior to addition of the ³H-ARA, the sole molecular type isolated on the endothelial, as well as the epithelial, side of the cornea was ARA-A.

IDU was degraded to dUd during the course of these corneal perfusion experiments. It was also observed that a small amount of IU was formed. In the presence of the corneal epithelium, the epithelial reservoir contained IDU and dUd in approximately the same ratio as the controls (Fig. 7, C). In the endothelial reservoir the relative percentage of dUd was increased by nearly twofold. IU was also found in greater concentrations on the endothelial side than on the epithelial side. Upon removal of the epithelium, the amount of dUd relative to the amount of IDU in the epithelial and endothelial media was similar to that in the control solutions. Little or no IU was found in the absence of the corneal epithelium.

**Rates of penetration of F₃TdR, ARA-A, and IDU.** The rates of penetration of the parent antiviral drugs through the cornea was calculated by multiplying the rate of penetration of total radioactive molecules by the percent of those molecules that were identified as being either F₃TdR, ARA-A, or IDU. This calculation is based on the assumption that the chemical breakdown and metabolic conversion of these antivirals to the respective degradation products identified during this study followed first-order rate constants. Ravin et al.⁹ have shown that the degradation of IDU due to pH is a first-order phenomenon. Nestler and Garrett¹⁰ have observed the breakdown of F₃TdR to 5-COOH-2'-dUd to be first-order. The rates of penetration calculated by this method are listed in Table III. These rates were based on an initial antiviral drug concentration of 25 μM, a concentration at which all three antiviral drugs were in true solution.

In the presence of the epithelium F₃TdR penetrated more rapidly than IDU, which penetrated faster than ARA-A. Removal of the epithelium increased the rate of penetration of both F₃TdR and IDU about twofold, whereas the rate of penetration of ARA-A was increased fivefold. The rate of ARA-A in the presence of covidarabine was nearly equal to the sum of the rates of penetration of ARA-A and ARA-HX.

**Discussion**

The linear kinetics of penetration and lack of saturation kinetics observed during these penetration experiments suggests that penetration of F₃TdR, ARA-A, and IDU are not carrier-mediated processes and that the data obtained at low or subclinical concentrations are directly related to the data obtained at relatively high or clinical concentrations.

The experimental controls conducted in the absence of corneal tissue provided a measure of the stability of F₃TdR, ARA-A, and IDU in BR at pH 7.6 at 34° C. The isolation of 5-COOH-2'-dUd as the sole degradation product of F₃TdR breakdown is consistent with the observations of Nestler and Garrett,¹⁰ who also observed that in ophthalmic solutions of F₃TdR the sole degradation product was 5-COOH-2'-dUd. The biological properties of 5-COOH-2'-dUd, such as antiviral activity and toxicity, have yet to be reported.
Metabolically F$_3$TdR has been reported to be converted to F$_3$T and 5-COOH-U following intravenous injection. F$_3$T and 5-COOH-U are formed by the sequential action of a pyrimidine nucleoside, orthophosphate deoxyribosyl transferase (nucleoside phosphorylase), and physiological pH. The antiviral activity of these compounds was reported to be low. The inability to isolate either F$_3$T or 5-COOH-U in the perfusion media of our system suggests the absence of nucleoside phosphorylases in the rabbit cornea. This observation was supported by the fact that F$_3$TdR and 5-COOH-2'-dUd were isolated from the aqueous after topical administration of F$_3$TdR drops or intracameral injection of F$_3$TdR. These data indicate that nucleoside phosphorylases with thymidine substrate specificity are not present in ocular tissues of rabbits. Therefore it is concluded that physiological pH, temperature, and ionic strength contribute significantly to the degradation of F$_3$TdR.

By comparison, ARA-A was stable at pH 7.6 and 34° C, and deamination occurred only in the presence of corneal tissue. ARA-HX was formed in both the presence and absence of the corneal epithelium, thus indicating that high levels of adenosine deaminase are present in corneal tissue. The deaminase did not appear to be exclusively of epithelial origin, since deamination occurred in its absence. The addition of cvidarabine to the epithelial and endothelial perfusion media resulted in the corneal penetration of only ARA-A, indicating that deamination is not required for the corneal penetration of ARA-A.

Unlike ARA-A, IDU is not stable at either alkaline or acid pH. Alkaline hydrolysis of IDU results in the formation of dUd, which does not absorb UV light because of pyrimidine ring cleavage. The isolation of dUd and a UV-nonabsorbing compound in our control experiments clearly indicate that mild alkaline hydrolysis does occur at physiological pH, temperature, and ionic strength. Neither of these degradation products have been reported to have antiviral activity or toxicity.

IDU can also be degraded to IU and uracil by acid hydrolysis or by the action of two enzymes. Enzymatically IU is formed by the action of nucleoside phosphorylase with uridine substrate specificity. IU is then converted to uracil by the action of an NADP-dependent dehalogenase. Oppelt et al. found the levels of these two enzymes to vary greatly, depending upon the organ from which they were isolated. Our studies and those of Itoi et al. suggest that the level of the latter enzyme is low in ocular tissue, since little or no uracil was isolated. The isolation of small amounts of IU in corneal perfusions suggests that nucleoside phosphorylases with uridine substrate specificity may contribute to the degradation of IDU by the cornea.

Our results differ from those of Pavan-Langston et al., who have reported tentative identification of uracil as the major breakdown product isolated upon topical administration of IDU to rabbits. Studies are currently underway to determine the levels of nucleoside phosphorylases and dehalogenating enzymes in ocular tissues in an effort to resolve these differences. The levels of IU present in ocular tissues are extremely important because of its antagonistic effects on the antiviral action of IDU and its extreme toxicity. Independent of the metabolic or chemical breakdown, F$_3$TdR was observed to penetrate cornea more rapidly than ARA-A or IDU. The uniform distribution of radioactive F$_3$TdR and 5-COOH-2'-dUd on either side of the cornea in both the presence and absence of the corneal epithelium suggests that the cornea is not selectively permeable to either compound.

On the other hand, ARA-A was observed to penetrate the cornea at the slowest rate of the three antiviral drugs. The presence of the corneal epithelium and deaminase associated with it resulted in the accumula-
tion of primarily ARA-Hx on the endothelial side. Removal of the epithelium resulted in the accumulation of more ARA-A on the endothelial side. This observation suggests that either the epithelium is a selective barrier to ARA-A or it is a primary source of deaminase. The increased penetration rate of ARA-A in the absence of the epithelium may explain why Pavan-Langston et al. observed increased efficacy of ARA-A in patients with damaged epithelium.

The total number of radioactive molecules which penetrated the corneas was not increased by removal of the epithelium when IDU was used. However, when the epithelium was present, the endothelial media contained nearly twice as much dUd as either the control or the endothelial media from the perfusion of the paired cornea with the epithelium removed. This observation suggests that either the epithelium is selectively permeable to dUd or its presence increases the rate of IDU degradation.

In summary, the data indicate that under idealized conditions, F3TdR penetrates the cornea faster than either IDU or ARA-A. Studies in this in vitro system permit us to determine the effects of physiological pH and ionic strength on the degradation of these three antiviral drugs as well as the effect of cornea-associated enzymes. Under physiological conditions F3TdR was observed to be degraded to 5-COOH-2'dUd via mild alkaline hydrolysis. Cornea-associated enzymes did not contribute significantly to its degradation. On the other hand, ARA-A was observed to be stable at physiological pH and ionic strength but was degraded by adenosine deaminase associated with the cornea. IDU was degraded to primarily dUd by mild alkaline hydrolysis at pH 7.6 and 34°C. It is suggested that the small amount of IU formed during corneal penetration was formed as a result of a cornea-associated nucleoside phosphorylase.

We gratefully acknowledge the counsel of Dr. R. A. Hyndiuk of the Medical College of Wisconsin, Department of Ophthalmology.

REFERENCES

Cornal penetration of F, TdR, ARA-A, and IDU


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