Effect of Azone and Propylene Glycol on Penetration of Trifluorothymidine Through Skin and Efficacy of Different Topical Formulations Against Cutaneous Herpes Simplex Virus Infections in Guinea Pigs

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Topical formulations of 5-trifluoromethyl-2'-deoxyuridine (TFT) containing different concentrations of TFT, Azone (Nelson Research and Development, Irvine, Calif.), and propylene glycol were evaluated for their potential efficacy in the treatment of cutaneous herpes simplex virus infections by in vitro studies of TFT penetration through skin and in vivo studies of therapeutic activity against herpes simplex virus type 1 infections in the dorsal cutaneous guinea pig model. Azone dramatically increased TFT penetration through human and guinea pig skin. Unexpectedly, high concentrations of propylene glycol were also associated with increased penetration. Studies in the guinea pig model revealed increased efficacy with Azone-propylene glycol-containing formulations, consistent with the in vitro drug diffusion results. A formulation containing 1% TFT, 5% Azone, and 80% propylene glycol decreased lesion area, in comparison to the drug vehicle control, more effectively than 5% acyclovir in polyethylene glycol (reduction of 70 versus 46%, P = 0.03). These studies demonstrate the value of penetration-enhancing agents and the need for careful preclinical evaluations in the development of topical antiviral agents.

5-Trifluoromethyl-2'-deoxyuridine (trifluorothymidine, trifuridine, [TFT]) is a fluorinated pyrimidine nucleoside synthesized by Heidelberger et al. (8). Although it possesses mild antineoplastic activity, it has attracted the most attention as an antiviral agent. After the demonstration by Kaufman and Heidelberger in 1964 (10) that topical TFT was effective against herpes simplex virus (HSV) type 1 (HSV-1) infections in the rabbit cornea model, TFT was formulated as a topical preparation for human herpetic keratitis (Viropptic; Burroughs Wellcome Co., Research Triangle Park, N.C.). The pharmacology of TFT and its activity against herpetic keratitis in rabbits and humans have been reviewed extensively elsewhere (2, 7). TFT is a competitive inhibitor of HSV-1 and cellular thymidine kinases (15) and probably acts at several other steps along the pathway of incorporation of pyrimidines into DNA. Despite having a reduced affinity for cellular thymidine kinase in comparison with the natural substrate (3, 15), TFT is generally considered to be a “nonselective” agent with a marginal toxic/therapeutic ratio and has been shown to be an effective inhibitor of thymidine kinase-negative strains of HSV-1 (5, 6). Studies of TFT in experimental animal models of HSV infections other than rabbit eyes have been limited. Clough and Parkhurst (4) demonstrated that intracerebral injections of TFT protected mice from HSV-1 encephalitis, and Burkhardt and Wigand (1) demonstrated that a topical preparation of 2% TFT in glycerol and Tween 80 was modestly effective against cutaneous HSV-1 infections in guinea pigs.

Skin penetration appears to be important for successful topical therapy of cutaneous HSV infections (11, 12). Dodecylazacycloheptan-2-one (laurocapram, Azone; Nelson Research and Development, Irvine, Calif.) is a smooth, oily, hydrophobic liquid which has been reported to be nonirritating to human skin and capable of enhancing the percutaneous penetration of a variety of compounds (13, 14). The concentration of Azone which maximally enhances penetration varies with different compounds and also with the nature of the formulation (14).

To develop an effective topical formulation of TFT, we conducted in vitro studies of the effect of Azone on the penetration of TFT through guinea pig and human skin and examined the in vivo efficacy of different TFT preparations in the Hubler guinea pig model of cutaneous HSV-1 disease (9, 12). The data show that Azone in combination with TFT enhances the penetration of TFT through skin and results in a highly effective formulation for the treatment of experimental infections. Unexpectedly, the concentration of propylene glycol (PG) was also an important aspect of the formulation.

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MATERIALS AND METHODS

Experimental animals and virus. Hartley strain outbred female albino guinea pigs weighing 200 to 250 g each were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. The virus used in these experiments was HSV-1 E115 (12).

Skin specimens for in vitro drug diffusion studies. Guinea pigs were shaved with electric clippers and sacrificed with ether. Two specimens (3 by 3 cm) of full-thickness skin, one
on each side of the spine at the mid-back level, were removed by dissection. The skin was clamped into glass diffusion cells and used immediately as described below.

Specimens of human skin were obtained from the arms of cadavers. The skin was not shaved or chemically treated before being harvested. The skin was removed by making a 0.3-mm-thick cut with a dermatome and was kept at 4°C and used in diffusion experiments within 48 h after acquisition.

Antiviral drugs. Powdered TFT and 12 topical formulations containing different proportions of TFT, Azone, PG, and polyethylene glycol (PEG) were obtained from Allergan Pharmaceuticals, Irvine, Calif. For treatment studies of experimental HSV-1 infections, the corresponding drug vehicles without TFT were also supplied. The composition of each formulation is detailed below. The PEG used consisted of mixtures of low (300)-, intermediate (4,000)-, and high (80,000)-molecular-weight molecules to produce either an ointment or a solution. [3H]TFT (15 Ci/mmol) was supplied by Moravek Biochemicals, Inc., Brea, Calif. The purity of the preparation was evaluated by thin-layer chromatography on a fluorescent silica gel plastic plate (Eastman Kodak Co., Rochester, N.Y.) with an upper phase of ethylacetate-formic acid-water in a ratio of 60:5:35. The single peak observed contained 98% of the activity. Radiolabeled TFT formulations were prepared by stirring [3H]TFT into the "cold" ointments and solutions. Ointments were liquefied by being heated to 70°C to permit stirring. Heating had no effect on [3H]TFT in thin-layer chromatography.

Acyclovir (ACV) (5%) in PEG (Zovirax ointment) and PEG placebo ointment were kindly provided by Burroughs Wellcome Co.

In vitro drug diffusion experiments. Diffusion experiments were conducted in single-chamber glass diffusion cells constructed by the Department of Chemistry, University of Utah, Salt Lake City. The receiver chamber had a volume of 5.7 ml and was filled with 0.15 M NaCl-0.1% thimerosal. Stirring was done with a magnetic bar. All experiments were performed at room temperature (25°C). TFT solution (100 μl) or ointment (250 mg) was applied at time zero, and samples were subsequently withdrawn through a side port in the receiver chamber over time and assayed for radioactivity in a Beckman scintillation counter. The quantities of formulations applied corresponded to the quantities used in the experimental animal model. The radioactivity in the receiver chamber in a representative diffusion experiment was studied by thin-layer chromatography and confirmed to be [3H]TFT. Drug flux through the skin (micrograms of TFT per square centimeter per hour) was determined from the slope of a plot of radioactivity in the receiver chamber versus time and the volume of the receiver chamber, the area of the treated skin surface, and the specific activity of each formulation (counts per minute per microgram of TFT). The permeability coefficient (centimeters per hour) was a measure of the inherent ability of the compound to cross the skin sample under the given experimental conditions and was determined by dividing drug flux by the concentration of drug in the formulation.

Experimental animal model of HSV-1 infection. Animals were anesthetized intraperitoneally with pentobarbital sodium. Hair was removed from the dorsum with electric clippers and a chemical depilatory (Nair; Carter-Wallace, Inc., New York, N.Y.). A grid of four areas was demarcated with a pen at the midbump and rump on either side of the spine. Undiluted virus stock (0.02 ml) was applied to each area and introduced into the skin with a vaccination instrument as originally described by Hubler et al. (9). The day of inoculation was designated day 0.

Tolerance studies (data not shown) indicated that even low-dose, infrequent applications of formulations containing Azone were inflammatory for guinea pig skin and could potentially obscure or confound evaluation of the effect of TFT on the course of the HSV-1 infection. To resolve this problem, we treated all infection sites with 250 mg of 0.1% betamethasone valerate cream (Valisone; Schering Corp., Bloomfield, N.J.) once daily 2 h before applying the antiviral formulations. Antiviral treatments were begun on day 1 (24 h after inoculation) and given once daily for a total of 3 days. The amount of antiviral agent applied to each infection site was ca. 250 mg of ointment. The drug and the corresponding drug vehicle were always tested opposite each other at the same rostral-caudal level. A drug or drug vehicle was tested only once on each animal. Studies of infected animals treated on one side with Valisone and on the other side untreated showed that Valisone treatment had no effect on the number, size, or viral titer of the lesions.

On day 4, the animals were sacrificed with ether, and the severity of the infection on drug- and drug vehicle-treated

FIG. 1. Diffusion of TFT through guinea pig and human skin for solutions A, B, C, and D containing Azone, PG, PEG, and TFT in the following percentages, respectively: (A) 30, 53, 45, and 5; (B) 5, 46, 46, and 2.5; (C) 10, 45, 45, and 0.1; and (D) 0.1, 47, 47, and 5. The formulations were applied at time zero, and serial measurements of the concentration of drug in the receiver chamber of the diffusion apparatus were made. The linear nature of the plots allowed an accurate determination of the slope by linear regression and calculation of drug flux (micrograms of TFT per square centimeter per hour).
sites was evaluated by counting the number of lesions, measuring the lesion area, and quantitatively determining infectivity by dissection of each skin site, homogenization, and plaque assay. These procedures have been described in detail elsewhere (12).

Statistical procedures. The slope of drug concentration in diffusion cells as a function of time was determined by linear regression. Paired data (drug versus drug vehicle) from in vivo experiments were evaluated by the Wilcoxon signed rank test, with the result for the drug expressed as a percentage of the result for the drug vehicle. The percent differences between the drug treatment and the drug vehicle control for the different formulations were compared by the Mann-Whitney rank sum test. All probability determinations were two tailed, and $P \leq 0.05$ was considered to be significant.

RESULTS

In vitro studies of TFT diffusion through guinea pig and human skin. The penetration of TFT from different topical formulations through guinea pig and human skin was studied in single-chamber glass diffusion cells by measuring the concentration of TFT in the receiver chamber as a function of time. The results of a representative experiment with four different TFT solutions are shown in Fig. 1. After a lag period, a linear increase in drug concentration in the receiver chamber was observed for two of the formulations containing Azone, and the slope could be determined and used to derive drug flux (micrograms of TFT per square centimeter per hour). Pretreatment of the skin with Azone markedly reduced the lag period (N. Sheth and S. L. Spruance, manuscript in preparation).

Figure 2 shows the derived values for TFT flux and permeability coefficients for nine different TFT solutions and ointments containing different concentrations of TFT, Azone, PG, and PEG for guinea pig and human skin. The penetration of TFT through guinea pig skin occurred at a rate ca. sixfold greater than that observed with human skin. However, the relative degrees of skin penetration of different formulations were the same for guinea pig and human skin. For each type of skin, the permeability coefficient for TFT was the same order of magnitude for solutions A, B, and C, which contained 5 or 10% Azone and 43 to 46% PG. The flux of TFT through guinea pig and human skin for solutions A, B, and C was proportional to the drug concentration, consistent with Fick's law (12).

Three pairs of formulations contained the same concentrations of TFT and PG but 10, 5, 1, or 0.1% Azone (A and D, E and G, H, and I). For each pair, the preparation with more Azone resulted in a markedly greater flux of TFT. Two pairs of formulations contained the same concentrations of TFT and Azone but 80 or 10% PG (E and H, G and I). These two

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**FIG. 2.** Penetration of TFT through guinea pig and human skin expressed as drug flux (micrograms of TFT per square centimeter per hour; open bars) and permeability coefficient (drug flux/drug concentration [centimeters per hour]; hatched bars). Nine different formulations (A through I) containing different proportions of TFT, Azone, PG, and PEG were tested. All experiments were performed in triplicate. Bars represent ±1 standard error of the mean.
comparisons showed that an 80% concentration of PG (with a correspondingly low proportion of PEG) also increased TFT penetration.

**Efficacy of TFT formulations against experimental HSV-1 infections in guinea pigs.** The foregoing drug diffusion experiments indicated that Azone enhanced the penetration of TFT through skin and that a marked effect on the permeability coefficient could be achieved with an Azone concentration of 5%. High concentrations of PG also influenced the permeability of skin to TFT. To determine the influence of different drug vehicles on topical TFT efficacy, we evaluated five TFT ointment formulations for the treatment of dorsal cutaneous HSV-1 infections in guinea pigs as described above. Four formulations contained 1% TFT, 5% Azone, and different concentrations of PG, and the other formulation contained 1% TFT and 80% PG but no Azone. ACV (5%) in PEG was evaluated concurrently. The compositions of the formulations and the therapeutic results are shown in Table 1.

Table 1. Effect of different topical antiviral formulations on experimental HSV-1 infections.

<table>
<thead>
<tr>
<th>Therapeutic agent (%)</th>
<th>Vehicle component (%)</th>
<th>Mean ±SD (n = 8)</th>
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<tr>
<td></td>
<td></td>
<td>No. of lesions</td>
</tr>
<tr>
<td>ACV 0%</td>
<td>TFT 1% Azone 0% PG 95%</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>0%</td>
<td>0%</td>
<td>44 ± 8</td>
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<td>41 ± 12</td>
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To develop a topical antiviral preparation potentially useful for the treatment of mucocutaneous HSV-1 infections, we formulated TFT with the penetration-enhancing agent Azone. Formulations containing different concentrations of TFT, Azone, PG, and PEG were first evaluated in vitro to determine the effects of different formulations on the diffusion of TFT through excised guinea pig skin; subsequently, five topical preparations were evaluated for their ability to reduce the severity of HSV-1 infections in the dorsal cutaneous guinea pig model. The results indicated that the in vitro diffusion of TFT through guinea pig and human skin was markedly enhanced by 5 to 10% Azone. Unexpectedly, the concentration of PG also appeared to be important. In vivo studies in the guinea pig model indicated that the activity of TFT could be potentiated by the combination of Azone and PG. A formulation containing 1% TFT, 5% Azone, and 80% PG was significantly more active than 5% ACV in PEG in reducing lesion area. Although the full antiviral activity of TFT in these formulations could not be explained because of the irritating effect of Azone on guinea pig skin, the present data provide evidence of potent efficacy as well as a rationale for the construction of a formulation for evaluation in humans.

In the only other prior study of topical TFT in the treatment of experimental cutaneous HSV infections, Burkhardt and Wigand demonstrated a modest effect of TFT against experimental HSV-1 infections (1). In their study, 2% TFT was prepared in a 10% solution of glycerol with 0.5% Tween 80. TFT reduced the relative cumulative lesion severity score by half, in comparison with a control; this moderate response was similar to the response that we observed in our model with 1% TFT and 80% PG in the absence of Azone. TFT (5%) and ACV (5%) in 95% dimethyl sulfoxide are both highly effective and equivalent in potency against dorsal cutaneous HSV-1 infections in guinea pigs (S. L. Spruance, unpublished data). The present studies show that the enhancement of TFT penetration with Azone and PG also yields excellent clinical results in the animal model. Azone has been used to enhance the efficacy of topical adenine arabinoside in the hairless mouse model of cutaneous HSV-1 infections (W. M. Shannon, L. Westbrook, W. I. Higuchi, R. Vaidyanathan, and D. C. Baker, Program Abstract. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, Las Vegas, Nev., abstr. no. 149, 1983).

Azone is an exciting new agent which appears to have a potential for decreasing the substantial inherent barrier properties of the stratum corneum in both guinea pig and human skin and for allowing the diffusion of multiple substances, including antiviral agents, into the deeper layers of the epidermis. The present study demonstrated the usefulness of Azone in combination with TFT, identified an appropriate concentration of Azone, and showed that high concentrations of PG are also important. The mechanism of action of PG in combination with Azone, TFT, and PEG is presently being investigated.

**LITERATURE CITED**


