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Effects of epidermal/dermal separation methods and ester chain configuration on the bioconversion of a homologous series of methotrexate dialkyl esters in dermal and epidermal homogenates of hairless mouse skin

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Summary

A comparison has been made regarding various methods of hairless mouse skin dermal/epidermal separation techniques using criteria such as soluble protein yield and rate of hydrolysis per unit soluble protein, as well as effectiveness of separation, in which histological techniques were employed. Also compared were the relative esterase activities of dermal and epidermal layers, in which the dermis exhibited higher activity. A homologous series of methotrexate dialkyl esters were studied as to their pseudo-first order degradation in homogenates of dermis and epidermis of hairless mouse skin. A logarithmic relationship between pseudo-first order rate constant and alkyl chain length was observed for both dermal and epidermal homogenates. The degree of bioconversion of the diesters was also studied and the enzymatic reaction failed to proceed beyond the level of the monoalkyl esters. Examination of the selectivity for the formation of α- and γ-monoalkyl products revealed that γ-monoester appeared to predominate over α-monoester in the case of the branched-chain dialkyl ester.

Introduction

A relative improvement in the permeability of methotrexate (MTX) dialkyl esters over MTX across full-thickness hairless mouse skin has been demonstrated (Fort et al., 1994). Additionally, the permeability and degree of simultaneous bioconversion of these compounds across tape-stripped hairless mouse skin have been described (Fort et al., 1993). In the tape-stripped system, all diesters during transport across cutaneous tissue (dermis and epidermis) underwent some degree of bioconversion to MTX monoalkyl esters. This bioconversion during diffusion was observed to cease at the level of the monoalkyl esters, and did not

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proceed further to the parent MTX. While the bioconversion did not progress completely to MTX, a relationship between the degree of bioconversion to the monoesters and alkyl chain length was observed.

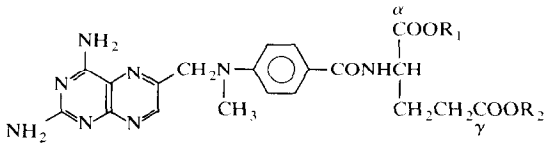
It is the goal of this present study to put the tape-stripped hairless mouse skin bioconversion data into proper perspective with a homogenate study. It is not known whether the apparent increase in reactivity of the diesters to the cutaneous esterases is a result of characteristics of the intact membrane and/or enzyme localization. It is also not certain whether the cessation of reaction at the monoester level is due to lack of affinity of the negatively charged compounds for the cutaneous esterases or due to the limited residence time of diffusing molecules in the membrane biophase.

In this study, the relative contribution of dermal and epidermal layers to enzymatic degradation of MTX diesters, the dependence of dermal/epidermal separation method on esterase activity, the kinetics of diester loss in dermal and epidermal homogenates, and the selectivity of the cutaneous esterases toward the formation of α- vs γ-monoalkyl esters have been individually examined.

Experimental

Materials

MTX was generously provided by Lederle Laboratories, Pearl River, NY. The preparation of some of the compounds used in this study, namely, dimethyl MTX (DMMTX), diethyl MTX (DEMTX), dipropyl MTX (DPMTX), dibutyl MTX (DBMTX), and diisopropyl MTX (DIPMTX), has been described previously (Johns et al., 1973; Rosowsky, 1973; Fort and Mitra, 1987). The isomeric monoalkyl MTX mixtures, namely, α- and γ-isomers of methyl (MMTX), ethyl (EMTX), propyl (PMTX), butyl (BMTX), and isopropyl (IPMTX), were synthesized by a slight modification of the method of Rosowsky et al. (1978). The preparation of individual monoester reference samples of α-EMTX, γ-EMTX, α-PMTX, γ-PMTX, α-BMTX, γ-BMTX, α-IPMTX, and γ-IMPTX was described elsewhere (Fort, 1989). Chemical structures of the diester compounds utilized in this study are shown in Scheme 1.



Compound	R_1	R_2
MTX	H	H
DMMTX	CH_3	CH_3
DEMTX	CH_2CH_3	CH_2CH_3
DPMTX	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$
DIPMTX	$CH(CH_3)_2$	$CH(CH_3)_2$
DBMTX	$CH_2(CH_2)_2CH_3$	$CH_2(CH_2)_2CH_3$

Scheme 1. Chemical structures of methotrexate and its dialkyl esters.

Trypsin, Na_2EDTA, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Double distilled water was used throughout the study.

HPLC assay procedure

The HPLC analyses of the MTX dialkyl esters were carried out with pH 3, 0.05 M KH_2PO_4/acetonitrile (ACN) mobile phases with compositions of 72.5:27.5 to 55:45 (v/v) containing 5 mM triethanolamine (TEA). The stationary phase consisted of a 15 cm Novapak C-18 column (Waters Associates). Flow rate was kept constant at 1.0 ml/min. Monoester and MTX assays, except for MMTX, required pH 7, 0.002 M KH_2PO_4/methanol (MeOH) mobile phases varying from 65:35 to 55:45 (v/v) proportions containing 5 mM tetrabutylammonium phosphate (TBAP). MMTX isomers were assayed with 72.5:27.5 (v/v) proportions of the above mobile phase on a 25 cm Phase-II C-18 column (Bioanalytical Systems). Mobile phase flow rate was set at 1.5 ml/min. The HPLC equipment assembly consisted of an M-6000A pump, a 440 single wavelength detector set at 254 nm, a U6K injector, all from Waters, an Omniscribe B-5000 strip chart recorder (Houston Instruments), and a Chro-

matopac E-1A integrator (Shimadzu). The sample concentrations were determined from standard curves of diesters, monoester mixtures, and MTX.

Epidermal / dermal separation methods

Physical scraping, heat separation, trypsin treatment and EDTA separation methods have been employed to determine an optimum method of separation and to compare relative enzymic activities of dermal and epidermal layers of hairless mouse skin. The skin of an HRS/J hairless mouse (12 weeks of age, Jackson Lab., Bar Harbor, ME) from neck to tail was removed after the animal was killed by cervical dislocation. Four equal sections of the skin were treated according to each of the following four separation techniques.

Physical scraping The separation of dermis and epidermis by physical scraping was performed by scraping the skin with a Bard Parker scalpel blade until no more superficial layer (epidermis) remained (Weibel et al., 1975). The complete removal of epidermis was verified by taking a similarly treated skin section and ascertaining that no further epidermal tissue sections could be removed following heat treatment. Also, a skin section separated by this method was studied by histological methods and a similar conclusion was drawn.

Trypsin treatment Trypsin separation (Yuspa and Harris, 1974) was performed by preparing a 0.2% solution of Difco trypsin 1:250 in isotonic pH 7.0 phosphate buffer-saline. The skin sample was placed on a Whatman filter paper and floated on the trypsin solution in a small Petri dish. The dish was placed in the refrigerator (approx. 4°C) for 6.5 h. The epidermis was easily scraped from the dermis and each tissue section was utilized for homogenate preparation and histology.

Heat separation Heat separation of epidermis from dermis was performed by placing the skin section in 60°C water for 45 s (Scribner and Slaga, 1973). The skin was then removed from the heat and placed in ice water for immediate cooling. The epidermis was then easily separated from the dermis by peeling with a scalpel blade.

EDTA separation EDTA separation was performed by preparing a 20 mM Na_2EDTA and 15 mM sodium phosphate buffer in normal saline and adjusting the solution pH to 7.2. The skin sample was immersed in this solution for 4 h at 37°C, and the epidermis was easily peeled from the dermis using a scalpel blade.

After each separation method was performed, the epidermal and dermal sections were homogenized in pH 7.4 phosphate buffer. The actual homogenization procedure is described below. Each homogenate was analyzed for protein content using a dye binding assay (BioRad). The rate of hydroloysis of DBMTX (initial concentration 10 μM) was determined for each epidermal and dermal homogenate preparation.

Histology of skin sections obtained by various treatments

The histological procedure was initiated with the fixation of skin samples for a minimum of 12 h in neutral buffered formalin (10%). The skin was precut, dehydrated in a series of alcoholic solutions, and embedded in liquid paraffin wax. The tissue was then microtomed into 5 μm slices and mounted on glass slides. The slides were stained with hematoxylin and eosin and then covered with a cover slip. Photomicroscopy was performed with a Nikon Labophot microscope, with an attached Nikon FX-35 camera. Magnification of up to 800 $\times$ was employed.

Homogenization procedure

Both epidermal and dermal tissues were placed in 5 ml of an ice cold pH 7.4 buffer (KH_2PO_4 200 mg, Na_2HPO_4 1.15 g in 800 ml of distilled water). The dermal tissue was precut with surgical scissors to facilitate homogenization. Then the tissue in buffer was homogenized with a Polytron PT 10/35 homogenizer with a PTA 10-S generator (Brinkmann) set at speed 6 for 2×30 s. The resulting milky suspension was then centrifuged for 6 min at 5000 rpm ($1030 \times g$) at 4°C in a TL-100 refrigerated ultracentrifuge (Beckman). The resulting supernatant was filtered through a glass wool plug in a pasteur pipette. This clearer supernatant was subsequently centrifuged and frozen for later use.

Determination of soluble protein content

The method by which the epidermal and dermal homogenates were assayed for their soluble protein content (a relative measure of potential enzyme protein) was the Biorad method, which utilizes a modified Bradford technique (Bradford, 1976).

Comparison of separation techniques (dermal enzyme activity)

The most suitable method for dermal/epidermal separation was determined by kinetic studies in homogenate medium. The ester hydrolase activities of each layer were evaluated and compared using DBMTX as the substrate. The comparison was based on the soluble protein yield in the homogenate in terms of activity per unit soluble protein, and activity per g tissue utilized. For simplicity (since epidermal homogenate has a much slower rate), only the dermal tissue was utilized, and the tissue weighing was carried out before the dermis and epidermis were separated (dermis is the predominant part of the tissue mass). The rate of loss of 10 μM DBMTX was compared for each of the methods and the degree of resulting esterase activity was calculated.

Kinetics of diester hydrolysis in dermal and epidermal homogenates

The ester hydrolase activity in the dermal and epidermal tissues was determined in terms of the relative rates of hydrolysis of the homologous series of MTX dialkyl esters. A uniform protein concentration in the homogenate was required for comparison. Each compound, i.e., DMMTX, DEMTX, DPMTX, DIPMTX and DBMTX (Scheme 1), was dissolved in DMF to generate a 2 mM solution in 20% DMF/homogenate. The presence of 20% DMF was found not to influence esterase activity to any measurable extent because the observed rate constant was not significantly different from that of 0% DMF, when DMMTX was used as the substrate. This finding tends to agree well with the fact that hydrophilic organic molecules are known to exert only minimal influence on the activity of esterases (Bogdanffy et al., 1987). The diester loss was followed as a function of time to determine the first-order rate constant. The regenerated monoester and MTX concentrations were also monitored simultaneously. Therefore, the relative degradative rate for each compound and the selectivity for the formation of the α- vs γ-monoester could be determined. Since no MTX was formed, the scheme for this enzymatic hydrolytic process is simplified considerably which could be easily fitted into a parallel degradative kinetics; i.e., $k_{obs} = k_1 + k_2$. The term k_{obs} represents the observed rate constant of diester disappearance and k_1 and k_2 denote the formation rate constants for α- and γ-monoesters, respectively.

Results and Discussion

Four different methods for epidermal/dermal separation have been compared as to their relative effectiveness in yielding uncontaminated layers. The effectiveness of each method in separating skin layers has been examined microscopically. The layer separation was apparent in each case (photomicrographs not shown). However, some separations appear to be more complete than others. The trypsin separation was shown to be easily discernable, while heat separation and EDTA methods required much higher magnification to demonstrate that in fact separation had been achieved. In all of the methods except physical scraping, the epidermal layer was easily removed from the dermis by peeling or gentle scraping. The scraping method may not be very precise, since the end point was very difficult to ascertain. Microscopic examination also confirmed that, although the removal of epidermis was complete, it was not known to what extent the epidermis was contaminated with dermis.

Once physical separation had been achieved, a comparison of the methods was necessary to examine how the various methods influenced enzyme activity in each of the layers. The first set of experiments began with the separation of a hairless mouse skin into four approximately equal sections, each subjected to one method of separation. After each separation procedure was performed, the entire epidermis and dermis from

TABLE 1

Effects of separation methods on soluble protein yield and dermal enzymatic activity using DBMTX as a substrate [a]

Method	Initial rate (M/min per mg protein) ($\times 10^7$)	Initial rate (M/min per mg tissue) ($\times 10^9$)	Soluble protein yield (mg/mg tissue) ($\times 10^3$)
Scraping	2.88	4.58	15.88
Heat separation	5.51	5.11	9.27
Trypsin	2.40	1.30	5.44
EDTA	6.64	5.69	8.57

[a] Data represent means of three determinations with variations less than 10%.

each section were homogenized in 5 ml of the pH 7.4 phosphate buffer as previously described. When the initial rate of loss of DBMTX was followed, it became apparent that the initial dermal rate was considerably higher than the epidermal rate (data not shown). The heat separation method yielded no usable data, but the other three methods all exhibited this trend consistently.

Each separation method was further examined according to the following three criteria: (1) initial rate of hydrolysis per unit soluble protein content; (2) initial rate of hydrolysis per unit weight of tissue; and (3) soluble protein yield per unit weight of tissue. Discrimination among these methods was rather difficult considering the fact that each method hydrates the skin to a different degree, and that epidermal degradation rates were too slow to allow for any practical comparison. Therefore, as described previously, skin from one mouse was removed and was separated in four approximately equal sections. The weighing of the tissue was performed before separation was achieved, since at this point, all of the skin sections were hydrated to the same degree. Only the dermis section was utilized for enzyme kinetic experiments, since these rates were relatively more rapid and hence can be more easily measured. As summarized in Table 1, EDTA treatment yielded the highest rates per unit concentration of soluble protein. Moreover, the highest rate per mg of tissue was also obtained from the EDTA treatment. Based on these criteria, EDTA treatment was chosen as the separation method for subsequent studies where several of the diester loss profiles were compared in each of the dermal and epidermal layers. Additionally, trypsin separation was unsuitable because of its considerably low protein yield value. Trypsin, being a serine protease, can conceivably destroy some of the enzymes contributing to ester hydrolase activity in the skin. The physical scraping method was eliminated from consideration since it was hard to determine if any cross-contamination of layers occurred. Heat separation was judged slightly inferior to the EDTA method as demonstrated by the lower rates of hydrolysis.

Since EDTA treatment has been determined to be a favorable method for dermal/epidermal separation, a comparison of the relative rates of hydrolysis for each of the diesters in homogenates of both layers has been made in order to determine the specificity of skin esterases. Preliminary experiments suggested that 2 μM was a reasonable starting concentration for all of the compounds. As illustrated in Fig. 1, both the dermis and epidermis first order rate constants increase logarithmically with chain length, up to the dibutyl ester. The branched-chain DIPMTX showed a rate constant in between those for the dimethyl and diethyl esters in both dermal and epidermal

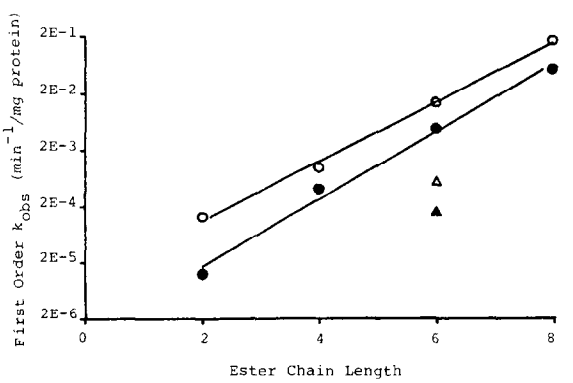


Fig. 1. Observed first order rate constants (k_{obs}) of diester hydrolysis in hairless mouse skin homogenates as a function of ester chain length. The initial concentration of diester was 2 μM in 20% DMF/homogenate at 37°C. (○) Dermal homogenate; (●) epidermal homogenate; (△) DIPMTX in dermal homogenate; (▲) DIPMTX in epidermal homogenate.

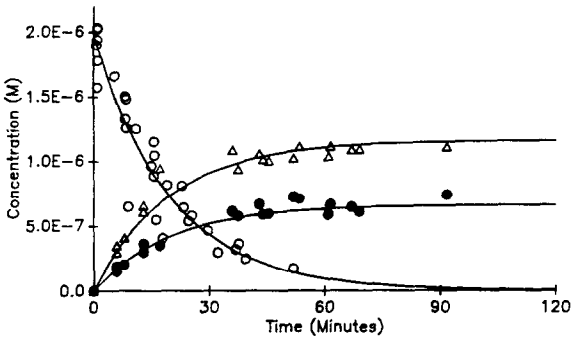


Fig. 2. DPMTX hydrolysis in a dermal homogenate (20% DMF). (○) DPMTX; (●) α-PMTX; (△) γ-PMTX.

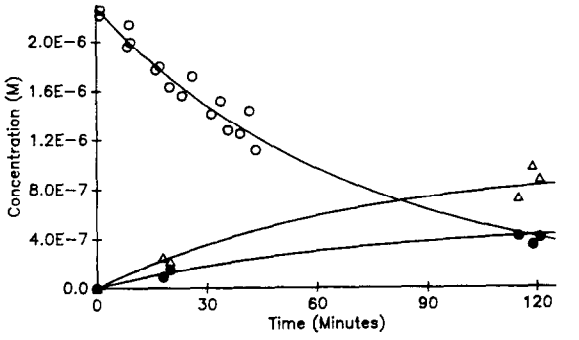


Fig. 3. DPMTX hydrolysis in an epidermal homogenate (20% DMF). (○) DPMTX; (●) α-PMTX; (△) γ-PMTX.

homogenates. For all compounds except DMMTX, the dermis showed 2–3-fold enhanced activity per unit protein weight compared with the epidermis.

In addition to following the loss of the diesters in the homogenates, the simultaneous formation of α- and γ-monoesters was also monitored. An example of a concentration/time profile for the di- and monoesters in dermal and epidermal homogenates is presented in Figs 2 and 3.

As with the stripped skin transport studies, the formation of MTX was not observed as well, at least over the experimental time period. This is consistent with another previous report (Krisch, 1971) suggesting that negatively charged compounds have little or no affinity for esterases, where the same linkage is cleaved rapidly when the compounds are neutral. From the homogenate data, the rate constants (k_1 and k_2) can be calculated and these values have been summarized in Table 2. Some observations can be made regarding the magnitudes of the individual rate constants obtained in these experiments. For the straight-chain compounds, i.e., DEMTX, DPMTX, and DBMTX, the relative proportions of α- and γ-monoester are not considerably different. However, a comparison of the branched-chain DIPMTX with the straight-chain DPMTX shows that there is more selectivity for the formation of the γ-monoester.

In conclusion, results obtained from this in vitro bioconversion study of MTX dialkyl esters in hairless mouse skin homogenates confirmed our previous observation that skin esterases play an important role in the cutaneous transport of MTX prodrugs. The activity of skin esterases correlates positively with the side-chain length, and therefore, the substrate lipophilicity. Branching of ester side-chains, on the other hand, retards the rate of cleavage significantly. The specific affinity for dialkyl esters rather than monoesters indicates that only neutral molecules can better serve as skin esterase substrates. The consequence of this property is unclear at present

TABLE 2

Apparent first order rate constants in the dermal and epidermal homogenates [a]

Compound	Dermis			Epidermis		
	k_1	k_2	k_2/k_1	k_1	k_2	k_2/k_1
DMMTX	7.198×10^{-5}	6.078×10^{-5}	0.84	–	–	–
DEMTX	4.064×10^{-4}	5.548×10^{-4}	1.36	–	–	–
DPMTX	4.918×10^{-3}	8.622×10^{-3}	1.75	1.594×10^{-3}	3.102×10^{-3}	1.94
DIPMTX	1.139×10^{-4}	4.358×10^{-4}	3.83	–	–	–
DBMTX	7.084×10^{-2}	9.310×10^{-2}	1.31	2.112×10^{-2}	3.080×10^{-2}	1.46

[a] Data represent means of three determinations. The unit of ks is in min^{-1} mg^{-1} protein.

concerning the transformation of MTX prodrugs to MTX.

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