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Transport and degradation characteristics of methotrexate dialkyl ester prodrugs across tape-stripped hairless mouse skin

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Summary

A series of methotrexate dialkyl esters were examined with respect to their permeability across tape-stripped hairless mouse skin. The dialkyl esters showed a parabolic permeability versus side chain length relationship with the dimethyl ester being the most permeable compound. These compounds were also found to undergo an increased degree of degradation with increased ester chain length during the diffusion process, while with substantially reduced degradation occuring with the branched chain diisopropyl ester. No measurable methotrexate was formed during the course of the experiment, apparently due to the chemical and enzymatic stability of the intermediate α- and γ-monoesters.

Introduction

Methotrexate (MTX), an antifolate chemotherapeutic agent, has been used for many years in the therapy of various forms of neoplasms including choriocarcinoma and acute lymphocytic leukemia (Farber et al., 1948; Kamen, 1987). More recently, MTX has been utilized successfully in the treatment of severe and otherwise unresponsive cases of psoriasis (Weinstein and Frost, 1971). This application, however, requires the drug to be administered systemically, thus exposing the patient to all of MTX's potentially dangerous toxicities (Weinstein, 1977). Attempts to deliver MTX topically to the psoriatic lesions have been less encouraging (McCullough et al., 1976). After considerable study it has been largely determined that the possible reasons of MTX's topical ineffectiveness are its inadequate penetration through the stratum corneum (Wallace et al., 1978a), as well as its rapid removal from the viable epidermis, the site of action, via a dermal clearance mechanism (Siddiqui et al., 1985).

The preparation of linear lipid soluble diester homologs of MTX has been described previously, as has been the study of their hydrophobic pa-

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rameters as a function of alkyl chain length (Fort and Mitra, 1987). Reasonable anti-psoriatic activity was noticed in skin plug assays for some of these diesters (McCullough and Weinstein, 1974; Weinstein and McCullough, 1975; McCullough et al., 1977). However, a very limited study on their permeability determination through human cadaver skin has been reported (McCullough et al., 1976) in which a single 20 h receptor sample, after finite dose application of dimethyl MTX (DMMTX), was measured along with sections of dermis and epidermis. No difference was shown in the amount of drug penetrated as compared to MTX, although higher dermal and epidermal levels were observed. The permeability of the compound has not been measured nor a study of its simultaneous degradation during transport has been undertaken.

This report describes an evaluation of a homologous series of MTX dialkyl esters across the tape-stripped hairless mouse skin with an effort to characterize their permeabilities and the extent of simultaneous degradation kinetics.

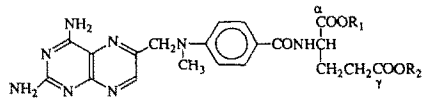
Materials and Methods

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) MTX, 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 and Mitra, 1993a). Chemical structures of the compounds utilized in this study are shown in Fig. 1.

HPLC assay procedure

HPLC analyses of the dialkyl esters of MTX were carried out with pH 3, 0.05 M KH_2PO_4/acetonitrile (ACN) mobile phases varying from 72.5:27.5 to 55:45 (v/v) proportions, and containing 5 mM triethanolamine (TEA). The separation system 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 tetrabutyl ammonium 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, 440 single wavelength detector set at 254 nm, a U6K injector (Waters), an Omniscribe B-5000 strip chart recorder (Houston Instruments), and a Chromatopac E-1A integrator (Shimadzu). Unknown concentrations were determined from standard curves of diesters, monoester mixtures, and MTX.



Compound	R_1	R_2
Methotrexate (MTX)	H	H
Dimethyl Methotrexate (DMMTX)	CH_3	CH_3
Diethyl Methotrexate (DEMTX)	CH_2CH_3	CH_2CH_3
Dipropyl Methotrexate (DPMTX)	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$
Diisopropyl Methotrexate (DIPMTX)	$CH(CH_3)_2$	$CH(CH_3)_2$
Dibutyl Methotrexate (DBMTX)	$CH_2(CH_2)_2CH_3$	$CH_2(CH_2)_2CH_3$
α-Methyl Methotrexate (α-MMTX)	CH_3	H
γ-Methyl Methotrexate (γ-MMTX)	H	CH_3
α-Ethyl Methotrexate (α-EMTX)	CH_2CH_3	H
γ-Ethyl Methotrexate (γ-EMTX)	H	CH_2CH_3
α-Propyl Methotrexate (α-PMTX)	$CH_2CH_2CH_3$	H
γ-Propyl Methotrexate (γ-PMTX)	H	$CH_2CH_2CH_3$
α-Isopropyl Methotrexate (α-IPMTX)	$CH(CH_3)_2$	H
γ-Isopropyl Methotrexate (γ-IPMTX)	H	$CH(CH_3)_2$
α-Butyl Methotrexate (α-BMTX)	$CH_2(CH_2)_2CH_3$	H
γ-Butyl Methotrexate (γ-BMTX)	H	$CH_2(CH_2)_2CH_3$

Fig. 1. Chemical structures of methotrexate, methotrexate dialkyl esters, and methotrexate monoalkyl esters.

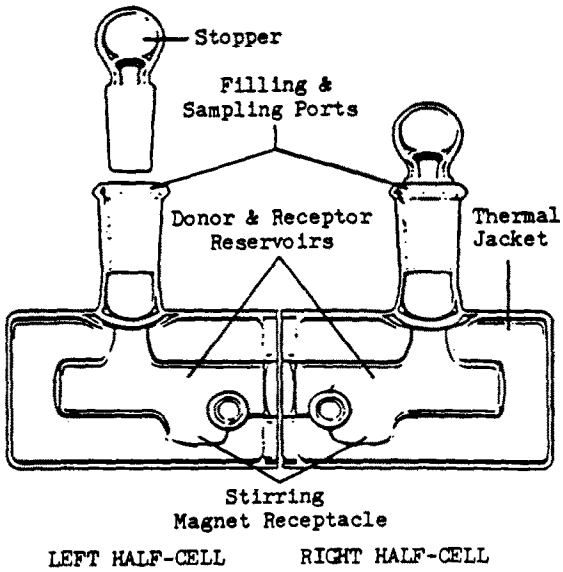


Fig. 2. Schematic diagram of the diffusion apparatus in which tape-stripped hairless mouse skin transport studies were performed.

Diffusion apparatus

The diffusion cells utilized in all of the skin transport studies are schematically illustrated in Fig. 2. These cells (DC-100B, Crown Glass) have a 3.4 ml internal volume, and a 0.636 cm^2 diffusion area, equipped with a built-in water jacketing system for constant temperature. Stirring was accomplished with the help of a magnetic stirrer (Scientific Products), and internally mounted star head magnets. The temperature was maintained with a small volume bath (MGW Lauda), circulating water through the external jacket of the diffusion cells via a tygon tubing.

Preparation of hairless mouse skin

The skin utilized in these studies was obtained from male hairless mice (HRS/J strain, Jackson laboratories) of 10–13 week age range. The animals were killed by cervical dislocation. A fresh piece of cellophane tape was rubbed across the desired area 25 times (Yu et al., 1979). Then, the skin was excised from the animal by lifting it from the underlying fascia. The dermal side of the skin was cleaned with blunt tweezers to remove any subcutaneous fat and other debris. Then, the skin section was divided into two approximately equal halves which were subsequently used in the transport and degradation studies.

Transport and degradation studies

The pre-harvested mouse skin was then mounted between two diffusion cells and the cells were clamped together. Each cell was filled with a diffusion medium, which consisted of 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4 in 800 ml of distilled water, adjusted to pH 7.4 to which 200 ml of dimethylformamide (DMF) was added, generating an apparent pH of about 8.1. This medium will be referred to as the diffusion medium. Both sides of the stripped skin were allowed to bathe in the diffusion medium for 4 h. The cells were maintained at 37°C and the medium was continuously stirred. At the end of this 4 h leaching period, the bathing solution was removed. Each cell was rinsed with fresh medium and then the receptor or dermal side was filled with blank diffusion medium.

The donor side of the diffusion cell was filled with a saturated solution of the diester dissolved in the diffusion medium being investigated. Excess solid was added to the donor side to ensure saturation over the time course of the experiment. Periodic samples were withdrawn from the receptor phases to establish forward diffusion of the diester and metabolites. The solution removed (2×50 μl samples) was replaced with fresh diffusion medium to maintain a constant volume. The samples were immediately frozen at −78°C and maintained at this temperature until analysis could be performed. The amount of each species diffusing per unit time was determined by assaying receptor samples for intact diester and hydrolytic products.

Results and Discussion

Each of the diesters, i.e., DMMTX, DEMTX, DPMTX, DIPMTX, and DBMTX, as well as the parent compound MTX were examined as to their simultaneous transport and degradation across tape-stripped skin. In each case, the compound was allowed to diffuse from a saturated donor solution in the diffusion medium, through

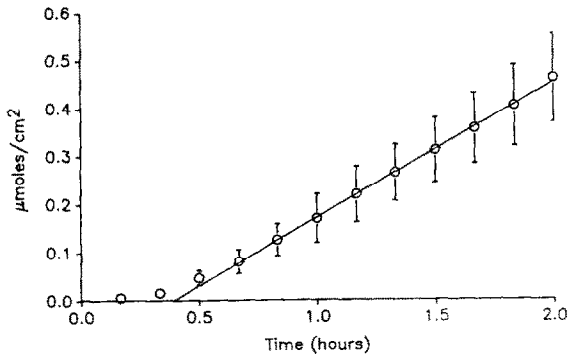


Fig. 3. MTX receptor appearance following transport across tape-stripped hairless mouse skin.

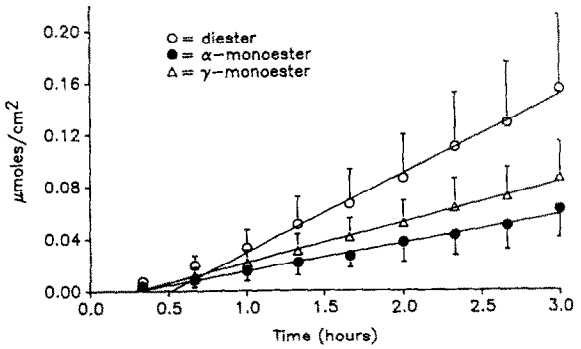


Fig. 4. Receptor appearance of DPMTX and simultaneous conversion products after diffusion through tape-stripped hairless mouse skin.

the tape-stripped membrane, into the receptor compartment containing the blank diffusion medium (20% DMF/pH 7.4 buffer). Before an experiment, the skin was also pre-leached for 4 h to minimize bulk phase metabolism of the compounds. The experiments were carried out for 2–4 h depending on the compound.

Fig. 3 graphically depicts the accumulation kinetics of MTX in the receptor phase following exposure to MTX solution in the donor compartment. It appears that the accumulation follows a typical linear transport behavior with a lag time of about 0.4 h. Diffusion studies involving diesters were found to be more complicated than MTX, since three species appeared in the receptor phase, i.e., intact diester and two cleaved monoesters. Therefore, the respective concentrations of all three species were individually measured as a function of time and the representative profiles are shown in Fig. 4.

After each diester had been examined, the flux data of these experiments were summarized and are listed in Table 1. The contribution of these three species in terms of percentages of total flux is also presented in Table 2. An important observation is that as the chain length of the series is ascended, the degree of conversion increases. Generally, at least in terms of receptor appearance, DMMTX and DEMTX are both shown to produce roughly 87% of the flux due to intact diester. For both of those compounds, the monoesters constitute the remainder of receptor flux.

With DPMTX, the degree of conversion increases dramatically causing about 46% of the total flux to be due to the monoesters. It is observed that the γ monoester predominates over α roughly in a 3:2 ratio. With the next higher homolog, DBMTX, none of the receptor flux has been found to be due to intact diester, i.e., conversion of this compound is complete during pas-

TABLE 1

Receptor steady-state flux obtained from stripped skin diffusion experiments

Compound	Apparent receptor flux (μmol/cm^2 per h)			
	Diester	α-Monoester	γ-Monoester	MTX
MTX	–	–	–	2.830×10^{-1}
DMMTX	4.869×10^{-1}	7.888×10^{-3}	6.149×10^{-2}	–
DEMTX	2.280×10^{-1}	6.662×10^{-3}	2.646×10^{-2}	–
DPMTX	6.078×10^{-2}	2.152×10^{-2}	3.092×10^{-2}	–
DIPMTX	1.102×10^{-1}	1.225×10^{-3}	3.509×10^{-3}	–
DBMTX	–	6.040×10^{-3}	7.772×10^{-3}	–

TABLE 2

Comparison of flux contribution from diesters, α-, and γ-monoesters in the receptor phases

Compound	Percentage of total receptor flux			
	Diester	α-Monoester	γ-Monoester	MTX
DMMTX	87.53	1.42	11.05	0
DEMTX	87.32	2.55	10.13	0
DPMTX	53.68	19.00	27.31	0
DIPMTX	95.88	1.07	3.05	0
DBMTX	0	43.73	56.27	0

sage through the membrane. The γ-butyl monoester predominates over the α isomer, however, only to a small degree. The degree of conversion of DIPMTX, in terms of receptor appearance of the monoesters, was found to be extremely small with only 4% flux due to monoesters. The predominance of the γ monoester over the α isomer is 3:1 as compared to a 3:2 ratio for DPMTX. No MTX was found in the receptor compartment with any of the compounds during the time course of the experiment.

Although the appearance of α and γ monoesters suggests simultaneous degradation of diesters along the in vitro diffusion process, the relative contribution of chemical degradation and skin esterase-mediated enzymatic cleavage cannot be easily accounted for. This is at least in part due to the rather complicated nature of this overall process. To separate these individual events, in vitro enzymatic degradation studies involving skin homogenates were performed (Fort and Mitra, 1993b). Computer simulation was then utilized to draw a clear picture of the individual kinetics (Fort and Mitra, 1993c).

For an approximate estimation, however, the degradation rate constants (k) of various diesters in the diffusion medium at 37°C which have been determined previously (Fort and Mitra, 1990) could be used. Taking into consideration that the k for DMMTX is 8.22×10^{-3} h^{-1} and the duration of a diffusion experiment was no longer than 4 h, the percentage of DMMTX hydrolysis can be calculated not to exceed 3.2%. Therefore, approx. 25% of the monoesters in the receptor phase may have been formed by chemical hydrolysis during DMMTX diffusion process. The percentage contribution of chemical hydrolysis to the overall diester degradation of other diesters with longer linear side chains will be significantly less due to their lower k values.

Some general observations can be made from the data presented in this report. First of all, the degree of conversion increases with chain length, i.e., a low degree of hydrolysis with DMMTX and DEMTX, to complete breakdown of DBMTX. Also, it appears that the degradation process always favors the formation of the γ monoester, with the ratio of γ/α decreasing with increasing chain length. However, the branching side chain of the diisopropyl ester results in a greater γ predominance over the straight chain dipropyl ester. Finally, no MTX was formed from the α and γ monoesters. This is not an unusual phenomenon, since it has been reported in the literature that negatively charged compounds have lesser affinity for esterases (Krisch, 1971)..

The stripped skin experiments also suggest that for those compounds that are minimally converted, namely, DMMTX, DEMTX and DIPMTX, the permeability values, calculated on the basis of diester flux only, exhibit a parabolic relationship with alkyl chain length (Fig. 5). The stripped skin permeability increases from MTX to DMMTX, and then apparently descends with increasing chain length.

The stripped skin membrane itself is essentially a bilaminate consisting of the viable epider-

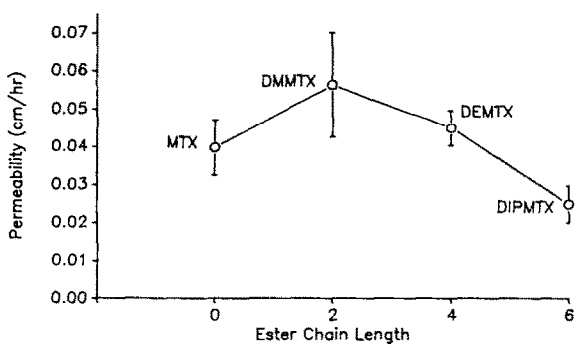


Fig. 5. MTX and MTX diester permeability through tape-stripped hairless mouse skin as a function of ester carbon chain length.

mis and dermis. Of the two layers, the viable epidermis is more lipoidal while the dermis is relatively polar (Barry, 1987). Methotrexate transport occurs primarily through a paracellular or appendageal pathway (Wallace and Barnett, 1978b). Upon diesterification, however, the transcellular component becomes more significant. As the series is ascended the molecules become more lipophilic and have less affinity for the highly solvated paracellular or appendageal pathway. Since DMMTX has the greatest permeability across this membrane, it can be stated that it exhibits the best biphasic affinity of all the compounds. In other words, it is more lipophilic than MTX, as shown by its partition coefficient, but still retains adequate affinity for the solvated regions of the membrane (its solubility in the diffusion medium was also the highest of all the compounds studied). Therefore, DMMTX adds transcellular transport through this thin epidermal membrane to MTX's paracellular component. Increasing the chain length further does not enhance the permeability since the solvated paracellular route offers more of a resistance to increasingly lipophilic molecules as seen especially with the diisopropyl ester. It can be suggested that in order to obtain optimum skin permeation a compound needs to possess adequate biphasic affinity, since the paracellular route is an extremely significant part of the total mass transport.

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