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The Metabolism of a Series of Ester Pro-drugs by NCTC 2544 Cells, Skin Homogenate and LDE Testskin

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Abstract—The metabolism of a series of substituted pyrazolopyridine ester pro-drugs was investigated using NCTC 2544 cells, human skin homogenate and LDE Testskin as model systems. The compounds were incubated in each system and the disappearance of drug and the production of the major hydrolysis product was observed with time and quantitated using HPLC. The toxicity of the ester pro-drugs and the metabolites was examined in NCTC 2544 cells using a cell viability assay procedure. Hydrolytic activity was slightly higher in the cell culture model than in skin homogenate solution but the rank order of activity for each pro-drug was similar. The metabolic activity of LDE Testskin was much reduced compared with the other systems, but again the overall pattern of metabolism was not dissimilar. These findings indicate that NCTC 2544 cells provide a reasonable model for human skin ester hydrolysis both in terms of rate and in terms of substrate specificity.

The fate of any drug applied to the skin surface, be it for topical or transdermal therapy, will be affected in one of several ways. If the skin is not capable of metabolizing the drug the local and systemic availabilities will be associated with the percutaneous absorption characteristics of the drug. If, however, the skin is capable of metabolically inactivating a pharmacologically active drug or changing the physicochemical properties of the drug, the percutaneous absorption characteristics and vehicle effects will still be important, but the rate of biotransformation will be of particular relevance as high levels of metabolic activity will reduce the local bioavailability of the drug. The skin may be capable of metabolically activating a drug with negligible pharmacological activity. In this instance, the local and systemic bioavailability is largely dependent on the metabolic activity of the skin. The role of biotransformation in topical and transcutaneous therapy has been discussed previously (Tauber 1989).

A pro-drug may be defined as an inactive agent that is enzymatically altered to the pharmacologically active drug in a predictable and controlled manner (Bucks 1984). The pro-drug approach has been employed in topical corticosteroid therapy and generally, corticosteroid esters are used to increase the lipophilicity of the drug and to facilitate penetration through the skin (Tauber 1989). Lipophilic pro-drugs of cromoglycic acid have been developed for anti-pruritic and anti-inflammatory activity (Bodor et al 1980). These pro-drugs enhanced skin penetration, particularly with the hexanoyloxymethyl ester. Pro-drugs of salicylic acid and aspirin have also been developed (Loftsson et al 1981) to overcome the problems of gastric irritation and bleeding. Other pro-drugs have been produced to increase absorption or otherwise enhance delivery (Yu et al 1979a,b; Mollgaard et al 1982; Bodor & Sloan 1983; Sloan et al 1983; Bundgaard 1988; Silver & Sloan 1988).

A spectrum of enzymes capable of metabolizing topically applied drugs and various substances is contained within the skin (Pannatier et al 1978; Tauber 1989). The metabolic role of the skin has been supported in studies with topically applied glyceryl trinitrate (Wester et al 1983) and it has also been suggested (Holland et al 1984) that the rate limiting step in percutaneous absorption of benzo[a]pyrene is the rate of metabolism within the skin. The skin performs reactions including phase I (functionalization) reactions and phase II (conjugation) reactions. These reactions have been outlined (Martin et al 1987; Martin 1988).

Methods employed in the extensive range of in-vitro studies for examining cutaneous metabolism have included the incubation of skin fragments (Smith & Holland 1981) or skin homogenates (Andersson & Ryrfeldt 1984; Guzek et al 1989) with test compounds followed by the subsequent characterization of the metabolites. Skin grafts on mice have provided an alternative in-vivo method of study (Guzek et al 1989). Short-term cultures of viable and structurally intact skin (Smith & Holland 1981; Kao et al 1983) have been used to study biotransformation reactions in the skin, removing the confounding effect of systemic metabolism which may inevitably be included in in-vivo studies. Cell culture offers a relevant, fully characterized and reproducible in-vitro system since a continuous cell line will reproduce the metabolic activity of the cells over a longer period than may be expected for a non-transformed cell line or primary culture.

In this study, the relative merits of cell culture are compared with those of the skin homogenates as models for skin metabolism. The basis of this comparison includes assessment of the enzymatic profile and the metabolic activity of both a skin epithelial cell line (NCTC 2544 cells) and homogenized skin. Furthermore, a commercially available product has recently been introduced as graft

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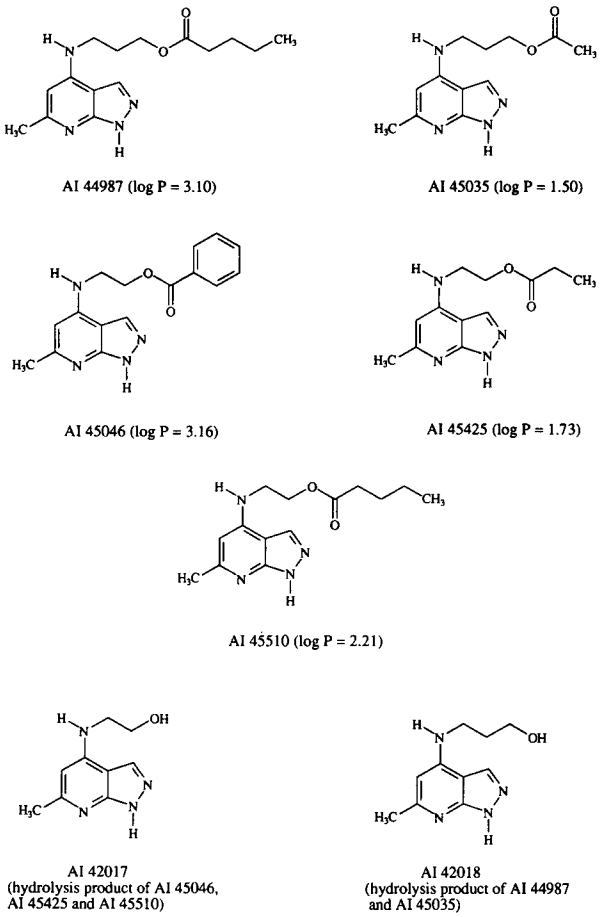

FIG. 1. Chemical structures and octanol/water partition coefficients (log P) of the AI ester pro-drugs and the major hydrolysis fragments.

replacement therapy for burns patients and is currently under development as an in-vitro skin penetration model. The potential of this LDE Testskin as a viable metabolizing skin substitute is evaluated here in comparison with the NCTC 2544 cells and the skin homogenate using a series of related ester pro-drugs intended as potential anti-inflammatory agents for topical administration (Fig. 1).

Materials and Methods

Chemicals, reagents and disposable materials

NCTC 2544 cells and Biorich II basal growth medium were obtained from ICN Flow, High Wycombe, UK. Dimethylsulphoxide (DMSO) and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (thiazolyl blue) were obtained from Sigma Chemical Co. Ltd, Poole, UK. Millicell-HA filter inserts were purchased from Millipore (UK) Ltd, Watford, UK. All other cell culture disposables were obtained from Northern Media, North Humberside, UK. Acetonitrile was HPLC grade and was obtained from May & Baker Ltd, Dagenham, UK. Phosphate-buffered saline (PBS) was obtained from Gibco Ltd, Paisley, UK. LDE Testskin kits were purchased from Organogenesis Inc., Cambridge, MA, USA. API ZYM test kits and reagents were obtained from API-bioMericieux (UK) Ltd, Basingstoke, UK. The series of ester pro-drugs and the hydrolysis products (Fig. 1) were synthesized and donated by SmithKline Beecham, Worthing, UK. The route of synthesis for the ester pro-drugs was by diacylation of the major hydrolysis product. For example, AI 45510 was prepared by diacylation of AI 42017 with either valeric chloride or a combination of valeric anhydride and valeric chloride. This yielded an intermediate (not isolated), the treatment of which with methanol provides the free base AI 45510 (after neutralization, extraction, chromatography on alumina and trituration with diethyl ether) which is a white crystalline solid. A scheme for the synthesis of AI 45510 is shown in Fig. 2.

Cell culture toxicity assay

Stock solutions (1000 μg mL^{-1}) of the ester pro-drugs were prepared in serum-free Biorich II growth medium containing 2% DMSO. These stock solutions were diluted where necessary to give final concentrations of 1000, 500, 100, 50 and 10 μg mL^{-1}. A cell suspension (0·5 mL) of NCTC 2544 cells (passage number between 283 and 286) at a seeding density of $2{\cdot}5 \times 10^5$ cells mL^{-1} was added to a Millicell-HA filter insert placed in a well of a Falcon 3047 24-well plate (containing 0·5 mL growth medium) and the cells were grown at 37°C for seven days, after which time the spent growth medium was removed and discarded. A sample (0·5 mL) of Biorich II containing the ester pro-drugs and the major hydrolysis products at each concentration was added both to the inside and the outside of three Millicell-HA filter inserts. Serum-free Biorich II was used in triplicate

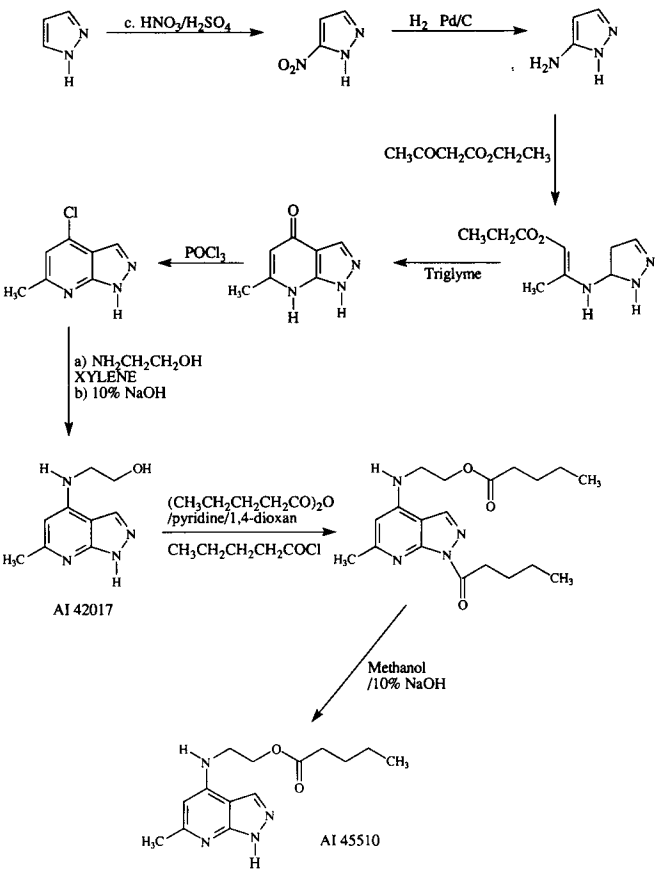

FIG. 2. Synthetic route for the production of AI 45510.

filter inserts to provide negative controls for cells not exposed to the drugs. Positive controls of 10 and 25% DMSO in Biorich II were also added to triplicate filter inserts to demonstrate toxicity. The filter inserts were incubated at 37°C for 48 h with 5% CO_2–95% air atmosphere. After the test exposure time of 48 h the viability of the cells was assessed using the thiazolyl blue mitochondrial conversion assay as described previously (Mossman 1983).

Cell culture metabolism

Seven days before initiation of metabolism experiments, NCTC 2544 cells (passage number 284-290) were seeded into 80-cm^2 tissue culture flasks at a density of $1–2 \times 10^5$ cells cm^{-2}. The cells were maintained at 37°C in an atmosphere of 5% CO_2–95% air and the growth medium (serum-free Biorich II) was changed every 72 h or sooner if required. Working solutions containing approximately 100 μg mL^{-1} of the ester pro-drugs were prepared in Biorich II growth medium. The working solutions were further diluted to 75, 50 and 25% of the initial concentration and the four solutions were used in triplicate cell-culture flasks for each of the ester pro-drugs. The metabolism experiments were initiated by replacing the spent growth medium in the tissue culture flasks with 15 mL Biorich II containing the ester pro-drugs. A volume (15 mL) of Biorich II containing the ester pro-drugs at each concentration was added to triplicate control tissue culture flasks without cells. Samples (0·5 mL) were removed and diluted with HPLC grade acetonitrile (0·5 mL). The samples were centrifuged at 12 000 rev min^{-1} for 5 min (Microcentaur, Fisons, Loughborough, UK) and the supernatant was then stored frozen (−20°C) until analysis. The sample volume was replaced with Biorich II at each sample point. The samples were thawed to room temperature (21°C) and assayed by HPLC to determine the parent pro-drug concentration and the concentration of the major hydrolysis product. Biorich II growth medium containing the major hydrolysis products AI 42017 and AI 42018 at 100 μg mL^{-1} was prepared and incubated in the presence of NCTC 2544 cells to determine if further metabolism of these compounds occurred.

Skin homogenate metabolism

Human abdominal full thickness skin was obtained following stomach reduction surgery (ethical permission was granted by the Ethics boards of the hospitals concerned). The skin was stored frozen (−20°C) between Perspex sheets and used within a period of three months. Any subcutaneous fatty tissue was cut from the dermal surface and discarded. The skin was cut into 2 × 2 cm squares and placed in a blender. PBS (50 mL) was added to the blender and the mixture homogenized for 10 min. A further volume of PBS (50 mL) was added to the blender and the mixture homogenized for an additional 5 min. The homogenate was centrifuged at 3500 rev min^{-1} for 20 min and the supernatant solution used in the following experiments. Skin homogenate solution (9 mL) was diluted to 20 mL in precooled (4°C) PBS and stored at 4°C. Working solutions of PBS containing 111·1 μg mL^{-1} of the ester pro-drugs were prepared and further diluted to 75, 50 and 25% of the initial concentration. Volumes (0·9 mL) of these solutions were added to clean test tubes and the temperature was allowed to increase to 37°C. The temperature of the diluted skin homogenate solution was allowed to increase to 37°C and a volume (0·1 mL) was added to each test tube. The test tubes were maintained at 37°C throughout the experiment. Control experiments were carried out in triplicate by incubating 9·9 mL PBS containing the drugs with 9·1 mL PBS. Three test tubes per time point were used for each concentration of the ester pro-drugs and were specific to each time point. The incubation was terminated at each time point by diluting a sample (0·5 mL) with HPLC grade acetonitrile (0·5 mL). The samples were centrifuged at 12 000 rev min^{-1} for 5 min and the supernatant was then stored frozen (−20°C) until analysis. The samples were thawed to room temperature (21°C) and analysed by HPLC to determine the parent ester pro-drug concentration and the concentration of the major hydrolysis product.

LDE Testskin metabolism

The LDE Testskin samples were grown on Millicell-HA filter inserts using LDE Testskin maintenance medium. Growth medium containing 50 μg mL^{-1} of the ester pro-drugs was prepared in LDE Testskin test medium. The metabolism experiments were initiated by replacing the spent growth medium surrounding the LDE Testskin samples with LDE Testskin test medium containing the ester pro-drugs. The medium was added to the inside of the LDE Testskin filter (5 mL) and also to the well (1·5 mL) in which the LDE Testskin filter was maintained. Millicell-HA filter inserts without cells were added to 6-well plates (Falcon 3046) and used as controls. LDE Testskin test medium containing the ester pro-drugs was added to the inside and outside of the control filters in the same proportions as the LDE Testskin samples. Samples (0·25 mL) were periodically removed from the inside of the LDE Testskin filters and from the control filters and were diluted with HPLC grade acetonitrile (0·25 mL). The samples were centrifuged at 12 000 rev min^{-1} for 5 min and stored frozen (−20°C) until analysis. LDE Testskin test medium was added after each sample was taken to replace the sample volume. The samples were thawed to room temperature (21°C) and assayed by HPLC to determine the parent ester pro-drug concentration and the concentration of the major hydrolysis product.

Total protein determination: cell culture

On completion of the metabolism experiments described above, the NCTC 2544 cell monolayers were rinsed in calcium- and magnesium-free Hank's balanced salt solution (CMF HBSS) and trypsin-EDTA solution (0·05 g trypsin, 0·02 g EDTA, 2·5 mL 1 M HEPES buffer in 97·5 mL CMF HBSS) was added to the culture flask, gently agitated, and removed to leave 1 mL in the flask. The flask was incubated at 37°C for 10–15 min to dislodge the cells. The cell sheet was then resuspended in growth medium and centrifuged through 1 mL serum at 200 g for 15 min. The cellular pellet produced after centrifugation was resuspended in sterile double-distilled water (10 mL) to produce a cell lysate. Lysis of the cells was aided by vortexing the solution and sonicating in an ultrasonic water bath for 5 min. The cell lysate was cooled (4°C) and diluted 5- and 10-fold with precooled (4°C) sterile double-distilled water after which the total protein content of these

solutions was determined using the method of Lowry et al (1951).

Total protein determination: cell culture: skin homogenate
The total protein content of the skin homogenate solution was determined (Lowry et al 1951).

Total protein determination: cell culture: LDE Testskin
The LDE Testskin sample mounted on the filter was cut from the filter housing and added to sterile double-distilled water (5 mL). The Testskin sample was physically disrupted using a glass rod, vigorous vortexing for 10 min, followed by sonication in an ultrasonic water bath for 10 min. The solution was centrifuged at 2000 rev min^{-1} (10 min) to remove the filter. The total protein content of this solution was determined (Lowry et al 1951).

Enzyme profiles
Representative samples of the cell culture lysate suspensions, the skin homogenate solution and of the LDE Testskin lysate solution, prepared as above for total protein determination, were used in the API ZYM test kit to show the enzyme profiles of the respective systems of the cell monolayers.

Analysis of the series of ester pro-drugs
The HPLC equipment consisted of a Gilson dilutor 401 and sample injector model 231, a Gilson pump model 302 with dynamic mixer model 811 and manometric module model 802C and a Gilson UV 116 detector (Gilson International, Villiers-le-Bel, France). The HPLC data were stored and integrated using Gilson 714 HPLC system controller software. The HPLC column was a Hypersil CPS 5 μm (Shandon Scientific, Runcorn, UK) (15 cm × 4·6 mm i.d.) and the column was eluted with a mobile phase consisting of acetonitrile (25%) and 0·01 M potassium dihydrogen orthophosphate adjusted to pH 2·5 using orthophosphoric acid (75%) delivered at a flow rate of 2·0 mL min^{-1}. The retention times (min) were as follows: AI 42017 1·7, AI 42018 1·8, AI 44987 4·4, AI 45035 2·2, AI 45046 4·1, AI 45425 2·4 and AI 45510 3·7. Duplicate or triplicate injections were made for each sample. The assay method was linear over the range used in the studies ($r^2 > 0.997$) and demonstrated good recovery, precision (variability < 3%) and accuracy.

Data analysis
An exponential model of the form $A_t = A_o e^{-kt}$ (where A_t is the amount of drug remaining in an incubation at time t, A_o is the initial amount of drug present and k is a rate constant) was fitted using nonlinear regression analysis (MINIM 1·9, R. D. Purves) to the drug concentration-time data obtained from the HPLC analytical procedure. The metabolic half-life was calculated from the division of the natural logarithm of 2 by the rate constant. Metabolic activity values were calculated from the initial metabolic rates corrected for protein concentration in the incubation.

Results

The toxicity of the ester pro-drugs and the hydrolysis products to NCTC 2544 cells is shown in Table 1. The DMSO positive controls demonstrated that the toxicity test using the thiazolyl blue assay was able to distinguish between the toxic effects of two different concentrations of DMSO. When the relative toxicity of the ester pro-drugs is examined it appears that the most toxic of the drugs is AI 45046. This result may have been influenced by the presence of DMSO in the growth medium to aid dissolution of the ester, but this compound was the only one of the series to contain an aromatic (benzoyl) substituent attached to the ester linkage. The hydrolysis products, AI 42017 and AI 42018, were seen to be the least toxic moieties towards NCTC 2544 cells. AI 44987 was considerably more toxic than AI 45510, both of which have a pentanoyl group attached to the molecule by the ester linkage (Fig. 1). However, AI 44987 has a propylamino chain on the pyrazolopyridine side of the ester linkage which may have increased the toxicity of the compound in comparison with AI 45510 which has an ethylamino chain in this position (Fig. 2). The complete side chain is likely to determine the relative toxicities of the molecules. AI 45035 also contains the propylamino chain on the pyrazolopyridine side of the ester linkage but has a much lower toxicity towards NCTC 2544 cells, particularly at 100 μg mL^{-1}, than AI 44987.

The effect of incubating AI 42017 and AI 42018 in the presence of NCTC 2544 cells was examined and showed that these likely hydrolysis products were not broken down by NCTC 2544 cells during the course of the experiment. Each ester pro-drug was incubated with NCTC 2544 cells at four different concentrations, and the disappearance of the parent drug and the production of the major hydrolysis compounds was examined and corrected for the dilution sampling effect which occurred at each sample point. All concentrations

Table 1. Effect of dimethylsulphoxide, the AI ester pro-drugs and their major hydrolysis products on the viability of NCTC 2544 cells.

Concn in medium (μg mL^{-1})	NCTC 2544 cell viability (% control)						
	42017	42018	44987	45035	45046	45425	45510
0	100·0	100·0	100·0	100·0	100·0	100·0	100·0
10	109·9	103·5	87·3	116·4	81·5	112·3	113·5
50	98·9	98·2	62·2	132·9	37·2	80·9	118·0
100	106·4	125·0	19·4	115·1	14·7	98·5	50·5
500	66·9	76·8	9·4	6·8	2·6	9·8	23·9
1000	3·2	2·8	3·0	11·2	2·3	3·0	9·9
10% DMSO	25·3	46·8	48·0	34·8	27·8	29·5	34·3
25% DMSO	4·9	5·1	8·8	11·8	5·2	7·6	6·3

Table 2. Metabolism of the ester pro-drugs by NCTC 2544 cells.

Compound		Study I	Study II	Study III	Study IV
AI 44987	Concn (μmol mL^{-1})	0·230	0·213	0·139	0·067
(log P = 3·10)	Rate constant (h^{-1})	1·049	1·216	1·247	1·171
	Half-life (h)	0·66	0·57	0·56	0·59
AI 45035	Concn (μmol mL^{-1})	0·395	0·301	0·211	0·085
(log P = 1·50)	Rate constant (h^{-1})	0·105	0·132	0·170	0·159
	Half-life (h)	6·61	5·25	4·08	4·36
AI 45046	Conc (μ mol mL^{-1})	0·189	0·125	0·076	0·043
(log P = 3·16)	Rate constant (h^{-1})	0·709	1·074	0·981	0·975
	Half-life (h)	0·98	0·64	0·78	0·71
AI 45425	Concn (μmol mL^{-1})	0·283	0·217	0·162	0·071
(log P = 1·73)	Rate constant (h^{-1})	0·428	0·472	0·753	0·489
	Half-life (h)	1·62	1·47	0·92	1·42
AI 45510	Concn (μmol mL^{-1})	0·325	0·241	0·161	0·088
(log P = 2·21)	Rate constant (h^{-1})	1·017	1·050	1·059	1·690
	Half-life (h)	0·68	0·66	0·65	0·41

were below 100 μg mL^{-1}, shown above to represent a toxic concentration for AI 44987 and for AI 45046. A certain amount of hydrolysis occurred in the absence of NCTC 2544 cells, but this control hydrolysis was found to be insignificant over the first 8 h of the incubation period, demonstrated to be the most important period for cell-mediated hydrolysis.

The total protein yields from the NCTC 2544 cell monolayers after 48 h incubation with various concentrations of the ester pro-drugs were calculated (data not shown). The lowest total protein concentration corresponded to the highest drug concentration for each of the ester pro-drugs. The lower yield in total protein was likely to be due to the loss of cell monolayer caused by the toxicity of the ester pro-drugs. This correlates well with the toxicity test results in rank order, where the toxicity of the drugs increased with concentration (Table 1).

The metabolic half-lives and rate constants for the hydrolysis of the ester pro-drugs by NCTC 2544 cells are shown in Table 2. The half-life (and rate constant) was relatively independent of concentration for each compound, with only minor variation being observed. AI 45510 was found to be the compound most susceptible to hydrolysis, with a half-life range of 0·41–0·68 h. The next most likely to be hydrolysed was AI 44987 with a half-life range of 0·56–0·66 h. The half-life of metabolism for the compound least susceptible to hydrolysis, AI 45035, was observed to be some eightfold longer with a range of 4·08–6·61 h. This rank pattern of metabolism can again be observed in Fig. 3, which shows the metabolic activity corrected for protein yield.

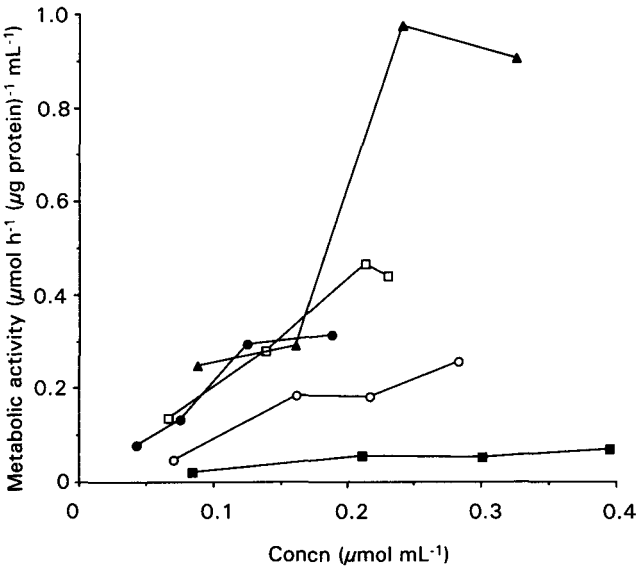

FIG. 3. Effect of AI ester pro-drug concentrations on the metabolic hydrolysis activity of NCTC 2544 cells. □ AI 44987; ■ AI 45035; ● AI 45046; ○ AI 45425; ▲ AI 45510.

This pattern of metabolism was repeated in a modified form when the compounds were incubated with a skin homogenate solution. The metabolic half-lives (Table 3) obtained were much longer than were observed with the NCTC 2544 cells, increasing to 45·8–83·8 h for AI 45035, for example. This compound was clearly the one least likely to be cleaved by hydrolytic enzymes within the skin homogenate solution. AI 44987 was the most susceptible to hydrolysis and had a half-life range of 1·8–2·5 h. This variation in half-life value from NCTC 2544 cells to skin homogenate is somewhat reduced if metabolic activity values are compared (Figs 3, 4). These results demonstrate that the metabolic activity of skin homogenate is generally close to that observed for NCTC 2544 cells, and that the pattern of metabolic susceptibility is also similar, save for the exchange of AI 44987 and AI 45510 as the compounds most likely to be hydrolysed in these test systems.

For reasons of economy, the LDE Testskin system was used to study each ester compound at one concentration only (Table 4). The half-lives for the hydrolysis of the ester pro-drugs were greatly extended in this series of experiments with values up to two orders of magnitude greater than was seen using the NCTC cells or the skin homogenate solution. This lack of activity was confirmed when the metabolic activities for each compound were calculated. These demonstrated that the maximum activity observed was one-fifth that seen in the other test systems. Nevertheless, the pattern of susceptibility to hydrolysis was largely conserved, with AI 44987 and AI 45510 the most sensitive to hydrolysis. There was little difference seen amongst results obtained for AI 45035, 45046 and 45425.

Table 3. Metabolism of the ester pro-drugs by skin homogenate.

Compound		Study I	Study II	Study III	Study IV
AI 44987	Concn (μmol mL^{-1})	0·429	0·291	0·217	0·102
(log P = 3·10)	Rate constant (h^{-1})	0·281	0·306	0·333	0·387
	Half-life (h)	2·5	2·3	2·1	1·8
AI 45035	Concn (μmol mL^{-1})	0·474	0·367	0·241	0·117
(log P = 1·50)	Rate constant (h^{-1})	0·008	0·010	0·013	0·015
	Half-life (h)	83·8	66·6	53.3	45·8
AI 45046	Concn (μmol mL^{-1})	0·301	0·222	0·200	0·067
(log P = 3·16)	Rate constant (h^{-1})	0·090	0·100	0·124	0·182
	Half-life (h)	7·7	6·9	5·6	3·8
AI 45425	Concn (μmol mL^{-1})	0·594	0·447	0·286	0·144
(log P = 1·73)	Rate constant (h^{-1})	0·026	0·027	0·027	0·028
	Half-life (h)	26·7	25·3	26·0	24·4
AI 45510	Concn (μmol mL^{-1})	0·408	0·312	0·200	0·103
(log P = 2·21)	Rate constant (h^{-1})	0·146	0·173	0·180	0·254
	Half-life (h)	4·8	4·0	3·8	2·7

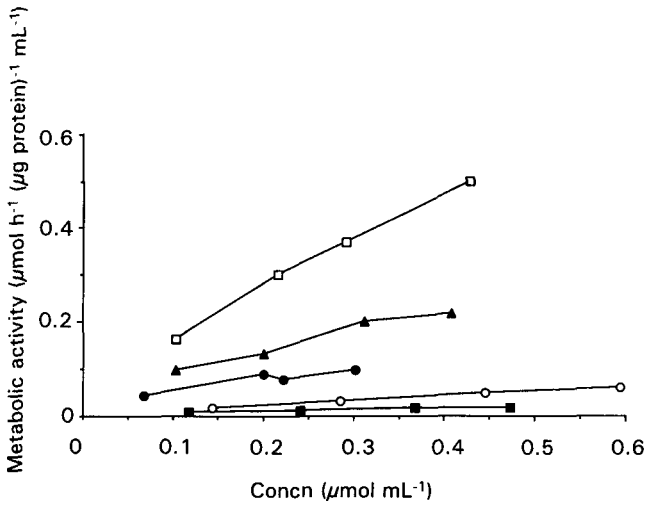

FIG. 4. Effect of AI ester pro-drug concentration on the metabolic hydrolysis activity of human skin homogenate solution. □ AI 44987; ■ AI 45035; ● AI 45046; ○ AI 45425; ▲ AI 45510.

The enzyme activity shown by the API ZYM test measured the activity of the enzymes towards the specific API ZYM substrates (2-naphthyl butyrate and 2-naphthyl caprylate for C4 esterase and C8 esterase lipase, respectively). The specificity of the enzymes in NCTC 2544 cells, skin homogenate solution and LDE Testskin for the API ZYM substrates and the ester pro-drugs was probably different. The range of enzymes detected using the API ZYM test was almost identical for all systems (Table 5).

Discussion

The skin has been shown to be capable of metabolizing a variety of substances (Pannatier et al 1978) and is regarded as being a metabolically active organ. Cutaneous metabolism is therefore likely to influence the bioavailability of topically applied drugs. The drugs which have been extensively studied for skin metabolism include the steroids (Bucks 1984) and polycyclic hydrocarbons such as benzo[a]pyrene (Fox et al 1975; Pannatier et al 1981; Kao et al 1983). Biotransformation of these compounds is enzyme-mediated and the reactions are typically integrated with redox cycles or ATP-generating systems (Kao & Carver 1990). The cutaneous metabolism of steroids involves the interconversion of oxo and hydroxyl groups in the steroid molecule by hydroxysteroid dehydrogenases and the reduction of steroid molecules with a 3-oxo-Δ-4 structure at the C5 position to produce the skin-specific 5 α-reduced derivatives. These reactions, which include the interconversion of cortisone to hydrocortisone, represent co-factor-dependent metabolism where NAD is the preferred co-factor (Kao & Carver 1990). The hydrolysis of esters catalysed by esterases is not dependent upon the presence of co-factors and may be considered as passive metabolism (Kao & Carver 1990). Non-specific enzymes capable of metabolizing esters have been shown to be present in the skin (Montagna 1955) and hydrolytic cutaneous reactions have been observed to occur during the evaluation of pro-drugs for topical delivery (Sloan & Bodor 1982; Sloan et al 1983). A pro-drug approach to transdermal drug delivery may increase cutaneous penetration of the drug and the active molecule may be released by exploiting the enzymes present

Table 4. Initial drug concentration, rate constant, half-life and metabolic activity for the metabolism of the ester pro-drugs by LDE Testskin.

Compound	Initial drug concn (μmol mL^{-1})	Rate constant (h^{-1})	Half-life (h)	Metabolic activity (μmol h^{-1} μg^{-1} mL^{-1})
AI 44987	0·096	0·019	36·5	0·112
AI 45035	0·200	0·004	173·3	0·050
AI 45046	0·076	0·008	86·6	0·044
AI 45425	0·120	0·005	138·6	0·037
AI 45510	0·139	0·017	40·8	0·150

Table 5. The API ZYM enzyme profile of NCTC 2544 cells, skin homogenate and LDE Testskin.

Enzyme	Numerical value for activity		
	NCTC 2544 cells	Skin homogenate	LDE Testskin
Control	0	0	0
Alkaline phosphatase	4	2	0
Esterase (C4)	5	4	2
Esterase lipase (C8)	5	3	1
Lipase (C14)	0	0	0
Leucine arylamidase	5	5	5
Valine arylamidase	2	1	1
Cystine arylamidase	1	1	1
Trypsin	0	0	0
Chymotrypsin	0	0	0
Acid phosphatase	5	5	1
Naphthol-AS-BI-phosphohydrolase	5	4	4
α-Galactosidase	0	0	0
β-Galactosidase	5	3	1
β-Glucuronidase	3	3	0
α-Glucosidase	0	0	0
β-Glucosidase	1	5	0
N-Acetyl-β-glucosaminidase	5	4	0
α-Mannosidase	0	0	0
α-Fucosidase	0	0	0
Total protein ($\mu g\,mL^{-1}$)	0·78	0·28	0·016

in the skin. Ester bonds may, therefore, be an ideal chemical modification of a drug to achieve both increased penetration and to render the agent susceptible to rapid hydrolysis.

The five anti-inflammatory ester compounds examined in this paper were tested for susceptibility to skin esterases as possible models of pro-drugs. All exhibited some toxicity towards NCTC 2544 cells but this was almost entirely absent in the hydrolysis products (AI 42017 and AI 42018). These toxicity studies enabled us to select a range of concentrations below those causing cellular damage when evaluating susceptibility to metabolism in cell culture.

The hydrolysis of the ester pro-drugs by skin homogenate solution was generally less rapid than that arising in cell culture. This is in contrast to the work of Martin (1988) who demonstrated a greater extent of metabolism of mupirocin and related analogues by skin homogenate compared with the metabolism by NCTC 2544 cells. The preparation of a skin homogenate solution (Andersson & Ryrfeldt 1984; Guzek et al 1989) is an established method for the assessment of metabolism and biotransformation reactions which may occur in the skin. Nevertheless, the homogenization of skin tissue is problematic and is not an entirely ideal method for extracting enzymes. Furthermore, the enzyme profile and activity may vary according to the site of the body from which the skin is removed and the condition of the skin sample.

The hydrolysis of methylthiomethyl ester pro-drugs of aspirin has been demonstrated (Loftsson & Bodor 1981) using hairless mouse skin and rat liver homogenate. Extensive metabolism of aspirin was also shown during skin penetration studies, with half-life values of 31 h. This represented metabolism of 70% of the applied dose which was detected as salicylic acid. The hydrolysis of various aliphatic morphine esters was recently observed in human serum (Drustrup et al 1991), the rate of hydrolysis being strongly influenced by the position of the ester linkage for mono- and diesters of morphine. The 3-monoesters and diesters were rapidly hydrolysed by both human serum and by skin esterases, but the 6-monoesters and diesters were susceptible only to skin esterases. This group also showed (Burr & Bundgaard 1984) that structural effects which influenced the rate of hydrolysis of oxazolidines were predominantly steric effects and stability of oxazolidines was increased with increasing steric effects within the β-amino alcohol moiety. The rate of hydrolysis was increased by the introduction of electron-withdrawing groups at the β-position to the nitrogen atom in the β-amino alcohol moiety. The hydrolysis of corticosteroid esters by the skin is well established. Skin esterases readily hydrolyse 21-esters (betamethasone 21-valerate) but 17 esters (betamethasone 17-valerate) are resistant to enzymatic attack. The corticosteroid is released from the 17-ester by non-enzymatic conversion to the 21-ester followed by enzymatic hydrolysis (Tauber 1989). Differences in the rate of hydrolysis of 6 α-methylprednisolone acetate was shown between animal species and man (Tauber & Rost 1987), the slowest hydrolysis being observed in man.

It would appear that there may be a possible relationship between hydrolysis of the alkyl esters and their respective log P value for all metabolic models tested, with metabolic activity increasing as the log P value rises (Tables 2, 3). These observations are tentative due to the limited number of drugs in the series within this narrow log P range. It is, however, apparent that the benzoyl derivative, AI 45046 (log P 3.16) does not fit this trend and shows a reduced rate of hydrolysis when compared with AI 45510 and AI 44987. The log P value is influenced by the length of the ester-linked side chain which may be the major influence on the susceptibility of the ester pro-drug to hydrolysis. The longer side chain molecules i.e. AI 44987 (pentanoyloxypropylamino side chain-8 carbon) and AI 45510 (pentanoyloxyethylamino side chain-7 carbon) were metabolized

more rapidly than AI 45035 (acetoxypropylamino side chain-5 carbon) and AI 45425 (propionyloxyethylamino side chain-5 carbon). The position of the ester bond is likely to be important and the length of the carbon chain connected to the ester bond may be the major influence. The more rapidly metabolized esters, AI 44987 and AI 45510, both contain a pentanoyloxy group (5 carbon) and AI 45035 and AI 45425, the less rapidly metabolized ester pro-drugs, contain acetoxy and propionyloxy groups, respectively. Structure-activity observations suggested that the presence of the five-carbon chain in AI 44987 and AI 45510 was compatible with rapid metabolism of the ester pro-drugs. To establish clearly a structure-activity relationship with metabolism, the ester pro-drug series would have to be extended; however, the observations made here are consistent with previous reports (Bundgaard et al 1983; Johansen et al 1986) which showed that the longer side chain derivatives of metronidazole greatly enhanced enzymic cleavage. Bodor et al (1980) showed significant cutaneous metabolism of hexanoylmethyl and pivalyloxymethyl nitrate esters of cromoglycic acid. A structure-activity relationship has been observed in the metabolism of a series of mupirocin analogues by NCTC 2544 cells (Martin 1988) and the most stable of the mupirocin analogues were benzyl monate and hexyl monate. The log P values for these drugs are between 2 and 2·5, similar values to the log P value for AI 45510. This would suggest that comparisons between unrelated structures may not be warranted. Martin (1988) suggests that the stability of benzyl monate was caused by steric hindrance of the ester bond due to the benzyl group. AI 45046 also contains a benzyl group which is a single carbon atom from the ester linkage and appears more stable than AI 44987 and AI 45510. However, the shorter chain ester pro-drugs, AI 45035 and AI 45425, were more stable than AI 45046, indicating that the length of the side chain and the position of the ester bond are most important in determining susceptibility to metabolism. A parabolic dependency between log P value and rate of metabolism of the mupirocin analogues has been proposed (Hadgraft 1991) and demonstrates the potential of using cell culture systems as predictive models of drug metabolism.

Esterase (C4) and esterase lipase (C8) enzymes were detected at similar activities in the API ZYM enzyme profile for both NCTC 2544 cell lysate and the skin homogenate solution. The esterase enzymes have often been grouped together as non-specific esterases due to the limited information on individual esterase species. Grouping of the esterases has been attempted (Nachmanson & Wilson 1955) and a range of esterases were detected in cultures of *Staphylococcus aureus* (Arvidson 1983). The hydrolysis of the ester pro-drugs in these studies probably occurred due to the action of both esterase (C4) and esterase lipase (C8) enzymes and the differences in the efficiency of metabolism and rank order of metabolism were determined by the relative specificity of the enzymes for specific chain lengths.

The three model metabolism systems investigated, skin homogenate, NCTC 2544 cells and LDE Testskin, have proven suitable for the determination of the susceptibility of a range of ester pro-drugs to enzymatic hydrolysis. The interpretation of the metabolism data for NCTC 2544 cells is likely to be as relevant to in-vivo understanding as the metabolism data for the skin homogenate solution. The extent to which metabolism of the ester pro-drugs will occur in-vivo cannot be predicted but the relative susceptibility to metabolism can be established. The cell-culture method offers the advantages of limited variability between samples and may also offer an indication as to the relative toxicity of the ester pro-drugs, but it is limited by strict aseptic techniques and the use of a compatible growth medium. The preparation of skin homogenate is relatively difficult and inefficient and the profile of enzymes and the enzyme activity is likely to be less reproducible than a cell-culture system. The LDE Testskin studies were limited in comparison with the skin homogenate studies and the cell-culture studies but they did provide an equally suitable model for studying the metabolism of the ester pro-drugs. However, the rates of metabolism were lower for LDE Testskin than NCTC 2544 cells or skin homogenate which may limit its application when studying drugs which are less susceptible to metabolism.

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