Pharmaceutical Research, Vol. 7, No. 3, 1990

Report

Carboxylic Ester Hydrolase Activity in Hairless and Athymic Nude Mouse Skin

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Received May 12, 1989; accepted September 23, 1989

The carboxylic ester hydrolase activity was compared in athymic nude mouse skin and hairless mouse skin with respect to hydrolytic ability, heat inactivation, pH optima, and substrate specificity. Five aliphatic 5′-esters of 5-iodo-2′-deoxyuridine (IDU) were incubated with skin homogenate preparations, and the effect of linear chain length and branching of the ester substituent on hydrolysis rate was evaluated. The ester hydrolase activity was three times higher in athymic mouse skin relative to hairless variety. In both mice skin preparations maximum hydrolysis rates were obtained with the valeryl ester followed by butryl, isobutyryl, propionyl and pivaloyl derivatives. Kinetic studies, however, revealed that higher ester hydrolase activity (V_{max}) in athymic mouse skin is also associated with higher K_m values, while the carboxylic ester hydrolases from these two different strains of mice have similar biochemical properties with respect to heat inactivation and pH optima. Athymic mouse skin resembled hairless mice skin in terms of cholinesterase content. A significant fraction (70–80%) of ester hydrolyzing activities in both strains of mice skin resulted from cholinesterases (true and/or pseudo). The remaining activity was attributed to different ester cleaving enzymes in the two strains of mice. Carbonic anhydrases and arylesterases contributed to the ester hydrolyzing activity of the athymic and normal hairless mice skins, respectively. Product inhibition by the regenerated hydrolytic product, free IDU, was also noticed which resulted in incomplete conversion of rapidly hydrolyzable 5′-esters such as the valeryl and butyryl derivatives.

KEY WORDS: carboxylic ester hydrolase activity; hairless and athymic nude mice skin; substrate specificity; enzyme multiplicity; effects of enzyme inhibitors; product inhibition.

INTRODUCTION

Cutaneous enzymatic activity is a subject of considerable current interest (1,2). The advantages of percutaneous drug delivery have been reviewed by Shaw *et al.* (3). Among them avoidance of variable absorption rates and first-pass hepatic metabolism associated with oral delivery and improved therapeutic activity are notable (3,4). Excised cadaver skin samples have been used (5,6), but difficulties in obtaining human tissue and its variability prompted the use of skins derived from laboratory animals, e.g., mice, rats, sheep, pig, and snake, as models for the human skin (7–14). Hairless mice skin, because of its ready availability and some degree of similarity with human skin, is often selected for *in vitro* skin transport and metabolism studies of drugs (11–13).

We have studied ester hydrolase activity in athymic mouse skin to evaluate the transdermal delivery potential of ester containing drugs and prodrugs. Since athymic nude mouse skin has exhibited some physiologic similarity to human skin and since skin allograft rejection is minimized, it is used in the long-term maintenance of human skin transplants (15,16). The athymic skin is also considered as a potential model in which human psoriatic skin can be maintained (17). Very little is known about the biochemical nature and enzymatic activity of athymic mouse skin (18), and the present study addresses its esterase content. Using 5-iodo-2′-deoxyuridine (IDU) as a polar antiviral agent with little topical activity (19), a series of lipophilic 5′-esters was synthesized to examine skin carboxylic ester hydrolyzing activity. The results can serve as a basis of selecting the optimal 5′-aliphatic ester(s) for dermal delivery and optimal therapeutic activity.

MATERIALS AND METHODS

Hairless mice (HRS/J strain) and athymic nude mice 6–12 weeks old were obtained from Jackson Laboratory, Bar Harbor, Maine, and Harlan Sprague Dawley, Inc., Indianapolis, Indiana, respectively.

The parent drug 5-iodo-2′-deoxyuridine (IDU), bovine serum albumin, physostigmine sulfate, acetazolamide, and *p*-chloromercuribenzoate were all obtained from Sigma Chemical Co., St. Louis, Missouri. The acid chlorides were procured from Aldrich Chemical Co., Milwaukee, Wisconsin. All other chemicals were of analytical reagent grade.

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0724-8741/90/0300-0251$06.00/0

Synthesis of 5'-IDU Esters

The synthesis of aliphatic 5'-esters of IDU has been described elsewhere (20). In short, a 10% molar excess of the appropriate acid chloride was added to a chilled solution of IDU in a 1:1 mixture of pyridine and *N,N'*-dimethylformamide. The reaction was continued for 2–3 days, occasionally checking for completeness of reaction by thin-layer chromatography using chloroform–methanol (95:5) as a developing solvent. The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in chloroform and the organic phase was washed twice with water to remove any unreacted IDU. The chloroform layer was evaporated to dryness and the corresponding 5'-ester derivatives were recrystallized from benzene. The identity and purity of the compounds were verified by IR, NMR, CI-MS, TLC, and HPLC as described in our earlier report (20).

Analytical Method

A high-performance liquid chromatographic (HPLC) method was developed for monitoring the formation of IDU in the skin homogenate incubation medium. The system comprised a Model 510 solvent delivery module, U6K injector and 480 UV-vis variable wavelength detector, and a 30-cm μ-Bondapak phenyl column, all from Waters Associates, Milford, Massachusetts. The mobile phase employed for the analysis of IDU consisted of 20% (v/v) methanol in water and was pumped at a flow rate of 1 ml/min. The detection was carried out at 261 nm. Temperature was ambient. Under the conditions IDU was found to have a retention volume of 6.5 ml. The presence of IDU in the incubation medium was verified by comparing the retention volume of the unknown peak to that of the pure sample. For IDU-5' esters mobile phases containing acetonitrile in water were used where the acetonitrile content progressively increased as the carbon chain of the ester series was ascended.

Preparation of Skin Homogenate

The animals were sacrificed by cervical dislocation and the skin was rapidly frozen in dry ice. About 0.5 g of skin (devoid of subcutaneous fat and visceral tissue) was placed in 9 vol of ice-cold sodium phosphate buffer (25 m*M*, pH 7.4). The mixture was homogenized for 90 sec using a motor-driven tissue grinder (Polytron) while keeping the tubes immersed in an ice–water mixture. The homogenate was then centrifuged at 12,000*g* in a refrigerated centrifuge (4°C) (TL100, Beckman Instruments). The supernatant was filtered through glass wool to remove any particulate matter and then stored in several aliquots at −20°C for further studies. The protein content of the supernatants was measured by Lowry's method (21) using bovine serum albumin as the standard.

Hydrolysis of 5'-IDU Esters by Skin Homogenate

The carboxylic ester hydrolyzing activity was determined by monitoring the formation of IDU from the 5'-ester derivatives in phosphate-buffered saline (25 m*M*, pH 7.4). One hundred microliters of a 5 m*M* solution of the ester was added to 1 ml of the skin homogenate supernatant solution (pH 7.4) previously equilibrated at 37°C. The final concentration of the esters in the incubation medium was 0.45 m*M*. The solution was incubated in a constant-temperature shaker bath at 37°C. At predetermined time points, 50 μl of the reaction mixture was withdrawn, and the reaction was immediately stopped by adding 350 μl of ice-cold methanol. Following thorough mixing, the solution was centrifuged again at 8000*g* for 10 min and the supernatant was analyzed for the regenerated IDU by HPLC as described before. The initial hydrolytic rate in units of nanomoles of IDU formed per milligram of protein was calculated from the initial slope of a formation plot of IDU versus time. No measurable chemical hydrolysis of the esters occurred during the time span of enzymatic hydrolysis studies as evident by the absence of IDU formation in the control experiment where the tissue homogenates were replaced with pure buffer solutions.

Enzymatic Hydrolysis in the Presence of Inhibitors

In the absence of a satisfactory classification of carboxylic ester hydrolases, the use of various known inhibitors can lead to the differentiation of ester hydrolyzing activity. Using valeryl IDU as the model substrate, the effects of acetazolamide, a carbonic anhydrase inhibitor, *p*-chloromercuribenzoate, a modulator of arylesterase activity, and physostigmine sulfate and neostigmine bromide, inhibitors of cholinesterases, were examined in both hairless and athymic nude mice skin homogenate preparations.

All inhibitors were used at a 1 m*M* concentration except acetazolamide, which was employed at a concentration of 20 μ*M*. The skin homogenate supernatant solution (0.05 ml) was incubated with an equal volume of a freshly prepared aqueous solution of the inhibitor for 15 min at 37°C. Following this initial incubation, 5 μl (0.005 ml) of valeryl ester (5 m*M*) was added to the mixture and a second incubation followed for 15 min. The hydrolytic reaction was stopped by the addition of (0.25 ml) ice-cold methanol. The mixture was subsequently centrifuged at 8000*g* for 10 min and the concentration of IDU resulting from the hydrolysis was measured by the HPLC technique described previously.

pH Optima

The effects of hydrogen ion concentration on the skin

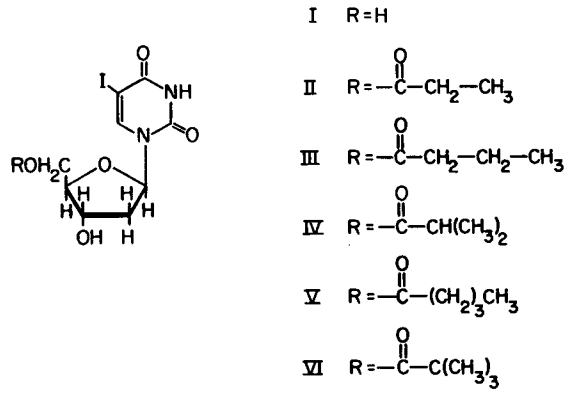

Fig. 1. Various acyl groups attached to the 5'-hydroxyl of IDU.

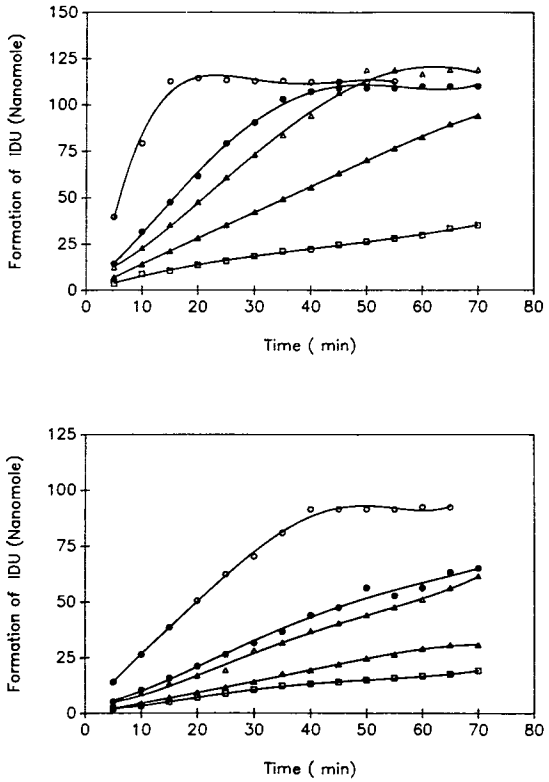

Fig. 2. (A) Hydrolysis of various 5′-IDU esters by athymic nude mice skin carboxylic ester hydrolases. (○——○) Valeryl; (●——●) *n*-butyryl; (△——△) *i*-butyryl; (▲——▲) propionyl; (□——□) pivaloyl. (B) Hydrolysis of various 5′-IDU esters by hairless mice skin carboxylic ester hydrolases. (○——○) Valeryl; (●——●) *n*-butyryl; (△——△) *i*-butyryl; (▲——▲) propionyl; (□——□) pivaloyl.

carboxyl ester hydrolase activity were studied at different pH values by following the hydrolysis kinetics of 5′-valeryl ester in skin homogenate supernatant solution at 37°C for 15 min. Different buffers (25 m*M*) were used for different pH values: pH 4.5–5.5, acetate; pH 6.0–7.5, phosphate; and pH 8.0–9.5, Tris–HCl. The incubation procedure was followed as described previously and the carboxyl ester hydrolyzing

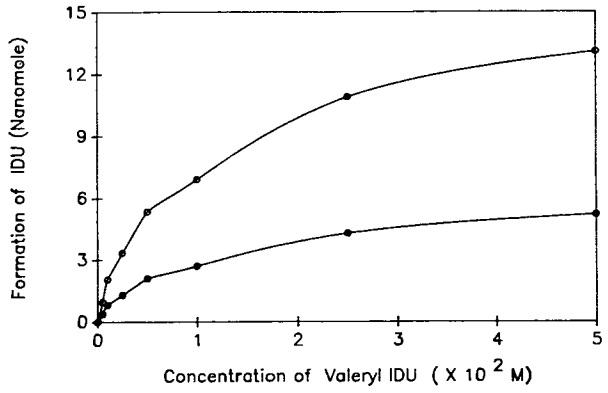

Fig. 3. Effect of increasing concentration of valeryl IDU on the rate of esterase-mediated hydrolysis in the presence (●——●, lower) and absence of IDU (○——○, upper).

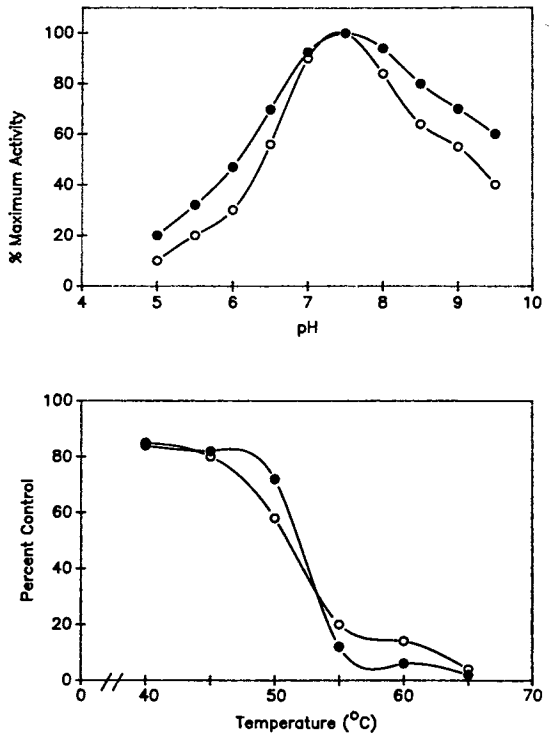

Fig. 4. (A) Effect of pH on the activity of carboxylic ester hydrolases in athymic nude mice (●——●) and hairless mice skin (○——○). (B) Effect of temperature on the activity of carboxylic ester hydrolases in athymic nude mice (●——●) and hairless mice skin (○——○).

activity was expressed as the percentage of maximum activity.

Temperature Effects

The effect of temperature on the carboxylic ester hydrolyzing activity was studied by preheating the supernatants to temperatures in the range of 40 to 65°C for 15 min followed by the measurement of hydrolytic activity using 5′-valeryl ester as the substrate.

Product Inhibition Studies of IDU-5′-Ester Hydrolysis

In order to test for product inhibition, the hydrolysis of valeryl-IDU by skin homogenate was determined in the presence and absence of added IDU.

A 5-μl solution of various concentrations of valeryl IDU (0.5 to 50 m*M*) was added to 50 μl (0.05 ml) of the skin

Table I. Michaelis-Menten Parameter Estimates for Esterase in the Athymic Nude Mouse and Hairless Mouse Skin

	$K_m \times 10^3$ *M*		V_{max} 10^3 unit/mg protein[a]	
Substrate	Hairless	Athymic	Hairless	Athymic
Butyryl-IDU	5	12.5	4.01	12.06
Isobutyryl-IDU	7.14	12.5	3.3	9.1
Valeryl-IDU	6.25	16.6	4.8	18.2

[a] Unit = picomoles of IDU produced per minute.

Table II. Effect of Different Inhibitors on the Esterase Activity Present in Athymic Nude and Hairless Mouse Skin[a]

	IDU formation (pmol/min)									
	Athymic nude mouse skin					Hairless mouse skin				
Substrate	None	I	II	III	IV	None	I	II	III	IV
Valeryl-IDU	530	99 (82)	118 (79)	529 (2)	421 (20)	203	60 (70)	67 (67)	131 (35)	213 (0)
Butyryl-IDU	416	74 (83)	83 (81)	420 (0)	315 (25)	151	42 (74)	42 (74)	105 (30)	143 (6)
Isobutyryl-IDU	392	66 (84)	85 (79)	400 (0)	293 (26)	142	33 (77)	35 (75)	100 (30)	151 (0)

[a] I, Physostigmine; II, Neostigmine; III, *p*-chloromercuribenzoate; IV, acetazolamide. Percentage inhibition is shown in parentheses.

homogenate (0.14 mg of protein) containing 5 μl of 1 m*M* IDU in a total volume of 0.06 ml. The entire mixture was incubated at 37°C for 15 min and the reaction was stopped by adding 350 μl of ice-cold methanol. Following thorough mixing, the solution was centrifuged and the supernatant was analyzed for the formation of IDU. The amount of added IDU was subtracted from the total value in order to obtain the actual amount of IDU formed by the skin homogenate.

RESULTS AND DISCUSSION

The chemical structures of the five aliphatic esters of IDU described in this report are shown in Fig. 1. The identification and physical–chemical characterization of these derivatives have been described in a previous report (20). The formation of IDU from these esters in the athymic mouse skin homogenate preparations (Fig. 2A) suggests the presence of carboxylic ester hydrolase activity. Similar activity was also observed with the hairless mouse skin preparations (Fig. 2B). No significant hydrolysis of the ester in the blank buffer solution was noted during the time course of the experiments. In spite of the observed differences in the hydrolysis rates of the esters by two skin types, a similar substrate specificity was achieved in both strains of mice. In both cases, maximum hydrolysis was obtained with the valeryl ester, followed by the butyryl, isobutyryl, propionyl, and pivaloyl derivatives. The enhanced enzymatic hydrolysis with increased linear chain length indicates the presence of a hydrophobic pocket at the active center of ester hydrolases, as previously shown for the hydrolysis of other ester prodrugs (22,23). However, increased steric hindrance of the 5′-acyl substituent decreased the hydrolysis rate, i.e., pivaloyl < *i*-butyryl < *n*-butyryl. As illustrated in Fig. 2, incomplete conversion of valeryl and butyryl esters to IDU could result from denaturation of the enzymes during the study or from IDU product inhibition. Enzyme denaturation is unlikely since linear increases in IDU formation with time were observed for the other three 5′-esters, while product inhibition may occur with accumulated IDU which does not get further metabolized in the skin homogenate preparations. Indeed the hydrolysis of the valeryl ester was inhibited by the presence of added IDU (Fig. 3). Rapid accumulation of IDU from the valeryl and butyryl esters, during the hydrolysis studies, can therefore inhibit the hydrolysis reactions.

The ester hydrolase activity in athymic mouse skin was similar to that in hairless mouse skin with respect to pH optima and heat inactivation. Figure 4A illustrates the effect of pH on the carboxylic ester hydrolase activity in both strains of mice. The pH–activity profile exhibits a parabolic relationship with the optimum activity around the physiological pH of 7.4. Buffer compositions and concentrations did not have any significant effect on enzyme activity. Figure 4B illustrates that the ester hydrolase activity in athymic mouse skin is sensitive to temperature variations. Thirty-five to 40% of the total enzyme activity is lost with pretreatment of the homogenate at 50°C.

The kinetics of carboxylic ester hydrolase activity can be described by a Michaelis–Menten model. The K_m and V_{max} values obtained from such experiments for three different esters are presented in Table I. For all three esters, the enzymatic capacity (V_{max}) for athymic mice skin ester hydrolases was at least three times higher than that of hairless mouse skin, although lower affinity, i.e., higher K_m, values were noted for athymic mice skin. Similar variations in V_{max} and K_m values were reported for albino and pigmented rabbit ocular esterases (24).

Since different values were obtained for the Michaelis–Menten parameters (Table I) for the esterases derived from the skin of the two strains of mice, it is possible that several of these esterases are unique to either athymic or hairless mouse skins. Problems of classifying carboxylic ester hydrolases have been discussed recently (25,26) and the absence of a satisfactory classification has led to the use of certain inhibitors to differentiate esterases (27,28). A similar study was also undertaken by Lee *et al.* (24), who used a single concentration of different inhibitors to differentiate esterases present in two different strains of rabbit eye. Table II summarizes the composition of different esterases present in the skin of both strains of mice. Athymic mice, when compared with hairless mouse, had a similar cholinesterase content. Seventy to 80% of ester hydrolyzing activities resulted from cholinesterase (true and/or pseudo) activity, and this activity was inhibited by pretreating the homogenate with either physostigmine sulfate or neostigmine bromide. The remaining activity was attributed to different ester cleaving enzymes in the two strains of mice. Carbonic anhydrases and arylesterases contributed to the ester hydrolyzing activity of the athymic and normal hairless mouse skins, respectively.

ACKNOWLEDGMENTS

This work was supported in part by a Merck Faculty Development Award to A.K.M. by Merck, Sharp and Dohme Research Laboratories. Instrumentation support was provided by NIH Grant NS 25284 and Biomedical Research Support Grant RR 05586.

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