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Research paper

Esterase activity in excised and reconstructed human skin – Biotransformation of prednicarbate and the model dye fluorescein diacetate

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ABSTRACT

Reconstructed human epidermis (RHE) is used in non-animal testing for hazard analysis and reconstructed human skin (RHS) gains growing interest in preclinical drug development. RHE and RHS have been characterised regarding their barrier function, but knowledge about biotransformation capacity in these constructs and in human skin remains rather poor. However, metabolising enzymes can be highly relevant for the efficacy of topical dermatics as well as genotoxicity and sensitisation. We have compared the esteratic cleavage of the prednisolone diester prednicarbate and the enzyme kinetic parameters (V_{max} and $S_{0.5}$) of the model substrate fluorescein diacetate (FDA) in commercially available RHS and RHE with excised human skin and monolayer cultures of normal and immortalised human keratinocytes and of fibroblasts. Formation of the main metabolite prednisolone and of fluorescein ranked as: RHS∼RHE > excised human skin and keratinocytes > fibroblasts, respectively. Because of the aromatic probe, however, V_{max} of FDA cleavage did not show a linear relationship with prednicarbate metabolism. In conclusion, RHE and RHS may be useful to quantitatively address esterase activity of human skin in drug development and hazard analysis, although an increased activity compared to native human skin has to be taken into account.

1. Introduction

To protect human health and the environment, compounds and products are subjected to standardised hazard assessment procedures. The efforts to develop and validate non-animal approaches such as *in vitro* testing in reconstructed human tissues have now become most urgent due to the implementation of the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) legislation in 2007 that requires testing not only of new but also of already existing compounds. Moreover, the 7th Amendment to the EU Cosmetics Directive in 2009 will completely ban animal tests for cosmetic ingredients from 2013 on. Finally, there is a clear need for efficient preclinical methods in drug development based on human-derived materials to overcome species differences.

Commercially available reconstructed human epidermis (RHE) – composed of differentiating keratinocytes – has been characterised regarding morphology, lipid composition, protein markers and barrier function [1,2]. Several RHE-based test procedures have been validated and adopted by the OECD, such as *in vitro* tests for skin corrosion [3–5], skin irritation [3,6–8], phototoxicity [9–11] and percutaneous absorption [12,13]. The protocols for percutaneous absorption of compounds in aqueous solution are both applicable in RHE and in reconstructed human skin (RHS; [14,15]) to compare

Abbreviations: Ac, acetone; BM, betamethasone; DMSO, dimethyl sulfoxide; CV, coefficient of variation; FDA, fluorescein diacetate; hCE, human carboxylesterase; HaCaT, human keratinocyte cell line; IL-8, interleukin-8; LDH, lactate dehydrogenase; NHDF, normal human dermal fibroblasts; NHK, normal human (epidermal) keratinocytes; OECD, Organisation for Economic Co-operation and Development; P17EC, prednisolone 17-ethylcarbonate; P21EC, prednisolone 21-ethylcarbonate; PBS, phosphate buffered saline; PC, prednicarbate (prednisolone 17-ethylcarbonate, 21-propionate); PD, prednisolone; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; RHE, reconstructed human epidermis; RHS, reconstructed full-thickness human skin; $S_{0.5}$, substrate concentration at half of V_{max}; SEM, standard error of the mean; SOP, standard operating procedure; V_{max}, maximum enzyme activity.

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various formulations [12,15–18]. In RHS, the differentiating keratinocytes that form an epidermis are grown on a dermal equivalent composed of collagen embedded fibroblasts. RHS therefore shows better morphological and functional similarities with native human skin.

Low molecular weight (< 500 Da) and moderately lipophilic agents (logP 1–3) can surmount the horny layer barrier and gain access to viable epidermis, dermis and finally the blood vessels to a low, yet relevant extent. Dermal biotransformation can result in the formation of less acutely toxic, but also mutagenic or sensitising metabolites and intermediates. A better characterisation of human skin and skin constructs in this regard could therefore promote an extended usage of RHE or – due to the inclusion of both dermal and epidermal cells – preferably RHS as alternative experimental systems. Over the last decade, a significant biotransformation potential of human skin has become obvious, because a broad spectrum of drug metabolising enzymes is expressed in this tissue [19–22]. Recent studies have expanded our knowledge on expression profiles of phase I and II metabolising enzymes in RHE [23–26] and RHS [27,28]. Enzyme activities have been described, as well [20,29–32].

The capacity of cutaneous ester hydrolysis [20,30,33] is of particular importance for efficacy and safety of, for example, many glucocorticoids for dermal application [34–37] and can influence also skin penetration [17]. Yet, ester hydrolysis in human skin and in reconstructed tissues has not yet been addressed systematically. Comprehensive data are available with respect to the skin penetration and biotransformation of betamethasone 17-valerate and the prednisolone diester prednicarbate (PC; Fig. 1). In human keratinocytes and fibroblasts [34,38,39], as well as in excised human skin and RHE [17,33,40,41], the 21-propionate ester of PC is enzymatically cleaved to prednisolone 17-ethylcarbonate (P17EC), which rearranges to the respective 21-ester (P21EC). This compound is finally converted by esterases forming prednisolone (PD). A corresponding process is followed with betamethasone 17-valerate [20,30,42]. In this study, we have aimed for a detailed insight into esterase activity of human skin and the respective constructs. For a full insight, we had to quantify the model drug prednicarbate and its metabolites within skin and constructs (penetration) as well as in the receptor medium (permeation), respectively. We have further characterised a probe that allows the quantification of esterase activity by a ready-to-use procedure in standardised and widely available human-derived materials.

2. Materials and methods

2.1. Compounds

Prednicarbate (prednisolone 17-ethylcarbonate, 21-propionate, PC; purity 98%; log*P* = 3.82; MW = 489 g/mol) and prednisolone 17-ethylcarbonate (P17EC) were a kind gift by Sanofi Aventis (Berlin, Germany). Betamethasone (BM, purity 98%) and prednisolone (purity 99%) were purchased from Sigma (Deisenhofen, Germany). Fluorescein (purity 95%) and fluorescein diacetate (FDA, purity 97%; log*P* = 3.6; MW = 416 g/mol) were obtained from Alfa Aesar (Karlsruhe, Germany). Standard compounds for the experiments were of the highest quality available.

PC 0.5% (w/v) solution was prepared with ethanol and diluted with phosphate buffered saline pH 7.4 (PBS) to the final concentration of 0.25% (w/v) immediately before the experiment. Glucocorticoid stock solutions (PC, BM, PD and P17EC, 10^{-2} M) in ethanol [38,43] were used for HPLC analysis and remained stable for 1 month when stored at −20 °C. Fluorescein stock solution (5×10^{-4} M, in 0.1 M tris-buffer pH 8.0) was diluted while adding tris-buffer supplemented with acetone to a final concentration of 0.2% (v/v; fluorescein 10^{-8} to 2.5×10^{-4} M). Immediately before usage, FDA stock solution (4×10^{-2} M, in acetone) was diluted to 8×10^{-5} M with tris-buffer. The stock solutions remained stable for 1 month at −80 °C.

2.2. Handling of human skin and reconstructed tissues

Surgically discarded human breast or abdominal skin (with permission) was handled according to standardised procedures [13].

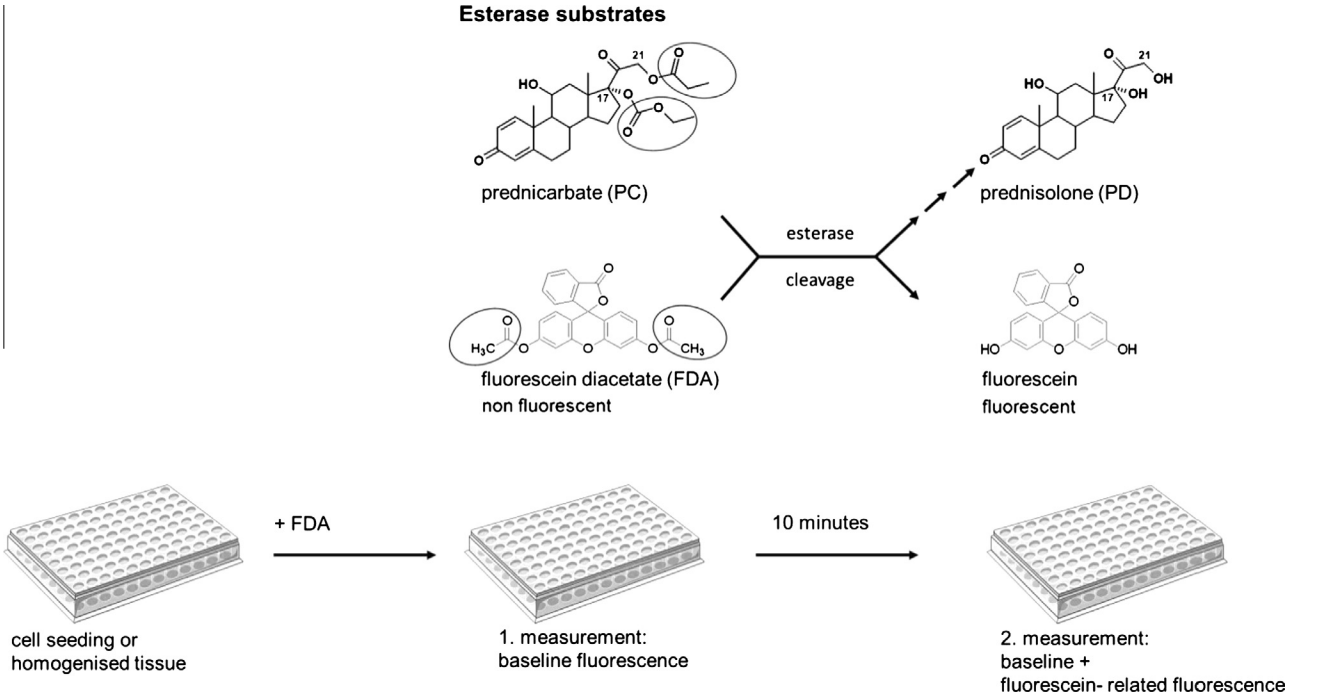

Fig. 1. Illustration of prednicarbate biotransformation and the experimental procedure for determination of esterase activity using fluorescein diacetate (FDA). The prednisolone diester prednicarbate (PC) is enzymatically hydrolysed by esterases at position 21, forming prednisolone 17-ethylcarbonate (P17EC). Following spontaneous rearrangement to prednisolone 21-ethylcarbonate (P21EC), the final metabolite prednisolone (PD) is released by esterases. Non-fluorescent FDA is enzymatically hydrolysed by esterases to fluorescent fluorescein. Figures were produced using Servier Medical Art.