Imaging the Prodrug-to-Drug Transformation of a 5-Fluorouracil Derivative in Skin by Confocal Raman Microscopy

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The widespread adoption of transdermal drug delivery has been limited by the barrier properties of the outermost layer of the epidermis, the stratum corneum (SC). A variety of approaches have been developed to overcome the barrier, including the use of a prodrug form of an active therapeutic agent to enhance transdermal delivery. Once in the epidermis, the pro-molecule is converted to the active drug by endogenous enzymes or simple chemical hydrolysis. The prodrug selected for the current studies, 1-ethyloxycarbonyl-5-fluorouracil, is known to enhance transdermal delivery of 5-fluorouracil, an important systemic antitumor drug. Using confocal Raman microscopy on pigskin biopsies treated with prodrug, we are able to image the spatial distribution of both prodrug and drug in the SC and viable epidermis, thereby providing information about permeation and metabolism. This approach may readily be extended to a variety of dermatological processes.

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INTRODUCTION

Transdermal delivery of drugs offers several important advantages for therapeutics, including ease of access, control of incorporation kinetics, and the relative non-invasiveness of application procedures (Barry, 2004; Prausnitz *et al.*, 2004). Various methods based on transient physical modification of the stratum corneum (SC) barrier have been developed to increase delivery of active agents, for example, iontophoresis, electroporation, and the use of chemical permeation enhancers. An alternative strategy, using prodrugs, entails modification of the physical and chemical properties of the active molecule. Evaluation of the success of transdermal delivery is limited by the absence of techniques that monitor *in situ* the spatial distribution of the drug, prodrug, or delivery vehicle. Any such method must be able to examine the outermost layers of intact skin in a non-perturbing manner and to distinguish closely related chemical species in the skin.

The Raman spectrum of a molecule provides a useful fingerprint for identification of substances without the necessity for external labels (which may perturb the physical properties of the molecules under study). Modern technology permits the acquisition of Raman spectra from skin in a microscopically resolved confocal manner (Caspers *et al.*, 2001), thereby providing a convenient, non-destructive method for monitoring the spatial distribution of exogenous materials and their biochemical transformations in intact skin.

5-Fluorouracil (5FU), an important systemic antitumor drug (Longley *et al.*, 2003), is also used to treat cancerous or precancerous conditions in skin including solar keratoses, actinic keratosis, superficial basal cell carcinoma, and Bowen's disease. Furthermore, 5FU is used to treat non-cancerous conditions in which cells are dividing rapidly, for example, psoriasis (Pearlman *et al.*, 1986). The delivery of 5FU in conventional topical preparations has been suboptimal, requiring methods such as light curettage to improve therapeutic utility (Epstein, 1985). A variety of prodrugs from 5FU have been prepared, one of which, 1-ethyloxycarbonyl-5FU (pro-5FU), provided a 25-fold increase in transdermal delivery (Beall *et al.*, 1994). The molecular structures of both prodrug and drug species are shown in the inset to Figure 1a. In this article, we describe the application of confocal Raman microscopy to image the spatial distribution of pro-5FU to 5FU conversion in skin.

RESULTS AND DISCUSSION

Differences in the Raman spectra (Figure 1a) of aqueous solutions of the prodrug and drug are significant in the low frequency region. Several bands likely arising from ring vibrations are diagnostic for each molecule, illustrating the potential for *in situ* differentiation of these molecules in the confocal Raman experiment. Included in Figure 1a is a spectrum of isopropyl myristate (IPM), the delivery solvent for the pro-5FU suspension. Only a few Raman bands in the

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Abbreviations: 5FU, 5-fluorouracil; IPM, isopropyl myristate; pro-5FU 1-ethyloxycarbonyl-5-fluorouracil; SC, stratum corneum

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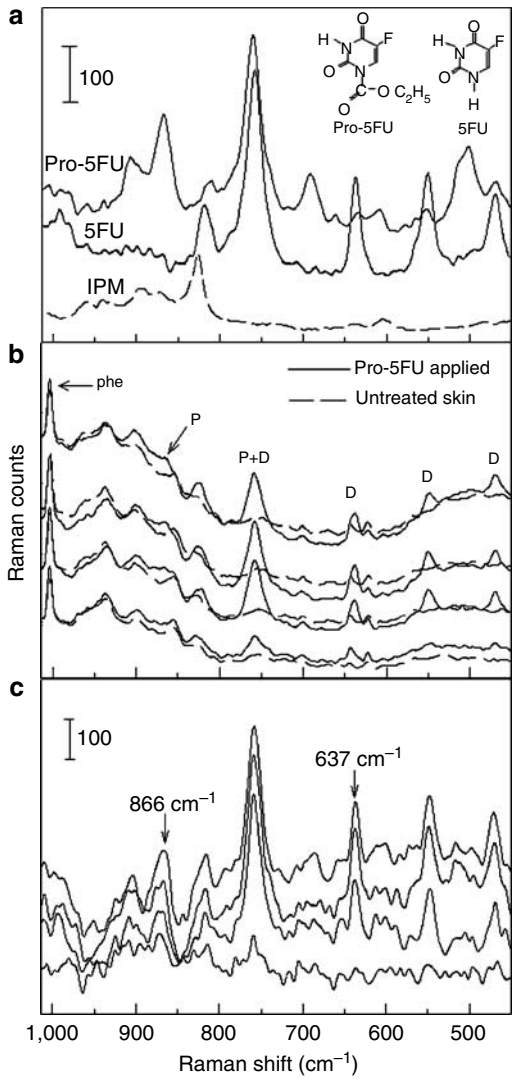

Figure 1. Raman spectra (450–1015 cm^{-1} region) probing pro-5FU (in IPM suspension) delivery of 5FU in pigskin. (**a**) Raman spectra of neat IPM (dashed line) and prodrug and drug solutions (solid lines, as noted). The molecular structures of pro-5FU and 5FU are shown in the inset. (**b**) Confocal Raman spectra of untreated (dashed lines) and pro-5FU-treated (solid lines) pigskin at different depths (2, 7, 12, and 17 μm) under the skin surface from top to bottom, respectively. Raman bands specific to prodrug (P) and drug (D), along with the phe ring-breathing mode are marked. (**c**) Confocal Raman spectra of pro-5FU-treated skin after subtraction of untreated skin spectra at equivalent depths. Spectra are stacked top to bottom as in (**b**). Raman bands used to quantify relative concentrations of prodrug (866 cm^{-1}) and drug (637 cm^{-1}) are annotated.

spectrum of IPM are observed in the same region as pro-5FU or 5FU. Bands at 866 and 637 cm^{-1}, free from interference from IPM bands, were chosen to monitor the relative concentrations of prodrug and drug, respectively. Spectra are presented over a higher wavenumber range in Figure S1a and S1b, supporting the selection of these two discrete bands in the low wavenumber region.

A suspension of pro-5FU was applied to intact pigskin (22°C) for 20 hours. Subsequently, Raman spectra were acquired at increasing depths beneath the skin surface in 5 μm increments. These were compared to spectra of untreated pigskin at equivalent depths (Figure 1b). In the Raman spectra of treated skin, features from prodrug and drug are observed along with Raman bands of the protein and lipid constituents of the SC. In a control experiment, pro-5FU was determined to be chemically stable in an IPM suspension monitored over a 1-month period. In an additional control experiment, when a suspension of 5FU in IPM was applied to skin, using the current experimental conditions, the drug permeated to a maximum depth of 5 μm. Thus, the presence of several bands arising from 5FU within the skin (Figure 1b) clearly indicates that *in situ* hydrolysis of the prodrug has taken place.

Evaluation of the spatial variation of the relative prodrug and drug concentrations requires an internal standard for Raman intensity. A useful spectral feature for this purpose, arising from endogenous skin proteins, is the ring-breathing mode of phe at 1,004 cm^{-1} (Figure 1b). This band diminishes in intensity with depth in the skin, and serves to calibrate for confocal scattering losses within the tissue (Figure S2). To determine the relative concentrations of pro-5FU and 5FU in skin, spectra of untreated skin are subtracted from spectra of treated skin at equivalent depths (see Materials and Methods). The difference spectra are shown in Figure 1c. The presence of both pro-5FU and 5FU is evident in spectra acquired at depths of 2, 7, and 12 μm beneath the skin surface. At a depth of 17 μm, bands arising from the drug are no longer visible, whereas the prodrug feature at 866 cm^{-1} is close to the noise level and is not considered significant. It is noted that the band parameters (positions and widths) of pro-5FU and 5FU in skin remain essentially unchanged from those in aqueous solution (Figure 1a). As Raman band parameters are often sensitive to environment, for example, solvent or intermolecular interactions, the similarity between the spectral features of the drug and prodrug in skin and in aqueous solution suggest that each molecule is located in an aqueous environment in skin. Consistent with this suggestion is the correlation between increased prodrug solubility in water and increased 5FU delivery through hairless mouse skin reported for this particular 5FU prodrug (Beall *et al.*, 1994).

An issue arises as to the depths and regions of the skin into which the prodrug or drug may permeate. More specifically, it is of interest to ascertain whether permeation is limited to the SC or whether the exogenous substances penetrate to the viable epidermis or dermis. Univariate analysis of spectral features or multivariate statistical algorithms such as factor analysis can be used to delineate native skin regions, that is, the methods are capable of differentiating the SC from the viable epidermis (Xiao *et al.*, 2004; Mendelsohn *et al.*, 2006; Zhang *et al.*, 2007). Factor analysis was applied to a set of confocal Raman spectra acquired from an untreated skin sample over the 800–1,015 cm^{-1} spectral region. Factor scores are shown as image planes (Figure 2a–c) for loadings that clearly differentiate the SC, viable epidermis, and dermis, respectively. The pigskin sample used for this analysis was taken from a biopsy similar to those used for pro-5FU permeation experiments. From the factor score images, the SC appears to be ~10–20 μm thick. Thus, for the data shown in Figure 1c, in which treated pigskin was incubated at 22°C,