Special Section on Emerging Novel Enzyme Pathways in Drug Metabolism

Biotransformation Capacity of Carboxylesterase in Skin and Keratinocytes for the Penta-Ethyl Ester Prodrug of DTPA

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ABSTRACT

The penta-ethyl ester prodrug of the chelating agent diethylene triamine pentaacetic acid (DTPA), referred to as C2E5, effectively accelerated clearance of americium after transdermal delivery. Carboxylesterases (CESs) play important roles in facilitating C2E5 hydrolysis. However, whether CESs in human skin hydrolyze C2E5 remains unknown. We evaluated the gene and protein expression of CESs in distinct human epidermal cell lines: HEKα, HEKn, HaCaT, and A431. The substrates p-nitrophenyl acetate (pNPA) and 4-nitrophenyl valerate (4-NPV) were used to access esterase and CES activity. C2E5 hydrolysis was measured by radiometric high-performance liquid chromatography after incubation of [14C]C2E5 with supernatant fractions after centrifugation at 9000g (S9) prepared from skin cell lines. CES-specific inhibitors were used to access metabolism in human skin S9 fractions with analysis by liquid chromatography–tandem mass spectrometry. We identified the human carboxylesterase 1 and 2 (CES1 and CES2) bands in a Western blot. The gene expression of these enzymes was supported by a real-time polymerase chain reaction (qPCR), pNPA and 4-NPV assays demonstrated esterase and CES activity in all the cell lines that were comparable to human skin S9 fractions. The prodrug C2E5 was hydrolyzed by skin S9 fractions, resulting in a primary metabolite, C2E4. In human skin S9 fractions, inhibition of C2E5 hydrolysis was greatest with a pan-CES inhibitor (benzil). CES1 inhibition (triglitazone) was greater than CES2 (loperamide), suggesting a primary metabolic role for CES1. These results indicate that human keratinocyte cell lines are useful for the evaluation of human cutaneous metabolism and absorption of ester-based prodrugs. However, keratinocytes from skin provide a small contribution to the overall metabolism of C2E5.

Introduction

Transdermal drug delivery is noninvasive, can be self-administered, avoids first-pass metabolism, and is well-suited to pediatric populations and particular patient groups who have trouble swallowing (Zemplsky, 1998). In addition, transdermal products are attractive owing to their sustained zero-order systemic release profile (Naik et al., 2000). However, to reach the systemic circulation, drug molecules need to pass through the skin’s multiple barriers, including the hydrophobic environment of the stratum corneum, the epidermis, and the dermis to reach the vascularized hypodermis. These barriers effectively limit direct transdermal delivery to molecules that possess aqueous solubility in the physiologic pH (~1 mg/ml, pH 5–9), a low molecular weight (usually <500 Daltons), moderate lipophilicity (oil-water partition coefficient Kow1 10–1000), and those that require a moderate daily dosage (<10 mg/day) (Naik et al., 2000; Perumal et al., 2013). A growing number of drugs that have many of the properties listed above have been approved for transdermal delivery. These include estradiol, fentanyl, lidocaine, and testosterone patches and ultrasonic delivery systems for analgesia (Nitti, 2003; Prausnitz and Langer, 2008).

In addition to the physical barriers, cutaneous metabolism via local phase I and phase II metabolic enzymes can also reduce bioavailability (Zhang et al., 2009; Esser and Gotz, 2013). Cytochrome P450 enzymes are clearly expressed in organotypic skin models (Saeki et al., 2002; Swanson, 2004). In human skin, cytochrome P450 families CYP1, 2, and 3 are responsible for the metabolism of the majority of drugs and other xenobiotics (Du et al., 2004). Xenobiotic-metabolizing enzymes are located in the epidermis and dermis, where hair follicles and sebaceous and sweat glands are located (Scheuplein and Blank, 1971; Sugiyashiki et al., 1999). The study of dermal metabolism is complicated by significant interspecies differences in xenobiotic metabolism (Inoue et al., 1999; Prusakiewicz et al., 2006; Fu et al., 2015). Therefore, ideally, metabolism should be investigated in human skin tissues.

Enzymatic metabolism in the skin can be used to bioactivate prodrug molecules and to improve dermal or transdermal delivery. For example, morphine propionate and morphine enanthe are two alkyl ester prodrugs of morphine that have been shown to enhance dermal delivery of...
morpine by 2- and 5-fold, respectively (Wang et al., 2007). Many prodrugs, including these two morphine prodrugs, are formed by esterification of the active molecule. The added ester moiety can be used to alter the physicochemical properties of the molecule and improve transdermal absorption (Wang et al., 2007). Once absorbed into the skin, enzymatic hydrolysis of the prodrug by esterases releases the active drug.

Carboxylesterases 1 and 2 (CES1 and CES2) are involved in the metabolism of xenobiotics. For example, CES1 activates prodrugs of angiotensin-converting enzyme inhibitors and CES2 activates the anticancer prodrug CPT-11 (Benchart et al., 2002; Thomsen et al., 2014). In humans, CES1 and CES2 expression is ubiquitous; however, CES1 predominates in most organs (Sato et al., 2002). Although CESs are known to be expressed in human skin, information on their role in the metabolism of topically applied drugs and prodrugs is limited (Zhu et al., 2007).

We have developed a penta-ethyl ester prodrug of the chelating agent diethylene triamine pentaacetic acid (DTPA), referred to as C2E5 (Fig. 1), to enhance clearance (decoration) of transuranic radionuclides (Zhang et al., 2013b). C2E5 is metabolized by CESs (Fu et al., 2015) and the physicochemical properties of C2E5 (CLogP of 4.7, with a molecular weight of 533 Daltons) suggest that it would be a good candidate for transdermal delivery. Evidence supporting transdermal application of C2E5 was reported in rat in vivo transdermal pharmacokinetics and efficacy studies (Zhang et al., 2013b). Therefore, the first objective of the current work was to assess the expression of CES isoforms in four different human skin cell lines. The second objective was to determine the capacity of the CESs in each cell line to metabolize the prodrug C2E5.

Materials and Methods

Materials. [14C]DTPA penta-ethyl ester ([14C]C2E5; 55 mCi/mmol, 1 mCi/ml) purchased from the Applied Radiolabeled Chemicals, Inc. (St. Louis, MO). Ultima-Fluor AP scintillation fluid was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Acetonitrile, 4-nitrophenyl valerate (4-NPV), was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Cell Culture. HaCaT cells, immortal human keratinocytes, and A431, an immortalized epidermoid carcinoma-derived cell line, were kindly provided by Dr. Zhi Liu (Lineberger Comprehensive Cancer Center, Chapel Hill, NC). Primary neonatal human epidermal keratinocytes (HEKn) and adult human epithelial keratinocytes (HEKa) cells were obtained commercially (Gibco/ThermoFisher Scientific, Waltham, MA). HaCaT and A431 were maintained in 10% fetal bovine serum and Dulbecco’s modified Eagle’s medium (DMEM)/high glucose medium (Gibco) containing 10% fetal calf serum, penicillin (10,000 IU/ml) and streptomycin (10 mg/ml) until subconfluence was reached (after 48 hours). HEKn and HEKa were cultured in EpiLife medium (Gibco), supplemented with 1% Epilife defined growth supplement and 0.1% calcium chloride (CaCl2) (Gibco). All incubations were conducted at 37 ± 1°C, 95% air/5% CO2, and saturated humidity.

Preparation of Cell Supernatant (S9 Fractions). For all cell lines, cytosolic S9 fractions were prepared as described in literature (Imai et al., 2013). Protein concentrations were determined by the Pierce BCA protein assay (ThermoFisher Scientific). Human liver S9 fractions (XenoTech, Kansas City, KS), human recombinant protein (BD Biosciences, Franklin Lakes, New Jersey), and human skin S9 fractions (BioreclamationIVT, Hicksville, NY) were purchased commercially.

Determination of the Gene Expression by Real-Time Polymerase Chain Reaction. HEKa, HEKn, HaCaT, and A431 cells were seeded and grown in 15 ml cell culture media in T75 tissue culture flasks. Expressions of the CES genes were evaluated by real-time polymerase chain reaction (qPCR). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Briefly, cell samples were lysed and homogenized using 1 ml/10 cm2 cells of TRIzol (Ambion/ThermoFisher Scientific) and then isolated by QA shredder columns (Qiagen) in a highly denaturing guanidine-thiocyanate-containing buffer. Ethanol (70%) was added and the samples were applied to an RNeasy Mini Spin Column (Qiagen). RNA was bound to the membrane of the column and contaminants were washed away. Subsequently, RNA was eluted from the column using 30 μl of water. The concentration and purity of the total RNA was determined using the Nanodrop 2000 method (Thermo Scientific, Wilmington, DE). cDNAs were prepared by reverse transcription of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and stored at −20°C until qPCR amplification. qPCR reactions were prepared using the 2x iTaq Universal Probes Supermix, TaqMan CES1, CES2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Taq-Man Gene Expression Assays Rack ID: 14429192; primers: Hs00275607_m1 for CES1, Hs01077945_m1 for CES2, and Hs02758991_g1 for GAPDH) (Applied

![Fig. 1. Structure of penta-ethyl ester DTPA prodrug (C2E5). 14C-radiolabel positions are indicated by asterisks.](image)

![Fig. 2. Log of relative expression of CES1 and CES2 mRNA in human epidermal keratinocyte HEKa, HEKn, HaCaT, A431 cells and human skin by qPCR analysis. (A) CES1 expression. (B) CES2 expression. Values represent mean ± S.E.M. (n = 3).](image)
Biosystems, Foster City, CA) and nuclelease-free water (Quagen). cDNA was diluted 5-fold, and qPCR was performed by the TaqMan Gene Expression Assay (Bio-Rad). The PCR amplification was conducted in a total volume of 20 µl containing universal PCR master mixture (10 µl), gene-specific TaqMan assay mixture (1 µl), diluted cDNA (5 µl), and nuclelease-free water (4 µl). The cycling profile was 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, as recommended by the manufacturer. Amplification and quantification were done with the Applied Biosystems 7900HT Real-Time PCR System (Foster City, CA). All samples were analyzed in triplicate and the signals were normalized to GAPDH and then expressed as relative levels of mRNA. The CES1 probe recognized both CES1A1 and CES1A2; these enzymes are identical although encoded by distinct genes encoded. GAPDH was included in the study as the loading control.

Quantification of Gene Expression. Relative RNA expression levels were determined from delta Ct values using the expression of the GAPDH gene as an internal control. The qPCR assay was performed in triplicate for each sample. For each replicate, the Ct was normalized to the GAPDH Ct (ΔCt = Ct[Target] – Ct[GAPDH]) before the mean and S.E.M. ΔCt was calculated.

Determination of the Protein Expression. Western blot studies were conducted to explore CES1 and CES2 expression in the different human skin cell lines. CES1 and CES2 antibodies were purchased from Abcam (Cambridge, United Kingdom). Protein concentrations were determined using the Pierce BCA Protein Assay (ThermoFisher Scientific). Total protein lysate (30 µg) was run on a NuPAGE 4–12% Bis-Tris Gel (Bio-Rad) at 120 V for 1 hour. Following electrophoresis, the proteins were transferred by blotting onto 0.45 µm polyvinyl difluoride membranes (ThermoFisher Scientific) at 350 mA for 1.25 hours in transfer buffer (Bio-Rad). The membrane was blocked in 5% skim milk powder in Tris-buffered saline 0.05% Tween for 1 hour before overnight incubation with primary antibody: monoclonal hCES1 or hCES2 (Sigma-Aldrich) at 1:10000 dilution or GAPDH (Abcam) at 1:10000 dilution. The membranes were washed and incubated with a secondary antibody, horseradish peroxidase–conjugated goat anti-rabbit (Sigma-Aldrich) at 1:10000 dilution or GAPDH (Abcam) at 1:10000 dilution. The chemiluminescent signal was registered with a FluorChem 8000 camera (Alpha Innotech Corp, San Leandro, CA). Human liver and intestine S9 fractions served as positive controls for CES1 and CES2, respectively.

Determination of Enzyme Activity by pNPA Assay and 4-NPV assay. Total esterase and CES-specific enzyme activity was measured using established substrates pNPA (100 µM) and 4-NPV (100 µM), respectively (Testa and Mayer, 2006). Hydrolysis of the freshly prepared substrates was carried out in 96-well plates with a total volume of 100 µl/well. Reactions were initiated by mixing 1 µl of substrate with diluted S9 samples (0.1 mg/ml). The rates of hydrolysis of pNPA and 4-NPV were determined spectrophotometrically using a UV spectrometer (BioTek, Winooski, VT) to measure reaction products at 405 nm (pNPA) and 402 nm (4-NPV) after a 10-minute incubation at 37°C, as previously described (Williams, 2008).

Determination of C2E5 Hydrolysis by High-Performance Liquid Chromatography–Radioisotopic Flow Scintillation Analyzers, [14C]C2E5 (1 µM, 0.55 nCi) was preincubated in 0.1 M phosphate buffer (pH 7.4) for 5 minutes at 37°C. Reactions were initiated by the addition of S9 fractions (1 mg/ml) from different cell lines (HEKa, HEKn, HaCaT, and A431) in a total volume of 100 µl; boiled S9 fractions were used as a blank to correct for nonenzymatic C2E5 degradation. The reactions were terminated by adding an equal volume of ice-cold acetoneitrile, followed by centrifugation at 14,000g for 15 minutes at 4°C. The supernatant was transfused to high-performance liquid chromatography vials for analysis as previously described (Fu et al., 2015). Representative radio-chromatograms illustrate a C2E5 peak generated from the boiled S9 fractions and C2E5 and the presence of a metabolite, C2E4, in human skin S9 fractions (Supplemental Figs. 1 and 2).

Sample Preparation for Human Skin S9 Fraction–Mediated C2E5 Hydrolysis with and without Inhibitors. Experiments were designed to examine the effect of specific inhibitors on C2E5 hydrolysis in human skin S9 fractions. The following inhibitors were selected: Benzil (10 µM) as a pan CES inhibitor (Wadkins et al., 2005), troglitazone (10 µM) as a CES1-specific inhibitor (Fukami et al., 2010), loperamide (100 µM) as a CES2-specific inhibitor (Williams et al., 2011), and bis-para-nitrophenylphosphate (BNPP; 1 mM) as a nonspecific esterase inhibitor (Li et al., 2007). Inhibitors were incubated with human skin S9 fractions for 30 minutes at 37°C before the reaction was initiated. All reactions were initiated by the addition of S9 fractions (0.5 and 1 mg/ml) to prepared C2E5 in water to result a final C2E5 concentration of 0.5 µM at 37°C. Reactions were terminated after 120 minutes by adding an equal volume of ice-cold acetoneitrile with 2% of formic acid, followed by centrifugation at 14,000g for 15 minutes at 4°C. Liver S9 fractions (1 mg/ml) were used as a positive control and boiled S9 fractions were used as negative controls. Reactions were performed in triplicate. The standards used to generate the liquid chromatography–tandem mass spectrometry (LC–MS/MS) C2E5 calibration curve were prepared by spiking boiled S9 fractions with C2E5 and processed as described above.

LC–MS/MS Chromatographic and Spectroscopic Conditions. Chromatographic separation from matrix components was achieved using reverse-phase chromatography on a YMC ODS-AM C18 (100 x 2 mm, 3 µm) column. Gradient elution was used on the basis of a combination of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The mobile phase was initiated at 13% B increasing to 40% B by 1 minute, to 60% B by 6 minutes, and to 95% B by 6.5 minutes. The mobile phase was held at 5% A and 95% B from 6.2 to 6.5 minutes when a post-run cycle that included isopropyl alcohol (C) was initiated. Between 6.5 and 7 minutes solvent B (95%) was gradually replaced with solvent C (95%). The mobile phase was then held at 5% A 95% C until 7.5 minutes before gradually being returned to initial conditions (87% A, 13% B, 100% C) before the reaction was initiated. Between 6.5 and 7 minutes solvent B (95%) was gradually replaced with solvent C (95%). The mobile phase was then held at 5% A 95% C until 7.5 minutes before gradually being returned to initial conditions (87% A, 13% B, 100% C) before the reaction was initiated.
0% C) after 8.5 minutes, which were maintained for 1.5 minutes prior to the next injection. The flow rate was 300 μl/min and the injection volume was 5 μl. The column oven temperature was set to 40°C. C2E5 was detected on a triple quadrupole mass spectrometer (TSQ Quantum Access; ThermoFisher Scientific) using electrospray ionization (ESI) in the positive-ion mode. The ionization source and collision parameters were optimized to give maximum analyte signal intensity (Spray Voltage 3500V; sheath and auxiliary gas nitrogen, 10 and 25 psi, respectively; collision gas argon at 1.5 mTorr; collision energy 35 eV). The mass spectrometer was set to carry out single-reaction monitoring (SRM) for the precursor → product ion transitions m/z 534 → 216 (C2E5) and m/z 506 → 188 (C2E4) at a retention times of 3.6 and 2.8 minutes, respectively. For C2E5 quantification, a calibration plot of analyte peak area against nominal C2E5 concentration (20–1000 ng/ml) was constructed from a quadratic equation with a weighting of one divided by concentration squared. Representative chromatograms showed formation of C2E5 metabolite, C2E4. Representative chromatograms illustrate a C2E5 peak generated from the boiled S9 fractions and C2E5 and the presence of a metabolite, C2E4, in human skin S9 fractions (Supplemental Figs. 3 and 4).

Data Analysis. The data were processed by Graph Pad Prism 5.0, and are presented as mean ± S.E.M.

Results

Gene Expression of Carboxylesterase in HEKa, HEKn HaCaT, A431 Cells, and Human Skin Tissue. The mRNA expression of CES1 was primarily detected in human skin tissue. A small amount was detected in HEKa and HEKn cells, and none was detected in HaCaT and A431 cells (Fig. 2A). In contrast, mRNA expression of CES2 was detected in all cells. Human skin CES2 expression was about 2-fold higher than HEKa, HEKn, and HaCaT and 10-fold higher than A431 expression (Fig. 2B). CES1 expression in the human skin was about 25-fold greater than CES2 expression. However, the human skin total RNA was obtained from only one human subject and interindividual variability could affect these comparative results.

Protein Expression of Carboxylesterase in HEKa, HEKn, HaCaT, A431 Cells, and Human Skin Tissue. Human liver and intestine S9 fractions were used as positive controls. CES1 protein expression was detected in human skin S9 fractions. The band for CES1 in human skin (30 μg of skin sample) was considerably lighter than the CES1 band in human liver S9 fractions (5 μg of liver sample). There was little evidence of CES1 in HEKa, HEKn, and A431 cells (Fig. 3A). Meanwhile, CES2 protein expression was detected in HEKn, HaCaT, human skin, and human intestine S9 fractions (Fig. 3B). The bands indicated that more CES2 was present in HEKn compared with HaCaT. Little evidence for CES2 in A431 was observed (Fig. 3B).

Hydrolysis Activity of pNPA and 4-NPV in HEKa, HEKn, HaCaT, A431 Cells and Human Skin Tissue. Enzymatic activity was determined using pNPA (esterases) and 4-NPV (CESs) assays with human liver S9 fractions as a control. The pNPA assay demonstrated that the S9 fractions from human skin cell lines exhibited esterase activity (Fig. 4A). pNPA hydrolysis activity in the cultured cells was slightly lower than in human skin S9 fractions and 5- to 10-fold lower than in human liver S9 fractions (Table 1). For example, HEKn displayed approximately 20% of the esterase activity of the liver. When the amount of protein was standardized across all the samples, the catalytic rate of esterase in HEKn was the highest among all cell lines. The 4-NPV assay showed that CES activity was present in all of the tested cells; the catalytic rate of CES in HEKn cell lines was the greatest (Fig. 4B).

Hydrolytic Activity of Penta-Ethyl Ester Prodrug of DTPA (C2E5) in Human Skin S9 Fractions. Near complete (98.6%) hydrolysis was seen in human liver S9 fractions, whereas human skin S9 fractions showed 62.8% loss of the parent drug (C2E5; Fig. 5). As expected, addition of the nonspecific esterase inhibitor bis-paramitrophenylphosphate totally blocked the hydrolysis of C2E5. Addition of the inhibitors benzil (pan CESs), troglitazone (CES1), and loperamide (CES2) resulted in 10.6%, 40.68%, and 77.68% loss of parent drug C2E5, respectively. These data suggest that both CES1 and CES2 hydrolyze C2E5, but the hydrolysis is primarily via CES1.

Discussion

As transdermal delivery technology becomes more common, questions remain as to how and whether ester-based prodrugs pass through the skin and whether hydrolysis during this transition affects the absorption, distribution, metabolism, and excretion of the drug. Because of the interspecies differences in hydrolysis profiles, skin derived from animals may be very different from human skin (Tauber and Rost, 1987; Hewitt et al., 2001; Prusakiewicz et al., 2006). Therefore, alternative methods to examine human-specific hydrolysis are needed during drug development. Previously, we reported that CESs play an important role in the metabolism of the ester-based prodrug C2E5, which is being investigated as a decoporation agent for contamination with transuranic elements (Zhang et al., 2013b). The present study characterized CES expression and activity in human skin tissue and different human keratinocyte cell lines and demonstrated the role of CES in facilitating C2E5 hydrolysis in skin during transdermal delivery. In addition, the prodrug C2E5 was hydrolyzed in all skin cell lines examined, and HEKa may be one of the most appropriate cell lines in which to study transdermal delivery.

<table>
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<tr>
<th>Table 1</th>
<th>Carboxylesterase activity in human skin cell cultures, human skin S9 fractions, and human liver S9 fractions</th>
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<tbody>
<tr>
<td>Cell Type</td>
<td>Hydrolytic Activity (nmol/min per mg)</td>
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<tr>
<td>---------</td>
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<tr>
<td>HEKa</td>
<td>20.2 ± 1.82</td>
</tr>
<tr>
<td>HEKn</td>
<td>31.6 ± 3.76</td>
</tr>
<tr>
<td>HaCaT</td>
<td>26.2 ± 1.97</td>
</tr>
<tr>
<td>A431</td>
<td>16.7 ± 1.11</td>
</tr>
<tr>
<td>Human skin</td>
<td>39.0 ± 11.54</td>
</tr>
<tr>
<td>Human liver</td>
<td>167.2 ± 5.30</td>
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Data are the Mean ± S.E.M. N = 3.

<table>
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<tr>
<th>Table 2</th>
<th>Carboxylesterase activity in human skin cell cultures, human skin S9 fractions, and human liver S9 fractions</th>
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<tr>
<td>Cell Type</td>
<td>Hydrolytic Activity (nmol/min per mg)</td>
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<tr>
<td>HEKa</td>
<td>40.9 ± 8.87</td>
</tr>
<tr>
<td>HEKn</td>
<td>125.5 ± 5.87</td>
</tr>
<tr>
<td>HaCaT</td>
<td>103.0 ± 9.65</td>
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<tr>
<td>A431</td>
<td>57.3 ± 7.56</td>
</tr>
<tr>
<td>Human skin</td>
<td>114.3 ± 5.60</td>
</tr>
<tr>
<td>Human liver</td>
<td>217.7 ± 13.83</td>
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Data are the Mean ± S.E.M. N = 3.
CES1 and CES2 proteins were expressed in human skin S9 fractions. qPCR clearly showed the greater expression of CES1 mRNA compared with CES2 mRNA, which supports a previous report of greater CES1 expression in human skin microsomes (Jewell et al., 2007). As expected, measurement of total esterase activity using the pNPA assay (Imai et al., 2013) revealed much less activity in the skin compared with the liver (Fig. 4A). However, the 4-NP assay, which measures CES activity (Williams et al., 2011), showed that total CES activity was only 2-fold lower in the skin than in the liver (Fig. 4B), confirming the potential for human skin to contribute to the metabolism of ester compounds when applied transdermally (Wang et al., 2007).

As an alternative to human skin, we assessed different human keratinocyte cell culture models for their utility as surrogates in investigation of transdermal drug metabolism. The keratinocyte tumor cell line A431 had no detectable CES1, and CES2 was expressed at barely detectable levels. Therefore, we concluded that A431 cell line is not a suitable model for investigating transdermal drug metabolism. Of the remaining keratinocyte cultures, our results demonstrated that whereas HEKa, HEKn, and HaCaT express CES1, CES2 was more abundant across the various keratinocyte cell lines. These findings are consistent with the work of Zhu et al. (2007), who identified CES2 as the main CES in HaCaT cells. Enzyme expression and activity in keratinocytes change over time in culture; cytochrome P450 enzymes are particularly vulnerable, but esterases and conjugated enzymes are also affected (Williams, 2008). This observation could explain our findings that CES2 expression was slightly lower in HaCaT cells compared with HEKa or HEKn cells, and that CES1 expression was greatly reduced or absent in all the cultured cells. HEKa and HEKn are primary cultures and, as such, may retain the greater enzymatic activity observed in human skin compared with the immortalized HaCaT cell line.

CES1 and CES2 are from the same family, known as 60-kDa serine esterases. Whereas these two isoforms have a similar molecular weight, (CES1 is 62.5 kDa and CES2 is 60.0 kDa), they are structurally quite different. The isoelectric point of CES1 is 5.8 and CES2 is 4.9 and the sequence homology between the two enzymes is only 48% (Pindel et al., 1997). These structural differences result in different substrate specificity. CES1 tends to hydrolyze molecules with a small alcohol moiety more efficiently, whereas CES2 is more efficient at metabolizing molecules with a larger alcohol moiety and more lipophilic molecules (Brzezinski et al., 1994; Williams et al., 2011). These differences in the substrate specificity of CES1 and CES2 could lead to differences in predicting skin absorption or metabolism. In different human cell lines and skin S9 fractions, we demonstrated CES expression by Western blot and qPCR and confirmed enzyme activity with pNPA and 4-NP assay; subsequently, we examined the metabolism of C2E5, a prodrug developed for transdermal delivery.

C2E5, the penta-ethyl ester of DTPA, is administered intravenously to treat plutonium, americium, and curium (Pu, Am, and Cm) contamination. In vivo studies in rats report de-esterification of C2E5 mainly into the tri- and di-ethyl esters C2E3 and C2E2 with some DTPA contamination. In vitro binding experiments using human, rat, and dog plasma, suggest that C2E2 is an effective chelator of Am (Huckle et al., 2015a), and this hypothesis is supported by efficacy study following oral administration of C2E2 in beagle dogs (Huckle et al., 2015b). Thus, to be effective, when applied transdermally, C2E5 needs to be metabolized to C2E2 in the body. Sustained plasma concentrations of C2E2 are observed in rats following transdermal application of C2E5 in a nonaqueous gel (Zhang et al., 2013b), and this is associated with effective Am decoporation (Zhang et al., 2013a), suggesting that transdermal delivery of C2E5 to the active C2E2 is possible. In the present study, we used the S9-fractions model to assess the potential translation of these preclinical observations to human tissues.

Previously, we used a human recombinant protein system to examine human CES1- and CES2-mediated C2E5 hydrolysis; the results demonstrated that both CES1 and CES2 were responsible for C2E5 hydrolysis. However, CES1 hydrolyzed C2E5 to a greater extent compared with CES2 (Fu et al., 2015). The results in the current study agree with our previous findings (Figs. 5 and 6).

Although complete metabolism to an active drug, C2E2, was not observed in human skin in the current study, once in the systemic circulation, further hydrolysis of C2E5’s metabolites can occur in the liver, mainly by CES1, and in plasma, possibly by paraoxonase and butyrylcholinesterase, as CES1 and 2 are not present (Bahar and Imai, 2013). Additionally, the metabolism of C2E5 by CESs in keratinocytes, which we report here, results in metabolites that are more hydrophilic than C2E5 and could potentially more readily enter the systemic circulation.

The differences in enzyme activity and expression among cell lines and skin tissue have important implications for future studies examining...
transdermal metabolism of ester-based prodrugs. HEKα, HEKn, and HaCaT cell cultures have potential for the examination of the metabolism of CES2 substrates. However, of the human cell lines we examined, only HEKα cells have the potential for establishing the metabolism of compounds that are substrates for CES1.

In summary, this is the first study to characterize the expression of CES isoforms in multiple human skin cell lines and human skin tissue with a view to using native -1 enzymes in skin to enhance transdermal delivery of C2ES. The differences in enzyme activity and expression among cell lines and skin tissue has important implications for future studies examining transdermal metabolism of ester-based prodrugs. We confirmed that CES activity is present in skin, albeit at lower levels compared with the liver, and that CES activity, but not CES1 activity, in HEKα, HEKn, and HaCaT cells is comparable to that of human skin. Consequently, human skin cell cultures may be useful in quantifying CES2-mediated drug metabolism. Of the human cell lines we examined, HEKα cells have the potential for establishing the metabolism of compounds that are substrates for CES1. However, precaution should be taken when human skin cell lines are used as alternative models for human cutaneous metabolism in transdermal drug delivery. Since the CES1 specific inhibitor reduced human skin S9 fraction–mediated hydrolysis of C2ES, CES1 appears to be crucial for C2ES metabolism; as a result, the HEKα cell line could be an appropriate model for metabolism of C2ES.

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Authorship Contributions

 Participated in research design: Fu, Sadgrove, Jay.
 Conducted experiments: Fu.
 Performed data analysis: Fu, Sadgrove, Marson, Jay.
 Wrote or contributed to the writing of the manuscript: Fu, Sadgrove, Marson, Jay.

References


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Article title:

Biotransformation capacity of carboxylesterase in skin and keratinocytes for the penta-ethyl ester prodrug of DTPA

Authors: Jing Fu, Matthew Sadgrove, Lesley Marson, Michael Jay

Journal title: Drug Metabolism & Disposition

Legends for Supplemental Figures

Figure S1. Representative HPLC-FSA chromatogram shows the separation of a mixture of C2E5 and others in control sample, boiled liver S9 fraction-mediated hydrolysis of C2E5 (25 µM) at 120 min. The result from chromatogram corresponding to the peak is parent drug C2E5.

Figure S2. Representative HPLC-FSA chromatogram shows the separation of a mixture of C2E5 and others in skin metabolism sample, human skin S9 fraction-mediated hydrolysis of C2E5 (12.5 µM) at 120 min. The results from chromatogram corresponding to the two peaks are metabolite (C2E4) and parent drug C2E5.

Figure S3. Representative LC/MS/MS chromatogram shows the separation of a mixture of C2E5 and others in a control sample, boiled liver S9 fraction-mediated hydrolysis of C2E5 (1 µM) at 120 min. The results from chromatogram corresponding to the two peaks are C2E4 (impurity in starting materials) and parent drug C2E5.

Figure S4. Representative LC/MS/MS chromatogram shows the separation of a mixture of C2E5 and others in skin metabolism sample, human skin S9 fraction-mediated hydrolysis of C2E5 (1 µM) at 120 min. The results from chromatogram corresponding to the two peaks are C2E4 (major metabolite and minor impurity) and parent drug C2E5.
HPLC-FSA chromatograms

Figure S1. Boiled liver S9 fraction-mediated hydrolysis of C2E5 (25 µM) at 120 minutes

Figure S2. Human Skin S9 fraction-mediated hydrolysis of C2E5 (12.5 µM) at 120 minutes
**LC/MS/MS chromatograms**

**Figure S3.** Human liver S9 fraction-mediated hydrolysis of C2E5 (1 µM) at 120 minutes

**Figure S4.** Human liver S9 fraction-mediated hydrolysis of C2E5 (1 µM) at 120 minutes