The distribution of esterases in the skin of the minipig

Christopher Jewell\textsuperscript{a}, Jeffery J. Prusakiewicz\textsuperscript{b}, Chrisita Ackermann\textsuperscript{b}, N. Ann Payne\textsuperscript{b}, Gwendolyn Fate\textsuperscript{b}, Faith M. Williams\textsuperscript{a,*}

\textsuperscript{a} Toxicology Unit, School of Clinical and Laboratory Sciences, 4th Floor, Devonshire Building, Devonshire Terrace, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4EA, UK

\textsuperscript{b} Pfizer Inc., Ann Arbor, MI, USA

Received 29 March 2007; accepted 13 July 2007
Available online 19 July 2007

Abstract

Skin esterases serve an important pharmacological function as they can be utilised for activation of topically applied ester prodrugs. Understanding the nature of these enzymes, with respect to their role and local activity, is essential to defining the efficacy of ester prodrugs. Minipigs are used as models to study the kinetics of absorption of topically applied drugs. Their skin has structural properties very similar to human skin. However, regional distribution differences in esterase activity from site-to-site could influence cross-species extrapolation. Investigation of the regional site variation of minipig skin esterase activity will facilitate standardization of topically applied drug studies. Furthermore, the characterization of regional skin variation, will aid in translation of minipig results to better predictions of human esterase activity. Here we report the variation in rates of hydrolysis by minipig skin taken from different regional sites, using the esterase-selective substrates: phenyl valerate (carboxylesterase), phenyl acetate (arylesterase) and \textit{p}-nitrophenyl acetate (general esterase). Skin from ears and back of male minipig showed higher activity than female. Skin from minipig ears and the back showed the highest level of esterase activity and was similar to human breast skin used in vitro absorption studies. These results suggest that skin from the minipig back is an appropriate model for preclinical human skin studies, particularly breast skin. This study supports the use of the minipig, with topical application to the back, as a model for the investigation of pharmacokinetics and metabolism of ester prodrugs.

© 2007 Elsevier Ireland Ltd. All rights reserved.

Keywords: Skin; Esterase; Minipig

1. Introduction

Prodrug esters have been developed to increase the absorption of a drug when topically applied. Many prodrug esters are activated by hydrolysis in the skin, which has been shown to contain esterases. Therefore, the hydrolysis rates in the skin determine, to some extent, the human exposure levels of active drug. Absorption through minipig skin has been found to be a more appropriate model of absorption through human skin than rodent. Rodents have much higher skin esterase activity, and therefore, higher prodrug activation than either the human or minipig skin (Prusakiewicz et al., 2006; Jewell et al., 2007). Esterases are members of the hydrolase family of enzymes, which primarily hydrolyse endogenous and exogenous esters with substrate specificity overlapping with lipases (Williams, 1985; Mentlein et al., 1988). They are ubiquitously expressed in mammalian liver and extra-hepatic tissues including blood,
skin, kidney, intestines, testes, brain, central nervous system and lung (Satoh and Hosokawa, 1998). Carboxylesterases with a serine active centre are inhibited by organophosphates, whereas arylesterases have a cysteine at the active centre and are not inhibited by organophosphates.

Regional variations in minipig esterase activity could influence the systemic delivery of topically applied ester prodrugs and confound the correlations to humans. Preclinical human dermal absorption studies have been performed primarily on skin from the female breast (mammoplasty) or abdomen (adominoplasty). In vitro absorption studies have generally been conducted using skin excised from the back of the pig (both domestic pig and minipig) or the isolated pig ear (Dick and Scott, 1992). For technical reasons, compounds are usually applied to the backs of the minipigs during in vivo pharmacokinetic studies. Investigation of the variation in metabolising capacity between different areas of minipig skin is warranted to assure that the back is an adequate model of human trunk skin, the usual site of a transdermal patch. It is also important to compare the skin with the liver, when considering all possible sites of prodrug activation. Two carboxylesterases have been identified in the pig liver and have been classified as CES1 and CES by Satoh and Hosokawa (2006). Human carboxylesterases are classified as CES1, CES2 and CES3. David et al. (1998) reported a pig intestinal carboxylesterase that showed high similarity with that of rat and human intestinal carboxylesterases. Carboxylesterases in pig skin have been shown to differ in isozyme distribution from the liver (Jewell et al., 2007). Esterases are present in endoplasmic reticulum and cytosol in keratinocytes of the skin and hepatocytes of the liver (Clark et al., 1993; McCracken et al., 1993a,b; Mutch et al., 2007). There are few reports describing cytosolic esterase activity. However, it is important to evaluate the contribution of cytosolic esterase activity to the tissue as a whole and to determine whether they differ from microsomal esterases in their activity and specificity.

In this study phenyl valerate (PV), phenyl acetate (PA) and p-nitrophenyl acetate (NPA) were used as esterase substrates to investigate the distribution of esterase activity in minipig skin microsomal and cytosolic fractions taken from various regional locations. PV is substrate for carboxylesterases, NPA for carboxylesterases and arylesterases and PA for arylesterases. By comparing hydrolysis of a range of substrates by microsomal and cytosolic fractions from skin and liver differences in regional esterase activity and specificity can be deduced.

2. Materials and methods

2.1. Reagents

Unless otherwise stated chemical reagents were purchased from Sigma–Aldrich (Poole, Dorset, UK).

2.2. Human skin preparation

Human skin was obtained following breast reduction surgery of healthy female individuals. Patients gave informed consent and ethical approval was obtained from University Hospital of North Durham, UK

2.3. Minipig tissue procurement

Minipig livers and skin from three male and three female, approximately 11 months old, were provided by Pfizer Inc., Framboise, France. A section of tissue from each of the five minipig liver lobes was provided, indicated as left and right lateral lobes, left and right medial lobes and the caudate lobe. Skin was taken from various minipig sites including the neck, back, shoulders, flanks and ears. All tissues were flash frozen, transported on dry ice and stored at −70 °C until further processed.

2.4. Skin preparation

After thawing human and minipig skin, the hair was gently removed using a scalpel before the skin was dermatomed (setting 6, 350 μm, Davis Miniplex Seven, Thackery, Leeds, UK). Weighed, dermatomed skin from each site of the three minipigs was minced separately and pulverised under liquid nitrogen. Ice-cold KCl/phosphate buffer (150 mM KCl, 100 mM K2HPO4, pH 7.4) was added to pulverised skin at 1 ml per 100 mg of tissue. Tissue slurry was further homogenised with three 10 s Ultra-turrax bursts. The homogenate was centrifuged for 10 min at 750 × g to remove macroscopic debris, followed by centrifugation of the supernatant for 10 min at 10,000 × g to remove mitochondria, nuclei and cell debris. Further centrifugation of the supernatant for 70 min at 100,000 × g separated the microsomes from the cytosolic fraction. The microsomal pellet was re-suspended and centrifuged at 100,000 × g for 70 min. The washed microsomal pellet was re-suspended in glycerol buffer (10% glycerol, 50 mM Tris, 100 μM KCl, 250 mM sucrose, pH 7.4) and stored at −70 °C for analysis. Replicate reactions were performed with each tissue fraction.

2.5. Minipig liver preparation

Minipig liver samples (approximately 1 g) from individual liver lobes were minced, and added to ice-cold KCl/phosphate buffer (1 ml per 100 mg tissue). Liver was homogenised (4 °C) with two 10 s bursts of an Ultra-turrax homogeniser. The procedure for homogenisation and centrifugation was repeated as described for skin. Microsomal and cytosolic samples were frozen at −70 °C until required. Protein concentrations of all
tissue fractions were measured by standard methods using bovine serum albumin as the protein standard (Smith et al., 1985).

2.6. NPA hydrolysis assay

Hydrolysis of NPA (0.5 mM) to p-nitrophenol was examined for skin and liver subcellular fractions. Using a 96-well plate, each well-contained microsomal protein (1 μg) or cytosolic protein (10 μg) in buffer (50 mM Tris, pH 8.0, 37 °C). NPA (0.5 mM) was added to each well and the change in absorbance at 406 nm, recorded for 6 min. This was a real-time assay that measured the formation of the yellow hydrolysis product, p-nitrophenol. Spontaneous hydrolysis of NPA was measured in the absence of protein (1.35 ± 0.06 nmol/min/ml) and was subtracted as background from initial rate determinations. A standard curve was constructed using p-nitrophenol in buffer. Reactions were linear for the duration of the assay and the slope was taken for determination of the rate of reaction. The substrate concentration used provided saturated substrate conditions for maximal enzyme kinetics.

2.7. PA and PV hydrolysis assay

PA or PV hydrolysis was examined in rat, minipig and human skin and liver subcellular fractions. Using a 96-well plate, each well-contained liver microsomal protein (1 μg), liver cytosolic protein (10 μg), skin microsomes (40 μg), or skin cytosol (20 μg) in buffer (100 mM Tris, pH 8.0). Each reaction contained aminoantipyrine (2.5 mM) and K3Fe(CN)6 (5 mM) as detection reagents. Hydrolysis reactions were initiated by additions of ester substrate (3 mM). The change in absorbance was recorded at 510 nm at 37 °C for 6 min. This is a real-time assay that measures the formation phenol, which then reacts with aminoantipyrine and K3Fe(CN)6 to form a red/orange product. Spontaneous hydrolysis of PA and PV was measured in the absence of protein. A standard curve was constructed using phenol. Hydrolysis over the 6 min of measurement was linear, with substrate concentration appropriate for maximal enzyme kinetics. The slope was taken for the determination of rate of reaction.

2.8. Statistics

Data was compared by ANOVA followed by Tukey’s post hoc test using prism, GraphPad Inc., San Diego, USA.

3. Results

3.1. Minipig skin preparation

Visually, minipig neck skin was the thickest, followed closely by back skin and then shoulder skin. These sections had thick fatty connective tissue below the skin surface and had thick course hair. Flank skin was much thinner and ear skin was thinnest. Flank and ear skin had far less fatty tissue associated with it and hair was more sparse and finer.

3.2. Minipig protein recovery

Overall, skin protein recovery was less than the liver (Table 1). Microsomal protein recovery from minipig skin was 1.40 ± 0.17 mg/g dermatomed tissue (mean ± S.E.M.) for male and 0.96 ± 0.08 mg/g for female. Cytosolic protein recovery from minipig skin averaged 16.11 ± 0.74 mg/g for male skin and 16.73 ± 0.85 mg/g for female skin. Microsomal protein recovery from minipig liver (male and female) was 12.62 ± 1.03 mg/g tissue and cytosolic protein was 107.61 ± 7.79 mg/g tissue and this compared to recovery from human skin of 1.93 ± 0.11 mg/g for skin microsomal protein and 22.93 ± 1.39 mg/g for cytosolic protein. There were no significant differences in protein recoveries in microsomal or cytosolic fractions from the five different minipig liver lobes (Supplementary Table 1). Skin from female neck and flank yielded significantly less microsomal protein than for the male (P < 0.05), but there was no significant difference in cytosolic protein.

3.3. Esterase activity

All samples of minipig liver and skin hydrolysed PV, NPA and PA indicating that carboxylesterases and arylesterases were present (Figs. 1 and 2). There were slight differences in activity for all substrates between the minipig skin sites analysed, both in microsomal and cytosolic fractions. For all three substrates, there

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein recovery from minipig skin preparations.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Male minipig</td>
</tr>
<tr>
<td>Neck</td>
</tr>
<tr>
<td>Back</td>
</tr>
<tr>
<td>Shoulder</td>
</tr>
<tr>
<td>Flank</td>
</tr>
<tr>
<td>Ear</td>
</tr>
<tr>
<td>Female minipig</td>
</tr>
<tr>
<td>Neck</td>
</tr>
<tr>
<td>Back</td>
</tr>
<tr>
<td>Shoulder</td>
</tr>
<tr>
<td>Flank</td>
</tr>
<tr>
<td>Ear</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.E.M. (n = 3).
Fig. 1. Rates of esterase hydrolysis in microsomes and cytosol for different sites of minipig skin ($n = 3$, mean ± S.E.M.). NPA—$p$-nitrophenyl acetate, PA—phenyl acetate and PV—phenyl valerate. MP—male minipig and FP—female minipig. Rates of hydrolysis expressed in terms of μmol/min/mg protein and μmol/min/g wet weight tissue.
was a trend for higher activity in the male, with male minipig back and ear skin showing the highest activity, although the differences were not statistically significant. There were no significant differences between the lobes of minipig liver. The liver activity (Fig. 2) was compared with activity from different skin sites (Fig. 1) and was significantly higher in liver for all substrates than in skin. Activity, in terms of protein, in minipig skin microsomal fractions was around 100 times lower than in liver microsomal fractions, whereas cytosolic activity was around 10 times less in skin than liver. For all substrates, rates of hydrolysis were similar in all skin fractions. However, in liver, PV hydrolysis was significantly higher than for NPA or PA hydrolysis ($P < 0.05$). PV hydrolysis by minipig skin was similar to human skin in both microsomal and cytosolic fractions. Additionally NPA hydrolysis in microsomal fractions was similar in minipig and human skin. PA hydrolysis in human skin was approximately three times higher than seen in the highest activity of minipig skin. In cytosolic fractions, rates of NPA and PA hydrolysis in human skin were approximately twice that of minipig skin.

4. Discussion

Phenyl valerate was hydrolysed by non-specific carboxylesterases in the skin and inhibited by paraoxon (Jewell et al., 2007) whereas phenylacetate has previously been shown to be a substrate for arylesterases in the liver and skin (McCracken et al., 1993a). There is little information on the nature of arylesterases in minipig skin. The relative importance of arylesterases in skin compared to carboxylesterases in the hydrolysis of ester prodrugs depends on the specificity of the substrate. It has been previously suggested for human skin that both arylesterases and carboxylesterases contribute to hydrolysis (McCracken et al., 1993b). Hydrolysis of NPA, a substrate for both arylesterases and carboxylesterases had been previously been studied in rat skin by Prusakiewicz et al. (2006). This substrate was previously considered to be a substrate for carboxylesterase and arylesterase in liver, and inhibition studies indicate similar involvement in pig skin (Jewell et al., 2007). Therefore NPA is probably not enzyme selective and is probably hydrolysed by both serine and cysteine esterases, supporting its use as a substrate to evaluate general esterase activity.

It was seen for all substrates that rates of hydrolysis was from various skin sites in the minipig were very similar, though there was a tendency for higher activity in back and ear skin, especially in the male. Expressing results as per gram wet weight of tissue takes into account the different protein yield between microsomes and cytosol. This information is important in evaluating the contributions to hydrolysis of an ester during percutaneous penetration. When expressed per gram of tissue the cytosolic component was up to 10 times greater than the microsomal component for all minipig skin sites. This illustrates the significant contribution and importance of skin cytosolic esterases in the hydrolysis of esters.

Skin from back and ear of male minipig were the most similar to human breast skin, in the ability to metabolise ester substrates. Therefore skin from male minipig back was an appropriate model for human breast skin for these esterase substrates. This supports the use of the minipig with topical application to the back as a model for investigation of the pharmacokinetics of ester prodrugs which could undergo local hydrolysis in the skin during absorption.

Acknowledgment

This research was supported by a grant from Pfizer Inc., Ann Arbor, MI, USA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2007.07.004.
References


