

RESEARCH ARTICLE

Effects of the genotoxic compounds, benzo[a]pyrene and cyclophosphamide on phase 1 and 2 activities in EpiDerm™ models

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Abstract

1. The micronucleus assay in the 3D human reconstructed EpiDerm™ skin model (RSMN) is a promising new assay for evaluating genotoxicity of dermally applied chemicals.
2. To complement the testing of metabolically activated chemicals, such as cyclophosphamide (CPA) and benzo[a]pyrene (B[a]P), we measured phase 1 (ethoxyresorufin O-deethylation (EROD) and testosterone metabolism) and 2 activities (UGTs and GSTs) in non-treated and genotoxin treated EpiDerm™ models in a study design which mimics the RSMN assay. The assay involved a three-dose dosing regimen over 72 h to take into account effects e.g. enzyme induction, which requires longer than the standard 2 dose 48-h assay.
3. These studies demonstrated the presence of basal phase 1 and 2 activities of EpiDerm™ models.
4. With the exception of GST, all of the activities measured did not reproducibly change over time.
5. It was possible to measure enzyme induction using this assay design. EROD activity was significantly induced by B[a]P but not by CPA. CPA and B[a]P had little or no reproducible effects on GST and UGT activities.
6. In conclusion, a number of metabolic enzyme activities were present in the EpiDerm™ skin model and at least the CYP1 family was inducible.

Keywords: Skin metabolism, CYP, 7ethoxyresorufin O-deethylation, induction, UDP-glucuronosyltransferases, glutathione S-transferases

Introduction

As a result of the 7th Amendment of the Cosmetics Directive (EU, 2003), cosmetic ingredients can no longer be tested for genotoxicity in *in vivo* assays. Therefore, much effort has been made on the development of a micronucleus assay in the 3D human reconstructed EpiDerm™ skin model (RSMN) (Curren et al. 2006, Dahl et al. 2011, Hu et al. 2009a, Munn et al. 2009). This is a promising new assay for evaluating genotoxicity of dermally applied chemicals and results to date demonstrate international inter-laboratory and inter-experimental reproducibility of the assay for chemicals that do not undergo metabolism

(Aardema et al. 2010). Ongoing studies in the Colipa-funded (European Cosmetics Association) project include the testing of chemicals which are metabolically activated to the genotoxic metabolite. Initial findings suggest that the EpiDerm™ model can also correctly identify a number of genotoxicants which have been shown to be metabolically activated in hepatic models and human lymphocytes (data not shown), e.g. cyclophosphamide (Cao et al. 1993, Elhajouji et al. 1994) and benzo[a]pyrene (B[a]P) (Elhajouji et al. 1994, Wu et al. 2003). The skin is the largest organ of the body and has multiple functions, not least of which is that of metabolism (Chuong et al. 2002). Chemicals that

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come into contact with the skin may be subject to metabolism as they penetrate through the skin and pass into the systemic circulation. It is therefore of great importance to appreciate and measure the metabolic capacity of skin and how this affects endpoints such as cytotoxicity, and this case, genotoxicity. EpiDerm™ (Hu et al. 2010) and other reconstructed skin models (Luu-The et al. 2009) are prepared from primary human keratinocytes and have been shown to exhibit a metabolic potential which is similar to native human skin, at least with respect to the mRNA expression of these enzymes (Hu et al. 2010). However, the presence of mRNA expression does not always infer that the respective enzyme protein is present and, moreover, functional (Pushparajah, 2008a). There are reports of functional enzyme activity measurements in native human skin models (Nohynek et al., 2005) and, indeed, EpiDerm™ models have been reported to have measurable N-acetyltransferase activities (Hu et al. 2009b). We have extended investigations of the metabolic capacity of EpiDerm™ models to include a study design which mimics the pre-validated RSMN (Dahl et al. 2011) with the measurement of a limited number of basal phase 1 and 2 activities. Few have reported whether the chemicals under investigation alter the metabolic enzyme levels; therefore, we have also investigated whether known genotoxicants alter (i.e. either increase or decrease) enzyme activities when they are applied to EpiDerm™ models under the same incubation conditions as those used in the RSMN assay. The design of these studies includes a 72h total treatment period (i.e. 3 daily doses) for two reasons (i) some metabolic enzyme activities (e.g. in human hepatocytes, PXR or CAR mediated CYPs) require an induction period of up to 72h (LeCluyse et al. 2000); therefore, we wanted to maximize the chances of detecting an alteration in skin enzyme activities and (ii) in certain circumstances, the extension of the RSMN incubation time from 48 to 72 h may be required, especially for metabolically activated genotoxicants (Dahl et al. 2011). A manuscript addressing the 48 and 72 h dosing regimen for metabolically activated genotoxicant is in preparation (Aardema et al. 2010).

Cyclophosphamide and B[a]P were chosen as model bioactivated genotoxicants for these studies. B[a]P is metabolized in human liver mainly by CYP1B1, CYP1A1 and microsomal epoxide hydrolase (Hvastkovs et al. 2007) and in part by CYP2C9, and CYP3A4 which produce the 3-hydroxy metabolite (Gautier et al. 1996). In skin, pro mutagen and mutagen formations are dependent on the CYP1 family, which is inducible by UV radiation and environmental poly aromatic hydrocarbons (Afaq et al. 2009, Swanson, 2004), to the ultimate carcinogen, B[a]P-diolepoxide. Modulation of CYPs has been shown to alter the amount of DNA adducts formed in HepG2 cells (Wei et al. 2009); therefore, metabolism plays an important role in the mechanism of B[a]P carcinogenesis. CYP1A1 is reported to be absent in native human skin but is induced by B[a]P (Oesch et al. 2007), making this an appropriate test chemical for our studies. Since CYP1A1 and CYP1B1 are responsible for the bioactivation of many

pro-genotoxicants in rodents and humans (Pelkonen and Nebert, 1982), the O-deethylation of the CYP1 substrate 7-ethoxyresorufin (EROD) was measured in EpiDerm™ models 24 h after each dose of test chemical. Rather than addition of the substrate to the medium underneath the model, 7-ethoxyresorufin was added in acetone to the surface – in the same way as the test chemicals. The presence of lipophilic solvents is known to enhance compound penetration into the skin (Guy et al. 1990); therefore, we dissolved both test chemicals and substrates in acetone to maximize penetration into the models. Previous studies have shown that treatment of the EpiDerm™ models with 10 µL acetone is not toxic and does not cause an increased frequency of micronuclei compared to non-treated models (Dahl et al. 2011). The penetration into the deeper epidermal layers of the relatively lipophilic 7-ethoxyresorufin from the model surface was considered to be greater than when it was added to the more hydrophilic medium. The incubation time was set to 6 h to allow for both penetration of the substrate into the model and metabolism (which is known to be much lower than in hepatic models (Oesch et al. 2007)). Others have shown that testosterone readily penetrates human RS models when applied to the model surface (Schäfer-Korting et al. 2008); therefore, we were confident that this application method would be suitable for this substrate. Indeed, we found that this was sufficient to allow metabolites from all substrates to be detected in the medium below the model. Further studies could include measurement of the test chemicals and substrates within the models to confirm the depth of penetration.

The metabolism of cyclophosphamide in human liver is mainly via CYP2B6, and this enzyme is also induced by cyclophosphamide in human hepatocytes (Gervot et al. 1999). Cyclophosphamide is also metabolized via human hepatic CYP2A6, 2C8, 2C9, 2C19, 3A4 and 3A5, but these CYPs have a much lower contribution than CYP2B6 (Xie et al. 2003). Notably, the expression of CYP2B6 is reported to be absent in the EpiDerm™ donors (donor 1188 and 254) used in these studies (Hu et al. 2010b). Of the other human hepatic CYPs involved in cyclophosphamide metabolism, only CYP2C9 and CYP3A5 were expressed (Hu et al. 2010b). We therefore used testosterone as a marker substrate for CYP2B6 (16β-hydroxide), CYP2C (producing 16α-hydroxide but also partly responsible for producing 16β-hydroxide) and CYP3A4 (6β-hydroxide and 2β-hydroxide) (Tachibana and Tanaka, 2001). The major metabolite of testosterone in skin is androstenedione (Beckley-Karty et al. 1997 reference), which is catalyzed by 17β-hydroxysteroid oxidoreductase and CYP2C19 in the liver (Tachibana and Tanaka, 2001). Therefore, this metabolite was also quantified in EpiDerm™ model incubates with testosterone. As with ethoxyresorufin, this lipophilic substrate was dissolved in acetone and added to the surface of the EpiDerm™ models.

B[a]P is reported to also induce glutathione S-transferase (GST) activities in HepG2 cells, which confers protection

against the formation of reactive metabolites (Wei et al. 2009). Human and rodent skin exhibit appreciable GST activity (Oesch et al. 2007), although still not in levels as high as those reported in human liver (300–500 nmol/min/mg protein in liver slices incubated with CDNB (Pushparajah et al. 2008b) and the EpiDerm™ models used in our studies have been shown to express certain GSTs (Hu et al. 2010b). Considering B[a]P induced GST activities in human liver slices (Pushparajah et al. 2008b), it was of interest to determine whether B[a]P also alters the levels of this detoxification enzyme under the conditions of the 72 h RSMN assay. The reactive metabolites of cyclophosphamide are also detoxified by human GSTs (Dirven et al. 1994); therefore, the effect of both B[a]P and cyclophosphamide on the metabolism of the broad spectrum GST substrate, CDNB, was also measured. The conjugation of CDNB with glutathione is catalyzed by a number of human GST isoenzymes, including the α , μ and π -classes (Sherratt and Hayes 2002) with π representing the predominant class in native human skin (Raza et al. 1991). It was not possible to measure GST activity by adding the substrate to the surface of the model because the conjugation reaction was too fast for this method and the homogenization method made it impossible to measure the absorbance of the metabolite (the reaction was best measured over 3 min). Therefore, CDNB metabolism was measured in S9 prepared from the models 24 h after test chemical dosing. This method ensured the sample did not contain cell debris that would quench UV light and cause interference.

The metabolites of B[a]P are also subject to conjugation by human recombinant UDP-glucuronosyltransferases (UGTs, (Mackenzie et al. 1993)), of which UGT1A6 is reported to be present in the EpiDerm™ donors used in our studies (Hu et al. 2010b). Therefore, basal UGT activities in acetone and test chemical treated EpiDerm™ models were measured using 4-methylumbelliferone as a broad spectrum substrate. As with 7-ethoxyresorufin and testosterone, this substrate was dissolved in acetone and added to the surface of the EpiDerm™ models and the amount of glucuronide metabolite formed was determined by measuring the amount of 4-methylumbelliferone consumed over 6 h of incubation.

These studies were designed to determine basal metabolic enzyme activities over time under the same conditions as those used in the RSMN assay and whether the test chemicals themselves altered the activities. In addition to metabolic activities, the morphology of the EpiDerm™ models was monitored over 72 h. This allowed a comparison of activities with the visual differentiated state of the models with respect to time.

Methods

Materials

The EpiDerm™ Skin Model, EPI-200-MNA-kit (including the phenol red-free New Maintenance Medium (containing keratinocyte growth factor) and Ca^{2+} - and Mg^{2+} -free

Dulbecco's phosphate-buffered saline (CMF-DPBS) was from MatTek (Ashland, MA, USA). The donors used were EPI-200-MNA-D2-254 (donor 254) and EPI-200-MNA-D2-2 1188 (donor 1188). All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (Germany) and were of the highest purity available.

Cryosectioning of EpiDerm™ models

EpiDerm™ models were retained for cryosectioning with hematoxylin-eosin staining at 24, 48 and 72 h after arrival. Briefly, the models embedded in tissue freezing medium (Sakura TissueTek, Torrance, CA, USA) and frozen in a cryostat. The models were then cut into sections (8 μm) and mounted onto microscope slides (Thermo Superfrost, Germany). The sections were fixed in ice-cold acetone, dehydrated and stained with hematoxylin-eosin.

Treatment of EpiDerm™ models

An overview of the treatment of the EpiDerm™ models is shown in Figure 1. The treatment design is based on that described by Dahl et al. (2011), with a few modifications. Unlike Dahl et al. our EpiDerm models arrived on a Wednesday (theirs arrived on a Tuesday) and in our studies, cytochalasin B was not added to the medium. On the day of receipt (Day 0), 1 mL of warmed New Maintenance Medium was transferred into the appropriate wells of 6-well plates. The EpiDerm™ models were then transferred into 6-well plates. The EpiDerm™ models were incubated at $37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ CO_2 in air (standard culture conditions) for 1 h before dosing with 10 μL of the test chemical, vehicle control, or reference compound on to the surface of the models. The term “reference compound” refers to compounds which are known to induce activities in human hepatocytes but not necessarily in skin models. The doses of test compounds were 2 and 50 $\mu\text{g}/\text{cm}^2$ B[a]P and 50 and 1000 $\mu\text{g}/\text{cm}^2$ cyclophosphamide. The top doses were based on those which are known to cause micronuclei in EpiDerm™ models (Aardema et al. 2010; Zhao et al. 1999). The vehicle control, acetone, has been shown not to increase the rate of micronucleus formation in this model (Dahl et al. 2011) and can therefore be considered to be a “true negative” chemical. The models were incubated under standard culture conditions. After 24 (Day 1), 48 (Day 2) and 72 h (Day 3), the medium from each model was collected and stored for lactate dehydrogenase measurement using the CytoTox 96® Non-Radioactive Cytotoxicity Assay from Promega Corp,

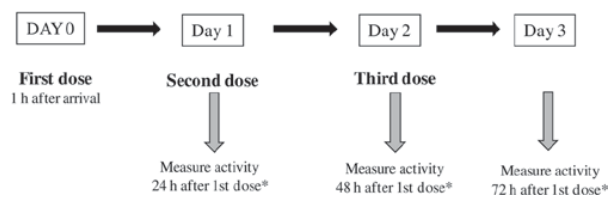


Figure 1. Overview of the dosing of test chemicals and enzyme activity determination.

Madison, WI, USA. The lactate dehydrogenase activity in the medium without models was also determined. On Day 1 and 2, the medium was replaced with fresh warm New Maintenance Medium, and the model was dosed again and incubated under standard culture conditions. Twenty-four hours after each dose of test chemical (on Days 1, 2 and 3), the phase 1 and 2 enzyme activities were measured in 2–3 models per experiment (two experiments per enzyme activity measured). For each activity measured, the medium was refreshed 1 h prior to the addition of the substrate.

EROD activity was measured by applying the substrate, 7-ethoxyresorufin, directly to the epidermal model surface and measuring the metabolite, resorufin, in the tissue and medium. The phase 1 and 2 inhibitors, dicoumarol (which inhibits DT-diaphorase (Nims et al. 1984) and salicylamide (which inhibits sulfate- and glucuronide-conjugation (Bennet et al. 1975; Heath and Dingell, 1974)) did not significantly affect the production of resorufin under the conditions used in these studies (data not shown). Ethoxyresorufin (10 μ L of 2.5 mM solution in acetone) was added to the surface of the models. The models were then incubated for 6 h in standard culture conditions. After this time, the medium and models were separated. The models were homogenized in 200 μ L homogenization buffer (0.25 M potassium phosphate, 0.15 M potassium chloride and 1 mM EDTA pH 7.25) using a Retsch TissueLyser and then centrifuged at 9000 \times g for 5 min at 4°C to remove debris. The fluorescence of a 100 μ L aliquot of the medium, the model S9 fraction and appropriate control samples (to account for background fluorescence from the 7-ethoxyresorufin and medium components) was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The amount of resorufin formed was calculated with respect to a standard curve. The limit of detection (LOD) of resorufin was determined by measuring the fluorescence of known concentrations of resorufin (0.001–0.1 μ M) in homogenization buffer and New Maintenance Medium (both with and without ethoxyresorufin) and determining the concentration at which fluorescence was above background levels. The buffer and medium did not fluoresce at the wavelengths used in this assay.

Testosterone metabolism was measured by applying 10 μ L of 50 mM testosterone (equivalent to a final concentration of 500 μ M when considering the medium and skin volumes) to the surface of the models and incubating for 6 h under standard culture conditions. The concentration of testosterone was in line with that used by others characterizing hepatic models (Brown et al. 2007) and was purposely high to take into account maximum exposure of this substrate to all layers of the EpiDerm™ models. Preliminary results showed that testosterone hydroxides were not significantly conjugated since inclusion of salicylamide in the incubation did not affect the amount of 6 β -hydroxytestosterone detected (data not shown). After 6 h, the medium and models were separated and immediately frozen and stored at –80°C until analysis.

The internal standard, 11 α -hydroxyprogesterone (50 μ L of 5 μ g/mL solution in methanol:water 55:45 (v/v)) was added to 450 μ L of medium. Dichloromethane (4 mL) was added and the sample mixed. The organic phase was transferred to a new tube and evaporated using an Eppendorf concentrator at 30°C for 30 min. The samples were reconstituted in 200 μ L methanol:water 55:45 (v/v). The EpiDerm™ models were homogenized in 500 μ L methanol:water (70:30 (v/v)) and then spiked with 50 μ L 11 α -hydroxyprogesterone. The homogenized samples were centrifuged at 9000 \times g for 10 min and the supernatant was extracted in the same way as for the medium samples. The samples were analyzed by HPLC (Beckman Gold Nouveau System) using EC 250/4 Nucleosil 100-5 C18 column and a CC 8/4 Nucleosil 100-5 C18 guard column (Macherey-Nagel, Germany) maintained at 37°C. The mobile phases were (A) 100% ultrapure HPLC water and (B) 100% MeOH (Carl Roth, Germany). The gradient was as follows: 0–13 min 55% B; 13–20 min 100% B; 20–29 min 55% B. Peak detection was carried out with a Shimadzu SPD UV detector set to 252 nm. The chromatograms were analyzed with the Beckman Gold Nouveau software. Testosterone, androstenedione and hydroxytestosterone metabolites were quantified using synthetic standards.

UGT activity was measured by adding the substrate, 4-methylumbelliferone, directly to the epidermal model surface and measuring the decrease in fluorescence in the skin and medium compared to control incubations (containing the same concentration of substrate in transwells without EpiDerm™ models). 4-Methylumbelliferone (10 μ L of a 10 mM stock solution in acetone) was added to the surface of the models which were then incubated for 6 h in standard culture conditions. After this time, the medium and models were separated. The models were homogenized in 100 μ L methanol:water 70:30 (v/v), using a Retsch TissueLyser and then centrifuged at 9000 \times g for 5 min at 4°C to remove debris. A 10 μ L aliquot of the samples (medium and the model S9 fraction) was added to 190 μ L 10 mM sodium hydroxide and the fluorescence was measured at 390 excitation and 460 nm emission using a microplate spectrofluorimeter. The amount of substrate remaining was calculated with respect to a standard curve of 4-methylumbelliferone. Background fluorescence from the medium was measured and confirmed to have no influence on the measurement of the substrate.

Unlike the other three activities, GST activity was measured by addition of the substrate, CDNB, to tissue S9 fractions. At each time point, the EpiDerm™ models were homogenized in 100 μ L homogenization buffer. The homogenized samples were centrifuged at 9000 \times g and 4°C for 10 min to remove debris. The resultant S9 fraction contained an average of 3.6 \pm 0.4 mg/mL protein. A 20- μ L aliquot was added to 180 μ L of the reaction mixture. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5 (at which pH non-enzymic conjugation is minimal (Habig et al. 1974)), 1 mM CDNB and 1 mM reduced glutathione. The reaction was started by

the addition of CDNB and the production of the conjugate was followed by measuring the rate of absorbance change over 5 min in a plate reader set at 340 nm. Rates were calculated using the extinction coefficient $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (Habig et al. 1974) adjusted to the path length of the solution per well ($5.03 \text{ mM}^{-1} \text{ cm}^{-1}$).

Statistical evaluation

Results were compared using the Student's unpaired *t*-test. $p < 0.05$ was considered significant. "*n*" is the number of models in each experiment.

Results

Morphology of EpiDerm™ models over 72 h

Figure 2 shows the cross section of the EpiDerm™ models at 24, 48 and 72 h in culture. After 24 h, the EpiDerm™ model contained basal, spinous, granular, and cornified layers, reflecting the structure of epidermis *in vivo*. There was notable stratum corneum thickening of the models over time. Apart from the thickness, there was little difference in the structure of the stratum corneum between 24 and 48 h, but by 72 h it was less compact than earlier time points. The basal, spinous, granular cells were still evident at 72 h.

Basal activities in EpiDerm™ models over 72 h

Table 1 summarizes the phase 1 and 2 activities measured in EpiDerm™ models treated with the vehicle control, acetone, over 72 h. These data are the average values from two experiments and up to six models and differences were based on the average value, as well as the reproducibility across the two experiments. EROD

activities at all three time points were considered to be at the limits of detection of the assay design ($0.025 \text{ pmol/min/model}$, considering the background fluorescence of the ethoxyresorufin present in either the EpiDerm™ models and medium, data not shown). The majority (97–90%) of the metabolite, resorufin, was detected in the medium at all incubation time points. The only hydroxy-metabolite, 6β -hydroxytestosterone, was present in all models at each time point measured. The majority (71–75%) of this metabolite was detected in the medium after the 6-h incubation at each time point. When testosterone 6β -hydroxylase activities from all models were averaged, this activity at 24 h was not significantly different from that at 48 or 72 h. Although there was a statistically significant difference ($p < 0.05$) in a single experiment (Figure 3A), between the 24-h and 72-h time points, the actual difference was $2 \text{ pmol/min/mg protein}$, which cannot be considered relevant when compared to the range of activities (thousands of pmol/min/mg) in human hepatocytes (LeCluyse et al. 2000). Androstenedione was also formed by all EpiDerm™ models and, like 6β -hydroxytestosterone, most of this metabolite was detected in the medium (80%). The production of androstenedione was similar in EpiDerm™ models incubated at 24, 48 and 72 h. Unlike the metabolites of testosterone, the parent compound itself was mainly detected in the EpiDerm™ models (66–73% of the total applied). The amount of testosterone remaining in the EpiDerm™ models was the same at all time points (73 ± 11 , 73 ± 15 and $66 \pm 18\%$ in the models at 24, 48 and 72 h, respectively).

The average UGT conjugation of 4-methylumbelliferone in six EpiDerm™ models was maintained over 72 h



Figure 2. Cross-sections of EpiDerm™ models incubated for 24, 48 and 72 h. The pictures represent one (Donor 254) of the two donors analyzed.

Table 1. Phase 1 and 2 activities over 72 h in EpiDerm™ models treated with acetone. The number of models (*n*) tested are shown.

Table 1. Phase 1 and 2 activities over 72 h in EpiDerm® models treated with acetone. The number of models (<i>n</i>) tested are shown.				
Activity	<i>n</i>	Time after first treatment		
		24 h	48 h	72 h
Phase 1				
EROD (pmol/min/model)	6	At LOD	At LOD	At LOD
Testosterone 6β-hydroxylation (pmol/min/model)	6	1.4±0.6	3.0±1.2	2.6±0.5
ASD formation (pmol/min/model)	6	18.2±5.3	19.4±10.3	17.1±7.3
Phase 2				
UGT (pmol/min/model)	6	110.4±12.7	86.2±19.4	97.3±19.8
GST (nmol/min/mg protein)	3–4	84.1±4.1	48.6±4.5	50.4±19.9

LOD, limits of detection ($0.025 \text{ pmol/min/model}$).

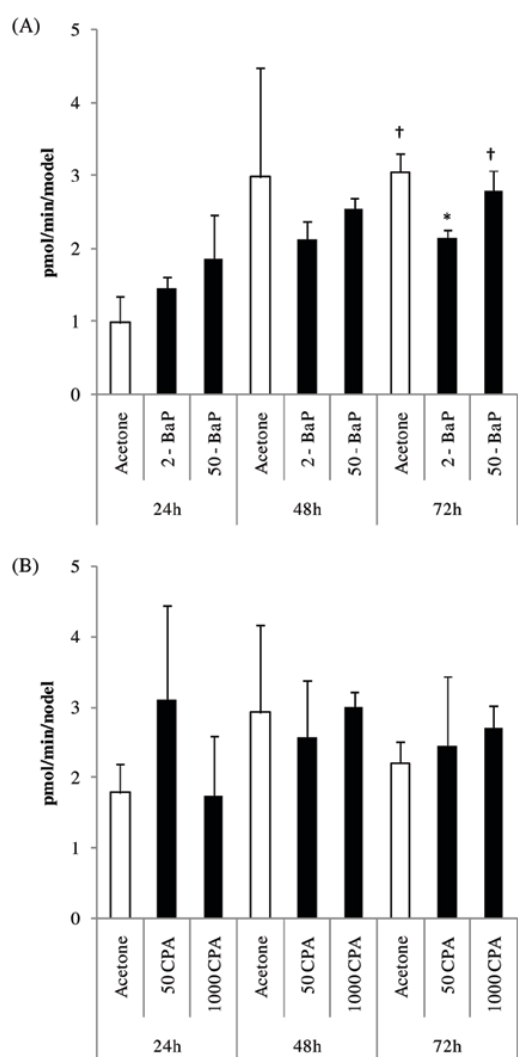


Figure 3. Effect of (A) B[a]P and (B) cyclophosphamide on testosterone 6 β -hydroxylase activities in EpiDerm™ models over 72h. The doses were 2 $\mu\text{g}/\text{cm}^2$ and 50 $\mu\text{g}/\text{cm}^2$ B[a]P (denoted as “2 BaP” and “50 BaP”, respectively) and 50 $\mu\text{g}/\text{cm}^2$ and 1000 $\mu\text{g}/\text{cm}^2$ cyclophosphamide (denoted as “50 CPA” and “1000 CPA”, respectively). Mean \pm SD, * = $p < 0.05$ difference from control at respective time point, † = $p < 0.05$ difference from 24h acetone control, ‡ = $p < 0.05$ difference between 2 $\mu\text{g}/\text{cm}^2$ and 50 $\mu\text{g}/\text{cm}^2$ B[a]P.

and any statistically significant changes within a single experiment were not reproducible (Figure 4A and 4B). As with resorufin and the testosterone metabolites, the majority of the substrate, 4-methylumbelliferone, was detected in the medium (between 80 and 100%). GST activity was highest in models at 24h and by 48h the activity had decreased from 84.1 nmol/min/mg to approximately 50 nmol/min/mg. The GST activity did not decrease further between 48 and 72h.

Effect of B[a]P on phase 1 and 2 activities

The effect of B[a]P on the basal EROD activities is shown in Figure 5A. Both 2 and 50 $\mu\text{g}/\text{cm}^2$ B[a]P caused a significant increase in EROD activity in EpiDerm™ models. The

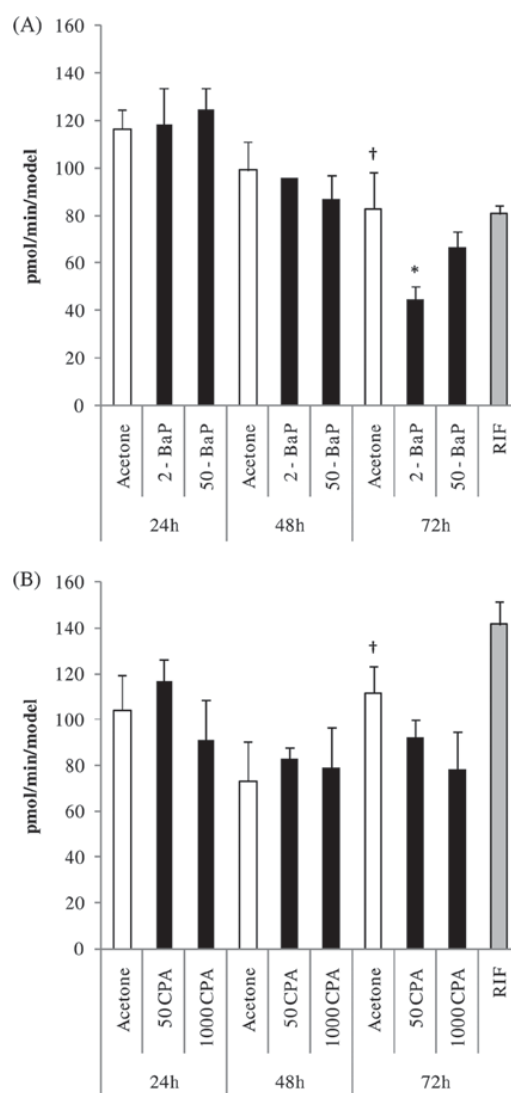


Figure 4. Effect of (A) B[a]P and (B) cyclophosphamide on UDP-glucuronosyltransferase activities in EpiDerm™ models over 72h. The doses were 2 $\mu\text{g}/\text{cm}^2$ and 50 $\mu\text{g}/\text{cm}^2$ B[a]P (denoted as “2 BaP” and “50 BaP”, respectively) and 50 $\mu\text{g}/\text{cm}^2$ and 1000 $\mu\text{g}/\text{cm}^2$ cyclophosphamide (denoted as “50 CPA” and “1000 CPA”, respectively). The reference compound was 25 $\mu\text{g}/\text{cm}^2$ rifampin (RIF) which was dosed in the same way as B[a]P and cyclophosphamide, but UDP-glucuronosyltransferase activities were only measured after 72h. Mean \pm SD, * = $p < 0.05$ difference from control at respective time point, † = $p < 0.05$ difference from 24h acetone control.

effect was evident after 24h and was still present after 72h. The increase in EROD activity was not dose-dependent, for example, after 24-h treatment with 2 and 50 $\mu\text{g}/\text{cm}^2$ B[a]P, EROD activity increased from approximately 0.02–0.12 \pm 0.06 and 0.16 \pm 0.02 pmol/min/model, respectively. After 48h, EROD activities in EpiDerm™ models treated with 2 and 50 $\mu\text{g}/\text{cm}^2$ B[a]P were marginally higher than at 24h (0.18 \pm 0.01 and 0.19 \pm 0.04 pmol/min/model, respectively, Figure 1A). After 72h, the increase in EROD activity above acetone treated controls was diminished, whereby the activity in 2 $\mu\text{g}/\text{cm}^2$ treated models was only

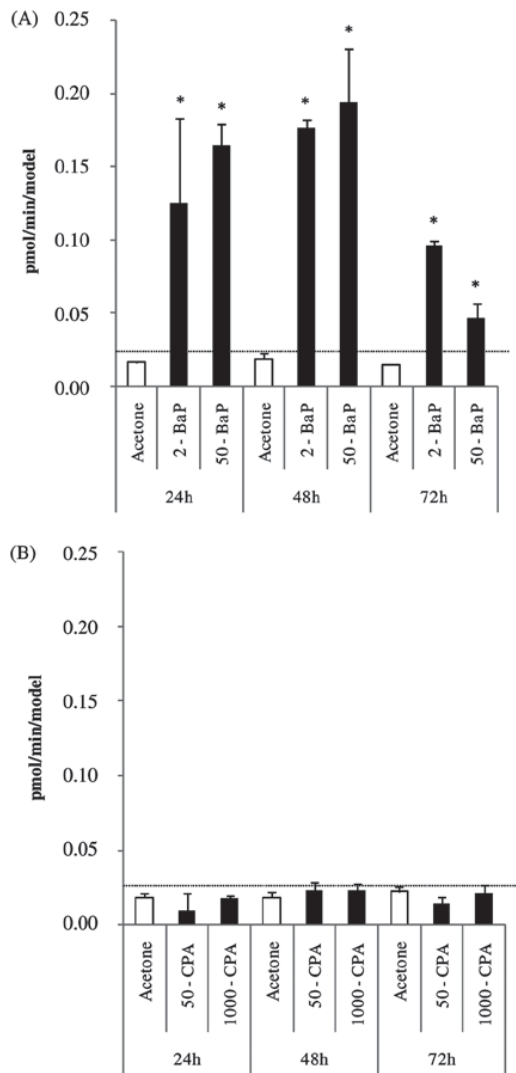


Figure 5. Effect of (A) B[a]P and (B) cyclophosphamide on ethoxyresorufin O-deethylase activities in EpiDerm™ models over 72 h. The doses were 2 µg/cm² and 50 µg/cm² B[a]P (denoted as “2 BaP” and “50 BaP” respectively) and 50 µg/cm² and 1000 µg/cm² cyclophosphamide (denoted as “50 CPA” and “1000 CPA”, respectively). Mean ± SD. * denotes a statistical significance of <0.05. The dotted line represents the LOD.

0.10 ± 0.00 pmol/min/model. EROD activity in 50 µg/cm² B[a]P treated models was lower than those treated with the lower B[a]P dose; however, this was not due to cytotoxicity of B[a]P. The lactate dehydrogenase release into the medium in models treated with both doses of B[a]P was the same as vehicle control models at each time point (Table 2).

There were no significant effects of 2 and 50 µg/cm² B[a]P on testosterone 6β-hydroxylase activities in EpiDerm™ models at any of the three time points measured (Figure 3A). The activities remained between 1.0 and 3.1 pmol/min/model. By contrast, the production of androstenedione was statistically increased by 50 µg/cm² B[a]P after 24 h of treatment (Figure 6A). This effect was

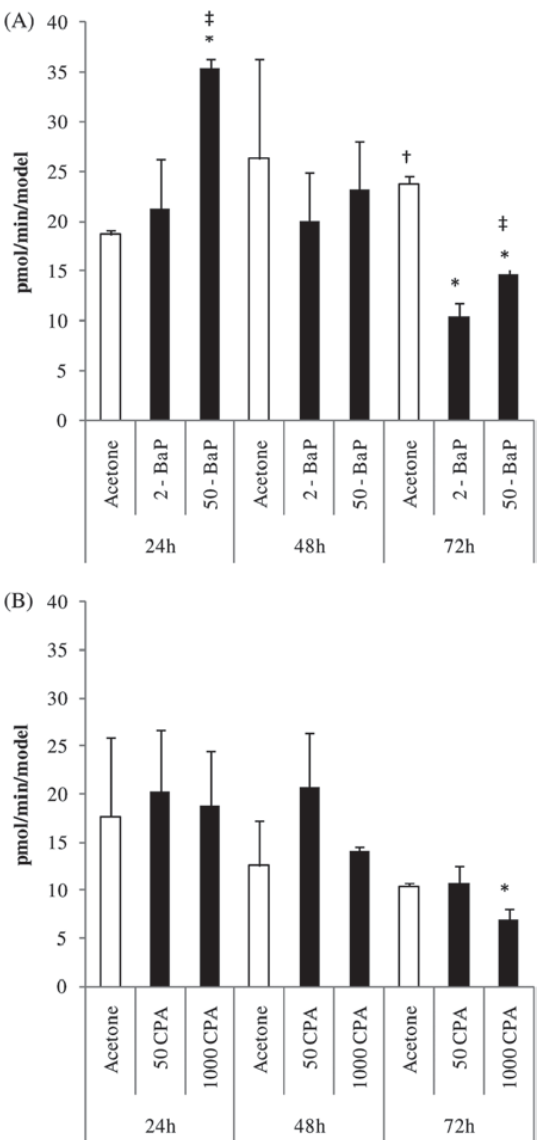


Figure 6. Effect of (A) B[a]P and (B) cyclophosphamide on androstenedione formation in EpiDerm™ models over 72 h. The doses were 2 µg/cm² and 50 µg/cm² B[a]P (denoted as “2 BaP” and “50 BaP” respectively) and 50 µg/cm² and 1000 µg/cm² cyclophosphamide (denoted as “50 CPA” and “1000 CPA”, respectively). Mean ± SD. * = *p* < 0.05 difference from control at respective time point, † = *p* < 0.05 difference from 24 h acetone control, ‡ = *p* < 0.05 difference between 2 µg/cm² and 50 µg/cm² B[a]P.

Table 2. Effect of (A) B[a]P and (B) cyclophosphamide on LDH release from EpiDerm™ models over 72 h. The viability was expressed as the % LDH released from treated models compared to vehicle control. The amount of LDH in the medium from vehicle control samples compared to medium without EpiDerm™ models was 164 ± 55, 122 ± 26 and 116 ± 20% at 24, 48 and 72 h, respectively.

Time (h)	B[a]P		Cyclophosphamide	
	2 µg/cm ²	50 µg/cm ²	50 µg/cm ²	1000 µg/cm ²
24	103 ± 5	98 ± 19	93 ± 1	105 ± 2
48	102 ± 3	102 ± 3	104 ± 2	109 ± 0
72	108 ± 2	114 ± 3	118 ± 8	110 ± 0

not evident after 48 h of treatment and by 72 h, both 2 and 50 $\mu\text{g}/\text{cm}^2$ B[a]P caused the production of androstenedione to significantly decrease compared to the acetone treated models (the production of androstenedione was 23.7 ± 0.9 pmol/min/model in acetone treated models compared to 10.4 ± 1.4 and 14.6 ± 0.7 pmol/min/model in 2 and 50 $\mu\text{g}/\text{cm}^2$ B[a]P treated models, respectively).

UGT activity was not significantly affected by 2 and 50 $\mu\text{g}/\text{cm}^2$ B[a]P at any time point tested (Figure 4). An additional reference compound was included to determine whether UGT activity could be induced by a known hepatic UGT inducer under the conditions applied in these studies. Rifampin, at a dose of 25 $\mu\text{g}/\text{cm}^2$ (equivalent to 20 μM if it was added to the medium) did not significantly alter UGT activities after 72-h treatment (80.7 ± 3.4 pmol/min/model in rifampin treated EpiDerm™ models compared to 87.7 ± 6.6 pmol/min/model in acetone treated EpiDerm™ models).

GST activities in EpiDerm™ models treated with 2 $\mu\text{g}/\text{cm}^2$ B[a]P were equivalent to those in the acetone control models (Figure 7A). GST activity was marginally higher in EpiDerm™ models treated with 50 $\mu\text{g}/\text{cm}^2$ B[a]P after 48 h (63.1 nmol/min/mg compared to acetone treated models with 48.6 nmol/min/mg), but it was not possible to determine the statistical significance because only two models were analyzed for this treatment. Additional EpiDerm™ models were treated with 5 $\mu\text{g}/\text{cm}^2$ β -naphthoflavone (equivalent to 50 μM if it was added to the medium) for 72 h, in which CDBN metabolism in duplicate models was increased 1.6-fold above the duplicate acetone control-treated models (from 36.2 to 57.5 nmol/min/mg).

Effect of cyclophosphamide on phase 1 and 2 activities

EROD activities in EpiDerm™ models treated with 50 and 1000 $\mu\text{g}/\text{cm}^2$ cyclophosphamide were equivalent to the corresponding acetone control models at each time point measured (Figure 5B), which did not exceed the limits of detection of the method. Likewise, these doses of cyclophosphamide had no effect on testosterone 6 β -hydroxylase activities at any of the time points measured and were between 1.8 and 3.0 pmol/h/model (Figure 3B). At 1000 $\mu\text{g}/\text{cm}^2$, cyclophosphamide did significantly decrease androstenedione production compared to the acetone treated models but this was evident at 72 h only (Figure 6B). UGT activities were not significantly affected by 50 and 1000 $\mu\text{g}/\text{cm}^2$ cyclophosphamide or 25 $\mu\text{g}/\text{cm}^2$ rifampin, at any time point tested (Figure 4B). UGT activities remained between 78.3 and 117.2 pmol/min/model. There was no dose-dependent effect of cyclophosphamide on GST activities (Figure 7B). There was a decrease in activity at 72 h in models treated with 1000 $\mu\text{g}/\text{cm}^2$ cyclophosphamide compared to acetone control models at the same time point, but it was not possible to determine the statistical significance because only two models were analyzed for this treatment. The decrease in GST activities measured in acetone treated EpiDerm™ models was also observed in cyclophosphamide treated models (Figure 7B).

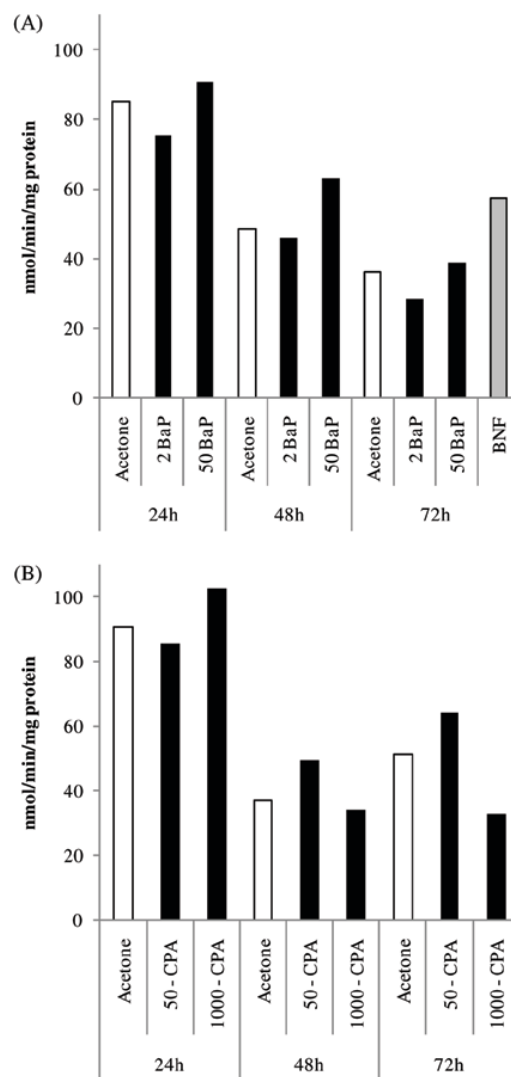


Figure 7. Effect of (A) B[a]P and (B) cyclophosphamide on glutathione S-transferase activities in EpiDerm™ models over 72 h. The doses were 2 $\mu\text{g}/\text{cm}^2$ and 50 $\mu\text{g}/\text{cm}^2$ B[a]P (denoted as “2 BaP” and “50 Bap” respectively) and 50 $\mu\text{g}/\text{cm}^2$ and 1000 $\mu\text{g}/\text{cm}^2$ cyclophosphamide (denoted as “50 CPA” and “1000 CPA”). The reference compound was 5 $\mu\text{g}/\text{cm}^2$ β -naphthoflavone (BNF) which was dosed in the same way as B[a]P and cyclophosphamide, but GST activities were only measured after 72 h. Mean of two models per treatment and time point.

Discussion

These studies were designed to determine a number of basal metabolic enzyme activities under the same conditions as those used in the RSMN assay. The reason for this was that we wanted to investigate whether (i) selected activities were present in the EpiDerm™ models during the course of a 72 h RSMN assay, (ii) they changed with time and (iii) the test chemicals themselves altered the activities. At least for UGT and GST activities, it was also possible to measure any decreases since the basal levels were well above the limits of quantification. Phase 2

conjugation pathways were clearly measureable in all models at each time point measured. Although there was some decrease in GST activities in acetone-treated models between 24 to 48 h, this drop did not continue between 48 to 72 h. The presence of GST activity is in agreement with mRNA expression data from the literature: GST Pi is the predominantly expressed GST in native human skin (Luu-The et al. 2009) and is also reported to be expressed in the EpiDerm™ models used in our studies (Hu et al. 2010). In addition, we have also measured marked levels of GST activities in native human skin and EpiDerm model cytosol (data not shown). UGT activities remained constant throughout the 72-h experiment. Although UGTs are expressed at low levels in human skin, two isoforms are expressed in the EpiDerm™ models used here, namely UGT1A6 and UGT1A8 (Hu et al. 2010). As with GSTs, we have also measured marked levels of UGT activities in native human skin and EpiDerm model microsomes (data not shown). In contrast to phase 2 activities, basal phase 1 functionalizing activities were at the limit of detection throughout the incubation. We also investigated EROD activities in native human skin and EpiDerm model microsomes, but the levels were below detection limits (data not shown). These findings are in agreement with others (Hu et al. 2010b) who found the overall expression of phase 2 conjugation enzymes to be more pronounced than that of phase 1 enzymes in the EpiDerm™ model (as well as in native human skin). The predominance of phase 2 conjugation over phase 1 functionalizing pathways supports the theory that skin acts as a barrier to toxins in terms of metabolic as well as a physical barrier. This is of significance for certain groups of cosmetic ingredients such as aromatic amines, which may be directly N-acetylated, rather than being hydroxylated to potentially genotoxic moieties (Skare et al. 2010; Goebel et al. 2009).

Although there was little change in the basal enzyme activities measured, there was some change in the morphology of the models. Over time, the stratum corneum increased in thickness. Despite this increase in the lipophilic nature of the upper layers of the models, the ratio of substrates and/or metabolites in the model and medium was unaltered. This is applicable to testosterone, which may have been expected to form a reservoir in the stratum corneum due to its highly lipophilic nature (van de Sandt et al. 2004). However, the amount of testosterone remaining in the EpiDerm™ models was the same at all time points. This may also have been true for B[a]P which is also highly lipophilic, but the amount of this test compound was not determined in these studies.

We have shown that B[a]P, at doses which are known to induce micronuclei in the RSMN assay (data not shown), markedly induced EROD activity after 24 h. The induced levels were highest at 48 h, after which, they decreased during the last 24 h of the assay. The induction we observed can be expected because B[a]P is known to bind with high specificity to the aryl hydrocarbon receptor, which in turn modifies human CYP1A1/2

and CYP1B1 expression (Li et al. 1998). Others have also shown significant induction of aryl hydrocarbon hydroxylase, ethoxycoumarin O-deethylase, and/or EROD in rat skin after dermal application of a number of chemicals including polycyclic aromatic hydrocarbons and 3-methylcholanthrene (Khan et al. 1989; Raza and Mukhtar, 1993). Topical application of B[a]P to rats has been shown to induce not only skin enzymes but also enzymes in other organs, although induction in the latter is lower than in the skin (Mukhtar and Bickers, 1981). The induction of the expression of CYP1A1 and aryl hydrocarbon receptor in human ex vivo skin after application of B[a]P to the epidermis has also been demonstrated (Costa et al. 2010). In their studies, the skin was placed epidermal side down in a 6-well plate containing 15 µL B[a]P but no medium for 18 h. The dose of B[a]P used was much lower (19 ng/cm²) than used here and it was shown to cause significant loss of viability and lipid peroxidation. However, there were measureable amounts of basal CYP1A1 and aryl hydrocarbon receptor proteins (using western blotting) which were both increased by B[a]P (by approximately 10-fold) under these conditions. The doses of B[a]P used in our studies were not toxic to the models at any time point, according to the marker of viability, lactate dehydrogenase release, which was used in our studies. The EpiDerm™ models may be more robust than ex vivo skin discs since they are continually growing in the presence of specialized medium; whereas the ex vivo model lacked this form of functional maintenance. As a result of the continuing growth, the structure of the EpiDerm™ models changed over time, such that more stratum corneum was present by 72 h, possibly causing the B[a]P-induced EROD activities in the upper layers to be lower than at earlier times. Despite this decrease, it was still possible to measure induction of EROD activity over the entire RSMN assay duration. The induction of EROD activity after 24 h in EpiDerm™ models was also demonstrated by Curren et al. (2007) who treated them by adding 10 µM 3-methylcholanthrene to the medium. EROD activity was measured by incubating the models with 2.5 µM ethoxyresorufin and 33.3 µM dicoumarol for 30 min at 37°C. The lack of effect of cyclophosphamide on EROD activities over time was therefore not due to a general unresponsiveness of the models to aryl hydrocarbon receptor mediated inducers – or due to cytotoxicity, since no increase of lactate dehydrogenase release was observed at either cyclophosphamide dose. It is important to demonstrate that a model exhibits induction responses similar to native skin since investigations in to the modulation of xenobiotic metabolizing enzymes will enhance our knowledge of not just genotoxicity but also general skin pathologies (Ahmad and Mukhtar, 2004).

Cyclophosphamide has been reported to induce a number of CYP activities in primary human hepatocytes, including CYP3A4, CYP2C8, CYP2C9 and CYP2B6 levels (Chang et al. 1997; Gervot et al. 1999; Lindley et al. 2002). We therefore used testosterone as a substrate in our studies since it is metabolized to a

number of metabolites via CYP-selective pathways. In humans, CYP2B6 catalyses the formation of 16 α -hydroxytestosterone, CYP3A3/4/5 catalyses the formation of 2 β - and 6 β -hydroxytestosterone and CYP2C9/19 catalyses the formation of androstenedione (Yamazaki and Shimada, 1997; <http://www.icgeb.org/~p450srv/>). In native human skin, which generally lacks CYP2C enzymes (Luu-The et al. 2009), androstenedione is most likely to be produced from the interconversion of testosterone and androstenedione catalyzed by 17 β -hydroxysteroid dehydrogenase 2, which is distributed around the basement layer of the epidermis (Hikima and Maibach, 2007). The kinetics of this enzyme have been investigated in cultured human keratinocytes (Milewich et al. 1986). Although we have focused on CYP activities here, it should be kept in mind that the skin plays a crucial role in steroid metabolism, which has been discussed in detail elsewhere (Chuong et al. 2002; Slominski et al. 2002 and 2004). The only metabolites detected in the EpiDerm™ models using non-radiolabelled testosterone were 6 β -hydroxytestosterone and androstenedione. Neither cyclophosphamide nor B[a]P altered the production rate of 6 β -hydroxytestosterone, suggesting that CYP3A4/5 is not affected by these test compounds during the course of the RSMN assay. Human CYP3A4 is responsible for the metabolism of over 50% of marketed drugs (Zuber et al. 2002) and is therefore an important CYP to investigate; therefore, future studies should include treatment of the EpiDerm™ models with known and FDA recommended human hepatic CYP3A4 inducers, such as rifampin (Huang and Stifano, 2006) to determine whether this CYP is also inducible in skin models, especially if the 72 h dosing regimen is employed in the RSMN assay. The production of androstenedione was significantly increased by the highest dose of cyclophosphamide after 24 h but not at later times. This may reflect an induction of CYP2C9/19 and/or 17 β -hydroxysteroid dehydrogenase, the levels of which decrease over time due to the increase in the number of corneocytes in the models. Further studies are needed to confirm the reproducibility of this effect and CYP selective inhibitors could be included to identify which CYPs, if any, are involved in the production of androstenedione.

GSTs play a role in metabolism of both B[a]P and cyclophosphamide; therefore, if GSTs are modulated by these test chemicals, their genotoxic effects may also be modified. The π -class is reported to be the predominant isoform in native human skin (Zhang et al. 2002) and is almost exclusively involved in the detoxification of the ultimate B[a]P metabolite, (+)-anti-benzo[a]pyrene 7,8-dihydrodiol 9,10-epoxide ((+)-anti-BPDE). Since EROD activity was significantly induced by B[a]P under the conditions used here, we investigated whether GSTs could also be induced. The reference control chemical, β -naphthoflavone, increased GST activity by 1.6-fold, the

magnitude of which may be expected since the extent of induction of this enzyme in human liver slices is known to be low compared to the CYP1 family (Pushparajah et al. 2008b). Likewise, B[a]P increased GST activities but only at 48 h and by 1.3-fold (the statistical significance of this result could not be made due to duplicates being taken for most of the samples). Cyclophosphamide also caused a 1.3-fold increase in GST activity after 72 h at the lower dose tested. Future studies will include the use of an isoform-selective substrate in order to elucidate whether the lower induction of GST was due to the non-specificity of CDNB. The aims of these studies were to determine whether selected phase 1 or 2 activities could be measured and/or altered during the course of the RSMN assay. B[a]P and cyclophosphamide were used as examples of chemicals which are bioactivated in the skin and caused significant micronuclei formation in EpiDerm™ cells under the conditions used (data not shown) i.e. a local effect. This shows that this model has the necessary metabolic capacity to bioactivate these chemicals and does not require additional metabolic supplements (such as S9 and NADPH regenerating system). Other dermal models may need additional S9-mix in the medium in order for this chemical to cause a genotoxic effect (Flamand et al. 2006). However, it may be that the skin does not take part in the bioactivation of a genotoxicant and that the site of bioactivation is systemic (e.g. via hepatic enzymes). In this case, the absorption of the chemical through the skin into the systemic circulation will affect the genotoxic potential of the chemical and the contribution of dermal metabolism will be negligible (Flamand et al. 2006). Conversely, some dermally applied chemicals may be extensively detoxified by the skin before entering the circulation (e.g. hair dyes by human N-acetyltransferase 1 (Goebel et al. 2009)); therefore, the presence of enzymes which may be involved in their detoxification (GSTs, UGTs, N-acetyltransferase 1 etc.) in the EpiDerm™ model is important to establish. In conclusion, we have demonstrated the presence of basal phase 1 and 2 activities of EpiDerm™ models incubated according to the RSMN assay. With the exception of GST, which decreased between 24 and 48 h, all of the activities measured did not change over time. It was possible to measure enzyme induction using this assay design. Of the enzymes tested, EROD activity was significantly induced by B[a]P but not by cyclophosphamide. Cyclophosphamide and B[a]P had little or no reproducible effects on GST and UGT activities. Since a number of metabolic enzyme activities are present and at least the CYP1 family is inducible in the EpiDerm™ skin model, the RSMN assay may not require an exogenous metabolic activation system for compounds that are activated or detoxified via these pathways. Further studies investigating the effect of inclusion of cofactor regenerating systems will help to elucidate the contribution of dermal metabolic enzymes to the genotoxicity of selected positive control chemicals.

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Declaration of interest

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