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# Evaluation of chemicals requiring metabolic activation in the EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay

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## ABSTRACT

The *in vitro* human reconstructed skin micronucleus (RSMN) assay in EpiDerm™ is a promising new assay for evaluating genotoxicity of dermally applied chemicals. A global pre-validation project sponsored by the European Cosmetics Association (Cosmetics Europe – formerly known as COLIPA), and the European Center for Validation of Alternative Methods (ECVAM), is underway. Results to date demonstrate international inter-laboratory and inter-experimental reproducibility of the assay for chemicals that do not require metabolism [Aardema et al., *Mutat. Res.* 701 (2010) 123–131]. We have expanded these studies to investigate chemicals that do require metabolic activation: 4-nitroquinoline-*N*-oxide (4NQO), cyclophosphamide (CP), dimethylbenzanthracene (DMBA), dimethylnitrosamine (DMN), dibenzanthracene (DBA) and benzo(a)pyrene (BaP). In this study, the standard protocol of two applications over 48 h was compared with an extended protocol involving three applications over 72 h. Extending the treatment period to 72 h changed the result significantly only for 4NQO, which was negative in the standard 48 h dosing regimen, but positive with the 72 h treatment. DMBA and CP were positive in the standard 48 h assay (CP induced a more reproducible response with the 72 h treatment) and BaP gave mixed results; DBA and DMN were negative in both the 48 h and the 72 h dosing regimens. While further work with chemicals that require metabolism is needed, it appears that the RSMN assay detects some chemicals that require metabolic activation (4 out of 6 chemicals were positive in one or both protocols). At this point in time, for general testing, the use of a longer treatment period in situations where the standard 48 h treatment is negative or questionable is recommended.

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## 1. Introduction

As a result of the 7th Amendment of the Cosmetics Directive [13], cosmetic ingredients can no longer be tested for genotoxicity in *in vivo* assays. Without the ability to conduct *in vivo* assays, the assessment of genotoxicity must rely solely on *in vitro* tests; however, these have a high rate of positive results that do not correlate with *in vivo* genotoxicity or carcinogenicity [29,30]. Reliance solely on *in vitro* assays would result in abandoning many safe new products. The Scientific Committee on Consumer Safety (SCCS) is the authoritative body that provides scientific evaluations of cosmetic ingredients for the European Commission. As described in

an SCCS position statement on genotoxicity testing [51], in the absence of *in vivo* assays “At present no validated replacement methods are available that allow the follow-up of positive results from standard *in vitro* assays without further animal experiments. Consequently, in many cases, it will not be possible to evaluate the mutagenic potential of cosmetic ingredients on a sound scientific basis. Because the potential genotoxicity of these ingredients is of major concern, an important part of the toxicological evaluation of cosmetic ingredients cannot be accomplished.” A recent review discusses the extent of this problem showing an analysis of the large number of hair dyes that would have been unable to progress to market without *in vivo* data [2].

It is important to note that the high rate of misleading positive results in current *in vitro* genotoxicity assays, along with the global interest in reducing/eliminating animal use, not only impacts cosmetic ingredients. This issue complicates large-scale chemical evaluation program, such as Registration, Evaluation,

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Authorisation and Restriction of Chemicals (REACH [14]) that require use of *in vitro* assays when they are available, such as for genotoxicity testing. Misleading positive data from *in vitro* assays result in the conduct of costly and time-consuming *in vivo* studies, which may be impractical for some registrants.

One approach to address these issues is to develop improved *in vitro* genotoxicity assays. To this end, we have been working on the development of a micronucleus (MN) assay in the 3D human reconstructed EpiDerm™ skin model (RSMN) [9,37,23,10]. Reconstructed skin (RS) models are prepared from primary human keratinocytes and have the advantage that they are not immortalised cells and, therefore, may be expected to have normal DNA repair and cell-cycle control. The structure of RS models mimics that of native skin [45]. The *stratum corneum* is an effective barrier to chemicals [46] and the deeper layers of the skin (especially the epidermis) are metabolically active [42] such that compounds applied to the skin may not enter the systemic circulation, or may be detoxified prior to systemic circulation. Since the skin is the first site of contact with maximum exposure to many different products, including cosmetics, assays that use RS models offer the potential for a more physiologically relevant approach to test dermal exposures compared with *in vitro* genotoxicity assays. Indeed, the use of RS models for genotoxicity assessment of dermally applied cosmetics was recently described [44].

Another approach to testing effects of dermal exposures is to use *ex vivo* human skin for the assessment of genotoxicity. Recently, induction of DNA damage measured by use of the Comet assay was reported for a variety of chemicals, with weak, but positive results for BaP and DMBA [49]. Previously, Reus et al. [48] had reported induction of micronuclei by UV and 8-methoxypsoralen in *ex vivo* human skin as an approach to photo-genotoxicity testing. There are no data at this time for the chemicals studied in the RSMN assay in EpiDerm™ to compare with the induction of micronuclei in *ex vivo* human skin. While studies in *ex vivo* human skin are important, there are difficulties with this approach for general testing, including limitations in laboratories being able to obtain fresh human skin for immediate use, source-dependent variability, variability in tissue handling and tissue quality that can affect the level of xenobiotic metabolising enzymes and responses in biological assays.

Many chemicals require oxidative metabolism in order to become genotoxic. Traditional *in vitro* genotoxicity assays include induced rat-liver S9 fraction to study the effect of metabolism on the outcome of the test. However, induced rat liver S9 is not ideal for assessing human dermal exposures because (a) it does not reflect the route of exposure, (b) there is an over-representation of CYP1A and CYP2B enzymes (the activity of which is maximised by the addition of high concentrations of co-factors), (c) there is a lack of comparable induction of normal detoxification components, and (d) there are potential species differences in metabolism. Therefore, to complement the genotoxicity project, we have investigated the metabolism of Phase I [21] and II enzyme [20] substrates in RS models and confirmed that they exhibit a metabolic capability similar to normal human skin, which is in agreement with the findings of others [31,23,24,39]. Phase I activities in microsomes from reconstructed epidermis were found to be very similar to those in human skin microsomes. EpiDerm™ models and native skin exhibited much higher and measurable Phase II activities and relatively little or absent Phase I activities (with the exception of cyclooxygenase), a finding that is in line with other reports [42]. Moreover, 3D epidermal models exhibited a xenobiotic metabolism that was more similar to that of human skin than to the metabolism in mono-layers of skin cell-lines, especially with respect to Phase I activities such as cyclooxygenase. Based on an analysis of Phase I (cytochrome P450 (CYPs), cyclooxygenase enzymes) and Phase II enzymes (*N*-acetyl transferases, UDP-glucuronosyl-transferases and glutathione *S*-transferases [20]), these investigations

concluded that EpiDerm™ tissue models may represent a more suitable model for dermal exposures than primary and keratinocyte cell-lines since they maintain Phase I native-like metabolic competence and are superior over cell lines with respect to metabolic capacity. Moreover, we have shown that a number of Phase I and II activities are present in the EpiDerm™ donors used in the RSMN assays in our studies [20], and that at least CYP1A/1B activities are inducible under these incubation conditions.

Since RS models exhibit a metabolic capability similar to that in normal human skin, and because we wanted to avoid issues with metabolism that are unspecific for the route of exposure, exogenous metabolic activation has not been used in the EpiDerm™ RSMN assay. It is thus important to determine if the RSMN assay correctly detects chemicals that require metabolism. Initial results from the RSMN assay with metabolically activated chemicals are promising but indicate that a longer duration of exposure may be needed, as reported recently by Kaluzhny et al. [27]. In this paper, we describe RSMN assay results for chemicals that require metabolic activation, namely, 4-nitroquinoline-*N*-oxide (4NQO), cyclophosphamide (CP), dimethylbenzanthracene (DMBA), dimethylnitrosamine (DMN), dibenzanthracene (DBA) and benz(*a*)pyrene (BaP). Comparative studies were performed in two laboratories, The Procter & Gamble Company (P&G) and the Institute for In Vitro Sciences, Inc. (IIVS) as part of a larger Cosmetics Europe study (trade association for the cosmetic industry, formerly known as COLIPA).

## 2. Materials and methods

The RSMN assay was conducted according to the protocol described in detail by Dahl et al. [10] with the exceptions noted below. The protocol was based on the principles in the OECD guideline 487 for the *in vitro* MN assay [41]. Acetone was selected as the solvent since it is commonly used in dermal carcinogenicity studies with rodents and this solvent has been used in previously published RSMN studies [9,37,23,10].

### 2.1. Chemicals

All the chemicals tested (Table 1) were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Acros Organics (Morris Plains, NJ, USA). Other reagents such as acetone, ethanol, cytochalasin B (cytoB), trypsin, acridine orange, etc. were obtained as previously reported [10]. Chemicals with limited solubility were tested up to the lowest precipitating concentration, in keeping with the OECD guideline for the *in vitro* MN assay [41].

### 2.2. Model constructs and transport

EpiDerm™ EPI-200-MNA kits and new maintenance medium (NMM) were obtained from MatTek Corporation (Ashland, MA, USA) and shipped overnight. Upon receipt, the models were transferred to 6-well plates containing 1 ml NMM at  $5 \pm 1\%$  CO<sub>2</sub> and  $37 \pm 1^\circ\text{C}$ , and allowed to recover during approximately 1 h for the 72 h treatment regimen, or 24 h for the standard 48 h treatment regimen.

### 2.3. RSMN assay

After the 1 h or 24 h recovery period, the models were re-fed with 1 ml of fresh NMM containing 3  $\mu\text{g}/\text{ml}$  cytoB (unless otherwise stated). The models were re-fed every 24 h. Test chemicals were dissolved in acetone and dosing solutions were prepared fresh each day. A dosing volume of 10  $\mu\text{l}$  was applied to the centre of the surface of each EpiDerm™ model. For the 48 h dosing regimen, the first dose of the test chemical was applied 24 h after the tissues were received and the second dose 24 h after the first dose. For the 72 h dosing regimen, the first dose was applied 1 h after the tissues were received and fresh media added, and the following doses were given 24 h and 48 h after the first dose. At the end of the treatment period, cells were harvested from the EpiDerm™ models and mounted onto slides using standard procedures described previously [10]. Briefly, the models were first incubated in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Dulbecco's phosphate-buffered saline at room temperature for 5–15 min, followed by a 15 min incubation in EDTA (0.1%, 1 g/L) at room temperature. After removal of the EDTA, the models were trypsinized in warm ( $\sim 37^\circ\text{C}$ ) trypsin–EDTA solution for 10–15 min at  $37^\circ\text{C}$ . The action of the enzyme was stopped by the addition of DMEM with 10% FBS and the models were gently agitated to release the cells. A few studies were also conducted with a 3-dose, 72 h harvest with cytoB present during the last 48 h of the protocol.

A third dosing regimen was tested by IIVS, whereby the EpiDerm™ models were treated with a single dose of test compound in the presence of cytoB, starting

**Table 1**  
List of compounds tested.

Compound CAS <sup>#</sup>	Metabolising enzyme(s) involved in bioactivation and expression in EpiDerm donor 254 <sup>a</sup>	Major genotoxic metabolite(s)	Enzymes involved in deactivation of metabolite and expression in EpiDerm donor 254 <sup>a</sup>
Mitomycin C (MMC) 50-07-7	Direct-acting genotoxin	NA	NA
7,12-Dimethylbenzanthracene (DMBA) 57-97-6	CYP1A1 – mRNA absent CYP1B1 – mRNA present [7] EROD activity (via CYP1A1/1B1) is inducible in EpiDerm <sup>TM</sup> models over 24–72 h [20,21]	3,4-diol-1,2-epoxides	GST – present [22]
Cyclophosphamide (CP) 6055-19-2	CYP2B6 – absent CYP2C8 – absent CYP2C9 – present [18,8]	4-HydroxyCP (intermediate)  Acrolein  Phosphoramidate mustard	4-HydroxyCP: GSTA1 – present GSTA2 – NM GSTM1 – present GSTP1 – present [12,21,22] Acrolein: GST P1 – present [5] Phosphoramidate mustard: GST1A1 – present [12]
4-Nitroquinoline N-oxide (4NQO) 56-57-5	NQO1 – present and inducible after 24 h [34,17]	4-hydroxylaminoquinoline N-oxide [60]	GSTP1-1 – present [36]
Benzo[a]pyrene (BaP) 50-32-8	CYP1A1 – absent but inducible over 24 h by BaP [42] CYP1B1 – present and inducible over 24 h by BaP [42]  EROD activity (via CYP1A1/1B1) is inducible in EpiDerm <sup>TM</sup> models over 24–72 h [22]  Microsomal epoxide hydrolase (mEH) – present	BP-epoxides and diol epoxides  7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-BP, (diol epoxide-2 (DE2) the ultimate genotoxin) [52]	GSTA1 – present [47] UGT2B1 [32] – NM UGT2B2 [32] – NM UGT1*06 [32] – present
1,2:5,6 Dibenanthracene (DBA) 53-70-3	CYP1A1 – absent  EROD activity (via CYP1A1/1B1) is inducible in EpiDerm <sup>TM</sup> models over 72–96 h [20]	5,6-Epoxy-5,6-dihydrodibenanthracene	GSTA1 [47,6] present
Dimethylnitrosamine (DMN) 62-75-9	CYP2E1 – absent [3] Cytochrome P450 reductase – present Cytochrome b5 – NM	DMN-hydroxide Diazonium ion	UGTs – present [35]

NA = not applicable; NM = not measured.

<sup>a</sup> The presence or absence of the expression of xenobiotic metabolising enzymes was according to Hu et al. [24] as well as others that are specified.

on Tuesday when the tissues were received, and then harvested after 72 h, on the Friday. This “single dose, 72 h” regimen was tested to evaluate the possibility of conducting the assay over a weekend since this might be more amenable to laboratories particularly in Europe and other regions where the tissue shipment typically does not arrive until late in the day on Tuesday, making the 72 h harvest late in the day on Friday impractical. In this scenario, tissues would be shipped on a Wednesday from the US, arrive in the laboratory late Thursday evening, dosing would occur on Friday, and the cells would be harvested 72 h later on the following Monday. This single dose, 72 h regimen was conducted in two different ways: (1) by refreshing the media on a daily basis with the standard model set-up or (2) without refreshing the media, in which case a platform (MatTek Corporation, part number EPI-WSHR) was placed in the well and the model was placed on top of the platform being careful to prevent air bubble formation under the model. Five ml of NMM medium containing 3 µg/ml cytoB was added to the well to support growth over the 72 h timeframe. A single dose, 72 h study was conducted with mitomycin C (MMC) and CP.

#### 2.4. Cytotoxicity and MN assessment

The percentage of bi-nucleated (BN) cells, i.e. the frequency of BN cells in 500 cells containing one, two, or more nuclei, was evaluated for each model. The relative % BN was calculated by comparing the % BN cells for each model with the average % BN of the solvent control. The measurement of % BN was used instead of other measures such as the replication index, or the CPBI (cytokinesis-block proliferation index) since this project was started prior to publication of the OECD guideline 487 in 2010, and it was considered important to keep the criteria the same across all the studies. The maximum cytotoxicity was set at 60%, based on the OECD guideline for the *in vitro* MN assay [41].

The percentage of BN cells with MN was assessed by means of a standard classification method. A one-sided Fisher's Exact Test was used to determine the statistical

significance of differences between solvent control and each of the treatments with the test chemicals, where  $p < 0.05$  was considered to be indicative of a statistically significant positive response. A Cochran–Armitage test  $p < 0.05$  was used to evaluate dose response.

#### 2.5. Evaluation criteria

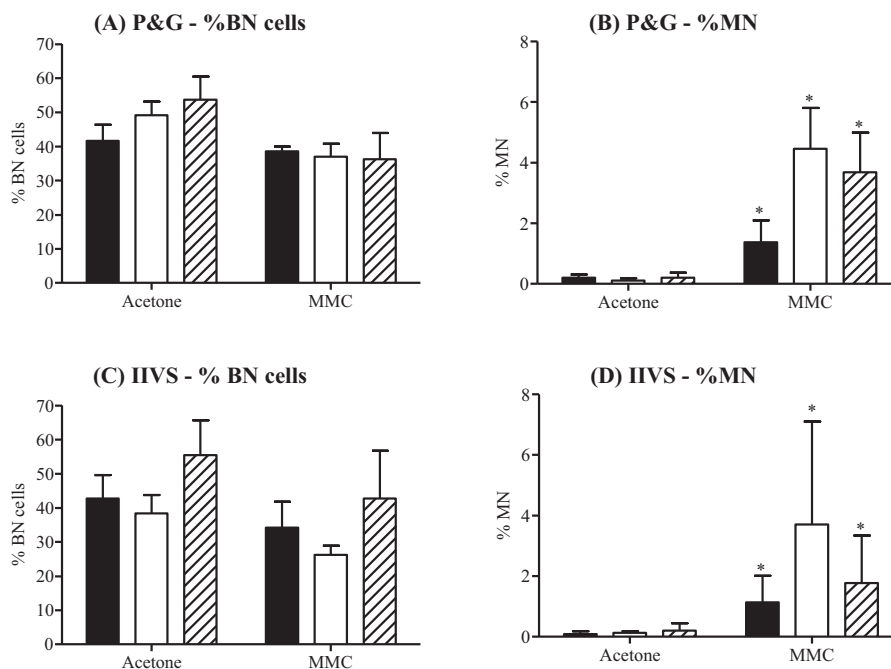
Assays were deemed valid if they passed the criteria described by Dahl et al. [10].

The criteria to judge the outcome of an assay as positive or negative were as described by Dahl et al. [10] with the following clarifications. A chemical was called positive for genotoxicity overall if there was at least one experiment in which two or more concentrations of the substance produced statistically significant increases in MN induction, or if only the highest concentration produced a statistically significant increase in MN induction, and a significant effect was reproduced in an independent study. If a chemical induced a significant increase at a mid-dose, it was considered positive overall if the increase was greater than the upper limit of the historical control range, the trend test was positive, and it was reproducible.

### 3. Results and discussion

#### 3.1. Effect of treatment regimen on the response to the positive control, MMC

Fig. 1 shows the effect of changing the dosing regimen from two doses (48 h treatment with cytoB present for 48 h; indicated



**Fig. 1.** Effect of the treatment time on the % BN cells (A and C) and % MN (B and D) in EpiDerm™ models treated at P&G (A and B) and IIVS (C and D), respectively, with the vehicle control (acetone), and with the positive control (MMC, 3 µg/ml). EpiDerm™ models were either treated twice with MMC in the presence of cytoB (48 h total treatment time + 48 h cytoB, ■), treated three times with MMC, in which time cytoB was present for the final 48 h (72 h + 48 h cytoB, □), or treated three times with MMC, in which time cytoB was present throughout the 72 h treatment period, ▨. Values are mean ± SD,  $n = 9$ –18 models for P&G and 20–26 models for IIVS; \* significant increase over concurrent vehicle control ( $P < 0.05$ ).

as 48 h + 48 h in Fig. 1) to three doses (72 h treatment) with cytoB present for the entire 72 h (72 h + 72 h in Fig. 1), or cytoB just for the last 48 h (72 h + 48 h in Fig. 1) on the responses to the vehicle control (acetone) and the positive control (3 µg/ml MMC). Studies conducted at P&G are shown in Fig. 1A and B and those performed at IIVS in Fig. 1C and D. As shown in Fig. 1A and C, the range of % BN observed in both laboratories with the different exposure regimens is consistent with previously published studies [23,1] with approximately 30–60% BN cells in both acetone-treated models and MMC-treated models.

The effect of changing the dosing regimen from two to three doses on the % MN is shown in Fig. 1B and D. In acetone-treated models, the % MN was reproducible and low (0.11–0.21% at P&G; 0.09–0.15% at IIVS) and in agreement with previously published studies [37,23,1]. In MMC-treated models, statistically significant increases compared with the acetone vehicle-control were observed in all studies regardless of the exposure regimen. At P&G, the % MN in MMC-treated models ranged from 1.38% to 4.46% at P&G (Fig. 1B), and from 1.14% to 3.70% at IIVS (Fig. 1D). Since all these values are in the range of previously published results with MMC in the 48 h standard assay (e.g., [23]), the differences likely reflect normal variation rather than an effect of the treatment regimen. Further studies with more direct-acting genotoxins would be required to investigate this more fully.

Results of a single-dose 72 h regimen to simulate an experiment conducted over a weekend are shown in Fig. 2. The % BN cells and % MN induced by MMC (3 or 10 µg/ml) were comparable to those observed with the standard two-dose 48 h and three-dose 72 h regimens, demonstrating the potential utility of this protocol. Studies with additional chemicals would be required to explore this option further.

Based on these results, studies with chemicals that require metabolic activation were focused on the two-dose, 48 h standard

protocol with cytoB present for 48 h, and the three-dose 72 h exposure with cytoB present for the full 72 h.

### 3.2. Effect of treatment regimen on the response to test chemicals that require metabolic activation

Six compounds (BaP, 4NQO, DMBA, DMN, CP and DBA) that require metabolic activation (Table 1) were chosen to be tested for their genotoxic potential in the EpiDerm™ model.

#### 3.2.1. Compounds that were positive after 48 h and 72 h treatments

**3.2.1.1. 7,12-Dimethylbenzanthracene (DMBA).** DMBA is an abundant environmental contaminant that is bio-activated mainly by CYP1A1 and CYP1B1 (Table 1). It is one of the most potent carcinogenic polycyclic aromatic hydrocarbons and known to cause skin tumours in rodents following dermal application (Table 2). DMBA is genotoxic *in vitro* and *in vivo*. Fig. 3 shows the relative % BN cells and % MN in EpiDerm™ models treated with DMBA for 48 h and 72 h. DMBA was non-toxic up to a dose of 30 mg/ml. The majority of DMBA doses tested caused statistically significant increases in the % MN after treatments during both 48 h and 72 h. The % MN induced at 48 h and 72 h were similar over the dose range 2.5–12.5 mg/ml (the latter is the lowest precipitating dose), suggesting that the additional 24 h incubation did not increase the genotoxic effect further. A dose of 30 mg/ml, which exceeded the lowest precipitating dose was evaluated in one study and induced a further increase in % MN cells.

The inducibility of CYPs is a major factor in the toxicity of a variety of chemicals. CYP1A1 and CYP1B1 are expressed at low levels or are not present in skin [24]; however, their expression has been shown to be induced in skin-cell lines and EpiDerm™ models [17,24]. We have also demonstrated that CYP1A/1B-mediated ethoxyresorufin *O*-deethylase activity in these models is induced

**Table 2**  
Test chemicals, historical genotoxicity and carcinogenicity data and the outcome in the RSMN assays.

Compound CAS <sup>#</sup>	Carcinogenicity and genotoxicity data	RSMN assay result	
		48 h	72 h
Positive control	• Carcinogenic in rats and mice (reviewed in Kirkland et al. [29])	+ in all tests	+ in all tests
Mitomycin C (MMC) 50-07-7	• Positive in <i>in vitro</i> genotoxicity assays and <i>in vivo</i> genotoxicity assays (reviewed in Kirkland et al. [29])		Also positive in 72 h + 48 h cytoB (IIVS, P&G)
Chemicals that require metabolic activation			
Positive at 48 h and 72 h			
7,12-Dimethylbenzanthracene (DMBA) 57-97-6	• Not classified by IARC with regard to human carcinogenicity • Skin tumours in mice, hamsters and gerbils following dermal application [38] • Bladder and kidney cancer [56] • Mouse Lymphoma-NCI Positive [40] • Positive in <i>in vitro</i> genotoxicity assays and <i>in vivo</i> genotoxicity assays [40]	+, + (P&G)	+, + (P&G)
Cyclophosphamide (CP) 6055-19-2		+, + (P&G) –, – (IIVS)	+, + (P&G) +, + (IIVS)  Also positive in 72 h + 48 h cytoB (IIVS, P&G)
Positive at 72 h only			
4-Nitroquinoline N-oxide (4NQO) 56-57-5	• Carcinogenic in rats and mice [40] • Positive in <i>in vitro</i> genotoxicity assays and <i>in vivo</i> genotoxicity assays [40]	+, – (P&G)	+, + (P&G)
Positive at 48 h only			
Benzo[a]pyrene (BaP) 50-32-8	• IARC Group 1, human carcinogen • Skin tumours in mice after dermal application [26,50]	–, +, – (P&G) +, + (IIVS)	–, – (P&G) – (IIVS)
Negative in all tests			
1,2:5,6 Dibenanthracene (DBA) 53-70-3	• Repeated dermal application produced skin papillomas and carcinomas in mice [59,57] but not Syrian golden hamsters [54]	–, – (IIVS)	–, – (IIVS)
Dimethylnitrosamine(DMN) 62-75-9	• IARC Group 2A, probable human carcinogen • Lung, liver and kidney tumours but no skin effects in rats after topical application [4]	–, – (IIVS)	–, +, – (IIVS)

“–” represents a negative result in one test, “+” represents a positive in one test.

by 3-methylcholanthrene (data not shown). Thus, since DMBA is a potent inducer of CYP1A1 [7] and CYP1B1 [33], it is likely that it was sufficiently bio-activated in the EpiDerm<sup>TM</sup> models to give rise to induction of MN.

**3.2.1.2. Cyclophosphamide (CP).** CP is genotoxic *in vitro* and *in vivo* and is often used as a positive control in genotoxicity assays. With respect to skin exposure, CP has been reported to induce MN in blood polychromatic erythrocytes in mice after topical application [43]. CP is a rodent carcinogen (see Table 2), but it has not been tested for carcinogenicity after exposure *via* the dermal route. The metabolism of CP is mainly *via* CYP2B6, which is also induced by this anticancer drug in cultured primary human hepatocytes [18]. However, this CYP was not detected in the EpiDerm<sup>TM</sup> model at the mRNA [24], protein or activity level (data not shown). Other CYPs involved in CP metabolism include CYP2C8 and CYP2C9, of which only CYP2C9 was shown to be present in the EpiDerm<sup>TM</sup> model (Table 1). The CYP-mediated hydroxylation results in the formation of 4-hydroperoxy-cyclophosphamide (Table 1). This metabolite is subsequently converted to acrolein and the potent alkylating agent, phosphoramidate mustard, both of which are genotoxic [19,11].

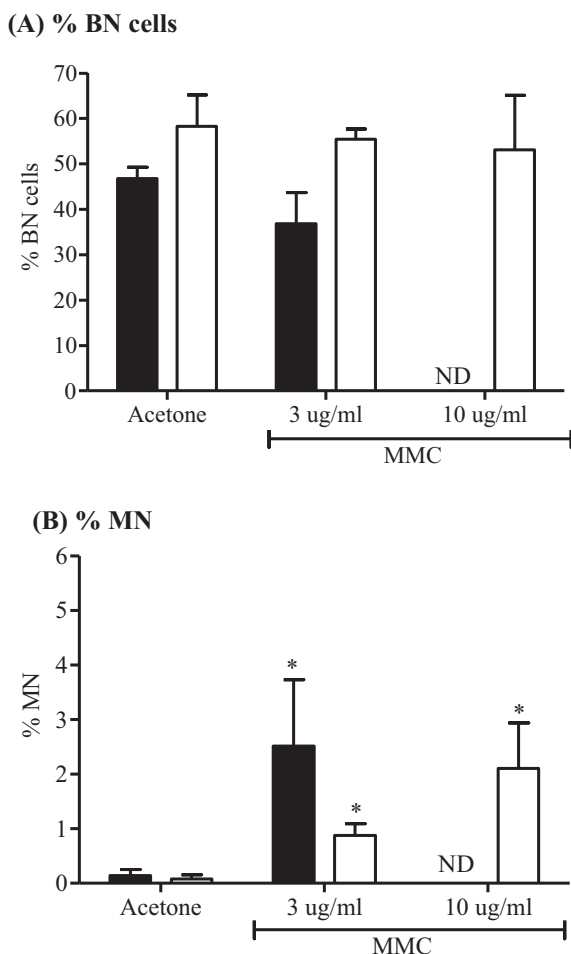
CP was tested at doses up to 100 mg/ml for 48 h and 72 h in the RSMN assay (Fig. 4). CP was positive after both treatment regimens in studies conducted at P&G, with statistically significant increases observed at every dose tested (Fig. 4A and C). CP induced statistically significant increases in MN formation with the 72 h regimen at IIVS, but was negative in the 48 h regimen, although only one study covering the complete dose range was conducted. The reason for this discrepancy is not known and further testing is needed to resolve this. However, it is noteworthy that data from Shambhu et al. [53] demonstrated a clear

dose-dependent increase in MN with CP in both the 48 h and the 72 h treatments. CP also induced statistically significant increases in MN formation in a protocol that combined 72 h treatment with 48 h cytoB treatment which was conducted at both P&G and IIVS (data not shown). Of the CYPs involved in CP metabolism only CYP2C9 was expressed in EpiDerm<sup>TM</sup> suggesting that this may have been the main CYP responsible for the bio-activation of CP in these studies.

CP (30–200 mg/ml) was evaluated in the single-dose, 72 h protocol in which the media was not changed during the entire 72 h period (data not shown). A statistically significant increase in MN formation was seen at 100 and 200 mg/ml CP, together with a positive trend test in one study, and one significant response at 60 mg/ml CP, but without a significant trend, in a repeat study. Overall, these results corroborate the positive results in the standard 72 h regimen observed at both P&G and IIVS, and demonstrate the potential of this alternative dosing regimen. Further work with additional chemicals is needed to determine more fully the utility of this approach.

In a different experimental design, Flamand et al. [15] reported that CP applied to EpiSkin<sup>TM</sup> did not induce MN in L5178Y target cells cultured in the media beneath the skin tissue unless 5% Arochlor-induced rat S9 was present. An assessment of MN in skin cells in a similar manner to ours was not conducted. The authors concluded that the metabolic capacity of EpiSkin<sup>TM</sup> does not ensure sufficient conversion of CP into its clastogenic metabolites, or that the required level of genotoxic metabolites in the culture medium is not reached.

Overall, the results in the studies reported here indicate that CP is reliably detected in the RSMN assay especially with a longer 72 h exposure regimen, since both laboratories measured reproducible positive results.



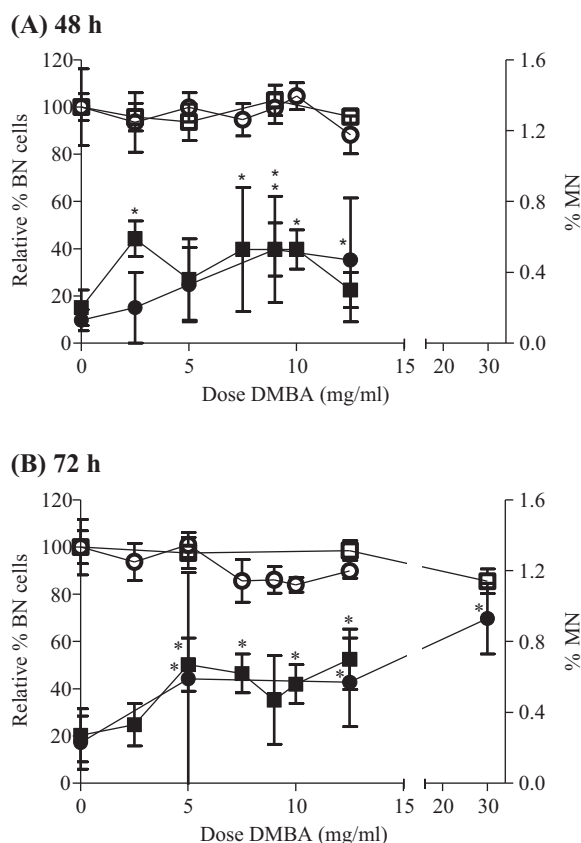
**Fig. 2.** Effect of a single-dose treatment on the % BN cells (A) and % MN (B) in EpiDerm™ models treated with the vehicle control (acetone), and the positive control (MMC), at IIVS. EpiDerm™ models were either treated with 3  $\mu$ g/ml MMC, in which time medium containing cytoB was replaced every 24 h over the weekend (■), or treated once with 3 or 10  $\mu$ g/ml MMC over the weekend, in which time media containing cytoB was not changed (□). Values are a mean  $\pm$  SD,  $n = 5$  models. ND, not determined. \* Significant increase over concurrent vehicle control ( $P < 0.05$ ).

### 3.2.2. Compounds that were negative after the 48 h and positive after the 72 h treatment

**3.2.2.1. 4-Nitroquinoline N-oxide (4NQO).** 4NQO is reduced by NADPH-quinone oxido-reductase (NQO1) to form 4-hydroxyaminoquinoline-N-oxide, a proximate carcinogen (Table 1). This activation enzyme has been reported to be present in rat epidermal cytosol at levels higher than in the liver [28]. It can be induced in the skin *in vivo* [60], and *in vitro* by AhR-mediated inducers [34]. The toxic effects of 4NQO (cytotoxicity and genotoxicity) in HepG2 cells have been shown to be dependent on the MRP2-mediated efflux of its glutathione conjugate and/or another toxic derivative of 4NQO, which confers resistance to this genotoxin [36].

There was a dose-dependent increase in the % MN in EpiDerm™ models treated with 4NQO for 48 h, with a statistically significant increase at the high dose only in one study; together with a negative trend test ( $p = 0.052$ ) (Fig. 5A). By contrast, the MN frequencies of most of the doses tested over 72 h (which were in the same range as for the 48 h incubations) were statistically significantly higher than those of the acetone-treated models (Fig. 5B).

Flamand et al. [15] reported a dose-dependent increase in DNA damage (statistical analysis not given) measured by means of the Comet assay, in EpiSkin™ treated with 4-NQO in PBS.

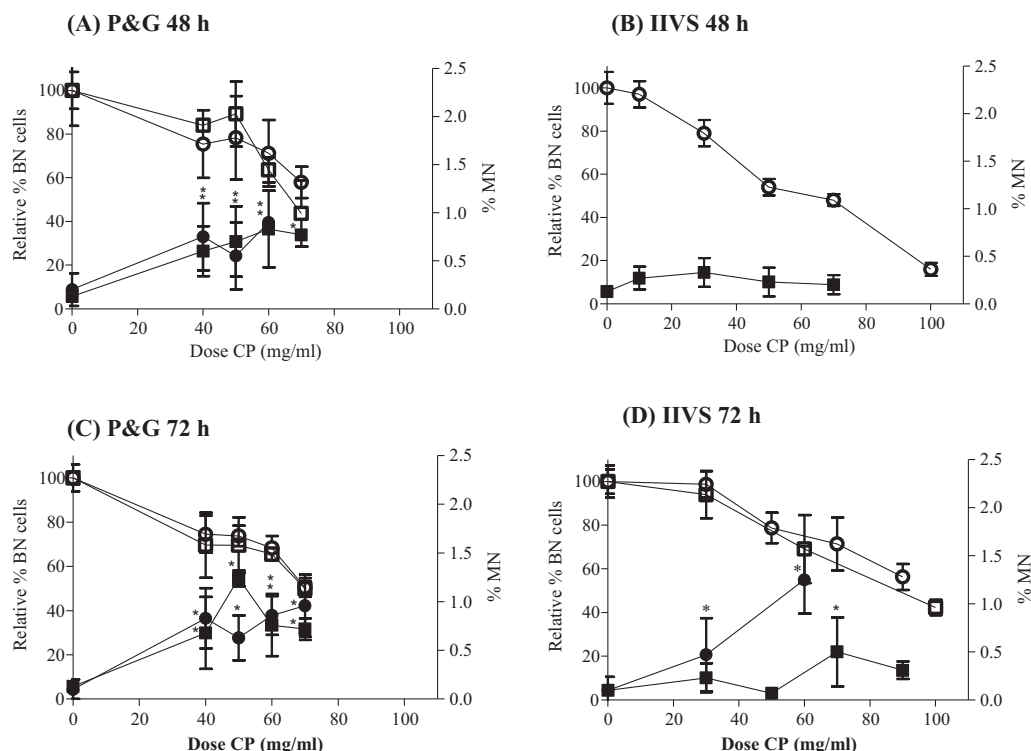


**Fig. 3.** % MN (closed symbols) and relative % BN (open symbols) after 48 h (A) and 72 h (B) treatments with DMBA. The symbols denote experiment 1 (○ or ●) and 2 (□ or ■). Values are mean  $\pm$  SD; \* significant increase over concurrent vehicle control ( $P < 0.05$ ).

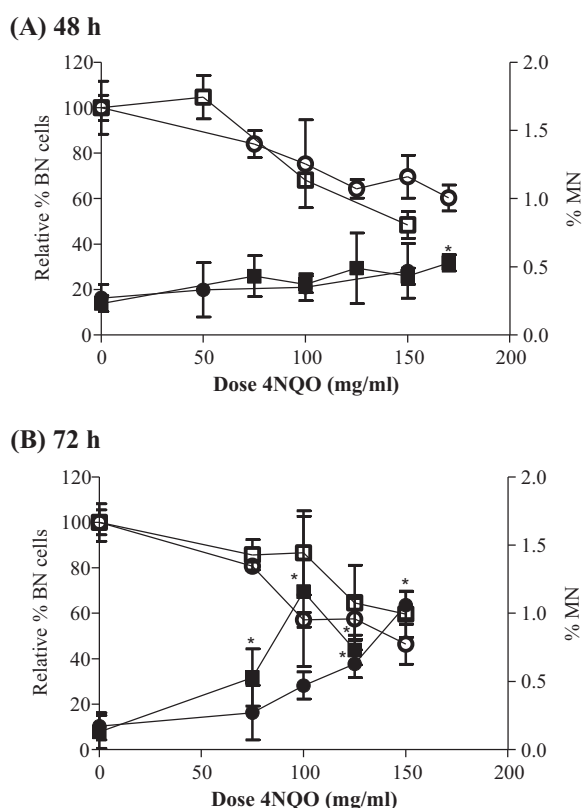
### 3.2.3. Compound(s) that were positive after the 48 h and negative after the 72 h treatment

**3.2.3.1. Benzo[a]pyrene (BaP).** BaP causes skin papillomas and carcinomas after topical application to mice and is a human carcinogen (IARC, Group 1) (Table 2). BaP is metabolised mainly by CYP1B1, CYP1A1 and microsomal epoxide hydrolase [25] and in part by CYP2C9, and CYP3A4 [16] to a number of products, of which BaP-7,8-diol is the intermediate that leads to the ultimate carcinogen, BaP-diolepoxide 2 (Table 1). Modulation of CYPs has been shown to alter the amount of DNA adducts formed [58]; therefore, metabolism plays an important role in the mechanism of BaP carcinogenesis. CYP1A1 is not present in native human skin or EpiDerm™ as discussed above, but it can be induced in human skin by BaP [42], a finding that is also observed in separate studies with EpiDerm™ models [20].

RSMN studies with BaP were complicated by the low solubility of this chemical, which leads to precipitation at high doses. Small, but statistically significant increases in MN formation were observed in the 48 h regimen in both P&G and IIVS studies, but only one of three studies at P&G (Fig. 6A) was also statistically significant for the trend test. However, both IIVS studies showed a significant dose response to this chemical (Fig. 6B). BaP induced small increases in % MN, which were not statistically significant in the 72 h regimen in studies conducted both at P&G and IIVS (Fig. 6C and D, respectively). We speculate that the low and variable responses with BaP in the RSMN assay may be due to concomitant induction of CYP1A and GST activities, the latter conferring protection against the formation of MN, as was reported to occur in HepG2 cells [58]. The skin shows extensive GST activity, and EpiDerm™ models have cytosolic GST activities of over 300 nmol/min/mg



**Fig. 4.** % MN (closed symbols) and relative % BN (open symbols) after 48 h (A and B) and 72 h (C and D) treatments with CP. Separate experiments conducted at P&G (A and C) and IIVS (B and D) are represented by different symbol shapes denoted as experiment 1 ( $\circ$  or  $\bullet$ ) and 2 ( $\square$  or  $\blacksquare$ ). Values are mean  $\pm$  SD; \* significant increase over concurrent vehicle control ( $P < 0.05$ ).



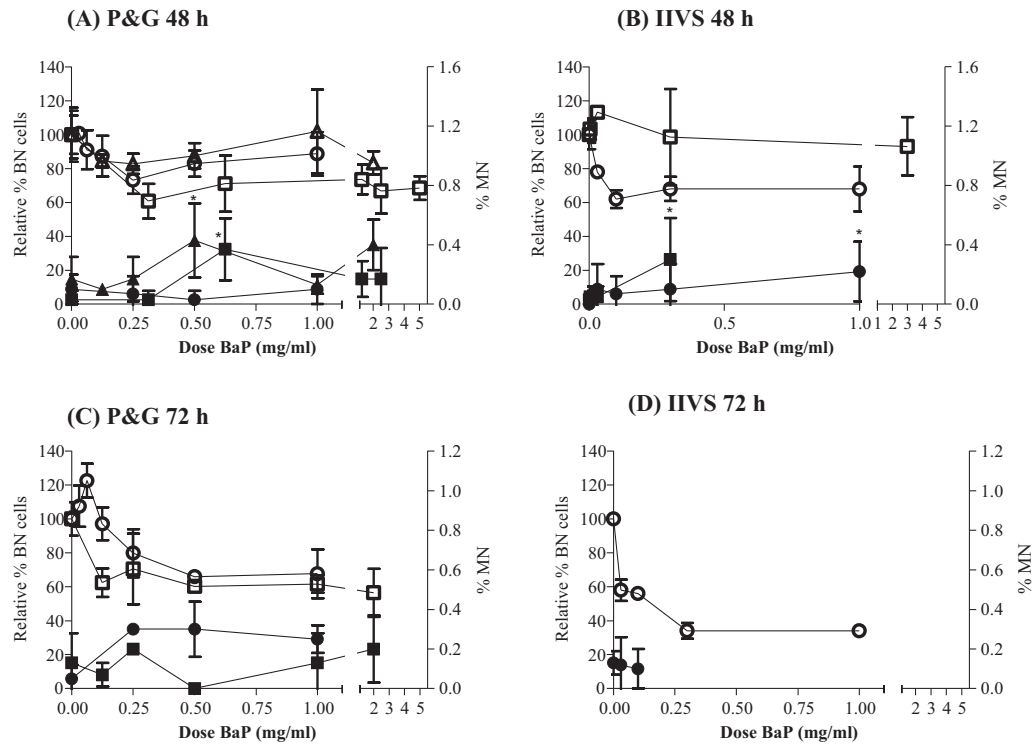
**Fig. 5.** % MN (closed symbols) and relative % BN (open symbols) after 48 h (A) and 72 h (B) treatments with 4NQO. The symbols denote experiment 1 ( $\circ$  or  $\bullet$ ) and 2 ( $\square$  or  $\blacksquare$ ). The dotted line shows the 40% cut-off for acceptable % relative BN cell values. Values are mean  $\pm$  SD; \* significant increase over concurrent vehicle control ( $P < 0.05$ ).

protein [20]. Therefore, it may be expected that a further increase in this detoxification enzyme may decrease the amount or longevity of BaP-diolepoxide 2. However, extended dermal exposures such as those in skin carcinogenicity studies in rodents did cause a carcinogenic effect in the skin. Overall, BaP is difficult to reliably detect in this assay since increases in % MN were small and not reproducible across laboratories. The responses with BaP measured in this assay would trigger further investigations.

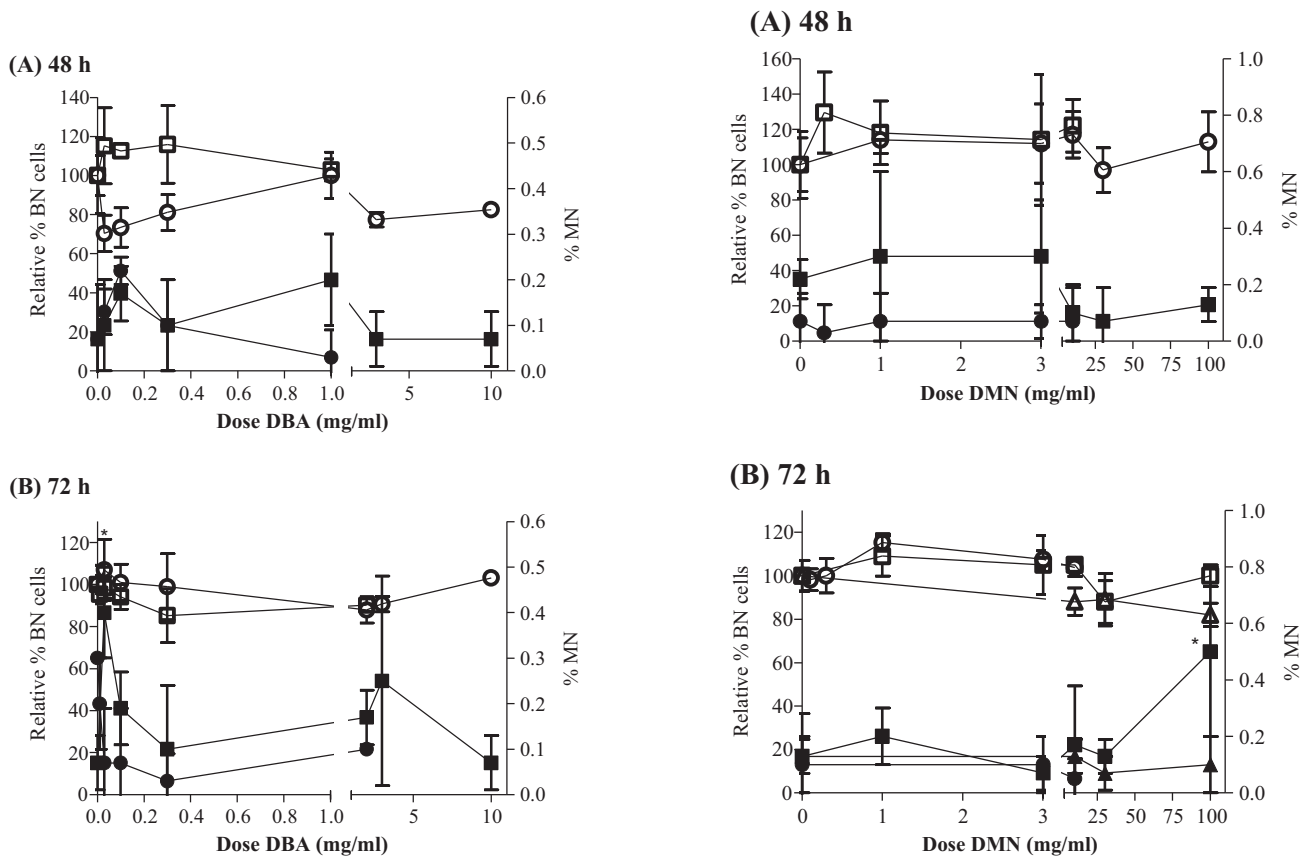
### 3.2.4. Compounds that were negative after the 48 h and 72 h treatments

**3.2.4.1. 1,2:5,6 Dibenanthracene (DBA).** DBA is a potent initiator of skin tumours in a two-stage system of carcinogenesis in the mouse and is metabolised by CYP1A1 (Table 2). Fig. 7 shows the effect of DBA on the relative % BN cells and % MN in EpiDerm<sup>TM</sup> models treated for 48 h and 72 h. There was no significant toxicity induced by DBA up to a dose of 10 mg/ml. All doses of DBA in the 48 h and 72 h regimens gave a negative result except the lowest dose of 0.03 mg/ml in one of the 72 h experiments ( $0.40 \pm 0.10\%$  MN compared with  $0.07 \pm 0.06\%$  in acetone-treated models). Since the response was not dose-dependent or reproducible, DBA was concluded to be negative in both the 48 h and 72 h regimens. Although CYP1A1 expression was absent in donor 254, it has the potential to be induced by DBA, since this chemical induced CYP1A1/2 in the human breast-cancer cell-line MCF7 [55]. The extent of enzyme activity, however, may not be sufficient to generate enough metabolite required to pick up a genotoxic effect in this model.

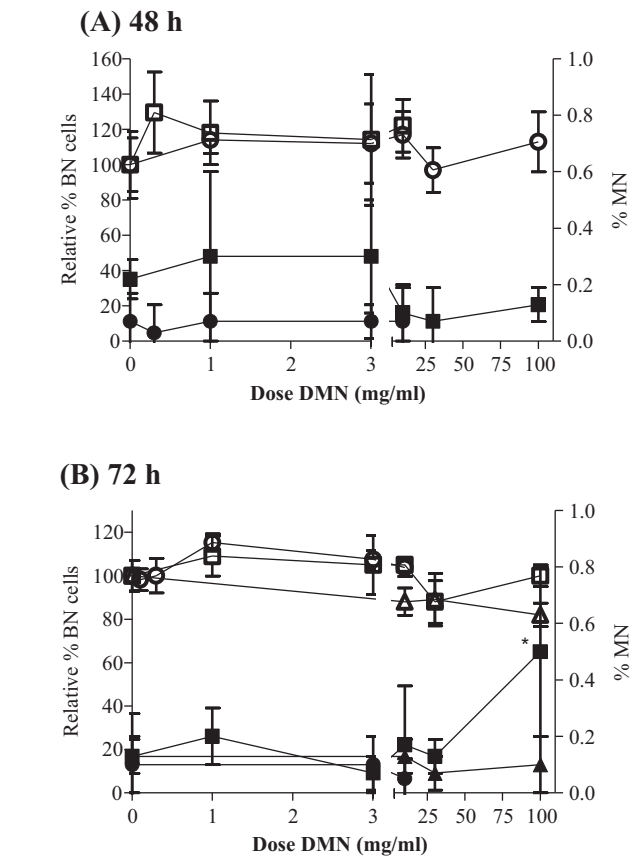
**3.2.4.2. Dimethylnitrosamine (DMN).** DMN is bioactivated by CYP2E1, which is present in native human skin [43] but not expressed in the EpiDerm<sup>TM</sup> (Table 1). CYP2E1 hydroxylates the  $\alpha$ -carbon atom to form an unstable intermediate, which decomposes to formaldehyde and an electrophilic methylating agent, the diazonium ion (Table 1). Fig. 8 shows the effect of DMN on the relative % BN cells and % MN in EpiDerm<sup>TM</sup> models treated for 48 h



**Fig. 6.** % MN (closed symbols) and relative % BN (open symbols) after 48 h (A) and 72 h (B) treatments with BaP. The symbols denote experiment 1 (○ or ●), 2 (□ or ■) and 3 (△ or ▲). The dotted line shows the 40% cut-off for acceptable % relative BN cell values. Values are mean  $\pm$  SD; \* significant increase over concurrent vehicle control ( $P < 0.05$ ).



**Fig. 7.** % MN (closed symbols) and relative % BN (open symbols) after 48 h (A) and 72 h (B) treatments with DBA. The symbols denote experiment 1 (○ or ●) and 2 (□ or ■). The dotted line shows the 40% cut-off for acceptable % relative BN cell values. Values are mean ( $\pm$ SD for % MN only); \* significant increase over concurrent vehicle control ( $P < 0.05$ ).



**Fig. 8.** % MN (closed symbols) and relative % BN (open symbols) after 48 h (A) and 72 h (B) treatments with DMN. The symbols denote experiment 1 (○ or ●), 2 (□ or ■) and 3 (△ or ▲). The dotted line shows the 40% cut-off for acceptable % relative BN cell values. Values are mean  $\pm$  SD; \* significant increase over concurrent vehicle control ( $P < 0.05$ ).

and 72 h. DMN was non-toxic up to a dose of 100 mg/ml. No significant increase in % MN was observed in the 48 h regimen. In the 72 h regimen, one of three experiments showed a statistically significant increase in % MN, but only at the highest dose, 100 mg/ml (0.50% MN compared with 0.1% MN in acetone-treated models). The overall trend test showed that the effect was significant in this single experiment. Since this result was not reproducible, DMN was concluded to be negative in the RSMN assay.

This outcome may be expected since application of DMN to the skin of rats resulted in tumours in the liver, lung and kidney, but not the skin [4]. This suggests that DMN is not bio-activated in the skin (or not to a sufficient degree), but it may be bio-activated if it is taken up in the systemic circulation and, consequently, it may cause genotoxic effects in other organs.

#### 4. Conclusions

As part of a continuing Cosmetics Europe project to validate the use of the RSMN assay, we have investigated whether genotoxic chemicals that require metabolic activation can be detected. To fully evaluate this, the standard protocol involving two doses over 48 h was compared with a longer, three-dose, 72 h treatment regimen.

Four of six chemicals evaluated in this study were positive in one or more of the dosing regimens (DMBA, CP, 4NQO, BaP), which shows that the RSMN assay is capable of detecting some but not all chemicals that require metabolic activation. Extending the treatment period to 72 h changed the result only for 4NQO, which was negative in the standard 48 h dosing regimen, but positive with the 72 h treatment. CP was also more reliably detected with the 72 h treatment. DBA and DMN were negative in both 48 h and 72 h dosing regimens. Based on these results, for general testing, the use of a longer treatment period is recommended in situations where the outcome of the standard 48 h treatment is negative or questionable. This approach is similar to the protocols for other *in vitro* genotoxicity assays that include a longer exposure regimen in addition to a shorter exposure regimen. While further work with additional chemicals requiring metabolism is needed to evaluate this fully, we feel that it is important to provide this recommendation so that it can be implemented in future studies, thereby helping to build the database to address this important question.

These data, as well as previously published results, support the conclusion that the RSMN assay is a valuable new *in vitro* method for the assessment of genotoxicity of dermally applied chemicals and drugs. The RSMN assay provides a useful approach for evaluating chemicals with positive or equivocal results in the standard *in vitro* genotoxicity tests, thereby filling a critical gap in the test battery for ingredients like cosmetics that cannot be tested in animals.

#### Conflict of interest statement

No conflict of interest.

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