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# Cutaneous biotransformation of N-(4-bromobenzoyl)-S,S-dimethyliminosulfurane and its product, 4-bromobenzamide, leading to percutaneous penetration enhancement of drugs: Initial evidence using hydrocortisone

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ABSTRACT

The role of the skin's metabolism of N-(4-bromobenzoyl)-S,S-dimethyliminosulfurane (DMBIS), an effective penetration enhancer, on its enhancement activity was investigated. It has been found that DMBIS hydrolyzes very fast in physiological buffer to 4-bromobenzamide (BBA), and even faster and almost completely in the presence of skin tissue. It was further shown that in the presence of skin from different species incubated at physiological conditions, the concentration of BBA (DMBIS' immediate product) dropped sharply to 70–80% in 10 min followed by a slower decrease of 0.35–0.50 μg/h. This metabolism was partially inhibited by a continuous application of iodine, and more profoundly, by iodoacetic acid (IAA) and dithiothreitol (DTT) combination treatment. This indicates that at least a part of the metabolism of BBA involves enzymes that are sensitive to reactions with their sulfhydryl groups. In an *in vitro* permeation study using human epidermis and conventional diffusion cells, we compared between the permeabilities of untreated epidermis and IAA/DTT-treated epidermis to hydrocortisone in the presence of BBA. Due to its metabolic inhibition, we noted a higher penetration of BBA through IAA/DTT-treated epidermis than through the untreated epidermis. Contrary to these results, the extent of the penetration of hydrocortisone was higher through the untreated epidermis with only 1.6 h lag time relative to its penetration through IAA/DTT-treated epidermis, which exhibited a lag time of 12.4 h. It is evident, therefore, that the skin enhancement activity of DMBIS/BBA depends on BBA metabolism in the skin, presumably through its *in situ* biotransformation into an active enhancer.

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## 1. Introduction

The skin is an excellent barrier to penetration of many substances, primarily due to the lipophilic properties of the outermost stratum corneum layer. Significant efforts have been made and numerous strategies have been proposed to overcome the impermeability of human skin in an attempt to develop and improve the transdermal drug delivery route and produce an effective alternative to oral delivery [1–12]. One of the strategies, which has been extensively studied, is the chemical penetration enhancement (CPE) by modification of the stratum corneum [13,14]. Many chemicals have been examined as enhancers including surfactants, terpenes, dimethylsulfoxide (DMSO), Azone analogs, alcohols, and water. It has been suggested [15,16] that the mechanisms by which these enhancers affect skin permeability are (a) disruption of the ordered lipid bilayers' structures such as fatty acids, Azone, or DMSO, and eventual fluidization of the lipid environment of the stratum corneum, (b) interaction with intracellular proteins of the stratum corneum, and (c) improvement of partitioning and solubility of the drug in the stratum corneum.

In previous reports [17–19], aromatic S,S-dimethyliminosulfurane derivatives have been introduced and studied for their CPE activities. These molecules are based on a DMSO molecule in which a nitrogen atom substituted to the arylsulfonyl, aroyl, or aryl group replaced the DMSO oxygen atom. Permeation studies using hairless mouse and human cadaver skin demonstrated that the bromo analog, S,S-dimethyl-N-(4-bromobenzoyl)iminosulfurane (DMBIS, Fig. 1), was the most effective penetration enhancer. These studies have also shown that the excellent enhancement effect of DMBIS could not be achieved with other various analogs including other halobenzoyl derivatives (i.e., F, Cl, I). Thus, *p*-bromo-substitution on the benzoyl imino-dimethylsulfurane molecule enables a dramatic enhancement effect while other halogeno- and various derivatives have a relatively

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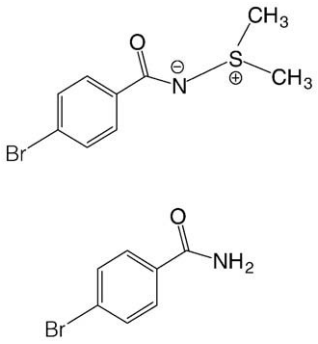


**Fig. 1.** The chemical structure of N-(4-bromobenzoyl)-S,S-dimethyliminosulfurane (DMBIS) and 4-bromobenzamide (BBA).

poor or no effect. It should be noted that atypical to most penetration enhancers, which usually cause skin irritation, none of the halogen-substituted analogs exhibited cytotoxicity on epidermal keratinocytes and dermal fibroblasts as determined by MTT assay [19]. Although effective, the enhancement effect of DMBIS was achieved at relatively high concentrations (i.e., 5–10%). These unexplained results have provoked the inevitable hypothesis that the enhancement effect, which is specifically attributed only to the bromo-substituted imino-sulfurane (DMBIS), might be related to an enzyme site selectivity, and the high concentration required to generate the effect may imply an *in situ* formation of a bio-product from DMBIS as a substrate of an enzymatic catalysis, while this bio-product is used as the active penetration enhancer. This hypothesis has also emerged due to the accumulating evidence of the skin's role as a highly metabolizing organ that acts as a frontal guard to protect the body from toxicants, reactive oxygen species (ROS), and various environmental pollutants. The metabolic role of this large organ includes cytochrome *P450*-dependent (*CYP* gene products) activation and disposition of natural and foreign compounds [20–22]. As a first example, skin metabolism of dinitrochlorobenzene to glutathione conjugate, while absorbed through mouse, rat, pig ear, and human skin, was related to skin glutathione-S-transferase activity and high levels of glutathione (GSH). The activity of the enzyme in human skin was the highest on the substrate, followed by rat, pig, mouse and neonatal rat skin. Levels of GSH were highest in mouse skin, followed by neonatal rat, adult rat, pig, and human skin, with pig and human skin showing similar levels [23]. As a second example, the skin metabolism of nitroglycerine to 1,2- and 1,3-glyceryl dinitrate was significantly enhanced by GSH, a cofactor of glutathione-S-transferase [24]. In addition to these metabolic roles, the skin tissues are also programmed to combat oxidative stresses induced by ROS. ROS mediate damage to proteins and induce their alterations. To prevent excessive accumulation of oxidized proteins and cell death, methionine in the skin reacts with ROS being oxidized to methionine sulfoxide (both R and S enantiomers). To prevent methionine depletion, methionine sulfoxide is recovered to free or protein-bound methionine by methionine sulfoxide reductases (MSR-A for S enantiomer and MSR-B for R enantiomer). In addition to the antioxidant function of MSRs, it has been shown that they can also regulate the function of methionine-containing proteins by alternating them between active and non-active form. It should be noted that a recent study showed both enzymes to be present in the human epidermis [25].

In the present report, we attempt to understand the mechanism by which DMBIS and its immediate degradation product, 4-bromobenzamide (BBA), enhance skin permeation of drugs from the view of its metabolism in the skin. Our specific objectives were (a) to elucidate whether DMBIS and BBA are metabolized by skin tissue taken from various species and (b) to evaluate whether the skin metabolism is correlated with the observed enhancement activity of a model drug.

## 2. Materials and methods

### 2.1. Chemicals

N-(4-bromobenzoyl)-S,S-dimethyliminosulfurane (DMBIS) was synthesized as previously described [17,18]. Briefly, dimethylsulfoxide was reacted with trifluoroacetic anhydride followed by the reaction of the resultant intermediate compound, S,S-dimethyl-S-(trifluoroacetoxy)sulfonium trifluoacetate, with 4-bromobenamide (BBA). The purified product (yield: 75%) was conformed and characterized by melting point determination and NMR spectral analysis. 4-Bromobenzamide, iodine, iodoacetic acid (IAA), DL-dithiothreitol (DTT), N-ethylmaleimide (NEM), propylene glycol, and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, methanol, and water were HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO).

### 2.2. Animals and skin

Skin excised from hairless mice (NCRNU/Mcr Nude homozygous, Taconic Farms, Inc., Germantown, NY) was provided by Dr. Minko from the Ernest Mario School of Pharmacy, Rutgers University (Piscataway, NJ), and used on the same day. Sprague Dawley rats (male, 200–300 g) were supplied by Charles River Laboratories (Wilmington, MA). Full-thickness skin was taken from the rat carcasses, and kept at −20 °C. Porcine skin was obtained from young Yorkshire pigs (26.5–28 kg, UMDNJ, Newark, NJ). The pig skin was excised and dermatomed using Padgett® Model B Electric Dermatome (Integra LifeSciences, Plainsboro, NJ). The thickness of the dermatomed skin ranged 500–600 μm. Human epidermis was separated from human cadaver skin (National Disease Research Interchange — NDRI, PA, # OD25080). The human cadaver skin piece was thawed at room temperature before immersion in phosphate buffer saline (pH 7.4) at 60 °C for 45 s to separate the dermis from epidermis. The epidermis was peeled off gently with a pair of forceps (average thickness of 100–150 μm) and was then cut into appropriate size and stored at −80 °C.

### 2.3. Skin metabolism

Excised skin (from mouse, rat or pig) was cut to small pieces and incubated at 37 °C within PBS (pH 7.4); the skin–PBS ratio was 1:5 (w/v) in all experiments. Just prior to incubation, 1 mg/ml solutions of DMBIS or 4-bromobenzamide (BBA) were introduced to produce an initial solution concentration of 10 μg/ml. Aliquots (0.1 ml) of the solution were withdrawn at 10, 20, 30, 40, 50, 60, 90, and 120 min. To stop further reaction, the samples were diluted with 0.2 ml of a cold methanol–water (1:1) mixture. Samples were kept at −20 °C until analyzed for remaining DMBIS or BBA, and formed BBA by HPLC. As BBA was found to be the major and immediate product of DMBIS, inhibition of BBA biotransformation was examined by adding various enzyme modifiers into the skin–PBS system prior to BBA introduction, as explicitly described below.

#### 2.3.1. Iodine

Appropriate aliquots of a concentrated iodine solution (2 mM $I_2$ in ethanol/propylene glycol/water 1:20:79%vol) were added into the skin–PBS system to produce initial concentrations of 4–25 μM before the introduction of BBA. It was noted that in the presence of skin, the brownish solution formed after iodine addition was very rapidly decolorized, and the obtained results were concentration independent within the working range of 4–25 μM. In seven control experiments (without iodine), the corresponding aliquots of the vehicle used to dissolve iodine were added.

#### 2.3.2. Alkylating agents

Into skin containing PBS (pH=8) solutions, appropriate aliquots of 0.4 M NEM solution (in PBS, pH 8) and 1 M IAA solution (in 1 M NaOH)

were incorporated to produce 10 mM and 20 mM final concentrations of NEM and IAA, respectively. The skin in the NEM solution was incubated for 1 h at 37 °C prior to BBA introduction. The skin in the IAA solution was left at ambient temperature for 1 h, then DTT solution was added to quench IAA (40 mM) and BBA was introduced.

### 2.4. Skin metabolism and influence of iodine using diffusion cells

Pieces of dorsal rat skin were mounted on vertical Franz diffusion cells (receptor volume 8.4 ml with a donor area of 3.14 $cm^2$) filled with PBS at pH 7.4. The receptor fluid was maintained at 37 ± 0.5 °C and stirred continuously at 600 rpm using a magnetic stirrer. The donor was filled with 1 ml iodine solution (100 mM $I_2$/300 mM KI) in water. In control cells, the donor chamber was filled with 1 ml water (i.e., the vehicle of the iodine solution). Two hours after iodine application, the receptor solution was replaced with PBS containing 10 μg/ml BBA. "Receptor" aliquots (200 μl) were then withdrawn at 0, 15, 30, 45, 60, 90, 120, 180, 240 min, and 22.5 h after BBA introduction. The samples were removed through the cell sampling port and immediately replaced with an equivalent volume of appropriate buffer solution. The samples were diluted with 200 μl of cold methanol–water (1:1) mixture and kept at −20 °C until analyzed for the remaining BBA by HPLC.

### 2.5. Mass balance calculation

At the end of each skin metabolism experiment, the skin pieces were extracted in 1 ml ethanol and the resultant mixture was shaken at room temperature for 1 h. Mass balance was calculated by the following equation:

$$\text{Mass Balance}(\%) = \left\{ \left[ Q_s + Q_T + \sum_{i=1}^{n} S_i \right] \times 100 \right\} \Big/ Q_0$$

where $Q_s$ is the DMBIS or BBA mass extracted from the skin, $Q_T$ is the amount remained in the PBS solution at the end of the experiment (at time $T$), and $S_i$ is the amount taken with sample $i$ from the PBS solution during the experiment, when $n$ is the number of samples. $Q_0$ is the initial amount of DMBIS or BBA in the solution (at time 0).

### 2.6. Penetration enhancement of hydrocortisone by DMBIS and BBA using dermatomed pig skin

The permeation of hydrocortisone through dermatomed pig skin was measured *in vitro* with eighteen vertical Franz diffusion cells (Permegear, Inc., Bethlehem, PA) filled with PBS at pH 7.4. The diffusion area was 0.64 $cm^2$, and the receptor compartment volume was 5.1 ml. The receptor fluid was maintained at 37 ± 0.5 °C and stirred continuously at 600 rpm using a magnetic stirrer. On the day of the experiment, skin pieces were thawed and mounted on the Franz diffusion cells. After 1 h of skin equilibration, 4 μl 0.4 M DMBIS or BBA solutions (in propylene glycol) or plain propylene glycol was pipetted onto the skin. Six cells were assigned for each group (DMBIS, BBA and vehicle control). The enhancers were allowed to permeate into the skin (and be metabolized) for 1 h. After 1 h, a 40 μl saturated solution of hydrocortisone in propylene glycol was added. Receptor samples of 300 μl were removed through the cell sampling port and immediately replaced with an equivalent volume of PBS (pH 7.4) at 0, 4, 7, 10, 18, 20, 22, and 24 h after drug application. The cells were checked for air bubbles at each sampling point. The samples were kept at −20 °C until analyzed by HPLC. After analysis, data were expressed as the cumulative permeation ($Q_t$) of BBA (only small levels of penetrating DMBIS were detected) and hydrocortisone per unit of skin surface area, $Q_t/S$ ($S$=0.64 $cm^2$). The slope of the linear portion of the permeation curve provided the flux value in $\mu g \cdot cm^{-2}\ h^{-1}$ at steady state. The lag time was determined by extrapolating the linear portion of the curve to the $x$-axis. Enhancement ratio (ER) for flux was calculated by dividing the flux for skin treated with enhancer by the flux obtained without enhancer treatment (control).

### 2.7. Penetration enhancement of hydrocortisone after epidermal treatment with iodoacetate

The permeation of hydrocortisone through human epidermis was measured *in vitro* with six vertical Franz diffusion cells (Permegear, Inc., Bethlehem, PA) filled with PBS at pH 7.4. The diffusion area was 0.64 $cm^2$, and the receptor compartment volume was 5.1 ml. The receptor fluid was maintained at 37 ± 0.5 °C and stirred continuously at 600 rpm using a magnetic stirrer. On the day of the experiment, epidermis pieces were thawed and mounted on the Franz diffusion cells. Twenty μl of 1 M IAA solution (in 1 M NaOH) was applied with 50 μl PBS (pH 8.5 in the donor compartment). After 45 min, 40 μl of DTT was added to the donor chamber and was allowed to react for 15 min. In control cells, 50 μl PBS (pH 8.5) and 20 μl water followed by another portion of 40 μl water were used instead of IAA/DTT. After treatment, the fluid in the donor compartment was removed and 4 μl 0.4 M BBA solution (in propylene glycol) was pipetted onto the epidermis in all cells. The solution was allowed to permeate into the skin (and be metabolized) for 1 h. After 1 h, a 40 μl saturated solution of hydrocortisone in propylene glycol was added. Receptor samples of 300 μl were removed through the cell sampling port and immediately replaced with an equivalent volume of PBS (pH 7.4) at 0, 1, 2, 7, 20, 22, and 24 h after drug application. The cells were checked for air bubbles at each sampling point. The samples were kept at −20 °C until analyzed by HPLC.

### 2.8. Simultaneous HPLC analysis of DMBIS, BBA and hydrocortisone

Samples were analyzed using Agilent 1100 automated HPLC with diode array detector (Agilent Technologies, Inc., Santa Clara, CA). Aliquots of 20 μl from each sample were injected into the HPLC system, which was equipped with a prepacked C18 column (Brava ODS, 5 μm, 250×4.6 mm, Alltech Associates, Inc., Deerfield, IL). The simultaneous detection of DMBIS, BBA and hydrocortisone was carried out at 242 nm. The samples were chromatographed using a mobile phase consisting of water–acetonitrile or 0.05% acetic acid solution–acetonitrile at a flow rate of 1.4 ml/min. The gradient system was programmed to linearly alter the solvents' mixture from water–acetonitrile ratio of 80:20 to 60:40 in 7 min. Calibration curves (peak area versus drug or enhancer concentration) were linear over the range 1–20 μg/ml (in PBS). The limit of quantitation was 0.1 μg/ml.

## 3. Results and discussion

It was the principal goal of this study to examine the hypothesis that effective skin penetration enhancement of drugs by N-(4-bromobenzoyl)-S,S-dimethyliminosulfurane (DMBIS) depends on the metabolic capability of the skin to transform this compound into an effective enhancer. Such a bioformed enhancer may better intercalate into the lipid bilayer structure of packed ceramides, create spatial disruption, or may even open aqueous channels to facilitate drug diffusion through the stratum corneum. It has been shown that an immediate degradation of DMBIS occurs in aqueous solutions, and it is even more intensive when incubating with skin tissue. Therefore, it has been reasonably deduced that it is 4-bromobenzamide, the immediate product, which can be the potential precursor for enhancer (i.e., pro-enhancer). This hypothesis was further supported by the drug enhancement effect elicited by 4-bromobenzamide as described in the following.

### 3.1. Elimination of N-(4-bromobenzoyl)-S,S-dimethyliminosulfurane in buffer solutions and in the presence of skin tissue

Fig. 2 demonstrates how fast DMBIS decomposes in aqueous solutions at four different pH values, i.e., up to 80% of the substance

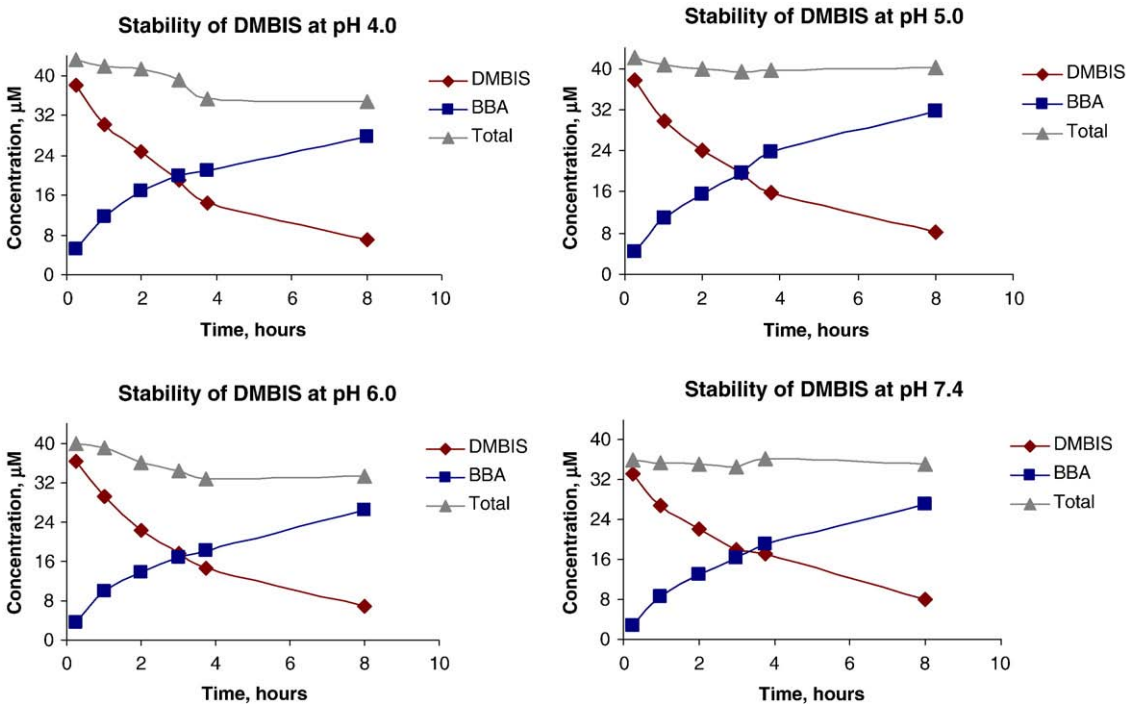


**Fig. 2.** DMBIS hydrolysis at pH values ranged 4 to 7.4. A simultaneous formation of BBA and the total molar concentrations of the remaining DMBIS and the formed BBA at each time point are also shown.

was eliminated in 8 h. The half-life of the reaction kinetics is approximately 3 h as shown from the point of intersection of DMBIS elimination–BBA formation lines. No difference in DMBIS elimination as well as in BBA formation was noted between the four buffer systems. A relatively more interesting phenomenon relating to DMBIS decomposition was presented in Fig. 3. The half-life of elimination in buffer solution (pH 7.4) decreased from about 3 h to 1 h when the substance had brought in contact with skin tissue at 37 °C. After 2 h, only 38.3% (±4.1%) of the initial quantity of DMBIS remained in the buffer system when it had been incubated with skin, compared to 74.8% (±4.3%) that remained with no presence of skin (Fig. 3b). It is also interesting to note that compared to the 100% mass balance (DMBIS plus BBA moles) obtained without the skin tissue, only 84% of total DMBIS and formed BBA molar quantities remained after 2 h of incubation with skin. This difference is obviously due to the skin metabolism of BBA as presented and explained further in the following.

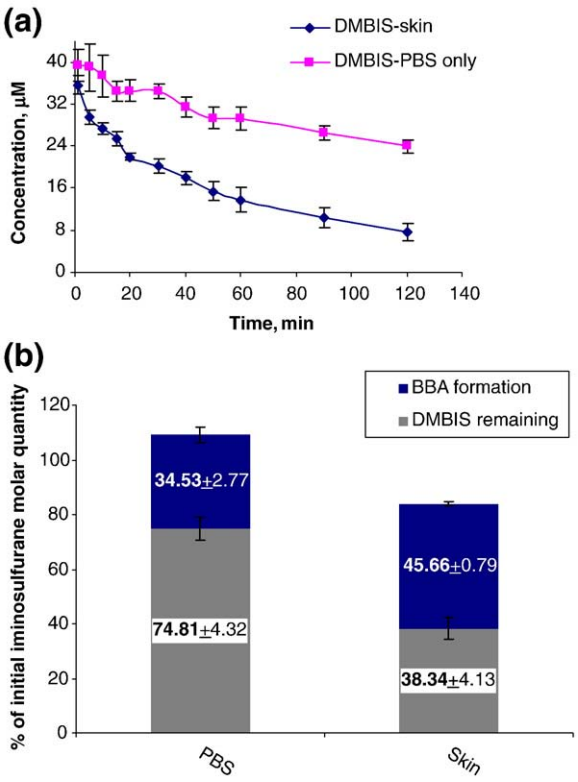


**Fig. 3.** Elimination of DMBIS in the presence of pig skin tissue compared to the elimination without skin. (a) The kinetic profiles of DMBIS elimination, and (b) the mass balance as calculated after 120 min incubation including the molar percentages of the formed BBA.

### *3.2. Percutaneous penetration of hydrocortisone co-applied with N-(4-bromobenzoyl)-S,S-dimethyliminosulfurane and 4-bromobezamide on pig skin in vitro*

Once it was obvious that DMBIS had rapidly been transformed to BBA by skin tissue and that BBA might serve as a pivotal factor in drug enhancement, the transdermal effects of both materials were evaluated and compared. Table 1 summarizes the parameters obtained for the

**Table 1**
Parameters of *in vitro* percutaneous penetration of hydrocortisone through dermatomed pig skin — enhancement by DMBIS and BBA

| Test groups | Flux (μg/cm²/h) | $Q_{24}$ (μg/cm²) | $T_{lag}$ (h) | ER[a] |
|---|---|---|---|---|
| Control (no enhancer) | 0.065 (±0.017) | 0.778 (±0.355) | 12.04 (±5.76) | 1 |
| DMBIS (4 μl 0.4 M sol) | 0.154* (±0.063) | 2.043* (±0.973) | 12.86 (±1.51) | 2.4 |
| BBA (4 μl 0.4 M sol) | 0.143* (±0.055) | 1.653* (±0.846) | 12.01 (±2.78) | 2.2 |

* Statistically different than the control group (ANOVA, $p<0.05$).
[a] Enhancement ratio (ER) was calculated by dividing the flux for skin treated with enhancer by the flux obtained without enhancer treatment (control).

percutaneous hydrocortisone delivery through dermatomed pig skin. As shown in the table, the co-application of DMBIS and BBA increased drug penetration by 2 fold (the enhancement ratios were 2.4 and 2.2 for DMBIS and BBA, respectively), relative to the control group without enhancer. Simultaneous analysis of the enhancers and the cumulative drug penetration has revealed that BBA was the major substance penetrating through the skin after DMBIS application, although it was in lesser extent than after its direct application (data not shown). Only small amounts of DMBIS were detected in the receptor solutions. These results indicate that either BBA itself implements the enhancement activity, or it may be used as a precursor for another *in situ* formed enhancer that makes the effect.

### *3.3. Elimination of 4-bromobenzamide in the presence of skin tissue obtained from mouse, rat and pig*

Fig. 4 presents the first evidence that BBA undergoes an intensive metabolic elimination by skin tissue. As seen, the concentration of the compound dropped sharply to 70–80% in 10 min followed by a slower decrease of 0.35–0.50 μg/h. Although dermatomed pig skin was more metabolically active than mouse skin (from dorsal or abdominal side), the concentration of BBA decreased to about 50% of the initial level after 6 h with both skin species. An increase in the skin–PBS ratio from 1:5 to 1:2 resulted in a further decrease in the initial concentration down to 30% after 6 h. Such a decrease (70%) in BBA concentration was observed only after 22 h if a 1:5 skin–PBS ratio had been kept (data not shown). Besides the observed decrease in the BBA peak ($R_t$=7 min) as analyzed by the HPLC assay, we have noted an increasing peak that was emerged at a longer retention time ($R_t$=16 min). This new peak possessed a UV absorbance that resembled the UV spectrum of BBA.

Metabolism of BBA was also observed in the supernatants from skin homogenates; however, its rate and extent were significantly lower than the metabolic elimination noted when sliced skin was used. The reduced activity of the cytosols (i.e., supernatants from skin homogenates) may indicate that the metabolism of this compound in the whole skin occurs mainly by the action of membrane-bound, rather than unbound, enzymes.

### *3.4. Inhibition of the metabolic elimination/transformation of 4-bromobenzamide in skin tissues — the influence of iodine*

Iodine has been proved for more than forty years as a non-irritant, safe and efficient topical antiseptic [26]. It is noteworthy that iodine is devoid of skin adverse effects, and is widely used as topical disinfectant in humans for many years without complaints on skin irritation. Moreover, recent studies have shown that iodine is counterirritant against chemical and thermal burns; namely, certain iodine formulations

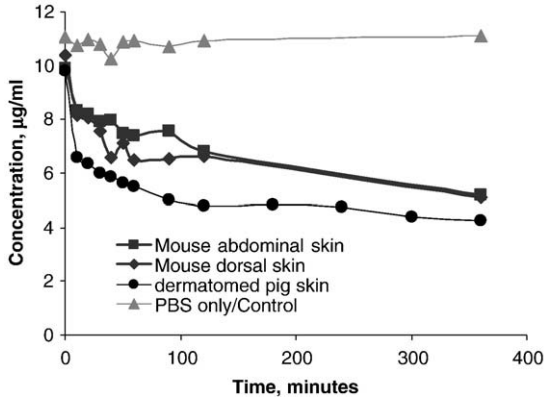


**Fig. 4.** Elimination profiles of BBA in the presence of mouse (dorsal and abdominal) and pig skin at physiological conditions *in vitro* compared to a reaction mixture without skin.

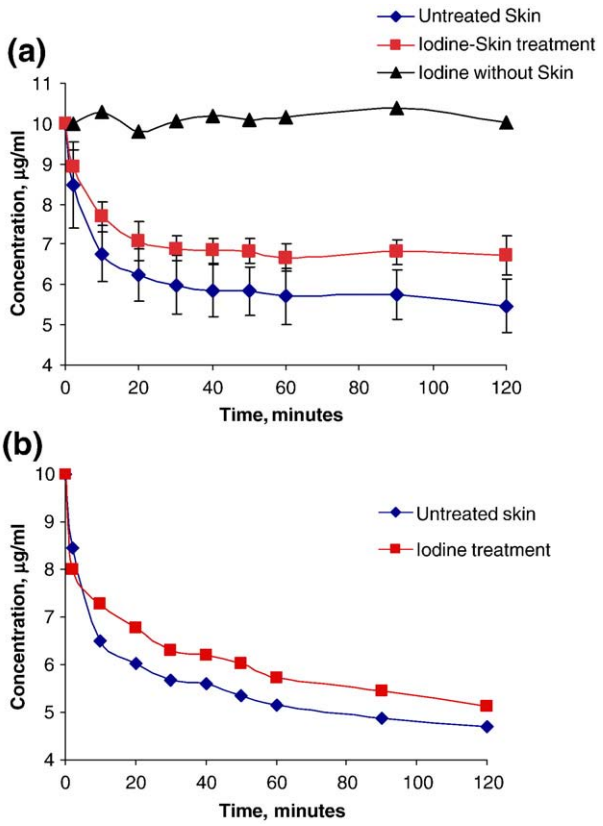


**Fig. 5.** Elimination profiles of BBA in the presence of (a) pig skin and (b) rat skin at physiological conditions *in vitro* with and without iodine pretreatment of the skin ($n$=7).

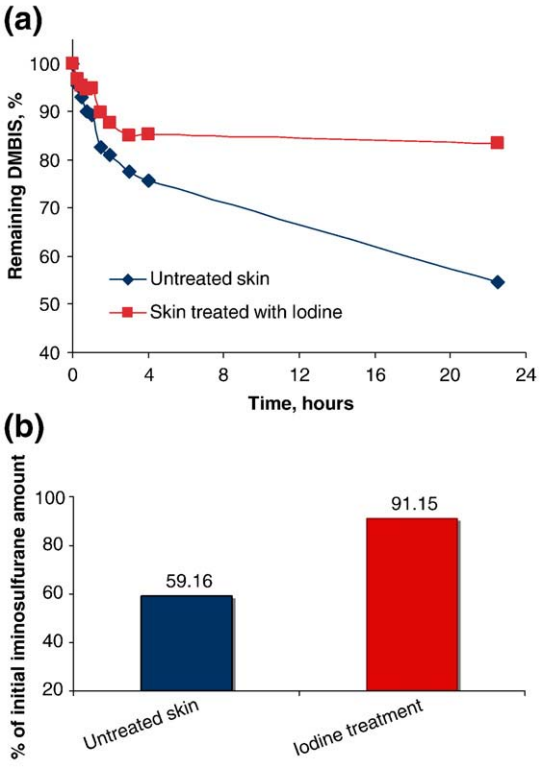


**Fig. 6.** Influence of iodine treatment of rat skin mounted in diffusion cells ($n$=2) on the elimination of BBA in the 'receptor' solution. (a) The kinetic profiles of BBA elimination, and (b) the mass balance as calculated after 22 h experiments.

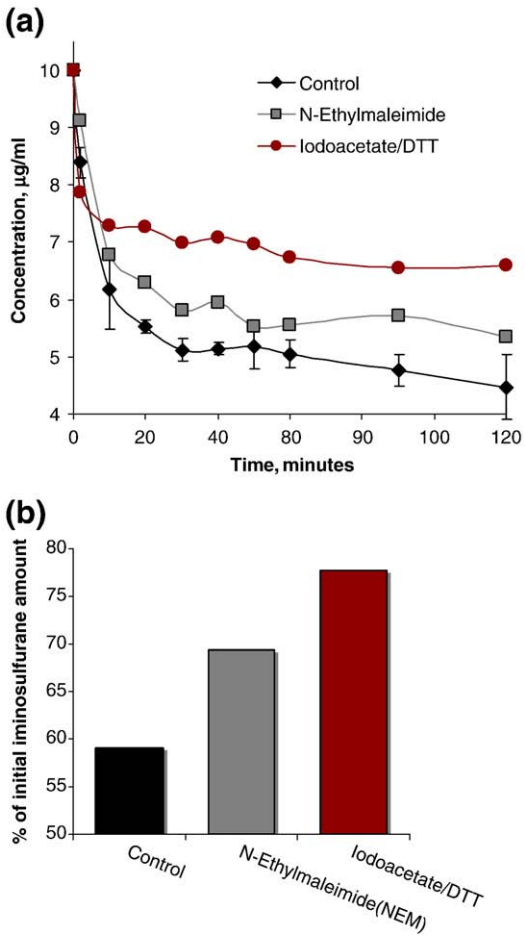


**Fig. 7.** Metabolism of BBA in the presence of rat skin tissue *in vitro* with and without pretreatment of skin with alkylating agents. The influence of iodoacetate and N-ethylmaleimide as illustrated by (a) the kinetic profiles of BBA elimination, and (b) the mass balance as calculated after 120 min incubation.

prevent or reduce heat- and irritant-induced skin damage in both humans and animals [27,28]. Iodine is a mild oxidant that can alter the properties and activity of proteins by a variety of reactions; however, when using small quantities of iodine at physiological conditions, its oxidation is limited primarily to cysteinyl residues (i.e., free thiol groups) [29]. The free thiol groups in the protein can be oxidized either to sulfenic acid (–SOH), sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$) groups, and even to a disulfide bridge (–SS–) in cases where two sulfhydryl groups exist in proximity within the protein molecule. Iodine oxidation may cause the inactivation of proteins such as urease and creatine kinase [30]. However, iodine may also result in increased protein activation such as the 16-fold enhancement of the hemolytic activity of component C2 [31].

As shown in Fig. 5, iodine slows down the metabolism of BBA in the presence of dermatomed porcine skin (Fig. 5a) or rat skin (Fig. 5b). It is interesting to note that we have never in any of our experiments observed a complete inhibition of the metabolic process by iodine. This may imply that iodine is involved in the inhibition of certain steps (probably the last step) in the enzymatic process, but it cannot modify all participating enzymes. As most, if not all, of the common metabolic reactions are reversible and become fixed when the next reaction step removes the product, it is reasonable to conceive that iodine intervenes in the last metabolic reaction.

A greater success was achieved when iodine (in $I_2$/KI aqueous solution) was applied in the donor compartment of vertical Franz diffusion cells mounted with rat skin for the entire experiment. BBA that had been exposed to the dermis side of the skin in the receptor compartment was rapidly eliminated without iodine in the donor, but its disappearance was completely halted after a few hours when iodine was present in the donor. Fig. 6a illustrates the significant difference between the elimination of BBA exposed to iodine-treated and untreated skin, while Fig. 6b presents the mass balance after 22 h, showing that $I_2$ inhibited approximately 30% of the metabolism of BBA by rat skin.

### *3.5. Inhibition of the metabolic elimination/transformation of 4-bromobenzamide in rat skin — the influence of alkylating agents*

Since the evident inhibition of the metabolic elimination of BBA by iodine is seemingly related to oxidation reactions with cysteinyl residues of the enzymes, we predict that covalent blocking of the free sulfhydryls by alkylating agents might be a better strategy to inhibit the enzymes involved in the process. Chemical modification of proteins by iodoacetic acid (IAA) and N-ethylmaleimide (NEM) has been known to involve reactions that occur specifically at the thiol groups of cysteinyl residues. The introduction of these S-alkylating agents to the reaction mixture of BBA and rat skin resulted in a significant inhibition of the metabolism by both agents (Fig. 7a/kinetics and Fig. 7b/mass balance). In particular, iodoacetate/dithiothreitol (IAA/DTT) demonstrated a greater inhibition of the enzymatic reactions than NEM. This may be a consequence of using DTT, which reduced existing disulfide bonds to free thiols, thus increasing the availability of more thiol groups to S-carboxymethylation by IAA. As demonstrated in Fig. 7, permanent chemical modification of enzymes by alkylating agents, especially IAA with DTT, inhibited the metabolism of BBA in the presence of skin tissue.

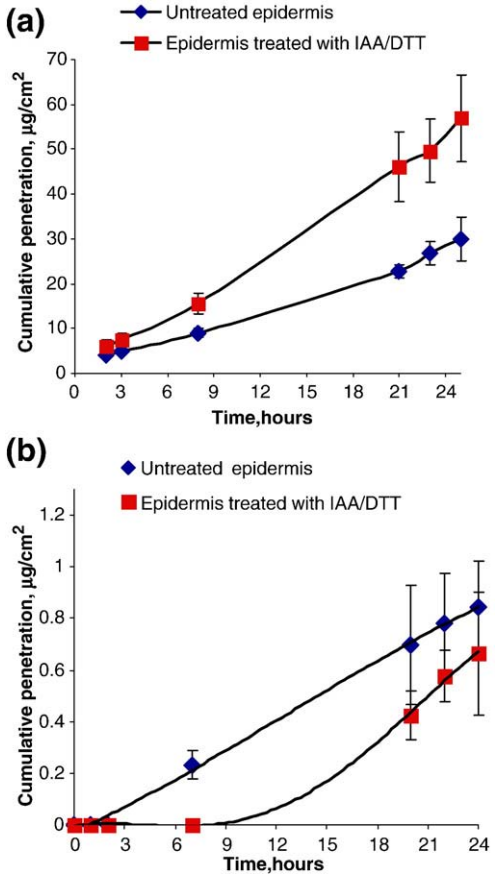


**Fig. 8.** *In vitro* percutaneous penetration of BBA (a) and hydrocortisone (b) co-applied on human epidermis. Comparison between untreated epidermis and pretreatment with iodoacetate/dithiothreitol ($n=6$).

**Table 2**
Influence of epidermal treatment with iodoacetate and dithiothreitol on the *in vitro* penetration enhancement of hydrocortisone by 4-bromobenzamide

| Test groups | Flux (μg/cm²/h) | $Q_{24}$ (μg/cm²) | $T_{lag}$ (h) |
|---|---|---|---|
| Untreated human epidermis | 0.039 (±0.012) | 0.845 (±0.175) | 1.62 (±0.708) |
| Human epidermis treated with IAA/DTT | 0.038 (±0.010) | 0.663 (±0.239) | 12.41 (±4.652)* |

* Significantly different as compared with $T_{lag}$ obtained from the untreated group ($n=3$, $p<0.05$ according to Student's *t*-test).

### *3.6. The influence of iodoacetate/dithiothreitol treatment on the penetration of BBA and on the enhancement of hydrocortisone penetration by BBA through human cadaver epidermis*

Once we have shown that BBA is extensively metabolized by skin, a 'proof-of-concept' is required to determine whether or not the skin metabolism is correlated with the observed enhancement activity of a model drug. Although bearing some limitations, skin penetration studies of hydrocortisone are commonly performed *in vitro* and their relevance to *in vivo* investigations is well established [32,33]. Therefore, in an *in vitro* permeation study using human epidermis, we compared between the permeabilities of untreated skin and IAA/DTT-treated skin to hydrocortisone in the presence of BBA. It is worth noting that a vehicle control was not used in these experiments since BBA has already been proved in a previous report to have a significant enhancing effect for hydrocortisone over propylene glycol as a vehicle control [17].

Fig. 8 and Table 2 present the results of these experiments. It was obvious from the previous experiments that, due to inhibition of the metabolism of BBA in the skin, more molecules would be retained and transported into the receptor than with untreated skin. Fig. 8a shows a higher penetration of BBA through the IAA/DTT-treated epidermis than the untreated epidermis. Conversely, the extent of the penetration of hydrocortisone through the untreated epidermis was higher (and had much shorter lag time) than its penetration through IAA/DTT-treated epidermis (Fig. 8b). Although the permeation fluxes were similar in both groups, hydrocortisone started to penetrate 2 h after its application onto the untreated epidermis in contrast to the 12 h delay demonstrated after the enzymes were inhibited, as calculated by a linear regression method (Table 2). It is evident, therefore, that at least one enzymatic reaction is involved in the enhancement activity of BBA; by inhibition of the enzymatic reaction, the enhancement activity is also inhibited.

In summary, this initial study using hydrocortisone as a model drug offers a new direction towards future development of non-toxic penetration enhancers. In fact, we have provided clear evidence that skin biotransformation of BBA is responsible for the enhancement effect of hydrocortisone only. Similar experiments, however, using drugs such as caffeine, lidocaine, bumetanide, and diclofenac failed to show true results due to a rapid metabolism of the drug itself in the skin while a significant inhibition of this metabolism occurred in the presence of the alkylating agent. Further work should be done with other well-selected model drugs, more specific enzyme inhibitors, and other bromoaromatic derivatives to substantiate the mechanism of action and the metabolic process in the skin.

## 4. Conclusions

The search for an ideal skin penetration enhancer has been the focus of a considerable research effort over the last decades. Unfortunately, no chemical enhancer has yet been found to possess ideal properties; furthermore, many enhancers are toxic, acting as skin irritants or allergens at some level (depending on their concentration and the frequency of their treatment). Generally, the toxicity of a potent enhancer is associated with its enhancement effect, indicating that its mechanism of action is related to chemical interaction with skin components or structures. In this paper, we described for the first time a new mechanism of action by which a non-irritant and powerless "enhancer" (i.e., a pro-enhancer) is activated by its own metabolism in the skin tissue. To understand the mechanism of enhancement by S,S-dimethyl-N-(4-bromobenzoyl)iminosulfurane, or more practically, by its immediate product, we evaluated its metabolic elimination of 4-bromobenzamide in the presence of skin from various species, inhibited its biotransformation by iodine and alkylating agents, and eventually proved that this biotransformation is essential for its enhancement activity. Relative to the commonly-known enhancers, greater advantages could be gained by this type of an *in situ* formed enhancer, such as (a) a continuous and slow release, (b) the presence of only small quantities of the active enhancer/metabolite at a given time, and (c) an enhancer's capability to being rapidly eliminated. Although it is now evident that an effective enhancement activity can be generated by a metabolite, an understanding of BBA metabolism, and in particular, the nature of the new active molecule/s is needed. Hence, it is of great interest to study and analyze the chemical structure of the active bioform of BBA; such research may trigger development of a new generation of potent enhancers and permitting a better understanding of their structure–activity relationships.

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