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# Topical application of Azone analogs to hairless mouse skin: a histopathological study

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**Abstract**

Eight dermal penetration enhancers were evaluated for irritancy potential on hairless mice. The enhancers included propylene glycol and isopropyl myristate as controls. Novel enhancers included: Azone or 1-dodecylhexahydro-2*H*-azepin-2-one (**1**); *N*-dodecyl-2-pyrrolidinone (**2**); *N*-dodecyl-2-piperidinone (**3**); *N*-dodecyl-*N*-(2-methoxyethyl)acetamide (**4**); *N*-(2,2-dihydroxyethyl)dodecylamine (**5**); and 2-(1-nonyl)1,3-dioxolane (**6**). The analogs were tested at concentrations of 10% (approx. 0.4 M) in the vehicle propylene glycol and at 100%. Plastic cups containing the solutions were attached to the dorsal side of the animals ($n = 3$) for 24 h. A biopsy technique was used and the treated skin and adjacent untreated skin were fixed in 10% buffered formalin, embedded in paraffin, and stained with haematoxylin and eosin. Histological examination coupled with visual observation allowed for assessment of damage to the epidermal and dermal layers of the skin. Propylene glycol and isopropyl myristate were found to have no discernible effects on the skin even at 100%. Enhancers **1** and **6** were found to have virtually no effect on the skin at 10% in propylene glycol. Enhancers **2**, **3**, and **5** at 10% were found to have some effects on the skin and are considered to be mild-to-moderate irritants. Enhancer **4** at 10% and enhancers **1**, **2**, **3**, and **6** at 100% were found to cause severe irritation to the skin.

*Keywords:* Azone; Penetration enhancer; Percutaneous absorption; Skin irritancy; Hairless mouse

## 1. Introduction

Penetration enhancers are chemical compounds that interact with stratum corneum lipids allowing better penetration of coadministered compounds through this relatively impermeable barrier. The outermost layer of the skin, the stratum corneum, is responsible for the protection of the body from xenobiotics and environmental hazards (Idson, 1975). The stratum corneum contains intercellular regions of lipids that are arranged in a lamellar phase (Elias, 1981; Bouwstra et al., 1992). These lipids consist of ceramides, cholesterol, glycerides, and fatty acids. Barry has graphically described the stratum corneum as a structure of 'protein bricks in a lipid mortar' (Barry and Bennett, 1987). It is thought that lamellar bodies within the epidermis secrete membrane coating granules and Odland bodies, which fill the upper epithelial interstices,

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effectively forming the lipid mortar between the flattened keratin bricks, which forms the barrier against the outward passage of polar substances (Elias and Friend, 1975; Elias et al., 1977).

There are three possible pathways of diffusion through the stratum corneum – the transappendageal route, the intercellular route, and the intracellular route (Idson, 1975). The transappendageal route consists of hair follicles, sebaceous glands, sweat glands, and apocrine glands which can provide entry ports for drugs applied topically. The impact of this route of transdermal delivery is considered to be insignificant as it represents only a small portion of the total skin surface area at $< 0.001\%$ (Scheuplein and Blank, 1971). It is thought that transient penetration occurs via the shunt pathway and that steady-state transport occurs via the intercellular pathway (Idson, 1975). Since the cells of the stratum corneum are packed so tightly together, it is thought that the intracellular pathway plays little, if any, part in the diffusion of drugs through this barrier (Idson, 1975).

Polar and nonpolar substances diffuse through the stratum corneum by different molecular mechanisms (Blank, 1967). The nonpolar or lipophilic pathway consists of penetration through the continuous intercellular lipid phase of the stratum corneum. The polar or hydrophilic pathway refers to the alternate passage through hydrophilic cellular protein and intercellular lipids of the stratum corneum. Barry suggests that the hydrophilic route is the main pathway by which drugs and other substances diffuse across the stratum corneum and subsequently through the layers of the epidermis and dermis and into the systemic circulation after treatment with the enhancer Azone (Barry and Bennett, 1987). The degree of hydrophilicity or lipophilicity of the drug being delivered transdermally determines how it will partition into and through the stratum corneum barrier. Azone is thought to accelerate penetration of a drug via the hydrophilic molecular mechanism. The structure of Azone is thought to be relevant to this role (Brain and Walters, 1993). It consists of a seven-membered ring and a long hydrocarbon side chain. This structure imparts both lipophilic and hydrophilic characteristics to the compound, allowing it to partition between the lipophilic mortar substance and the hydrophilic protein domains, probably fluidizing the lipids in the skin and hence allowing drug to pass through (Lewis and Hadgraft, 1990). As the enhancers in this study were based on the structure of Azone, it is thought that they may function via a similar mechanism.

This study evaluated and measured cutaneous irritation caused by the topical application of Azone analogs. It has been postulated that these analogs reversibly alter the skin structure, allowing penetration of a drug through the stratum corneum. Histological examination was utilized since it is able to distinguish between mild and moderate irritants (Lashmar et al., 1989). Visual patch test reactions have been utilized as an early indicator of irritation in the past, but these tests usually rely on dermal reactions, which effectively give responses that indicate that there is no irritation or severe irritation present and therefore, do not take into account the mild to moderate irritants (Landsdown, 1972). The developed method presented in this study is able to distinguish between epidermal and dermal changes in histological structure after application of a potentially irritating compound, thereby allowing differentiation between mild and moderate irritants. It was first developed by Ingram and Grasso (1975) and later modified by Lashmar et al. (1989). The method was further modified by the use of hairless mice as the model to evaluate a new series of penetration enhancers that have been evaluated in vitro and showed relatively good enhancement (Michniak et al., 1993a,b, 1994a,b, 1995a,b).

There have been several reports on the toxicity of Azone. Lashmar et al. (1989) report that Azone is at least a moderate irritant when applied topically. Ismail et al. (1992) found Azone to be unacceptably irritating to ocular tissues when used at concentrations as low as 0.7%. Barry and Bennett (1987) state that Azone is not irritating, except at higher concentrations. In a thorough review of toxicity potential of Azone, the authors concluded that Azone was not toxic in humans, although animal models had indicated the potential for irritation (Vaidyanathan et al., 1987). This method was a more objective way of evaluating

irritancy potential and should clarify the question of Azone's potential for irritancy.

The purpose of this study was to determine the relative irritancy of new compounds based on the structure of Azone. The data also gave an indication of the general location where these enhancers may function and hence may give some insight into their possible mechanism of action.

## 2. Materials and methods

### 2.1. Materials

Several penetration enhancers were selected for this study. Propylene glycol (PG) and isopropyl myristate (IPM) were chosen as controls. The compounds (Fig. 1) investigated include the following: Azone or 1-dodecylhexahydro-2*H*-azepin-2-one (**1**); *N*-dodecyl-2-pyrrolidinone (**2**); *N*-dodecyl-2-piperidinone (**3**); *N*-dodecyl-*N*-(2-methoxyethyl)acetamide (**4**); *N*-(2,2-dihydroxyethyl)dodecylamine (**5**); and 2-(1-nonyl)-1,3-dioxolane (**6**).

Propylene glycol (lot no. 116F-0371) and isopropyl myristate (98% purity, lot no. 62H0421) were purchased from Sigma Chemical Co. (St. Louis, MO). Enhancer **2** was purchased commercially from Aldrich Chemical Co. at 99% purity (lot no. 04629CV). Compounds **1**, **3**, **4**, **5**, and **6** were synthesized in our laboratory with purity of 99 + % as assessed by elemental analysis by Atlantic Microlabs (Atlanta, GA). The compounds were found to be within 0.4% of theoretical. Synthesis of these compounds has been presented previously (Michniak et al., 1993a,b, 1994a,b).

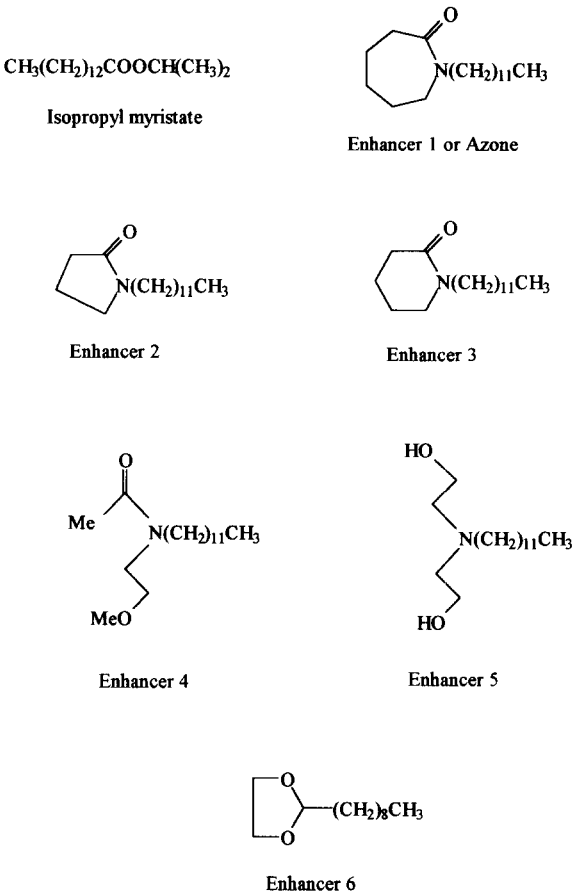


Fig. 1. Chemical structures of Azone analogs evaluated.

### 2.2. Methods

This study was based upon one conducted by Lashmar et al. (1989) in which athymic nude mice were used. Hairless mice were utilized in this study with PG and IPM as controls. Enhancer **1** or Azone (originally patented by Rajadhyasksha, 1976) was chosen.

The compounds were tested as 10% v/v solutions in propylene glycol, a common vehicle for topical formulations. Enhancers, such as Azone, are generally effective at concentrations < 10% (approx. 0.4 M). The compounds were also tested at 100% to assess their maximum irritancy potential.

Male hairless mice (strain SKH1 hr/hr, Charles River Laboratories Inc., Wilmington, MA) at the age of 8 weeks were used. The animals were not pretreated in any way before the experiment.

The test compounds were filled into a plastic cup (modified from Nunc caps for microfuge tubes by InterMed, Denmark) of surface area 1 $cm^2$ and volume of 0.3 $cm^3$. One cup was fastened to the dorsal side of each animal using surgical tape (Dermiform * hypo-allergenic knitted tape, John-

son and Johnson Medical Inc., Arlington, TX). The mice were held upside down when the cup was fastened to the dorsal side. Superglue (Devcon Corp., Wood Dale, IL) was placed on the edges of the cup prior to placement on the skin to ensure a tight seal. Three animals were exposed to each preparation and were maintained in separate cages with food and water freely available throughout the experiment. The materials were kept in contact with the skin for 24 h.

Immediately after the mice were killed with $CO_2$, specimens of the exposed areas and of an adjacent untreated skin area were taken for histological examination. Gross visual evaluation of the exposure site was not recorded or evaluated; however, there did not appear to be significant signs of erythema or edema. Animals whose cups were dislodged, or showed signs of leakage were not used (rejection rate was approx. 10%). The skin pieces were immediately fixed in 10% buffered formalin for 72–96 h. The specimens were pre-embedded in agar to ensure orientation. After the tissue was processed, it was treated

Table 1
Histological scoring system

| | Epidermal changes | |
|---|---|---|
| A | Epidermal thickening | |
| | 2× normal in places | 1 |
| | 2× normal generally | 2 |
| | 2–3× normal in places | 3 |
| | 2–3× normal generally | 4 |
| | More than 3× normal | 5 |
| B | Increase in the cell layers of the stratum granulosum | |
| | by 1 cell layer | 1 |
| | by 2 cell layers | 2 |
| | by 3 cell layers or more | 3 |
| C | Hyperkeratosis (formation of skin overgrowth) | |
| | mainly loose | 1 |
| | mainly severe | 2 |
| | half loose, half compact | 3 |
| | compact | 4 |
| | compact severe | 5 |
| D | Spongiosis (intercellular edema) | |
| | slight | 1 |
| | extensive | 2 |
| | microvesicle formation | 3 |
| | bullae formation | 4 |
| E | Intracellular edema | 1 |
| F | Destruction of the epidermis (loss of cells and structure) | |
| | superficial | 15 |
| | 1/4 of sectioned area | 18 |
| | 1/2 of sectioned area | 20 |
| | 3/4 of sectioned area | 25 |
| | whole of sectioned area | 30 |
| G | Hyperemia (increased blood supply) | |
| | slight | 5 |
| | moderate | 10 |
| | extensive | 15 |

Table 1 (continued)

| | Dermal changes | |
|---|---|---|
| A | Increase in the density and thickness of the collagen bundles | |
| | slight (an increase in places) | 1 |
| | slight-moderate (an increase almost throughout) | 2 |
| | moderate (an increase throughout) | 3 |
| | moderate-severe (bundles appear as continuous mass in places) | 4 |
| | severe (bundles appear as continuous mass throughout) | 5 |
| B | Fractured collagen | |
| | slight (fractured in places) | 1 |
| | moderate (more than half the layer fractured) | 2 |
| | severe (fractured throughout) | 3 |
| C | Infiltration of the dermis (cellular infiltration; mixing of epidermal elements) | |
| | slight in the uppermost layer | 1 |
| | slight diffuse | 2 |
| | moderate in the uppermost layer | 3 |
| | moderate diffuse | 4 |
| | severe in the uppermost layer | 5 |
| | severe diffuse | 6 |

with xylene as a transitional medium, and embedded in paraffin (Histomatic Tissue Processor Model 166MP by Fisher Scientific). The tissue cassettes were removed from the processor and re-embedded in paraffin to increase the infiltration of the tissue (HistoEmbedder by Jung). Sections of 5 $\mu$m thickness were cut from each sample (Reichert-Jung 2030 Cutter with Leica Biocut blade), the slides were dried in a Lipshaw Electric Laboratory Dryer (Model 207), dehydrated through a graded series of alcohols, and stained with haematoxylin-eosin for microscope observation. Three sections were selected randomly and examined using a scoring system as designed by Ingram and Grasso (1975) and later modified by Lashmar et al. (1989) (Table 1). The final score was the average score from at least three animals. Scores from epidermal changes were added to the scores from dermal changes to determine the compound's relative irritancy score. An overall score of 0–10 indicates that the preparation is minimally irritating to the skin of hairless mice. A score of 11–25 (mild irritant) indicates that the preparation does have an effect on the epidermal and dermal layers of the skin, but this does not necessarily preclude their inclusion in topical and/or transdermal products. A score greater than 25 (moderate irritant) indicates that the preparation is modestly irritating to the skin and should undergo further testing before their inclusion in topical and/or transdermal products.

## 3. Results and discussion

The results from each preparation are summarized in Table 2 and are illustrated in Figs. 2–11. These scores allow for comparison of irritation or inflammatory effects due to the application of the different enhancers. Based on this scoring system, we found that propylene glycol and isopropyl myristate have no discernible effects on the skin even at 100%. Enhancers **1** and **6** had virtually no effect on the skin at 10% in propylene glycol. Enhancers **2**, **3**, and **5** at 10% had some effects on the skin, their scores falling right at the border distinguishing mild from moderate irritants. Enhancer **4** at 10% and enhancers **1**, **2**, **3**, and **6** at 100% were found to cause the most severe irritation to the skin. There was no evidence of erythema, edema, or dryness of the skin, which are the visual signs of skin irritation. Treatments of enhancers **4** and **5** at 100% were not conducted as the compounds were found to be moderately to severely irritating to the animals at 10%. It was thought that these treatments would be unnecessarily irritating to the animals in the study.

Table 2
Irritation scores of Azone analogs on the skin of hairless mice ($n = 3$ for each preparation)

| Azone analog | Concentration (%) | Epidermal score | Dermal score | Total score |
|---|---|---|---|---|
| PG | 100 | 3.7±0.9 | 3.3±0.5 | 7.3±1.2 |
| IPM | 10 | 7.0±0.8 | 3.3±0.5 | 10.3±0.5 |
| IPM | 100 | 5.3±1.2 | 3.0±0.0 | 8.3±1.2 |
| **1** | 10 | 5.7±0.9 | 3.3±0.9 | 9.0±0.0 |
| **1** | 100 | 27.3±2.4 | 3.0±0.0 | 30.3±2.4 |
| **2** | 10 | 25.0±0.0 | 1.0±0.0 | 26.0±0.0 |
| **2** | 100 | 29.0±1.4 | 2.0±0.0 | 31.0±1.4 |
| **3** | 10 | 19.3±1.2 | 4.7±1.2 | 24.0±1.6 |
| **3** | 100 | 30.0±0.0 | 2.3±0.5 | 32.3±0.5 |
| **4** | 10 | 35.0±0.0 | 0.0±0.0 | 35.0±0.0 |
| **5** | 10 | 25.0±0.0 | 1.3±1.2 | 26.3±1.2 |
| **6** | 10 | 7.3±0.5 | 2.0±0.0 | 9.3±0.5 |
| **6** | 100 | 29.0±0.0 | 3.0±0.0 | 32.0±0.0 |

As can be seen from Table 3, there does not appear to be any correlation between efficacy of these enhancers with their relative irritancy. Two model drugs were evaluated with the compounds presented in this study. 5-Fluorouracil is a hydrophilic drug that benefited greatly from co-administration with dermal penetration enhancers. Ibuprofen is a lipophilic drug and the co-administration of enhancers had virtually no effect on the drug's penetration through hairless mouse skin. There was no correlation between efficacy of these enhancers and their relative irritancy for either model drug ($r = 0.12$, $r^2 = 0.015$ for 5-fluorouracil and $r = -0.27$, $r^2 = 0.072$ for ibuprofen) (Table 3). It has been proposed that the most effective enhancers are also the most irritating (Lashmar et al., 1989). These analogs do not appear to follow that pattern. This could indicate that these enhancers cause irritation by different

Fig. 2. Hairless mouse skin: untreated dorsal area showing clear delineation between epidermis and dermis. H&E × 10.

Fig. 3. Dorsal area treated with 10% enhancer **1** for 24 h under occlusion. Section shows slight epidermal thickening and an increase in the cell layers of the stratum granulosum. Moderate hyperkeratosis is present. There is a slight increase in the density of the collagen bundles and there is some infiltration into the dermis. H&E × 10.

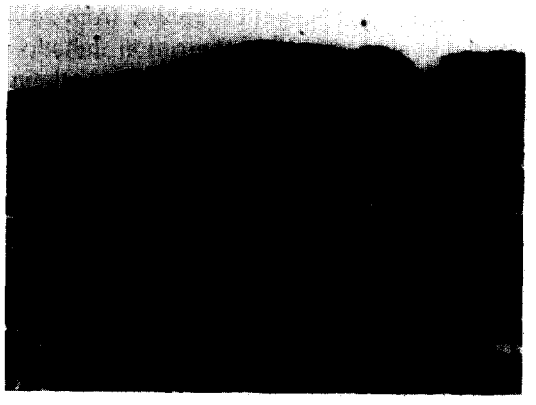

Fig. 4. Dorsal area treated with 10% enhancer **3** for 24 h under occlusion. Section shows compact hyperkeratosis and moderate destruction of the epidermis. The density of the collagen bundles is increased in places and appears slightly fractured. There is some infiltration into the dermis. H&E × 10.

Fig. 5. Dorsal area treated with 10% enhancer **4** for 24 h under occlusion. Section shows severe hyperkeratosis and complete destruction of the epidermis. Collagen appears normal and there does not appear to be any infiltration into the dermis. H&E × 10.

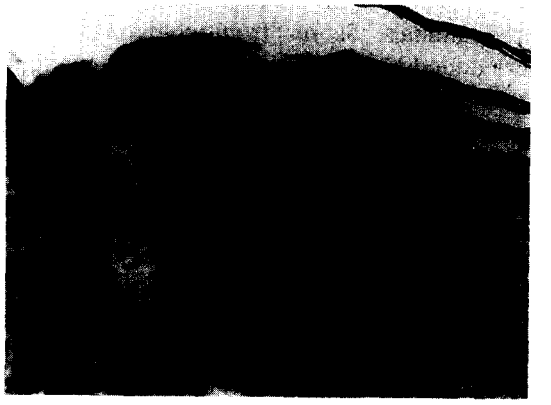

Fig. 6. Dorsal area treated with 10% enhancer **5** for 24 h under occlusion. Section shows severe hyperkeratosis with moderate destruction of the epidermis. Collagen appears normal but there is extensive infiltration into the dermis. H&E × 10.

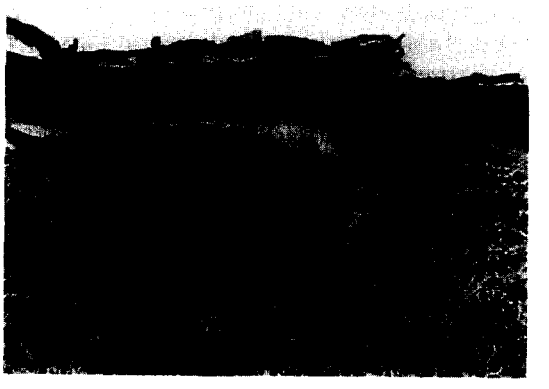

Fig. 7. Dorsal area treated with 10% enhancer **6** for 24 h under occlusion. Section shows some swelling in the epidermis and increase in the cell layers of the stratum granulosum. Hyperkeratosis is present to a slight extent. There is slight infiltration into the dermis. H&E × 10.

Fig. 8. Dorsal area treated with 100% enhancer **1** for 24 h under occlusion. Section shows compact hyperkeratosis and almost complete destruction of the epidermis. There appears to be some infiltration into the dermis. H&E×10.

Fig. 10. Dorsal area treated with 100% enhancer **3** for 24 h under occlusion. Section shows severe hyperkeratosis and acanthosis as well as almost complete destruction of the epidermis. There appears to be moderate infiltration into the dermis. H&E×10.

mechanisms of action than those reported in the aforementioned study.

Overall, it appears that hairless mouse skin gives a lesser response to irritants than nude mouse skin. In the study conducted by Lashmar et al., propylene glycol received an irritancy score of 11 at 50% concentration, whereas it received a score of 7.3 at 100% in this study. Additionally, Azone received a score of 45 at 10% concentration in study by Lashmar et al. whereas it received a score of 9 in this study. This could be due to a number of factors. The method is highly subjective, so evaluation of scores should be conducted by a single person, as it was in this study. In the study of Lashmar et al., it is not stated whether one person evaluated all of the data. The animal model used was different in the studies, which could indicate strain differences in tolerance to these compounds. Also, in the study of Lashmar et al., the authors applied the compounds in water or in combination with neutral-

Fig. 9. Dorsal area treated with 100% enhancer **2** for 24 h under occlusion. Section shows severe hyperkeratosis and acanthosis and almost complete destruction of the epidermis. This is slight infiltration into the dermis. H&E×10.

Fig. 11. Dorsal area treated with 100% enhancer **6** for 24 h under occlusion. Section shows almost complete destruction of the epidermis as well as fracturing of collagen bundles. Acanthosis is present. H&E×10.

Table 3
Correlation of efficacy of enhancers with relative irritancy score at 10%

| Enhancer | Relative irritancy score ($n = 3$) | $ER_{flux}$ for 5-fluorouracil [a] ($n = 4$) | $ER_{flux}$ for ibuprofen [a] ($n = 4$) |
|---|---|---|---|
| 1 | 9.0±0.0 | 15.91±3.00 | 1.13±0.09 |
| 2 | 26.0±0.0 | 34.73±3.31 | 1.15±0.13 |
| 3 | 24.0±1.6 | 15.56±1.14 | 1.19±0.26 |
| 4 | 35.0±0.0 | 6.68±0.81 | 1.30±0.24 |
| 5 | 26.3±1.2 | 2.44±0.43 | 1.20±0.18 |
| 6 | 9.3±0.5 | 1.01±0.14 | 1.53±0.16 |

Linear regression analysis comparing relative irritancy with efficacy of enhancers with two model drugs: for 5-fluorouracil: $r = 0.12$, $r^2 = 0.015$; for ibuprofen: $r = -0.27$, $r^2 = 0.072$. ER, enhancement ratio.
[a] Phillips and Michniak, unpublished data.

ized Carbopol 940. A direct comparison of the methods was not performed.

Most of the irritation caused by these compounds occurred in the layers of the epidermis. Close examination of the sections revealed the presence of neutrophils, precursors to an inflammatory response. It was expected that enhancers would produce some kind of an inflammatory response, but the extent of this reaction is unknown. Long-term testing and repeated exposures would determine the severity of these responses.

Cyclic enhancers are more effective as penetration enhancers (Michniak et al., 1993a,b, 1994a,b). This could be due to the fact that the cyclic structures possess the seemingly required 'spoon shape' necessary for penetration enhancement. Molecular modeling analysis has shown that Azone possesses an electronegative site at the carbonyl moiety with no complementary positive site. Intercalation of Azone into the ceramide matrix will therefore leave an unbalanced electronegative site on the ceramide, which could give rise to a permeable defect. Azone analogs that possess two electronegative sites, i.e., cyclic analogs, are more effective enhancers owing to increased electrostatic repulsion. It has also been reported that linear alkyl chain analogs of Azone are less effective in permeation enhancement (Brain and Walters, 1993).

Cyclic enhancers seem to have been tolerated better than the acyclic structures. There have been no reports in the literature to indicate a correlation between mode of action of these compounds and their potential for irritancy. It is possible that the cyclic analogs were tolerated to a greater extent because of their electrostatic properties, which also allow them to function as better enhancers. Since the acyclic analogs do not possess the necessary electrostatic properties to be good enhancers, it is possible they do not intercalate into the ceramide matrix as readily, leading to their recognition as a foreign body and therefore eliciting a stronger immune response. All of the enhancers had limited effects on the dermis after a 24 h exposure, indicating that the enhancers did not penetrate far into the dermis and therefore, would be less likely to be taken up systemically.

Histological examination of skin is a more objective means of determining irritancy potential and allows for greater precision in localization of the effects. Although this system is a satisfactory indicator of relative irritancy, it should be used in conjunction with long-term exposure studies and repeated exposure studies to more fully evaluate irritancy of compounds.

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