In situ chemical behaviour of methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI) in reconstructed human epidermis: a new approach to the cross-reactivity issue

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Summary

Background. Methylisothiazolinone (MI) [with methylchloroisothiazolinone (MCI) in a ratio of 1:3, a well-recognized allergenic preservative] was released as an individual preservative in the 2000s for industrial products and in 2005 for cosmetics. The high level of exposure to MI since then has provoked an epidemic of contact allergy to MI, and an increase in MI/MCI allergy. There are questions concerning the MI/MCI cross-reaction pattern.

Objectives. To bring a new perspective on the MI/MCI cross-reactivity issue by studying their *in situ* chemical behaviour in 3D reconstructed human epidermis (RHE).

Methods. MI and MCI were synthesized with ^{13}C substitution at positions C-4/C-5 and C-5, respectively. Their *in situ* chemical behaviours in an RHE model were followed by use of the high-resolution magic angle spinning nuclear magnetic resonance technique.

Results. MI was found to react exclusively with cysteine thiol residues, whereas MCI reacted with histidines and lysines. The reaction mechanisms were found to be different for MI and MCI, and the adducts formed had different molecular structures.

Conclusion. In RHE, different MI/MCI reactions towards different nucleophilic amino acids were observed, making it difficult to explain cross-reactivity between MI and MCI.

Key words: cross-reactivity; HRMAS NMR; methylchloroisothiazolinone; CAS no. 2682-20-4; methylisothiazolinone; reaction chemistry; reconstructed human epidermis; CAS no. 26172-55-4.

Methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI) are the active ingredients of a preservative (MI/MCI 1:3 combination) that has been used since the 1980s and is one of the most common sources of allergic contact dermatitis caused by preservatives (1, 2). Following the introduction in the EU of a 15 ppm use limit in cosmetics, contact allergy to MI/MCI significantly decreased to a prevalence of ~ 2% after the 1990s (3). The sensitizing potential of the mixture was mostly attributed to the chlorinated derivative MCI, which was shown to be the stronger sensitizer, with the non-chlorinated MI being reported to be a much weaker allergen (4–6). Thus, MI alone started to be used as a preservative in the early 2000s in industrial products and in 2005 in cosmetics, but at higher concentrations than in the MI/MCI mixture, owing to its lower preservative potential. As a

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consequence, in recent years there has been an alarming increase in the prevalence of allergic contact dermatitis caused by MI (7, 8). Occupational cases of contact dermatitis caused by MI started to be reported with the use of paints (9), followed by non-occupational cases essentially seen with the use of wet wipes for hygiene and cosmetics (10, 11). Severe cases of airborne and systemic allergic dermatitis have appeared recently, caused by exposure to MI present particularly in water-based wall paints (12, 13). At the same time, the frequency of MI/MCI contact allergy has increased significantly over the past few years (7, 8, 14, 15). It has been proposed that the increased frequency of MI/MCI contact allergy is linked to the higher consumer exposure to MI, and is most probably attributable to previous sensitization of individuals to MI.

There is a critical issue concerning the use of MI and MCI in consumer products. Whereas MI was reported to be a weak sensitizer in the guinea pig (4), it has been classified as a strong sensitizer in the local lymph node assay (16). MI is currently allowed in leave-on and rinse-off cosmetic products at a maximum concentration of 100 ppm (17), whereas, so far, there are no limitations in industrial products. MI/MCI is patch tested at 200 ppm (50 ppm MI/150 ppm MCI) in water in the European baseline series. It was previously tested at 100 ppm, but, at this concentration, a significant increase in the percentage of contact allergy reactions to MI was not detected, as the mixture contained a concentration of MI that was too low. MI has been recently included in the European baseline series for patch testing at 2000 ppm in water (18). The Scientific Committee on Consumer Safety of the European Commission adopted an opinion on December 2013 in which it was stated that 100 ppm MI in cosmetic products was not safe for the consumer, and that a maximum concentration of 15 ppm was considered to be safe but only in rinse-off products (19).

Because of the presence of preservatives containing only MI on the market, questions have been raised among clinicians about the potential cross-reaction pattern between MI and MCI, but only a few studies reporting cross-reaction data of MI and MCI exist in the literature. This is probably directly related to the limited experimental approaches available so far to investigate cross-reactions between chemicals. A first approach is to use experimental animal models such as the guinea-pig maximization test (GPMT) and to perform challenge studies on animals previously sensitized to individual chemicals. On the basis of such challenge studies, it was reported that cross-reaction between the non-chlorinated and the chlorinated isothiazolinones was not relevant when MCI was the initial sensitizer, and only possible, probably non-significant, cross-reactivity was indicated when MI was the initial sensitizer (5). However, transposition of animal data to humans is always a matter of debate, owing to interspecies variability (differences in skin penetration, metabolism, detoxication pathways, etc.). On the other hand, investigation of cross-reaction patterns in humans is very difficult, especially when a concomitant exposure can be foreseen (MI/MCI, 1:3). The retest method proposed by Rustemeyer et al. (20) and based on the presence of memory T cells at the patch test side could give valuable information, but is not often used. From the clinical point of view, patch test studies have been performed recently with recruited patients reacting to MI/MCI and MI being additionally patch tested with MI/MCI, MI, and MCI (21). The studies concluded that high patch test reactivity to MCI was in support of MCI being the primary sensitizer with cross-reactivity to MI, and that high patch test reactivity to MI was in support of MI being the primary sensitizer with cross-reactivity to MCI. Nevertheless, despite the importance of such studies, true cross-reactivity between MI and MCI has not yet been clearly shown, and still remains unclear.

The first key step in the process leading to sensitization to chemicals, and subsequent skin allergy development, is the formation of stable antigenic entities by reaction with amino acids of epidermal proteins (22). The sensitization potential of a chemical is thus directly related to its chemical reactivity behaviour towards skin proteins and amino acid specificity. From a chemical point of view and in the strict sense, different allergens can cross-react when the same T cells are activated by similar epitopes. In other words, cross-reacting allergens should produce chemical protein modifications similar enough that the antigenic determinant, presented at the surface of antigen-presenting cells in association with major histocompatibility complex molecules, will activate the same T cell receptors. However, in most cases, the nature of the chemical modifications to epidermal proteins is based on speculations derived from experiments carried out on model nucleophiles, peptides, or proteins.

In this article, we report our investigations on the *in situ* chemical behaviour of MI and MCI in a reconstructed human epidermis (RHE) model, which is histologically similar to the *in vivo* human epidermis, by the use of high-resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR). This new approach provides information on the structures of adducts formed *in situ*.

Materials and Methods

Chemicals and reagents

MI ^{13}C-substituted at position 4 or at position 5, namely 4-(^{13}C)-MI and 5-(^{13}C)-MI, respectively, and MCI

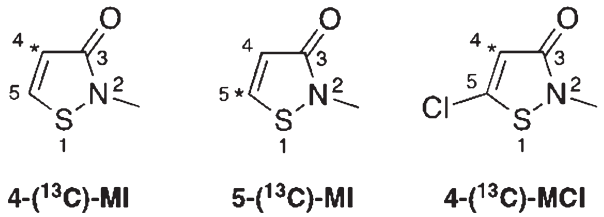

Fig. 1. Chemical structures of 4-(^{13}C)-methylisothiazolinone (MI), 5-(^{13}C)-MI and 4-(^{13}C)-methylchloroisothiazolinone (MCI) (asterisks indicate the ^{13}C-substituted positions).

^{13}C-substituted at position 4, namely 4-(^{13}C)-MCI (Fig. 1), were synthesized with a previously reported method (23). The reagents for introduction of the ^{13}C atoms, namely 2-(^{13}C)-acetic acid and (^{13}C)-formaldehyde, and deuterated solvents, were purchased from Euriso-Top (Saint Aubin, France). All other reagents and chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) and used without further purification. Aqueous solutions were prepared with deionized water.

Reconstructed human epidermis

The SkinEthic™ RHE model (4-cm^2 format) was chosen for the studies (SkinEthic, Lyon, France). It consists of normal human keratinocytes cultured for 17 days on an inert polycarbonate filter. The RHE units were received on day 18, and aseptically removed from the transport medium. They were then maintained for at least 2 h in growth culture medium (SkinEthic), at 37°C in a 5% CO_2 and humidified atmosphere, following SkinEthic's procedure.

Incubation of RHEs with 4-(^{13}C)-MI, 5-(^{13}C)-MI, and 4-(^{13}C)-MCI

RHEs were treated with either 4-(^{13}C)-MI, 5-(^{13}C)-MI or 4-(^{13}C)-MCI in acetone (0.4 M, 100 µl), with incubation times of 24 and 48 h. RHE negative controls were treated only with acetone (100 µl), and incubated for 24 h. After treatment, the RHEs were washed with deionized water, and separated from the polycarbonate support with dispase II (neutral protease, grade II; Roche, Manheim, Germany) in HEPES buffer. They were then stored at −80°C before NMR sample preparation.

HRMAS NMR analysis

Each sample was prepared at −20°C by introducing 15–20 mg of frozen, treated RHEs into a disposable 30-µl Kel-f® insert, as described previosuly (24). For the NMR spectrometer lock frequency, 10 µl of D_2O with 1% wt/wt trimethylsilyl propanoic acid was added to the insert. Just before the HRMAS analysis, the insert was placed in a 4-mm ZrO_2 rotor closed with a cap. The whole system was then introduced into an HRMAS probe precooled at 3°C. HRMAS spectra were recorded on a Bruker Avance III 500 spectrometer (Bruker BioSpin Corporation, Billerica, MA, USA) operating at a proton frequency of 500.13 MHz. The spectrometer was equipped with a 4-mm double resonance (^{1}H, ^{13}C) gradient HRMAS probe. A Bruker cooling unit regulated the temperature at 3°C by cooling the bearing air flowing into the probe. Experiments were conducted on samples spinning at 3502 Hz in order to keep the rotation sidebands out of the spectral region of interest. The shimming procedure used is described elsewhere (25). In order to detect the formation of adducts, two-dimensional ^{1}H–^{13}C heteronuclear single quantum correlation (g-HSQC) experiments were performed. Echo–antiecho gradient selection for phase-sensitive detection was used (26). Spectra were acquired by use of a 73-ms acquisition time with GARP ^{13}C decoupling and a 1.5-second relaxation delay. One hundred and thirty-six transients were averaged for each of 256 t1 increments, corresponding to a total acquisition time of 15 h. NMR data analysis and processing of spectra were carried out with Bruker's TOPSPIN™ software (version 3.2). Spectra were referenced by setting the lactate (present in the RHE) doublet chemical shift to 1.33 ppm in ^{1}H and to 22.7 ppm in ^{13}C. New NMR signals were identified in treated RHEs in comparison with untreated control RHEs. Then, ^{1}H and ^{13}C chemical shifts were assigned to potential metabolites and/or adducts of MI and MCI with amino acids by comparison with known chemical shifts of adducts obtained in previous studies in solution (23, 27, 28). Also, the measured chemical shifts were compared with those calculated for suspected structures by the use of CHEMDRAW™ Ultra software (version 12.0).

Results

NMR techniques in association with ^{13}C-substituted molecules have been shown to be very efficient tools for the investigation of hapten–protein interaction mechanisms in buffered or semi-organic solutions (29–31). In order to move forwards to an understanding of the chemical interactions taking place in a system closer to that of human skin, we have reported the usefulness of keratinocyte-based RHEs in association with the HRMAS NMR technique (24). The HRMAS NMR technique, which was initially developed to analyse the metabolome of normal and pathological tissues without time-consuming extraction (32–34), has now been combined with ^{13}C-substituted molecules to investigate,

in situ and in a non-invasive way, chemical interactions between haptens and epidermal cells. As keratinocytes constitute the major cell type in the epidermis and play a key role in skin inflammatory reactions, we used the SkinEthic™ RHE model, which consists of a normal human multilayered keratinocyte culture that is similar histologically to that of human epidermis.

MI was prepared ^{13}C-substituted at C-4 or C-5 to give 4-(^{13}C)-MI and 5-(^{13}C)-MI, respectively (Fig. 1). The C-4 and C-5 positions were chosen according to the reaction chemistry previously observed in solution (23, 27, 28). Position 5 corresponds to a potential site of adduct formation, and position 4 gives useful NMR information on the cyclic or open structure of adducts. As C-4 and C-5 are tertiary carbons bearing hydrogen atoms, the two-dimensional ^{1}H–^{13}C g-HSQC NMR sequence could be used. This sequence allows the distinction between CH and CH_3 signals, which are phased up, and CH_2 signals, which are phased down. This is, of course, very useful for characterizing the chemical structure of the adducts formed. MCI was prepared ^{13}C-substituted only at C-4 to give 4-(^{13}C)-MCI, as C-5 is a quaternary carbon atom substituted by chlorine and ^{1}H–^{13}C g-HSQC NMR correlation experiments cannot be applied (Fig. 1).

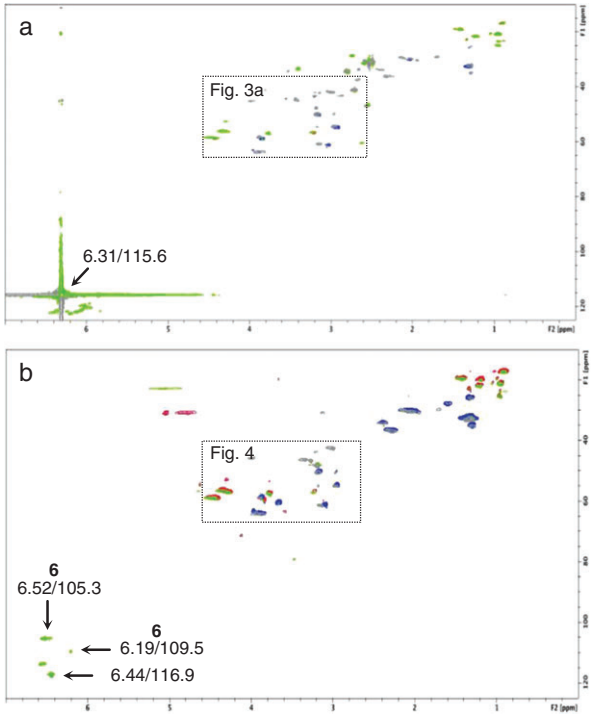

Fig. 2. (a) Full heteronuclear single quantum correlation (g-HSQC) spectrum of reconstructed human epidermis (RHE) treated with 4-(^{13}C)-methylisothiazolinone (MI) after 24 h, superimposed on native RHE. A residual signal of 4-(^{13}C)-MI at 6.31 (^{1}H)/115.6 (^{13}C) ppm is visible. The spectral region where interesting reactions were observed is detailed in Fig. 3a. (b) Full g-HSQC spectrum of RHE treated with 4-(^{13}C)-MCI after 24 h, superimposed on native RHE. A residual signal of 4-(^{13}C)-MCI at 6.44 (^{1}H)/116.9 (^{13}C) ppm is visible. Adducts that are **6**-like are indicated. The spectral region where more reactions were observed is detailed in Fig. 4.

Studies with MI

RHEs were treated separately with 4-(^{13}C)-MI and 5-(^{13}C)-MI, and this was followed by 24 and 48 h of incubation. ^{1}H–^{13}C NMR spectra of treated RHEs were compared with the spectrum of native RHE (used as a control) by superimposition to identify new signals in addition to the Skin Ethic™ RHE metabolome already characterized in a previous study (24).

Characteristic signals of 4-(^{13}C)-MI at 6.31 (^{1}H)/115.6 (^{13}C) ppm and 5-(^{13}C)-MI at 8.47 (^{1}H)/145.6 (^{13}C) ppm were still present after 24 h of incubation, as shown in Fig. 2a in the case of 4-(^{13}C)-MI. No residual signal of MI was detected in the spectra obtained after 48 h of incubation. This means that the MI still present after 24 h was completely consumed, hydrolysed or detoxified after 48 h.

MI was found to be reactive in RHEs, with the formation of several new signals. The region of the g-HSQC spectra shown is limited to the one where changes were observed after 24 h of incubation with 4-(^{13}C)-MI (Fig. 3a) or with 5-(^{13}C)-MI (Fig. 3b). No evolution was observed after 48 h of incubation. In the case of 4-(^{13}C)-MI, new signals (grey) were of a CH_2 nature, whereas for 5-(^{13}C)-MI, the new signals (green) were of a CH nature. Thus, well-defined CH_2 signals appeared at 2.56 (^{1}H)/46.8 (^{13}C) ppm, 2.71 (^{1}H)/41.0 (^{13}C) ppm, 2.87 (^{1}H)/43.0 (^{13}C) ppm, 3.18 (^{1}H)/47.5 (^{13}C) ppm, and 2.60–2.80 (^{1}H)/30.9–37.2 (^{13}C) ppm (Fig. 3a), whereas new CH signals appeared at 3.34 (^{1}H)/33.3 (^{13}C) ppm, 4.27 (^{1}H)/60.1 (^{13}C) ppm, and 4.27 (^{1}H)/80.1 (^{13}C) ppm (Fig. 3b). On the basis of the reported reaction chemistry of MI towards model nucleophiles, *N*-acetyl amino acids, and model peptides (23, 27, 28), the chemical shifts were characteristic of reactions with the nucleophilic thiol groups of cysteines leading to the formation, through complex chemical reactions, of adducts **1–3** (Fig. 5). Structures were identified by combining the NMR data obtained from the reactions of 4-(^{13}C)-MI and 5-(^{13}C)-MI. The C-4 chemical shift (δ) value of 46.8 ppm, correlated by HSQC data to protons at 2.56 ppm, and the C-5 δ at 33.3 ppm, correlated to a proton at 3.34 ppm, were characteristic of **1** bearing a cysteine unit and a thiol chemical group bound at C-5. As described for MI in solution with an excess of amino acid, in a situation similar to that of RHEs, a new disulfide bond can be formed through oxidation of the C-5 thiol chemical

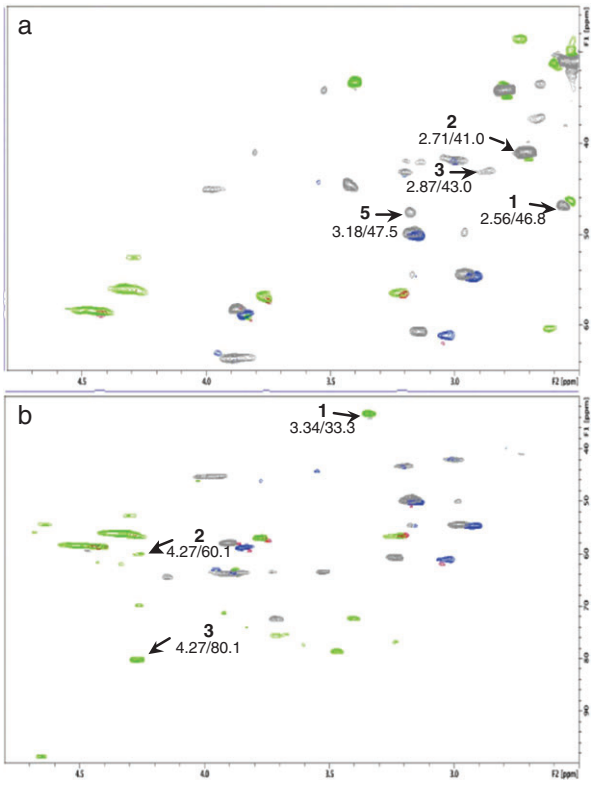

Fig. 3. High-resolution magic angle spinning $^{1}H-^{13}C$ heteronuclear single quantum correlation spectra for methylisothiazolinone (MI). (a) Reconstructed human epidermis (RHE) treated with 4-(^{13}C)-MI after 24 h, superimposed on native RHE. (b) RHE treated with 5-(^{13}C)-MI after 24 h, superimposed on native RHE. Treated RHE: green, CH/CH_3; grey, CH_2. Native RHE: red, CH/CH_3; blue CH_2.

group and further reaction with a new unit of cysteine, giving **2** with distinctive δ values at 41.0 (^{13}C)/2.71 (^{1}H) ppm for CH_2-4 and at 60.1 (^{13}C)/4.27 (^{1}H) ppm for CH-5 (28). We also observed adduct **3**, for which the C-5 δ at 80.1 ppm, correlated to a proton at 4.27 ppm, was characteristic of a hemithioacetal structure. For adducts **1–3**, the theoretical δ values for the methylene group of the lateral chain of linked cysteine would be around 2.60–3.00 (^{1}H)/30.0–35.0 (^{13}C) ppm. As indicated above, in this region we detected new CH_2 signals at 2.60–2.80 (^{1}H)/30.9–37.2 (^{13}C) ppm, this being therefore also in favour of **1–3** formation. In parallel, another new CH_2 signal was observed in the experiments with 4-(^{13}C)-MI, with δ values of 3.18 (^{1}H)/47.5 (^{13}C) ppm. The δ at 3.18/47.5 ppm was characteristic of a methylene group bearing a carboxylic acid and an amide chemical function. Most likely, water present in RHEs could compete with thiol groups and proceed to MI hydrolysis. The hydrolysis product **4** (not observed in the spectra) appeared to be oxidized *in situ*, most probably through an aldehyde dehydrogenase, to form acid **5** (Fig. 6).

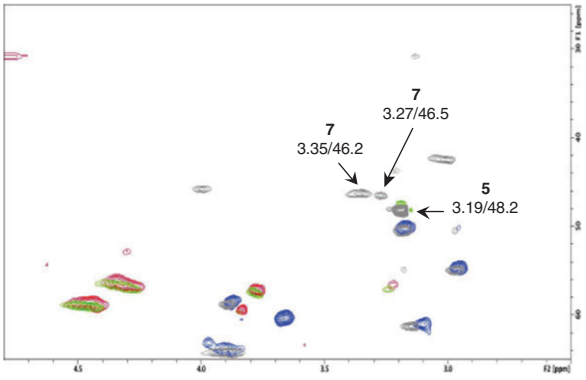

Fig. 4. High-resolution magic angle spinning $^{1}H-^{13}C$ heteronuclear single quantum correlation spectra of reconstructed human epidermis (RHE) treated with 4-(^{13}C)-methylchloroisothiazolinone after 24 h, superimposed on native RHE. Treated RHE: green, CH/CH_3; grey, CH_2. Native RHE: red, CH/CH_3; blue CH_2.

Studies with MCI

RHEs were treated with 4-(^{13}C)-MCI, and this was followed by 24 and 48 h of incubation. The signal of 4-(^{13}C)-MCI at 6.44 (^{1}H)/116.9 (^{13}C) ppm was still present after 24 h of incubation, as shown in Fig. 2b. As for MI, after 48 h of incubation no residual signal of MCI was detected.

MCI was reactive in RHEs but with different chemical reactions taking place and involving several amino acids. First, two groups of new signals at 6.52 (^{1}H)/105.3 (^{13}C) ppm and 6.19 (^{1}H)/109.5 (^{13}C) ppm (Fig. 2b) may correspond to **6**-like adducts on histidine (Fig. 5). The δ values were indeed very similar to those that we previously reported for MCI adducts on histidines of human serum albumin (35). Also, new signals appeared in the same region of the g-HSQC spectra that we showed for MI (Fig. 3a versus Fig. 4). No evolution was observed after 48 h of incubation. Well-defined signals (grey) were of a CH_2 nature at 3.35 (^{1}H)/46.2 (^{13}C) ppm, 3.27 (^{1}H)/46.5 (^{13}C) ppm, and 3.19 (^{1}H)/48.2 (^{13}C) ppm. On the basis of the reported reaction chemistry of MCI (23, 27, 28, 35), these δ values were characteristic of **7**-like adducts and of carboxylic acid **5** (Fig. 5). MCI was thus able to also react with the amino groups of lysines. The C-4 δ values of 46.2 and 46.5 ppm, correlated to protons at 3.35 and 3.27 ppm, respectively, are both representative of adduct **7** possessing an amide chemical function obtained by reaction with the amino group of the lysine side chain.

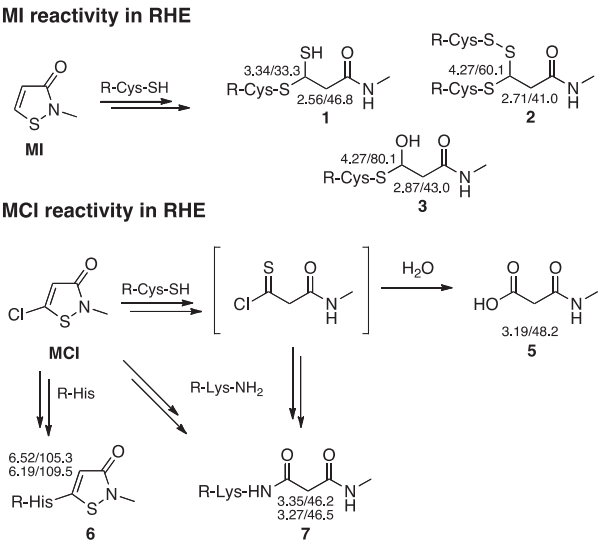

Fig. 5. Reactions of methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI), and the adducts characterized. Characteristic ^{1}H and ^{13}C-NMR chemical shifts for ^{13}C-substituted carbon atoms are indicated. RHE, reconstructed human epidermis.

Reactivity of MCI towards thiol groups cannot, however, be excluded, as **5** is most probably formed via hydrolysis of a thioacyl chloride derivative that we have shown, in previous studies in solution, results from reaction of MCI with cysteine thiol units (Fig. 5) (28).

Discussion

MI is currently responsible for a growing epidemic of skin contact allergy reactions. Contact allergy to MCI/MI 3:1 was mainly attributed to the chlorinated derivative MCI, which is considered to be a stronger sensitizer than MI. When MI alone was introduced in industrial products in the early 2000s and in cosmetics in 2005 at a maximum concentration of 100 ppm, there was an increase in its use that produced a dramatic increase in the number of cases of contact allergy to MI and to the MCI/MI mixture. At this time, in relation to this issue, a question has once again arisen regarding the potential cross-reactivity between MI and MCI. Although challenge and patch test studies have been undertaken with pure compounds and MCI/MI (5, 21), the answer to this question is still not clear.

Cross-reactivity is observed when an individual who is initially sensitized to allergen A reacts to a second allergen, B, which is different from A and to which the individual has not been previously exposed. The first compound is considered to be the primary sensitizer, and the second, eliciting a reaction, is a secondary allergen. However, the term cross-reactivity is often misused, and should be restricted to the well defined-cases that can be called true cross-allergens (36). In many cases, the identification of cross-allergic responses is difficult, and they may be confused with co-sensitization. At the molecular level, the main factors controlling antigen recognition are the nature of the chemical reactive group and its pattern of reactivity towards nucleophilic amino acids, and the molecular shape and spatial geometry of the formed adducts (22). Thus, even though the chemical group is very important, serving to define what are commonly called group allergies, the volume and shape of the activated T cell receptors are essential. Cross-reacting molecules must have similar sizes and spatial geometries to be recognized by the same T cell receptor. MI and MCI are structurally very similar chemicals, and cross-reactivity could thus be postulated. However, their chemical reactive sites are somewhat different, owing to the chlorine substitution at position 5 of MCI resulting in different chemical characteristics. The aim of this study was to bring, from a chemical viewpoint, a new perspective on the possibility of MI and MCI cross-reacting.

For many years, the reactions of MI and MCI with protein nucleophilic amino acids have been studied in solution, buffers, or semi-organic media (23, 27, 28). In all studies, MI was found to react exclusively with thiol groups. This was further confirmed with the synthetic peptides used in the direct peptide reactivity assay (DPRA) for screening contact allergens (28, 37). Peptides containing cysteine (Pep-Cys) or lysine (Pep-Lys) as nucleophilic amino acids confirmed the highly specific reactivity of MI towards cysteine, as MI extensively depleted Pep-Cys (98% depletion) but was completely inert towards Pep-Lys (38). On the other hand, MCI, apart from reacting with thiol groups, was found to also react with the imidazole side chain of histidine and the amino group of lysine (28, 35), through different reaction mechanisms from those described for MI. Reactions were described to be apparently faster with thiols than with amines. It was thus reasonably logical to observe depletion of both DPRA peptides, namely Pep-Cys (96% depletion) and Pep-Lys (35% depletion), at different rates (38). So far, such investigations carried out in buffer or semi-organic solutions have constituted the main source of information on how chemical sensitizers behave in the presence of nucleophiles, and the only basis for prediction of potential cross-reactions. However, the epidermis is a 3D tissue in which other factors, especially regarding bioavailability and potential metabolic transformations, need to be considered.

Since the 1980s, RHE models based on keratinocyte cultures have been developed for the assessment of skin penetration, the evaluation of skin irritancy of chemicals,

and predicting epidermal responses to irritants and skin sensitizers (39–41). In this study, we used the SkinEthic™ RHE model, which has high similarity with *in vivo* human epidermis morphologically and in metabolic activity (42), and the HRMAS NMR technique (43), which allows, in combination with carbon-substituted molecules, for the first time, the *in situ* chemical behaviour of skin sensitizers to be followed (24). Heterogeneous samples (i.e. RHEs) do not typically produce high-quality NMR spectra, but the combination of sample spinning and fast dynamics of some of the sample constituents allows the acquisition of highly resolved spectra. By spinning the sample at the magic angle θ_m 54.74° with respect to the direction of the magnetic field, the normal line-broadening effects resulting from dipolar interactions and susceptibility differences within the RHE sample are removed, resulting in high-resolution spectra (44).

In this study, by incubating RHEs with 4-(^{13}C)-MI/5-(^{13}C)-MI and 4-(^{13}C)-MCI, we observed that penetration, bioavailability and reactions were quite fast, as all adducts/products are present in <24 h. The spectra obtained after 48 h of incubation were strictly the same, except that MI and MCI were then completely consumed. On the one hand, MI was found to react in RHEs exclusively with cysteine thiol residues, confirming the results of our previous studies in solution. Reaction with a thiol group starts with a nucleophilic attack on the electrophilic sulfur atom of MI, leading to opening of the ring and the formation of an adduct where a cysteine is linked to the molecule through a disulfide bond. A new molecule of cysteine can then react with the disulfide bond and form an electrophilic thioxopropanamide intermediate, which can further react with cysteine thiol groups to form the observed adducts **1–3** (Fig. 5). On the other hand, MCI was found to react in RHEs with histidines and lysines, in good agreement with model studies in solution and also with human serum albumin (28, 35). The reaction of MCI with cysteine will lead to a highly electrophilic thioacyl chloride derivative that can then react with lysine to give **7** or with water to give **5** (Fig. 5). The reaction between MCI and lysine can therefore occur either by direct addition–elimination at the C-5 electrophilic carbon atom of MCI followed by opening of the ring, or by a direct reaction of the lysine side chain with the intermediate thioacyl chloride derivative. The imidazole ring of histidine can also react by direct addition–elimination at the C-5 electrophilic carbon atom of MCI, giving **6,** which is not subject to ring opening.

Thus, the final adducts that were identified in RHEs were different for MI and MCI. Different chemoselectivity (cysteine versus lysine and histidine) was found. The spatial volumes of these potential antigenic adducts were also different. Taking into consideration these finding, MI/MCI cross-reactivity would not be expected from the chemical point of view. However, on examination of the overall chemical reactions producing **1–3** and **6–7**, it seemed to be difficult to be definite about this. Putting aside the fact that MCI reacted with lysine and histidine by direct addition–elimination at the C-5 electrophilic carbon atom, which was not the case for MI, MI and MCI can initially react with cysteine. Similar first intermediates are then obtained, in which a cysteine unit is linked to the molecule through a disulfide bond. They only differ in the presence of a chlorine atom in the MCI product (28). We do not know whether these intermediates could be at the origin of similar antigenic determinants, supporting cross-reactivity. Besides, further reaction of another cysteine unit on the disulfide bond produced a thioxopropanamide intermediate derived from MI, and a thioacyl chloride derived from MCI. We know that the two intermediates had dissimilar associated reactions and afforded, as reported above, the different adducts with different chemoselectivity that we observed in RHEs. However, a by-product of this reaction is a modified protein in which two cysteine units have been converted to a cystine unit. Supposing that the proteins involved in MI and MCI reactivity were the same, these modified proteins would be the same with MI and MCI, and we do not know whether they could lead to similar antigenic determinants, in which case cross-reactivity would be expected.

The formation in RHE of the carboxylic acid **5** was shown for both MI and MCI. This clearly illustrates the further value of using RHE as compared with classic peptide/protein in solution for reaction chemistry studies. Indeed, RHEs have been shown to have metabolic activity similar to that of *ex vivo* human skin (45). In the case of MI, acid **5** is most probably the metabolic oxidation outcome of MI hydrolysis product **4**, possibly through the action of an aldehyde dehydrogenase (Fig. 6). This *N*-methyl-malonamic acid **5** was reported to be one of the main metabolites found in the urine of rats orally exposed to MI (46), but can obviously also be formed in the epidermis. This confirms that RHE is metabolically active and that our approach allows observation of the metabolic behaviour of haptens in addition to their chemical reactions. Interestingly, acid **5** is also formed by direct chemical hydrolysis of MCI in RHE, as already reported in solution studies (23, 27, 28). This metabolite **5** is the only common chemical formed in RHE after exposure to either MI or MCI, but its involvement in sensitization to MI and to MCI is not expected (47).

Very complex chemistry was shown by these studies in RHE. Multistep reactions have been highlighted, leading to the formation of various potential antigenic adducts.

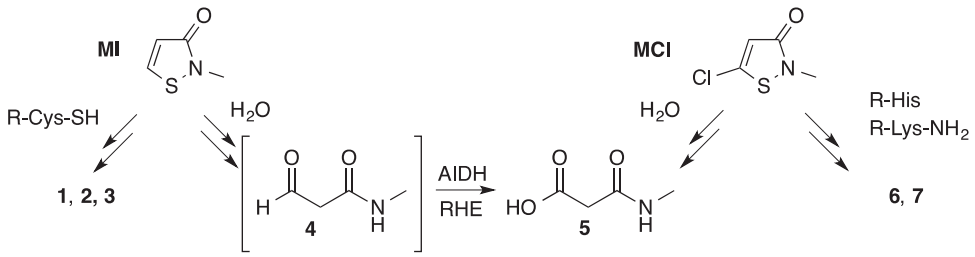

Fig. 6. Adducts and hydrolysis products formed from methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI).

These did not result from a simple reaction between one molecule of MI or MCI and a single cysteine or a single residue such as lysine or histidine; their formation involved reactions with several residues, with the formation of multiple adducts. As highlighted in Figs. 5 and 6, the chemistry of MI is different from that of MCI, which also reacts with lysines and histidines to form final adducts with very different structures (35). Knowledge of these adducts will now allow the performance of modelling studies to evaluate potential statistical associations based on molecular structures. Despite the similar structures of these two biocides, it is thus still difficult to draw a conclusion regarding the risk of cross-reactivity from the chemical point of view, although a common hydrolysis/metabolic non-sensitizing product **5** is formed.

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