Contact Sensitizers Induce Keratinocytes to Release Epitopes

Tools for *In Vitro* Tests and Implications for Autoimmunity

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Contact Sensitizers Induce Keratinocytes to Release Epitopes

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Cover picture: Blebbing human keratinocyte after exposure to monobromobimane (mBBr).

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I am among those who think that science has great beauty.
A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

– Marie Curie *(1867 - 1934)*

ABSTRACT

Contact allergy and its clinical manifestation, allergic contact dermatitis, affect approximately 20 % of the population in the Western world. It is caused by small reactive chemical compounds, called haptens. Haptens are thought to react with proteins in the skin and create immunogenic hapten-protein complexes. However, little is known about which proteins are covalently modified by haptens.

In this thesis, using caged bromobimanes as chemical probes, the basal keratinocytes and their cytoskeletal keratin intermediate filaments were shown to be hapten targets in *ex vivo* human skin. Furthermore, the first exact hapten target site detected against the backdrop of the entire proteome of the skin was presented.

Human cultured keratinocytes were found to release hapten modified keratins, as well as other possibly modified proteins, in blebs (plasma membrane vesicles) when exposed to haptens. The exact hapten target site found in skin samples were again found in blebs released by living cultured keratinocytes, indicating that the finding in *ex vivo* skin has *in vivo* relevance. Since the blebs contain hapten modified proteins, the hypothesis that blebs may play a role in sensitization in contact allergy is proposed.

In response to the European Union ban of testing cosmetic products or ingredients of cosmetic products on animals, the blebbing response of keratinocytes was utilized in a pilot study toward developing a new *in vitro* test for assessing the sensitizing potency of chemicals. The results are promising and it is hoped that the bleb response will be able to form the basis of a new, alternative, non-animal based test.

Further investigations of the bleb content revealed that several of the proteins present in blebs are known autoantigens in a variety of autoimmune disorders. Analysis of serum from hapten-exposed mice showed antibodies against a selection of the identified proteins as well as another marker of autoimmunity. However, the clinical relevance of the detected autoantibodies is unknown.

As keratins were found to be hapten targets, potential antibody responses against keratins in serum of hapten-exposed mice were analyzed in the final part of this thesis. The positive identification of antikeratin antibodies supports the previous results obtained from skin and cultured cells. Epitope mapping along the primary keratin sequences revealed different antibody binding patterns for different hapten exposures, thus providing new insights in which part of the protein that trigger a specific immune response.

In conclusion, the work presented in this thesis gives new, exciting insights into the mechanisms behind contact allergy as well as new tools for *in vitro* testing. It also demonstrates the power of combining chemistry and biology with high-tech microscopy and proteomic techniques when studying hapten-protein interactions in skin and cultured cells.

Keywords: Contact allergy, Bromobimanes, Caged fluorophores, Hapten targets, Hapten-protein complex, Keratin 5, Keratin 14, Blebs, Keratin bodies, Sensitizing potency, *In Vitro* assay, Alternative Methods, Bleb content, Autoimmunity, Epitope mapping
ISBN: 978-91-628-8376-8

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by the Roman numerals I-V. The papers are appended at the end of the thesis.

I. **Caged Fluorescent Haptens Reveal the Generation of Cryptic Epitopes in Allergic Contact Dermatitis**
Carl Simonsson*, Sofia I. Andersson*, Anna-Lena Stenfeldt, Jörgen Bergström, Brigitte Bauer, Charlotte A. Jonsson, Marica B. Ericson, and Kerstin S. Broo.
Journal of Investigative Dermatology, **2011**, 131(7), 1486-1493.

II. **Modification and Expulsion of Keratin by Human Epidermal Keratinocytes upon Hapten Exposure *in Vitro***
Brigitte Bauer, Sofia I. Andersson, Anna-Lena Stenfeldt, Carl Simonsson, Jörgen Bergström, Marica B. Ericson, Charlotte A. Jonsson, and Kerstin S. Broo.
Chemical Research in Toxicology, **2011***,* 24(5), 737-743.

III. **Contact Sensitizers of Different Reactivity Trigger the Release of Keratin in Membrane Vesicles**
Sofia I. Andersson, Anna-Lena Stenfeldt, Brigitte Bauer, Carl Simonsson, Jörgen Bergström, Marica B. Ericson, Charlotte A. Jonsson, and Kerstin S. Broo.
Submitted for publication

IV. **Contact Sensitizers Induce Release of Autoantigens and Formation of Autoantibodies**
Sofia I. Andersson, Brigitte Bauer, Charlotte A. Jonsson, Jörgen Bergström, and Kerstin S. Broo.
Submitted for publication

V. **Epitope Mapping of Antikeratin Antibodies in Contact Allergy**
Sofia I. Andersson, Jörgen Bergström, Charlotte A. Jonsson and Kerstin S. Broo.
Manuscript

*contributed equally to this work

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PENDING PATENTS

I. Method for screening the sensitizing properties of chemical compounds
US Appl. No:13/065,692
PCT/SE2011/000057
Sofia I. Andersson, Anna-Lena Stenfeldt, Brigitte Bauer, Marica B. Ericson and Kerstin S. Broo

II. Method for diagnosing and creating immunogenic tolerance in contact allergy and other epithelial, immunotoxicological ailments
US Appl. No:13/065,690
PCT/SE2011/000058
Sofia I. Andersson, Anna-Lena Stenfeldt, Charlotte Jonsson and Kerstin S. Broo

CONTRIBUTION REPORT

Paper I Contributed to the formulation of the research question and interpretation of the results. Performed the proteomic and animal experiments and contributed to the writing of the manuscript.

Paper II Contributed to the formulation of the research question and interpretation of the results. Performed the proteomic experiments and contributed to the writing of the manuscript.

Paper III Major contribution to the formulation of the research question; performed or supervised the experiments, and wrote the manuscript.

Paper IV Major contribution to formulating the research question; performed the experiments; interpreted the results; and wrote the manuscript.

Paper V Formulated the research question; performed the experiments; interpreted the results; and wrote the manuscript.

ABBREVIATIONS

A	Alanine
Aa	amino acid
Ab	Antibody
ACD	Allergic contact dermatitis
ACN	Acetonitrile
ANA	Anti-nuclear antibodies
C	Cysteine
CID	Collision-induced decomposition
D	Aspartic acid
dBBr	Dibromobimane
DC	Dendritic cell
DIA	Drug-induced autoimmunity
DMSO	Dimethyl sulfoxide
DNCB	1-chloro-2,4-dinitrobenzene
dpm	Disintegration per minute
E	Glutamic acid
EC3	Estimated concentration to induce a threefold stimulation index compared to control
ECVAM	European Centre for Validation of Alternative Methods
ESI	Electrospray ionization
F	Phenylalanine
G	Glycine
GSH	*γ*-Glutamylcysteinylglycine or Glutathione
GSSG	Oxidized dimer of glutathione
H	Histidine
HEKn	Human epidermal keratinocytes derived from neonatal foreskin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPC	Hapten-protein complex
I	Isoleucine
K	Lysine
K1	Keratin 1
K5	Keratin 5
K6	Keratin 6
K10	Keratin 10
K14	Keratin 14
K16	Keratin 16
K17	Keratin 17
KIF	Keratin intermediate filament
L	Leucine
LC	Liquid chromatography
(HP)LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLNA	Local lymph node assay
LSCM	Laser scanning confocal microscopy

LTQ-FT-ICR	Linear trap quadrupole-fourier transform-ion cyclotrone resonance
M	Methionine
mBBr	Monobromobimane
MHC	Major histocompatibility complex
MS	Mass spectrometry
MS/MS	Tandem coupled mass spectrometry/mass spectrometry
N	Asparagine
OECD	Organisation of economic co-operation and development
Oxazolone	4-ethoxymethylene-2-phenyl-2-oxazolin-5-one
P	Proline
PBS	Phosphate buffered saline
Q	Glutamine
R	Arginine
RA	Rheumatoid arthritis
S	Serine
SI	Stimulation index
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gelelectrophoresis
SLE	Systemic lupus erythematosus
SS	Sjögren's syndrome
T	Threonine
TPM	Two-photon microscopy
V	Valine
W	Tryptophan
Y	Tyrosine

CONTENTS

1

Introduction

1.1 THE SKIN

The skin is our largest organ, spanning approximately two square meters and accounts for about 7 % of total body weight in adults. It is the ultimate barrier between the human body and the surrounding environment and it protects us against e.g. mechanical stress, cold, heat, dehydration, UV irradiation from the sun, microorganisms and some chemical compounds. The skin is by no means a quiet, inert barrier; it is highly active and performs many different important tasks to maintain body homeostasis.
The skin consists of two main layers; the epidermis and dermis. The majority of cells in the epidermis are keratinocytes tightly connected by desmosomes; but melanocytes (cells synthesizing melanin), dendritic cells (antigen-presenting cells) and Merkel cells (tactile cells) are also present in this skin layer. The epidermis is generally divided into four differentiation layers: the stratum basale (basal cell layer), stratum spinosum, stratum granulosum and stratum corneum (Figure 1.1). The basal cell layer is the bottom layer of the epidermis. It contains the dividing keratinocytes and skin stem cells, responsible for maintaining the epidermis. When leaving the basal cell layer and moving upward through the suprabasal layers (stratum spinosum and stratum granulosum), the keratinocytes start to differentiate. In this process, the cytoskeletal proteins become more bundled (keratinization), the cell membrane thickens and a substantial impermeable cornified envelope starts to develop. In addition, the cells flatten and the nuclei and organelles disintegrate. The lipid content of the extracellular matrix also increases through the differentiation layers of the epidermis. These events ultimately result in the corneocytes, i.e. dead keratinocytes, residing in the stratum corneum. The stratum corneum is the outermost protective layer of the epidermis and of the skin in total. It contains corneocytes, surrounded by lipids and degraded proteins. This dense lipid rich layer prevents water loss and hinders microorganisms and hydrophilic chemical compounds from entering the skin. The full differentiation of the epidermis from the basal layer to the stratum corneum takes approximately 25-45 days, after which the dead cells in the stratum corneum are shed, e.g. in response to rubbing (*1, 2*).

Directly below the epidermis lies the dermis (Figure 1.1), which is a flexible and strong connective tissue that mainly consists of cells like fibroblasts. Mast cells, macrophages, dendritic cells and other white blood cells can also be found in this layer. Dermis is highly equipped with nerves, arteries, veins and lymphatic vessels as well as hair follicles, sweat glands and oil glands which originate from the dermis and stretch up through the epidermis. The subcutaneous tissue just below the dermis stores fat and anchors the skin to the underlying muscles. Together, the dermis and subcutaneous tissue provide important features e.g. stretch-coil properties and insulation (*1, 2*).

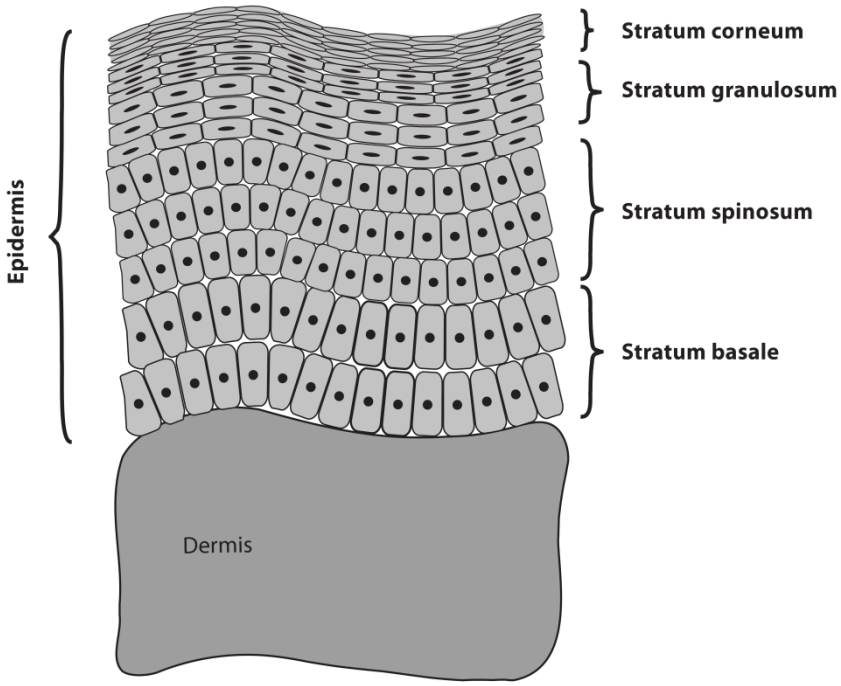


Figure 1.1. Epidermis and dermis. Epidermis: The basal layer (stratum basale) contains the dividing keratinocytes and stem cells and is responsible for maintaining the epidermal layers. As the keratinocytes move upward through the suprabasal layers (stratum spinosum and stratum granulosum) they differentiate; the cytoskeletal keratins get more bundled, the cell membrane thickens and the cornified envelope is formed. Ultimately the cells die and form the stratum corneum where they gradually detach from the skin surface. Dermis: This layer mainly consists of collagen-rich fibroblasts. Blood vessels, lymph vessels and nerves span this layer. Hair follicles and glands can also be found here.

1.1.1 Keratin intermediate filaments

The keratinocytes in the epidermis contain high amounts of proteins called keratins (*1*).
The keratins belong to the intermediate filament (IF) family. The keratin intermediate filaments (KIFs) are cytoskeletal proteins, spanning the cytoplasm and connecting with desmosomal proteins at the desmosomes, the epithelial cell-cell junctions. Traditionally, keratin proteins are divided into two classes: acidic (type I) and basic to neutral (type II). The different keratin classes all share some common structural characteristics: a central rod domain with α-helical conformation consisting of subdomains 1A, 1B, 2A and 2B connected by linkers 1, 12 and 2; and variable length "head and tail" domains of non-helical conformation. The keratins turn into filaments by first forming heterodimers consisting of one type I and one type II keratin where the rod domains form an coiled-coil (*3, 4*). These heterodimers then form heterotetramers which are the building blocks of the cell-spanning filaments (Figure 1.2).

The KIFs are expressed in a cell-specific manner, e.g. the keratin 5 (K5)/keratin 14 (K14) pair is specific for basal epithelial cells whereas the more bundled pair of keratin 1 (K1)/keratin 10 (K10) is expressed in the suprabasal layers, corresponding to the formation of the cornified envelope in the suprabasal layers of the epidermis (*3, 5, 6*). In the proliferating basal layers, keratin accounts for 25-35 % of the total protein content of the cells. This amount rises to about 80 % in the fully differentiated keratinocytes (*7*).

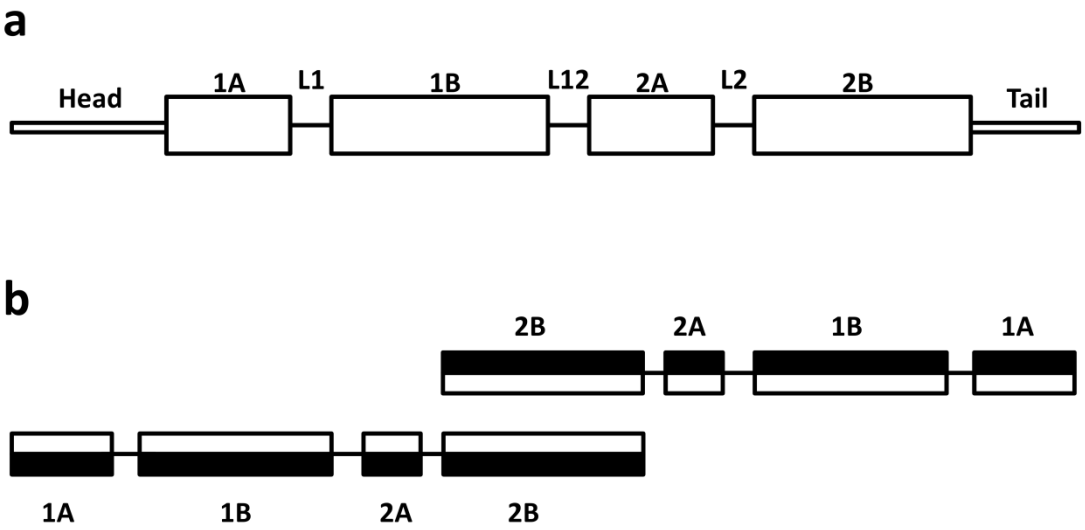


Figure 1.2. An overview of the structure of keratin intermediate filaments. (a) The domains of a keratin intermediate filament, (b) KIF heterodimer and tetramer. Other types of alignments of the heterotetramer have been shown, but discussing these is beyond the scope of this thesis.

1.1.2 Skin protective features

The skin features chemical, immunological and physical protection. Briefly, it protects us chemically by secreting the so-called acid mantle that results in a low pH at the skin surface. The low pH prevents microorganisms from multiplying. Also, bacteria are killed off by anti-bacterial compounds secreted in e.g. sweat and sebum. The pigment melanin is also included in the chemical protective system, as it protects skin cells from UV damage.
The immunological protection mainly consists of innate immune cells that can kill microorganisms, present processed antigens, and call for "reinforcement" (recruite more immune cells). The physical (mechanical) barrier is maintained via the tightness and the hardness of keratinized cells. Also, the lipid layer between cells in the epidermis prevents water from both exiting and entering the skin (*1, 2*).

Although amazingly ingenious in its construction, the skin cannot protect us against everything we expose it to in our modern daily life. Small reactive chemical compounds can penetrate the physical barrier of the skin and lead to undesired effects, such as allergic contact dermatitis. In this thesis, new understandings of the molecular processes occurring in the skin when reactive chemical compounds enter are presented.

1.2 ALLERGIC CONTACT DERMATITIS

Allergic contact dermatitis (ACD) is the clinical manifestation of contact allergy. It is the most prevalent form of immunotoxicity reported in humans as it is estimated that approximately 20 % of the population in the Western World is affected (*8*). ACD is caused by skin exposure to small reactive compounds called haptens (*9*) and is characterized by papules, redness and vesiculation followed by dry skin and scaling (*2, 10*). The most common haptens are metals, fragrance compounds and preservatives (*11, 12*) and the growing list of contact sensitizers comprises more than 4000 compounds (*13*). Contact allergy is a chronic, lifelong condition and once an individual is sensitized, the offending compound must be avoided to escape bothersome inflammation and eczema.

Contact allergy is a common occupational disease with a great socioeconomic impact. The disease can lead to serious changes in both working and everyday life as many haptens encountered in occupational settings are also used in everyday products such as perfumes, lotions, makeup, etc.

1.2.1 Hapten characteristics and protein interactions

Haptens are reactive chemical compounds of low molecular weight (< 1000 Da) and appropriate lipophilicity (Log*P* ~2). These properties are critical for the compounds to be able to penetrate the skin. Haptens are in themselves too small to trigger an immune response. They bind to proteins in the skin, thereby creating immunogenic hapten-protein complexes (HPCs). This protein-binding feature of haptens in ACD was presented by Landsteiner and Jacobs as early as 1935 (*14*). Nevertheless, no exact hapten target site in skin has been presented.

Most haptens are electrophiles, reacting with nucleophilic amino acid residues in skin proteins. Some of the most frequent types of electrophilic-nucleophilic reactions in hapten-proteins interactions are S_N2 reactions, nucleophilic addition to carbonyls (e.g. Schiff base formation) and Michael additions (Figure 1.3) (*9*). A majority of electrophilic haptens are directed against thiols (-SH) i.e. cysteine, and amines ($-NH_2$) i.e. lysine (*15*). In addition to electrophilic compounds, metals can also act as haptens (*9*).

Some compounds are so-called pro- or prehaptens. These type of compounds are not haptens in their original forms, but can be transformed into potent sensitizers by metabolic activation in the skin (prohaptens) (*9, 16*) or by air oxidation before skin contact (prehaptens) (*9, 17*).

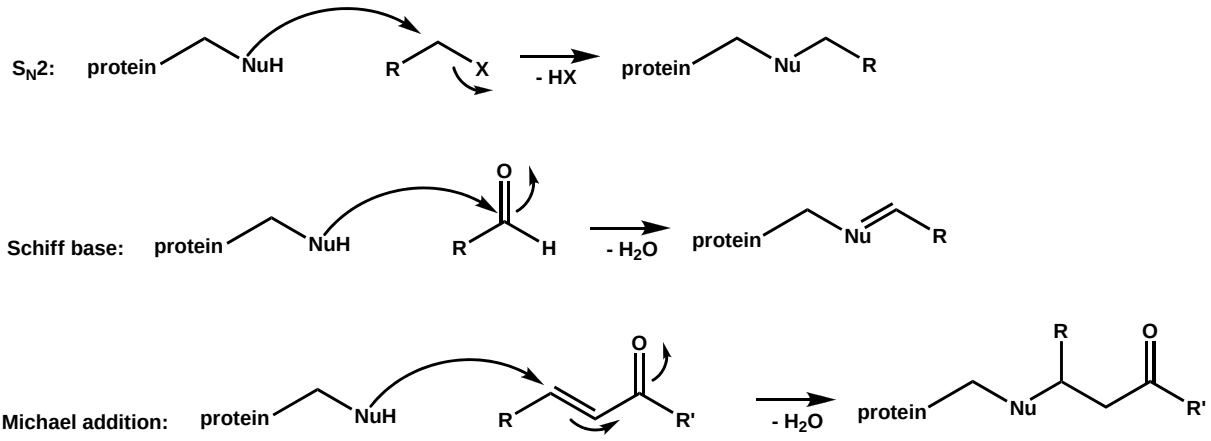


Figure 1.3. Overview of the most frequent electrophilic-nucleophilic reactions in hapten-protein interactions. X: Br, Cl, I (good leaving group), R and R': H, alkyl or aryl.

1.2.2 Immunological mechanisms

ACD is one of the most studied immunologically mediated toxicities and much is known about the immunological mechanisms. It is a hapten-specific T-cell mediated skin inflammation classified as a type IV delayed hypersensitivity reaction. ACD consists of two main phases: sensitization and elicitation (Figure 1.4). ACD also includes a third, less understood phase; the regulation phase of the inflammatory response (*10, 18*).
An immunological memory is created in the sensitization phase, which is also known as the induction phase. Briefly, haptens penetrate into the epidermis where they bind to and modify skin proteins. Haptenated/non-haptenated new epitopes of peptides/proteins are then taken up and processed by cutaneous dendritic cells (DCs). The DCs subsequently display the new epitope with/without hapten in the MHC I or II groove at the cell surface. These DCs migrate from the skin to the regional lymph nodes where specific, naïve T-cells are primed. Hapten-specific T-cells proliferate and migrate to the circulatory system, circulating between blood, lymph and skin (Figure 1.4) (*10, 18*). The sensitization phase normally takes around 8-15 days in humans and 5-7 days in mice (*10, 19*).

When a sensitized individual encounters the same or cross-reacting hapten again, the elicitation phase (challenge phase) of ACD is initiated (Figure 1.4). In this phase, the hapten penetrates the skin once again and modifies skin proteins. Skin cells expressing major histocompatibility complex (MHC) I and/or II present the hapten-specific epitope in the MHC groove. The hapten-specific T-cells, which were produced in the sensitization phase, are recruited and activated in the skin, triggering the inflammatory response. The manifestation of ACD appears within 48-72 h of re-exposure in man and 24-48 h in mouse (*10, 18*).
Primary ACD, where inflammation and eczema are already displayed at the very first encounter of a hapten in a previously non-sensitized individual, has also been reported. In this case, the individual goes through the sensitization phase and the specific T-cells start to circulate, just like described above. However, the hapten is still present in the skin, thereby triggering the newly formed hapten-specific T-cells and starting the elicitation phase immediately (*19*).

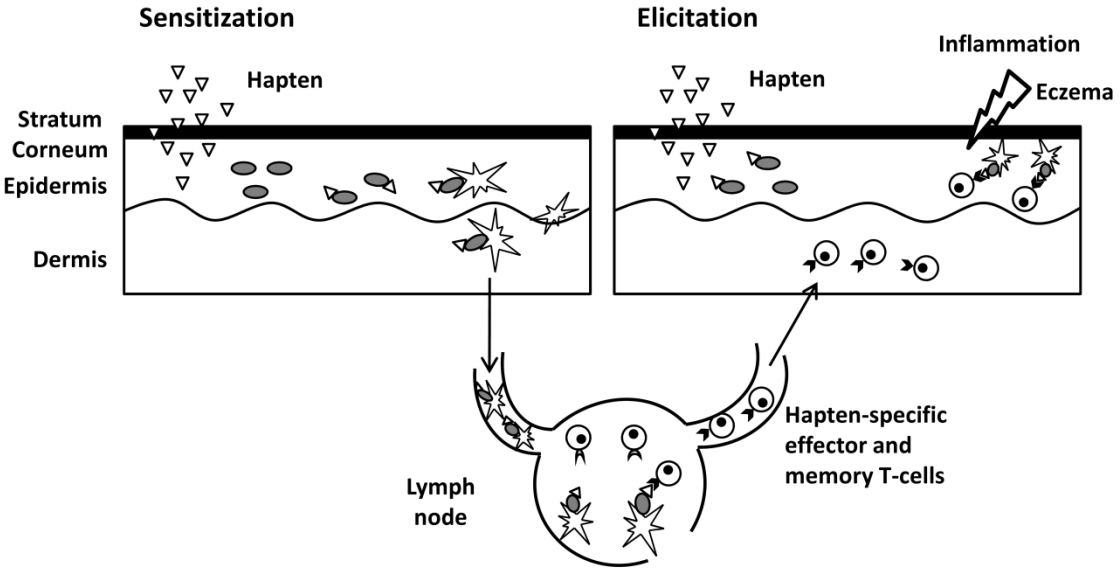


Figure 1.4. Schematic overview of the immunological mechanism in ACD. In the sensitization phase the hapten penetrates the skin, binds to skin proteins which are taken up by dendritic cells which in their turn process and display hapten-specific epitopes in the MHC I or II groove on the cell surface. The dendritic cells migrate to the regional lymph nodes where naïve, specific T-cells are primed. These hapten-specific T-cells enter the circulatory system and circulate between the lymphatic system and the skin. When the same or crossreacting hapten is encountered, the haptenated proteins are processed and displayed by skin cells. The immunological memory is activated and hapten-specific effector T-cells promote inflammation.

Although ACD is one of the most studied immunologically mediated toxicities, several details about its mechanisms are still lacking. For example, it has not been known where in the skin the haptenation reaction occurs, if the cellular targets are keratinocytes or antigen-presenting cells, or if intra- or extra-cellular proteins are targeted. The work presented in this thesis provides new knowledge on these details.

1.3 HAZARD IDENTIFICATION AND RISK ASSESSMENT

With the potential risk of developing contact allergy upon topical exposure to chemical compounds in mind, it is vital to make proper hazard identifications and risk assessments. Today, the recommended Organisation of Economic Co-operation and Development (OECD) guideline method for assessing contact sensitizing potency of chemical compounds is the murine local lymph node assay (LLNA) (*20*) (described in detail in section 3.2.1). The LLNA gives graded results, classifying haptens into weak, moderate, strong and extreme contact sensitizers (*21, 22*). However, since 2009, testing cosmetic products and their components on animals are forbidden in the EU according to the amendment to the EU Cosmetics Directive (2003/15/EC) (*23*). This directive is inspired by the three Rs; to refine, reduce and replace animal experiments (*24*) and calls for rapid development of reliable *in vitro* methods.

In the cosmetics industry, an *in vitro* test with a graded result is needed to allow for the assessment of safe concentration limits for ingredients, both separately and in combinations in finished products. A lot of time, money and effort have been put into this matter during the past years. However, no sensitization prediction *in vitro* model has yet reached validation status through the European Centre for Validation of Alternative Methods (ECVAM).

1.4 AUTOIMMUNITY

Autoimmunity is characterized by failure of self-tolerance where the immune system attacks the organisms own proteins and structures. The undesired immune response consists of autoreactive T-cells and/or B-cells prodicing autoantibodies against self-antigens (autoantigens). An autoimmune disease is defined as a disorder where autoimmunity is a causative or contributing factor. In combination, over 60 known autoimmune diseases have a population prevalence of 3-5 % (*25, 26*). However, a higher prevalence of approximately 5-8 % is proposed for developed countries, based on data from the USA (*26*).
Intensive research on why the immune system can no longer tell the difference between healthy body tissues and threatening antigens has been conducted during the last two decades (Reviewed in (*26*)). Several plausible causative routes have been proposed, e.g. deficiency in apoptotic cell clearance, genetic predispositions, mutations, and microbial infections (*26-28*).

The potency of haptens, especially metal ions, to induce autoimmunity has been discussed previously (*29, 30*). More precisely, it has been shown that nickel-complexing proteins are potential Toll-like receptor ligands and that T-cell clones induced by endogenous peptides with hapten attached on a peripheral position will also react with the unmodified peptide, thus initiating self-reactivity (*29*).

2

Aims of the Thesis

The overall aim of the present study was to gain knowledge regarding the identity of hapten-protein complexes in human skin and cultured human skin cells and the effects of protein modifications by haptens.

The specific aims of the thesis were:

1. To investigate what cells and proteins are targeted by haptens in human skin (Paper I)

2. To study the hapten targets in cultured human epidermal keratinocytes when exposed to contact allergens (Paper II)

3. To further investigate the findings in Paper II and begin to develop an *in vitro* test for assessing the sensitizing potencies of compounds, based on the keratinocyte response (Paper III)

4. To analyze the proteins released in keratinocyte blebs upon hapten exposure and to study a potential connection between hapten exposure and autoimmunity (Paper IV)

5. To study antikeratin antibodies in serum of hapten-exposed mice (Paper V)

3

Methods and Techniques

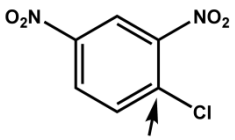
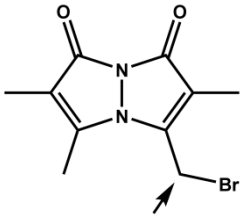
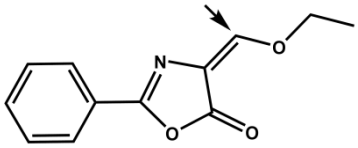
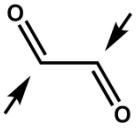
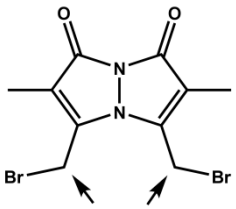
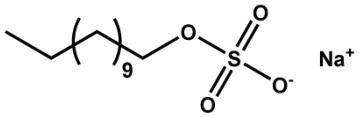
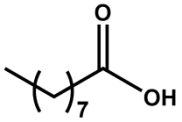
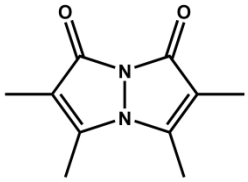
3.1 TEST COMPOUNDS

The compounds used in this thesis are listed in Table 3.1.
The caged fluorophores monobromobimane (mBBr), dibromobimane (dBBr) and the non-caged fluorophore methylbimane were used in Papers I and II (chapter 4) and are referred to as bromobimanes (mBBr and dBBr) or bimanes (mBBr, dBBr and methylbimane). The thiol-reactive bromobimanes were found to be strong contact sensitizers according to the Local lymph node assay (LLNA), whereas the non-reactive methylbimane was classified as a non-sensitizer at the tested concentrations. In Papers IV and V (chapters 6 and 7), the extremely potent sensitizers 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) and 1-chloro-2,4-dinitrobenzene (DNCB), the strong sensitizers glyoxal and the bromobimanes were used, whereas all of the above mentioned compounds (except oxazolone and methylbimane) plus the strong sensitizer formaldehyde and the non-sensitizers sodium dodecyl sulfate (SDS) and nonanoic acid were used in Paper III (chapter 5). The bromobimanes were used for their caged fluorescent properties, which are described below. The other compounds were used because of their known sensitizing capacities and common use in contact allergy research.

3.1.1 Bromobimanes

Bromobimanes are so-called caged fluorophores. The term “caged” refers to that the compound is non-fluorescent in itself, but it becomes fluorescent when covalently linked to a nucleophile. The bromobimanes are thiol-specific and become fluorescent when the bromine (bromines in the case of dBBr) is replaced by sulfur in a S_N2 reaction with a thiol, e.g. cysteine (*31, 32*). Since most of the clinically relevant contact sensitizers preferably react with thiols or amines (*15*), these compounds would be suitable as model haptens. Also, the log*P* values, calculated according to Tekot *et al* (*33*) (1.45 ± 0.85 for mBBr and 1.87 ± 0.86 for dBBr) and molecular weights (271.1 and 350.0) makes them ideal sensitizers according to reported hapten characteristics (described in section 1.2.1) (*9*).

Table 3.1 Structures and properties of compounds used in this study

Name	Structure & reactive site(s)	Nucleophile	EC3 (%, mM)
1-chloro-2,4-dinitrobenzene (DNCB)		Cys	0.015, 0.74 (*34*)
monobromobimane (mBBr)		Cys	0.12, 4.4 (*35*)
Oxazolone		Lys, α-NH_2	0.003, 0.14 (*36*)
Formaldehyde		Lys, α-NH_2	0.7, 233 (*36*)
Glyoxal		Arg	0.7, 127 (*37*)
dibromobimane (dBBr)		2 Cys	0.17, 4.9 (*35*)
Sodium dodecylsulfate (SDS)		NA	NA (*38*)
nonanoic acid		NA	NA (*38*)
methylbimane		NA	NA (*35*)

The stated nucleophile is the preferred one in the formation of a hapten-protein complex. Arrows indicate reactive group(s). EC3: the estimated concentration of test compound required to induce a 3-fold stimulation index (T-cell proliferation) in mice compared to control mice in the Local Lymph Node Assay (*39*). NA, Not Applicable.

3.2 PREDICTIVE TESTING

The local lymph node assay was used in the present studies to assess the sensitizing potencies of caged fluorophores mBBr and dBBr and control compound methylbimane (Papers I and III). All animal tests were approved by the local ethics committee.

3.2.1 Local lymph node assay (LLNA)

The murine LLNA is the OECD guideline for predicting the sensitizing potencies of chemical compounds (*20, 39*). Female CBA/Ca mice (8-12 weeks *n*=3 in each treatment group) were used in the experiments according to the following standardized protocol (Figure 3.1):

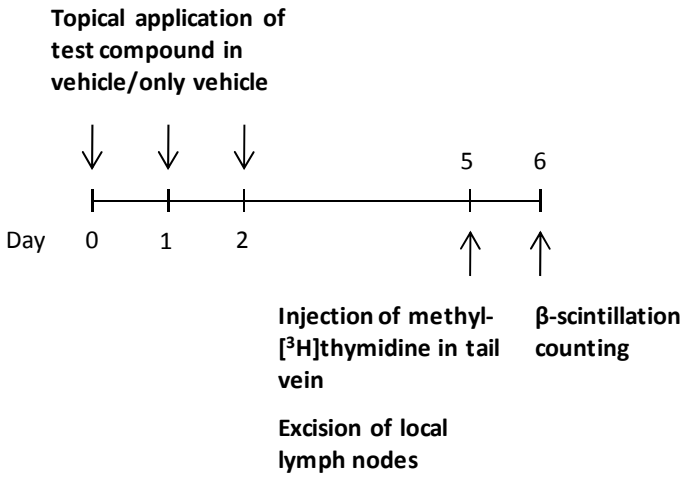


Figure 3.1. Outlined LLNA protocol.

On day 0, 1 and 2, 25 µL of the test compound dissolved in dimethyl sulfoxide (DMSO) was applied to the dorsum of each ear. The control group received only DMSO. On day 5, the mice were intravenously injected in the tail vein with 250 µL phosphate buffered saline (PBS) containing 20 µCi methyl-[^{3}H]-thymidine. After resting for 5 h, the mice were first anesthetized by inhalation of isoflurane and subsequently euthanized by inhalation of carbon dioxide (CO_2). The draining auricular lymph nodes were excised and pooled for each treatment group and single cell suspensions of lymph node cells were prepared. The incorporation of [^{3}H]-thymidine was measured using β-scintillation counting. The stimulation index (SI) of each treatment group relative to the control group was calculated using Equation 3.1.

$$SI = (dpm_A / \text{n lymph nodes}_A) / (dpm_B / \text{n lymph nodes}_B)$$

Equation 3.1. Calculation of SI in the LLNA. A=treatment group, B= control group, dpm: disintegrations per minute.

A test compound is considered to be positive in the LLNA if one or more of the tested concentrations results in a SI > 3. The estimated concentration required to induce a

threefold increase in SI (SI=3) compared to the control group (the EC3 value) is calculated using linear interpolation (Equation 3.2) (*40*).

$$EC3 = (A\text{-}C)\,[(3\text{-}D) / (B\text{-}D)] + C$$

Equation 3.2. Calculation of EC3 value in the LLNA. A = lowest concentration which gives SI>3, B = SI of A, C = highest concentration which gives SI<3, D = SI of C.

The sensitizing potency of test compounds was classified according to the following scale of EC3 values: extreme: < 0.1 % w/v; strong: ≥ 0.1 – < 1; moderate: ≥ 1 – < 10; weak: ≥ 10 – ≤ 100 (*21, 22*).

3.3 STUDIES OF CHEMICAL REACTIVITY TOWARD PEPTIDES

Most contact sensitizers react with thiols or amines (*15*). To mimic this *in vivo* reaction of haptens, model nucleophiles are used in *in vitro* reactivity experiments. Glutathione (GSH) is commonly used as a model nucleophile in these kinds of studies (*9*). This small model peptide contains both one thiol and one amine (Figure 3.2), allowing determination of which of these groups a hapten prefers to react with. Liquid chromatography coupled to mass spectrometry (LC-MS) is a frequently used analysis method for these types of experiments. This analytical technique first separates the hapten-peptide conjugates, unreacted hapten and peptide on a column. The eluting compounds are subsequently ionized and analyzed in the mass spectrometer, detecting the mass over charge ratio (*m/z*) of analytes. The methods described below were used in Paper I.

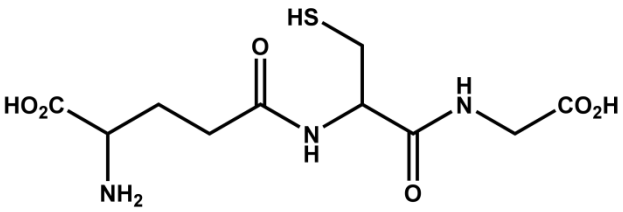


Figure 3.2. The structure of GSH (*γ*-Glutamylcysteinylglycine).

3.3.1 LC-MS

3.3.1.1 Reactions with glutathione

A solution consisting of 1 mM mBBr or dBBr, 5 mM GSH, 2 % acetonitrile (ACN) in pH 7.4 100 mM NaP_i buffer was prepared and immediately analyzed using LC-MS. The concentration of GSH corresponds to the physiological intracellular GSH concentration (up to 10 mM) (*41, 42*).

3.3.2 Fluorescence detection

Since the bromobimanes fluoresce when covalently linked to a nucleophile, the reactivity of these compounds towards glutathione can be monitored by measuring the fluorescence. mBBr becomes fluorescent when linked to one thiol, whereas dBBr must be linked to two thiols to become fluorescent.
A solution of consisting of 5 µM of mBBr or dBBr, 25 µM GSH and 0.01 % ACN in pH 7.4 100 mM NaP_i buffer was prepared and immediately transferred to a an opaque 96 well plate and analyzed in a plate reader (mBBr: excitation 388 nm, emission 475 nm; dBBr: excitation 390 nm, emission 480 nm) with one scan every 30 s for 7.5 h.

3.3.3 Hydrolysis

The bromobimanes were to be dissolved in buffer and applied on human *ex vivo* skin for 20 h. Therefore, it was important to determine the rate of hydrolysis of these compounds in buffer. The same reaction conditions (however without GSH) and analytical method as in 3.3.1.1 was used in the experiments. The amount of unhydrolyzed compound was monitored for 24 h.

3.4 IMAGING OF HAPTENS IN EPIDERMIS OF INTACT HUMAN SKIN

Two-photon microscopy (TPM) and laser scanning confocal microscopy (LSCM) were used in Paper I to detect bimane reaction sites in *ex vivo* human skin.

TPM presents major advantages compared to LSCM in analysis of whole tissue. Briefly, the use of two-photon excitation gives reduced scattering and increased light penetration owing to the fact that the samples are only exposed to the laser at the focal plane. This means that intact skin can be used directly without compromising the integrity of the tissue (*43-48*). The skin does not have to be cryofixed and sectioned, which is a great advantage since these treatments may cause mechanical and freeze damages to the tissue (*46*). Also, photobleaching of fluorophores is greatly reduced due to a more confined excitation volume (*45, 46, 48*). Using TPM, epidermis and upper parts of dermis can be visualized (*46, 47*), making it an ideal method to follow hapten binding in the skin.

The main disadvantage of LSCM is its limitation in scanning depth (a few tens of µm) because of higher light scattering compared to TPM. Also, the sample is exposed to the laser both above and under the focal plane, increasing photobleaching and phototoxicity (*43, 46*). However, this method works very well on tissue sections and was thus used to confirm the bromobimane binding pattern found with TPM and to analyze the colocalization experiments.
Please see Supplementary Methods in Paper I for complete details of TPM and LSCM experiments.

3.5 *IN VITRO* TESTS USING CULTURED KERATINOCYTES

Normal human undifferentiated epidermal keratinocytes (HEKn) were used in experiments in Papers II-IV. Because of its resemblance to undifferentiated basal keratinocytes, this cell type was used to mimic the basal keratinocytes found to be hapten targets in *ex vivo* skin (Paper I). Also, this cell type possesses normal cell cycles and metabolism compared to immortalized keratinocyte cell lines such as HaCaT. Taken together, these properties were judged to be important for investigating the consequences of hapten exposure in a living system.

Please see Paper II-IV for experimental details.

3.6 PROTEIN IDENTIFICATION

The methods described below were used in Papers I-IV.
Sample preparation, SDS-PAGE and immunoblotting were performed as described in Papers I-IV.

3.6.1 Nano-LC-LTQ-FT-ICR

This analytical method was used to identify proteins in human skin samples and in keratinocyte blebs (Papers I, II and IV).
A Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometer gives ultrahigh resolution of peaks and mass accuracy in the low ppm range (*49, 50*). In addition, the peak capacity is increased, resulting in more signals being detected than in lower resolution instruments (*49*). One major advantage with FT-ICR spectrometers is the linear ion trap (the LTQ, linear trap quadrupole) in front of the ICR cell (*49, 50*). A linear ion trap can store, isolate and fragment ions and subsequently transfer them to the ICR or to an off-axis electronmultiplier inside the ion trap (*50*). There is, however, a drawback of this instrument and that is the slow acquisition rate (several s per cycle) (*49*). Also, the ICR has quite a small dynamic range (*m/z* 200-2000) but is favored by electrospray ionization (ESI), which produces ions with multiple charges in this area (*50*).

The nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments in Papers I, II and IV were performed on a hybrid linear ion-trap FT-ICR mass spectrometer equipped with a 7 T magnet. The mass spectrometer was operated in data-dependent mode, automatically switching to tandem mass spectrometry (MS/MS) mode. One-dimensional MS spectra were obtained in the FT-ICR, while 2D MS/MS spectra were acquired in the LTQ-trap. For each scan of FT-ICR, the three most intense ions (double or triple charged) were sequentially fragmented in the linear trap by collision-induced dissociation (CID) (described in 3.6.6).

3.6.2 MASCOT search parameters

All the MS/MS spectra were searched by MASCOT (Matrix Science, Boston, MA, USA) against the Swiss prot database 57.1. For protein identification the minimum criteria were: two tryptic peptides matched at or above the 95 % level of confidence (Papers I, II and IV).
When analyzing proteins run on polyacrylamide gel, it is important to remember that proteins can get modified during electrophoresis. For example, there is always some amount of unpolymerized monomeric acrylamide. Cysteines (C) are potent nucleophiles which are readily alkylated by acrylamide, creating the propionamide modification. Methionine (M) is also known to undergo modifications during electrophoresis, but this amino acid is typically oxidized instead (*51*).
For proteins derived from hapten-exposed skin and keratinocyte blebs, the search parameters were set to: All species, MS accuracy 5 ppm, MS/MS accuracy 0.5 Da, one missed cleavage by trypsin allowed, fixed modification: propionamide modification of C, variable modification: oxidized M.
In addition, proteins are often subject to several posttranslational modifications during physiological processes. For example, deamidation of arginines (R) is known to occur during epithelial differentiation (*52*). Hence, deamidated R was included as a variable parameter for proteins obtained from keratinocyte blebs.

3.6.3 Peptide fragmentation

In CID of a peptide chain, y- and b-ions are the most common peptide fragments (*53*). The y-ion is a *C*-terminal fragment (charge retained on *C*-terminus) which is caused by protonation at the amine, followed by cleavage at the amide bond in the peptide backbone, resulting in an ammonium ion. The b-ion is also caused by cleavage at the peptide amide bond, but the charge is retained on the carbonyl, resulting in an acylium ion on the *N*-terminal fragment. Other fragments, such as x-, z-, c- and a-ions can also be detected. X- and z-ions are *C*-terminal fragments whereas a- and c-ions are *N*-terminal fragments (Figure 3.3) (*53, 54*).

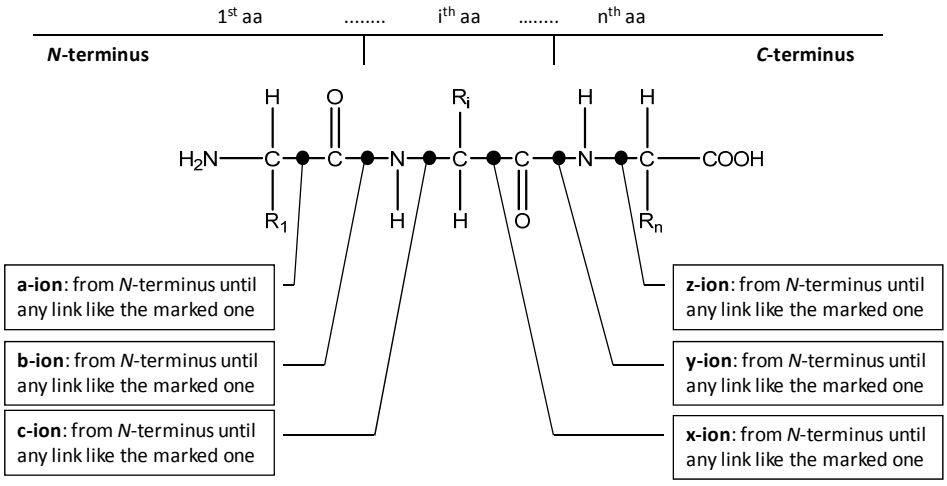


Figure 3.3. Overview of possible fragmentation of a peptide chain. Y- and b-ions are most common in collision-induced disintegration (CID) of peptides.

Each ion has a specific mass to charge ratio depending on the amino acid sequence and number of charges. The formulae for calculation of fragment ion molecular masses are listed in Table 3.2. To obtain *m/z* values, the exact mass of the required number of protons is added to the molecular weight and divided by the number of charges.

Table 3.2. Formulas to calculate fragment ion molecular masses.

Ion type	Neutral M_r
a	[N]+[M]-CHO
b	[N]+[M]-H
c	[N]+[M]+NH_2
x	[C]+[M]+CO-H
y	[C]+[M]+H
z	[C]+[M]-NH_2

[N] is the molecular mass of the neutral *N*-terminal group; [C] is the molecular mass of the neutral *C*-terminal group; [M] is the molecular mass of the neutral amino acid residues.

Another, perhaps more simple way to understand how to calculate fragment ion *m/z* value is described below (all calculations corresponding to monocharged ions): Each molecular mass of the component amino acids is decreased by one H_2O. To obtain a b-ion, add one proton to the sum of the amino acids. An a-ion has the same *m/z* as the corresponding b-ion minus CHO (≈ −28); a c-ion has the same *m/z* as the corresponding b-ion plus NH_3 (≈ +17). To obtain a y-ion, add one proton to the sum of the amino acids. An x-ion has the same *m/z* as the corresponding y-ion plus CO minus H_2 (≈ +26); a z-ion has the same *m/z* as the corresponding y-ion minus NH_2 (≈ − 16) (*54*).

3.7 ANTIBODY IDENTIFICATION

In Paper IV and V, antibodies against a selection of identified bleb proteins were detected. This was done using the Epitope Mapping service of LC Sciences in Houston, TX, USA (www.lcsciences.com), as described in the papers. This method was chosen because it allows for quantitative high-throughput screening of thousands of peptide sequences in one single experiment. One drawback of the methods used in the papers is that only peptides derived from the linear amino acid sequences of the proteins are screened for antibody epitopes. Thus, no conformational epitopes derived from the secondary, tertiary or perhaps the quaternary structures of the proteins are detected. However, trying to find these conformational epitopes was beyond the scope of this thesis.

4

Hapten Targets in Skin and Cultured Cells

4.1 STUDY OF HAPTEN-MODIFIED SKIN PROTEINS (PAPER I)

A previously used method to study penetration of compounds into the different layers of the skin is to apply solutions of fluorescent sensitizing compounds, such as fluorescein isothiocyanate, to the top of full skin extracts and let the solutions diffuse through the skin *ex vivo*. The penetration patterns of the compounds are then visualized by e.g. two-photon microscopy (TPM) or laser scanning confocal microscopy (LSCM) (*43-48*). The compounds used these previous studies are constantly fluorescent and do not show where hapten-protein binding take place. Therefore, a class of compounds called bromobimanes (described in section 3.1.1) was used to visualize hapten binding sites in the present study.

Before applying the bromobimanes to skin extracts, some control experiments had to be performed.
First, the sensitizing potency of the compounds needed to be investigated. To be able to find hapten targets which are relevant for contact allergy, the model compounds must be potent contact sensitizers. This was done using the murine LLNA (described in section 3.2.1) (Figure 4.1) (*20*). The compounds monobromobimane (mBBr) and dibromobimane (dBBr) were both strong sensitizers according to the LLNA (*21, 22*) with EC3 values of 0.12 % (4.4 mM) and 0.17 % (4.9 mM) respectively. The permanently fluorescent non-reactive control bimane, methylbimane, was a non-sensitizer in the LLNA. See Appendix 1 for complete experimental data of the LLNA experiments.

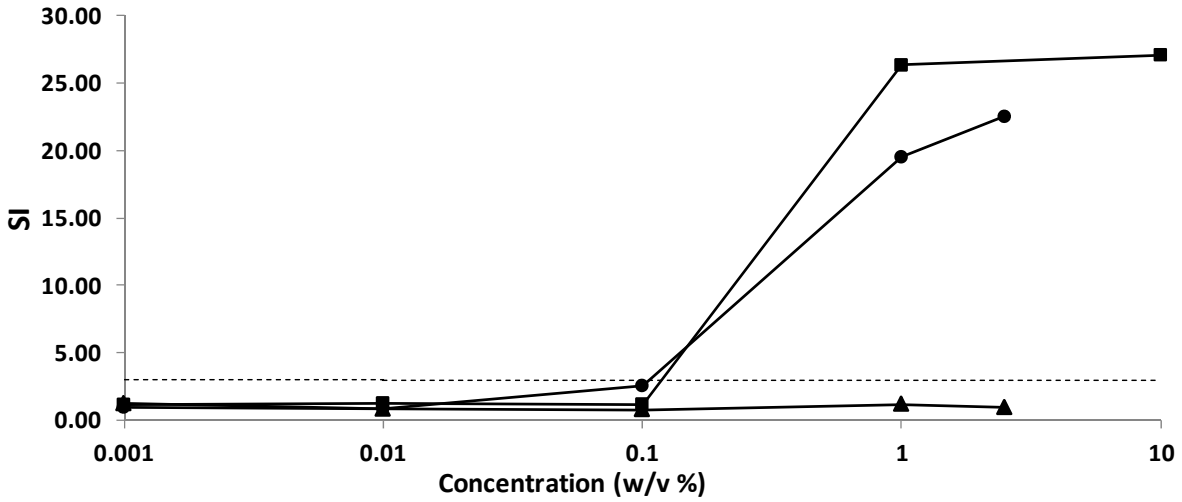


Figure 4.1. LLNA data for dBBr (■). mBBr (●) and methylbimane (▲). All compounds were tested in DMSO. SI, stimulation index. The dotted horizontal line marks SI = 3. The concentration for which a dose-response curve cross this line is the EC3-value (the estimated concentration which is required to give a SI three times higher than the control). mBBr and dBBr were found to be strong sensitizers whereas methylbimane was non-sensitizing at tested concentrations.

With the sensitizing potencies of the model haptens in place, the next step was to investigate the reactivity of mBBr and dBBr *in vitro*.

For these compounds to be suitable for diffusion into human *ex vivo* skin for 20 h, the majority of the applied amounts of the compounds should react with proteins within a similar time frame. Therefore, the bromobimanes were allowed to react with an excess of the model nucleophile glutathione (GSH) in buffer. The reactions were monitored with LC-MS/MS for 24 h. All of the monodentate mBBr had reacted within 2.5 h (not shown). For the bidentate dBBr the same approximate reaction rate with two GSH was observed (not shown), indicating that it is capable of cross-linking proteins.

The reactivity was further investigated using the caged fluorescence properties of the bromobimanes. Since the mBBr and dBBr are non-fluorescent in themselves but the mBBr-GSH and dBBr-2GSH conjugates are fluorescent, the bromobimane + GSH reaction can be monitored by measuring the fluorescence of the conjugates. mBBr and dBBr were once again allowed to react with an excess of GSH. As in the LC-MS experiments, the reactions reached their maxima at around 3 h. The results are plotted in Figure 4.2.

Together, the reactivity experiments showed that the reactions of the two model haptens with a model nucleophile occured within a suitable timeframe for the subsequent diffusion experiments.

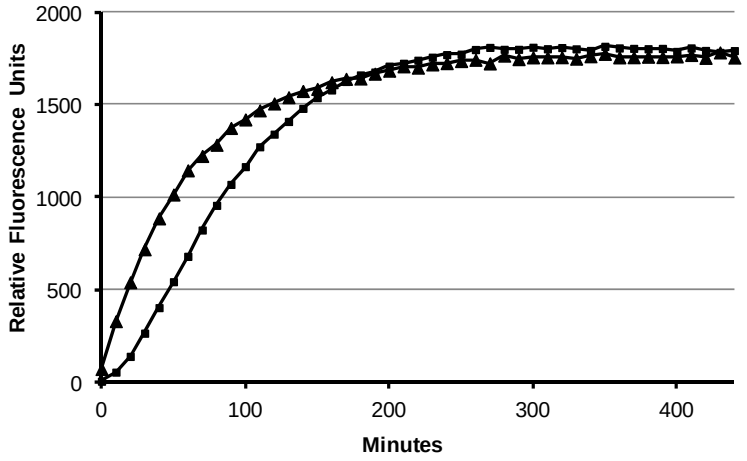


Figure 4.2. Reactivity of bromobimanes with GSH. dBBr and GSH (■); mBBr and GSH (▲). mBBr fluoresce when covalently linked to one thiol-containing compound (here GSH). dBBr must bind to two thiols (here two GSH) in order to become fluorescent. Both reactions were complete after approximately 2.5-3 h.

Considering that the bromobimanes were to be dissolved in buffer and allowed to penetrate excised skin for 20 h at room temperature, the hydrolysis rate in buffer was investigated. These tests were performed using the same experimental setup as in the reactivity study used above (without GSH). The hydrolysis rate was very slow for both mBBr and dBBr and considered non-significant for the penetration studies (data not shown).

4.1.1 Exposure of human skin to bromobimanes and visualization of skin targets

After the reactivity and hydrolysis rate of the bromobimanes had been determined, the compounds were tested on human *ex vivo* skin.
Full-thickness human skin, obtained from breast reduction surgery, was incubated with bromobimanes and methylbimane in a skin permeation system (*55*). The localization of fluorescent HPCs in intact skin was visualized by TPM. The TPM images showed intense labeling of the stratum corneum (Figure 4.3) as has been shown earlier by Samuelsson *et al* using FITC as a fluorescent model hapten (*47*). The stratum corneum is designed to be a protective barrier with high amounts of degraded proteins and reactive free amino acids (*56*) and it was no surprise that the bromobimanes reacted in this layer. Interestingly, the bromobimanes did not react in the suprabasal layers of the epidermis (the spinous and granulous layers). The non-reactive suprabasal layers have less available protein targets due to increased protein crosslinking combined with decreased cell membrane fluidity, e.g. formation of the cornified envelope, across the epidermis (Figure 4.4) (*7, 57*). However, in the basal layer the bromobimanes have clearly reacted. The cells in the basal layers have a high membrane fluidity which allows for compounds to enter the cells and react with free intracellular thiols, forming HPCs (*58*). A similar distribution pattern has also been noted in skin biopsies from human volunteers exposed to the extreme contact sensitizer 1-chloro-2,4-dinitrobenzene (DNCB) (Table 3.1) (*34, 36, 59*).

Approximately 95 % of the cells in the epidermis are keratinocytes, whereas dendritic cells make up about 1-3 % (*60*). The low abundance of bromobimane-labeled cells suggested that

dendritic cells were the targets. To address this question, colocalization studies of cryosectioned mBBr-exposed human excised skin was performed with antibodies directed against CD1a, a marker for Langerhans cells, a dendritic cell type present in the skin (*18*) (Figure 4.5). Using LSCM, no colocalization of mBBr and CD1a was detected, thus the Langerhans cells were ruled out as being bimane targets. Instead, colocalization with antibodies specific to the basal keratinocyte proteins keratin 5 (K5) and keratin 14 (K14) showed that the labeled cells were in fact basal keratinocytes (Figure 4.5).

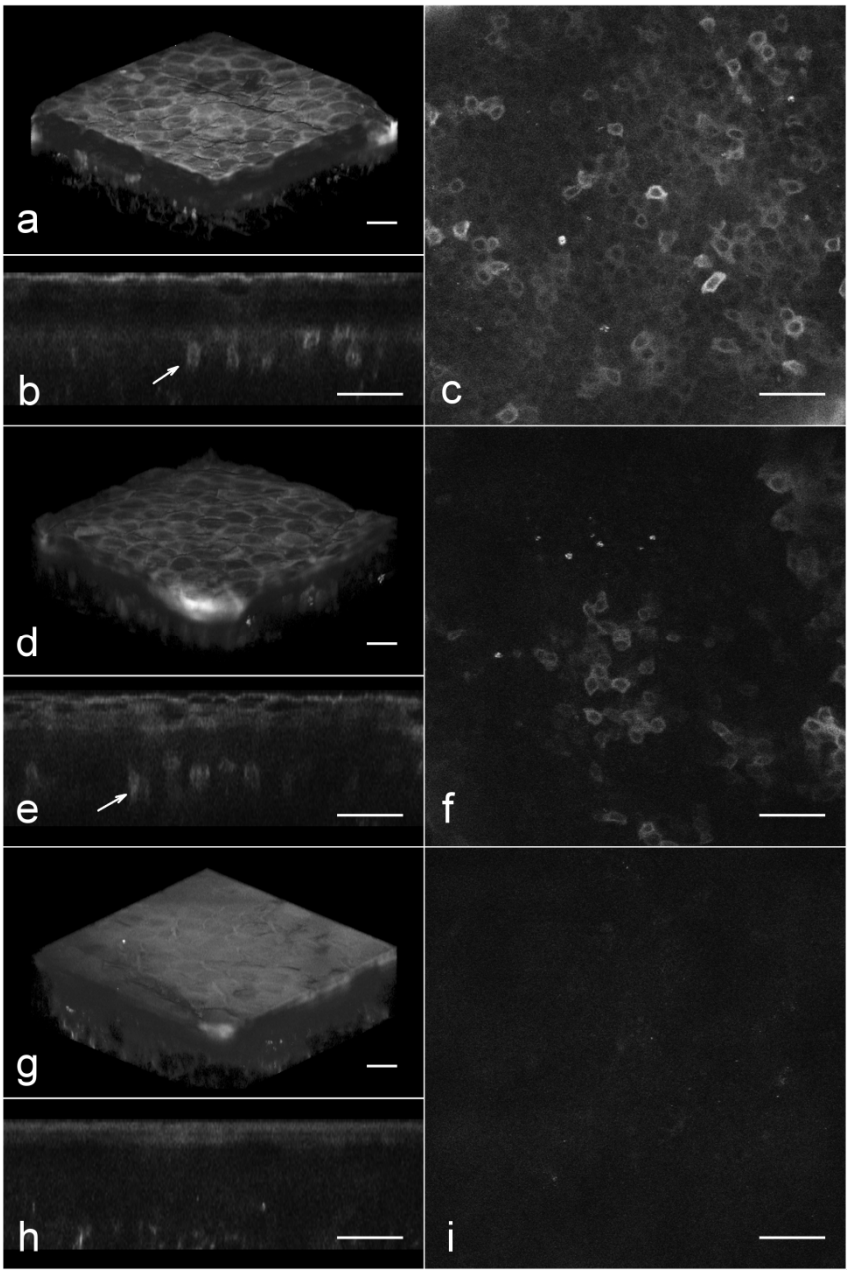


Figure 4.3. TPM images of human skin exposed to bromobimanes. The skin extracts were incubated with: a-c, mBBr; d-f, dBBr; g-i, methylbimane. Left panel shows 3D representations and optical cross-sections. Right panel shows the xy-plane of epidermis after incubation with mBBr (c, z = 52 µm), dBBr (f, z = 62 µm) and methylbimane (i, z = 50 µm). Scale bar = 50 µm. Arrows point at fluorescent bimane-labeled cells in the epidermal basal layer.

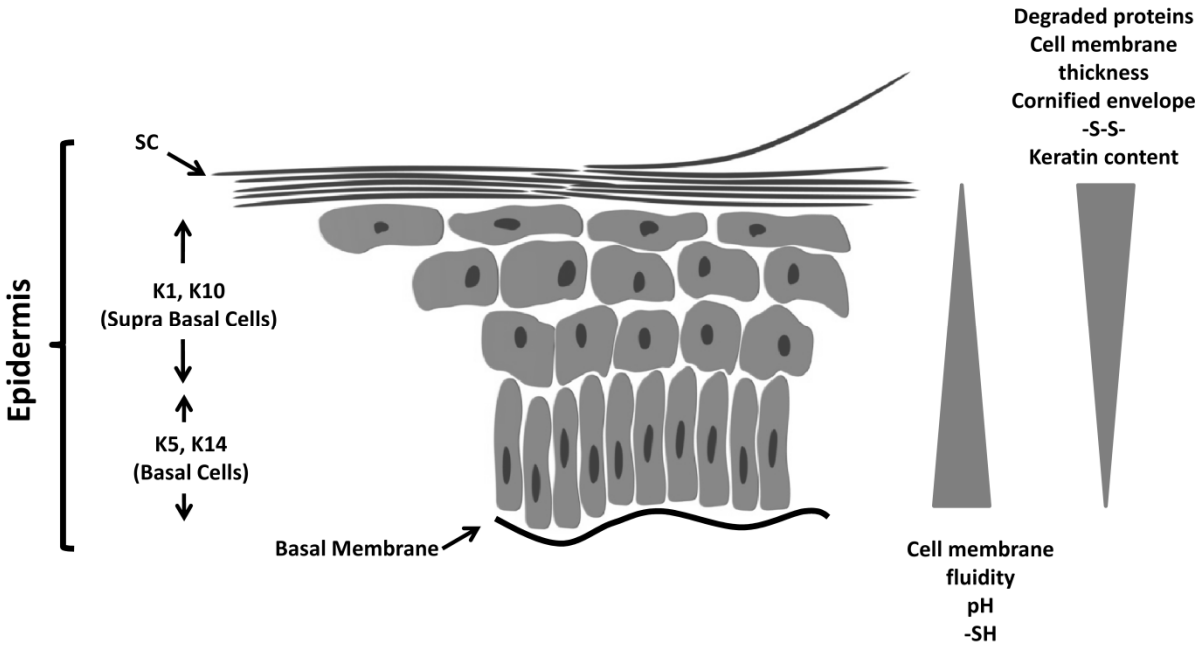


Figure 4.4. Changes in skin composition and structure during keratinocyte differentiation. Briefly, the keratin content and the amount of oxidized, crosslinked cysteines increase toward the skin surface. The cell membrane gets thicker due to the formation of the cornified envelope. Cell membrane fluidity, pH and amount of free thiols increase toward the dermis boundary.

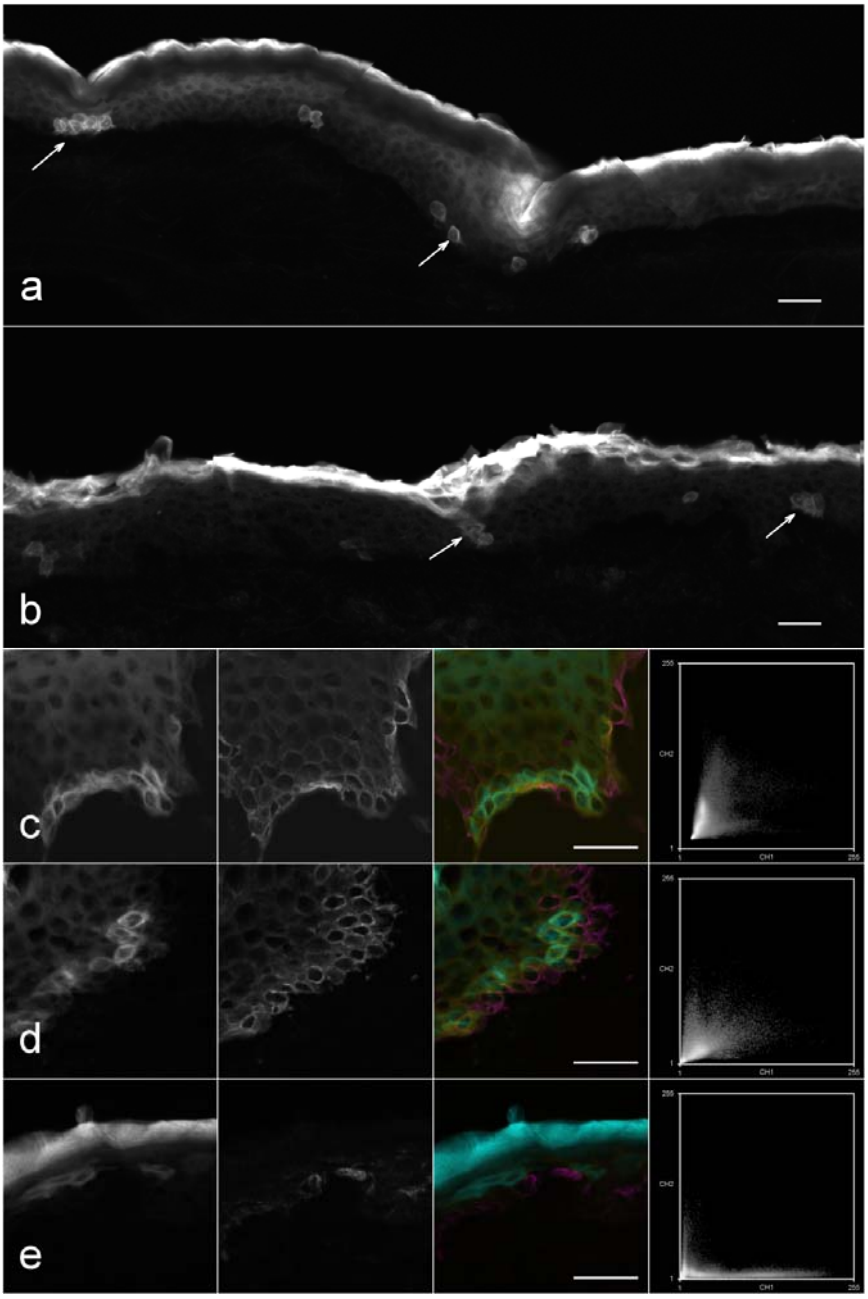


Figure 4.5. Confocal and immunohistochemistry images of human skin incubated with bromobimanes. a, skin incubated with mBBr and **b,** dBBr. **c-e**, immunofluorescence using K5, K14, or CD1a antibodies, respectively, of skin exposed to mBBr. Images from left to right: mBBr fluorescence, immunofluorescence, combined fluorescence images (magenta channel = immunofluorescence and cyan channel = mBBr), and intensity correlation plots. The Manders overlap coefficients for mBBr and immunofluorescence were 0.92, 0.80, and 0.40 for K5, K14, and CD1a, respectively. Arrows indicate fluorescent clusters of cells. Scale bars = 30 µm for all images.

The basal layer is the only dividing cell layer in the epidermis and is known to contain roughly 10 % progenitor keratinocytes (stem cells). These progenitor cells form clusters, so called stem cell niches, and are responsible for continuous renewal of the epidermis (*61*). These clusters may correspond to the mBBr localization pattern, but the question if the targeted keratinocytes are progenitor cells remains to be answered.

4.1.2 Proteomic studies of bromobimane protein targets in human skin

Having determined that basal keratinocytes were labeled by the bromobimanes, the next step was to find out which proteins were targeted by the haptens. Thus, another set of skin extracts were exposed to the bromobimanes in the skin permeation system. After 20 h, dermal and epidermal proteins were extracted and samples were analyzed with sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE) (Figure 4.6). The gels were first scanned for fluorescence to visualize the protein band labeled by the bromobimanes, and subsequently stained with coomassie to compare with the total protein content in the gels. The dermis sample showed no significant bromobimane labeling. The tape-stripped epidermis showed clearer, more distinct labeled protein bands than the full epidermis samples and hence, this study continued with only the tape-stripped epidermis samples.

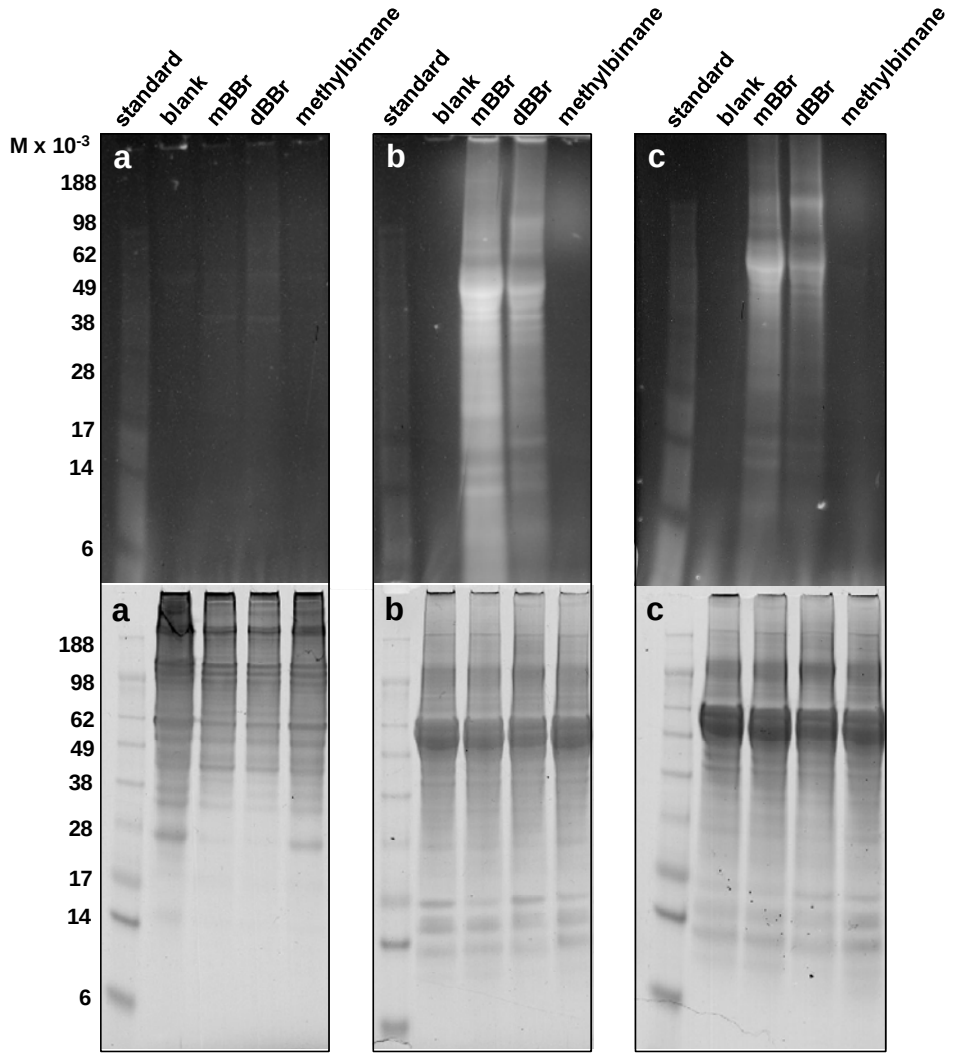


Figure 4.6. SDS-PAGE of skin exposed to bromobimanes. (a) dermis, (b) epidermis with stratum corneum, (c) tape-stripped epidermis. Upper panel: fluorescence images of the gels. Lower panel: coomassie-stained gels. Blank: skin exposed to buffer only. Standard: molecular weight marker.

Another SDS-PAGE of the tape-stripped epidermis samples was performed, using a fluorescent molecular weight marker. The fluorescent scanning of the gels showed two prominent fluorescent protein bands; one at ~ 55-60 kDa in the mBBr lane and one at ~ 100 kDa in the dBBr lane (Figure 4.7).

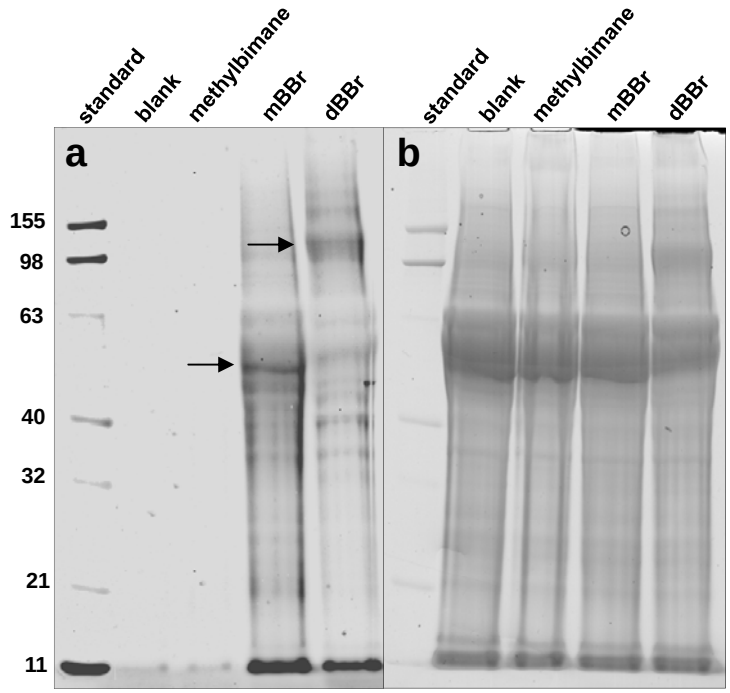


Figure 4.7. SDS-PAGE of tape-stripped epidermis samples from skin exposed to bromobimanes. (a) fluorescence (b) coomassie-staining. Arrows point at the two most prominently fluorescent protein bands.

The protein band marked with an arrow in the mBBr lane (Figure 4.7) corresponds to the basal keratinocyte protein keratin 5 (K5, 58 kDa). K5 is a type II, basic, intermediate filament, which always exists as a heterodimer with the 42 kDa protein keratin 14 (K14); a type I, acidic, intermediate filament (*3*).
The second protein band marked with an arrow in the dBBr lane (Figure 4.8) corresponds to dBBr-crosslinked K5/K14, K5/K5 or K14/K14. However, since K5 and K14 are present as a coiled coil heterodimer in the basal keratinocytes, it is more likely that dBBr crosslinks these two proteins than two identical proteins. K5/K14 crosslinking by sulfur mustard has been shown previously (*62*).

To confirm the presence of K5 and K14 in the fluorescent protein bands discussed above, a western blot using anti-K5 and anti-K14 antibodies was performed. Indeed, K5 was identified in the 58 kDa protein band, and both K5 and K14 were present in the 100 kDa protein band (Figure 4.8), suggesting that these proteins are crosslinked by dBBr.
The "snail-trail" appearance of the blot may be explained by the polyclonal properties of the antibodies used and the high sequence similarities of different keratins present in the skin. For example, K17, which is present in hair follicles (*3*), has 82 % sequence identity with K14. K16, also present in hair follicles (*3*), show 88 % sequence identity with K14. For K5, hair

follicle keratins 6a, b and c show 90 %, 90 % and 89 % sequence similarity, respectively (sequence similarities were calculated using blastp (NCBI BLAST2).

As almost all skin on the human body is covered with hair, it was no surprise that keratins present in hair follicles could be present in the blot samples.
As can be seen on the blot, both antibodies also bound to the 100 kDa protein band in the mBBr and blank lanes. Whether this is due to the presence of K5 and K14 in these locations or to the polyclonal properties of the antibodies remains to be investigated. Also, the anti-K14 antibody reacted with a protein band in the ~110 kDa of the K14 positive control. Reasons for this behavior may be that the K14 positive control contains or forms K14 dimers. However, the true reason is still unknown.

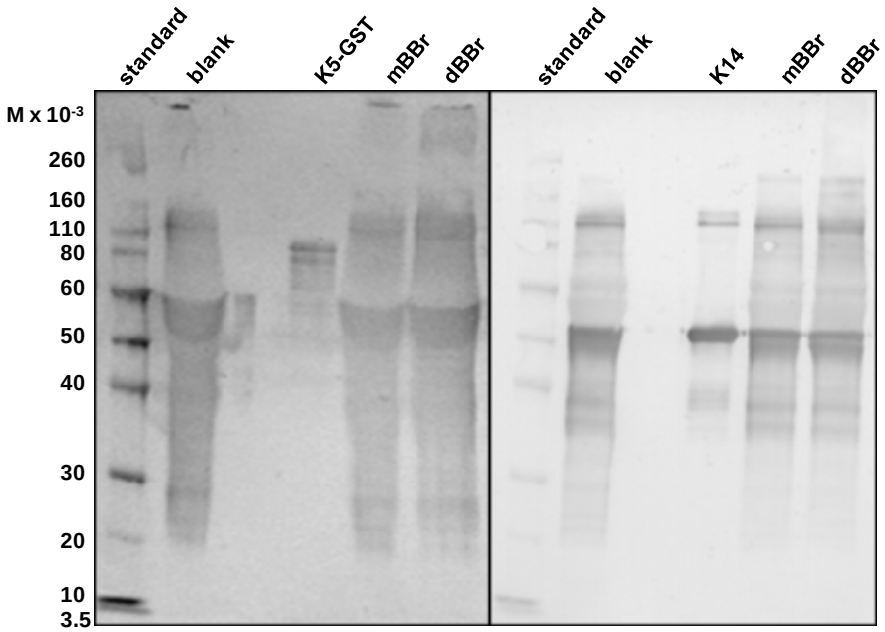


Figure 4.8. Western blot of tape-stripped epidermis samples from skin exposed to bromobimanes. Left panel: anti-K5. Right panel: anti-K14. Blank, buffer-exposed skin. The K5 positive control is equipped with a GST tag, explaining the high molecular weight.

The 58 kDa protein band in the mBBr lane and the 100 kDa protein band in the dBBr lane were cut from the gel (Figure 4.7), trypsinated and analyzed by LC-MS/MS. The mass spectrometry (MS) data confirmed the Western blot results i.e. the presence of K5 and K14 in the fluorescent protein bands (Figure 4.9).

So far, the bromobimane labeling had been tracked to K5 and K14 in the basal keratinocytes in the epidermis. But the question was to which amino acid residues they bind.

Both mBBr and dBBr bind to cysteines. K5 and K14 contain four and six cysteine residues, respectively (Figure 4.9). As the head region (N-terminal) of K5 resides close to the cell membrane (*63*), cysteine 54 (C54) is likely to be targeted by a hapten penetrating the cell membrane. Interestingly, the tryptic peptide containing C54 (V48-R62) was not found in the

MASCOT (Matrix Science) search of the MS data (Figure 4.9), suggesting that this peptide had been modified.

58 kDa protein band. Keratin 5.
Sequence coverage: 60 %

(M)SR**QSSVSFR**S GGSR**SFSTAS AITPSVSRTS FTSVSRSGGG GGGGFGR**VSL
AGACGVGGYG SR**SLYNLGGS K**R**ISISTSGG SFRNR**FGAGA GGGYGFGGGA
GSGFGFGGGA GGGFGLGGGA GFGGGFGGPG FPVCPPGGIQ EVTVNQSLLT
PLNLQIDPSI QRVR**TEEREQ IK**TLNNK**FAS FIDK**VR**FLEQ QNK**VLDTK**WT**
LLQEQGTKTV R**QNLEPLFEQ YINNLRRQLD SIVGERGRLD SELRNMQDLV**
EDFKNKYEDE INKRTTAENE FVMLKKDVDA AYMNKVELEA KVDALMDEIN
FMKMFFDAEL SQMQTHVSDT SVVLSMDNNR NLDLDSIIAE VKAQYEEIAN
RSRTEAESWY QTKYEELQQT AGRHGDDLR**N TKHEISEMNR** MIQR**LRAEID**
NVKKQCANLQ NAIADAEQR**G ELALKDAR**NK **LAELEEALQK AKQDMAR**LLR
EYQELMNTKL ALDVEIATYR KLLEGEECRL SGEGVGPVNI SVVTSSVSSG
YGSGSGYGGG LGGGLGGGLG GGLAGGSSGS YYSSSSGGVG LGGGLSVGGS
GFSASSGR**GL GVGFGSGGGS SSSVKFVSTT SSSRK**SFKS

100 kDa protein band. Keratin 14.
Sequence coverage: 69 %

(M)TTCSR**QFTSS SSMKGSCGIG GGIGGGSSRI SSVLAGGSCR APSTYGGGLS**
VSSSRFSSGG ACGLGGGYGG GFSSSSSSFG SGFGGGYGGG LGAGLGGGFG
GGFAGGDGLL VGSEK**VTMQN LNDRLASYLD K**VR**ALEEANA DLEVKIRDWY**
QRQRPAEIKD YSPYFKTIED LRNK**ILTATV DNANVLLQID NARLAADDFR**
TKYETELNLR MSVEADINGL RRVLDELTLA RADLEMQIES LKEELAYLKK
NHEEEMNALR GQVGGDVNVE MDAAPGVDLS RILNEMRD**QY EKMAEK**NR**KD**
AEEWFFTKTE ELNR**EVATNS ELVQSGKSEI SELRRTMQNL EIELQSQLSM**
KASLENSLEE TKGRYCMQLA QIQEMIGSVE EQLAQLRCEM EQQNQEYKIL
LDVK**TRLEQE IATYRRLLEG EDAHLSSSQF SSGSQSSR**DV TSSSRQIR**TK**
VMDVHDGKVV STHEQVLRTK N

100 kDa protein band. Keratin 5.
Sequence coverage: 53 %

(M)SR**QSSVSFR**S GGSR**SFSTAS AITPSVSRTS FTSVSRSGGG GGGGFGR**VSL
AGACGVGGYG SR**SLYNLGGS K**R**ISISTSGG SFRNR**FGAGA GGGYGFGGGA
GSGFGFGGGA GGGFGLGGGA GFGGGFGGPG FPVCPPGGIQ EVTVNQSLLT
PLNLQIDPSI QRVR**TEEREQ IK**TLNNK**FAS FIDK**VR**FLEQ QNK**VLDTK**WT**
LLQEQGTKTV R**QNLEPLFEQ YINNLRRQLD SIVGERGRLD SELRNMQDLV**
EDFKNKYEDE INKRTTAENE FVMLKKDVDA AYMNKVELEA KVDALMDEIN
FMKMFFDAEL SQMQTHVSDT SVVLSMDNNR **NLDLDSIIAE VKAQYEEIAN**
RSR**TEAESWY QTKYEELQQT AGR**HGDDLR**N TKHEISEMNR** MIQR**LRAEID**
NVKKQCANLQ NAIADAEQR**G ELALKDAR**NK **LAELEEALQK AKQDMAR**LLR
EYQELMNTKL ALDVEIATYR KLLEGEECRL SGEGVGPVNI SVVTSSVSSG
YGSGSGYGGG LGGGLGGGLG GGLAGGSSGS YYSSSSGGVG LGGGLSVGGS
GFSASSGR**GL GVGFGSGGGS SSSVKFVSTT SSSRK**SFKS

Figure 4.9. Sequence coverages of K5 and K14 in the MASCOT search of the MS data. Tryptic sequences in bold were identified. The first methionine residue is shown in parentheses because this residue is cleaved off by aminopeptidases.

Manual investigation of the MS data did not find the unmodified, native peptide (*m/z* 677.33, 1353.66). However, the same peptide modified by one mBBr (*m/z* 772.37) was found. The tandem mass (MS2) spectrum of this peptide was investigated further to see if the peptide fragmentation pattern could reveal if this peptide was indeed modified by one mBBr. In the MS2 spectrum, the peptide is fragmented one amino acid at a time. Using the known monoisotopic masses of each amino acid inside a peptide, the peptide sequence was calculated manually. This was performed according to the method described in section 3.6.6.

Manual investigation of the MS2 spectra of this peptide revealed that C54 was indeed modified by one mBBr (Figure 4.10), and the y_9 and a_7-ions confirms that mBBr is covalently linked to C54.

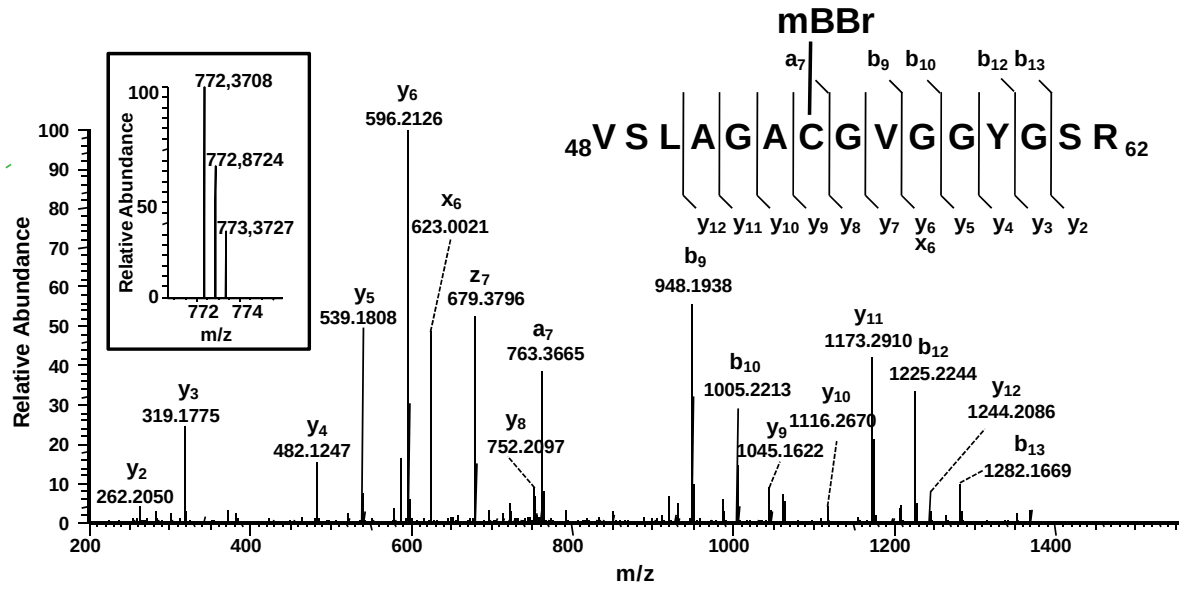


Figure 4.10. Tandem mass spectrometry (MS/MS) fragmentation spectrum. Tryptic fragment V48-R62 of K5 modified by mBBr at C54. Inset shows the M^{2+} ion with an m/z of 772.37. The mBBr-modification adds 190.0748 Da to the molecular mass of cysteine.

The band corresponding to the molecular weight of dBBr-crosslinked K14 and K5 was also cut from the gel and analyzed by LC-MS/MS. The protein band did indeed contain K5 and K14 with sequence coverages of 53 % and 69 %, respectively (Figure 4.9). However, no dBBr-modified peptides could be found, probably because all possibly linked tryptic fragment had too a large *m/z* ratio to be detected in the MS/MS (*m/z* 2000 maximum).

Finally, an ELISA experiment was performed showing anti-K14 antibodies in serum from bromobimane exposed mice. This indicated that there is *in vivo* relevance to the results presented in this chapter. See Appendix 1 for experimental details.

4.1.3 Concluding discussion

This study joins chemistry and biology together in a powerful combination to study biological processes. By employing chemical tools with known properties, the first exact hapten target site derived from human skin tissue could be reported.
As the SDS-PAGE showed that K5 may be a target for mBBr, the sequence of this protein was investigated further. The C54 residue is located in the head region of K5, which is positioned close to the cell membrane (*63*). If a hapten diffuses through the cell membrane, this region of K5 would be one of the first things it would encounter. This cysteine may thereby be a good target for haptens entering the intracellular space. It was very rewarding to find that this residue had indeed reacted with mBBr.
Other researchers have previously identified hapten targets by using e.g. human serum albumin and K14 as model proteins *in vitro* (*64-68*). Compared to earlier studies, the strength of the present study is that the haptens were allowed to react with any proteins available in the skin samples and that an exact hapten target site was detected against the entire backdrop of all proteins in the epidermis.

One drawback of this study is that only model compounds were used. However, as the bromobimanes were strong sensitizers and most clinically relevant haptens target either thiols (cysteines) or amines (lysines and α-NH_2) (*15*), the thiol-specific bromobimanes were considered to be give relevant results for haptens reacting with cysteines. It would be very interesting to investigate if amine-reactive contact sensitizers give the similar binding patterns.
Also, human *ex vivo* skin that had been frozen was used. This implies that all active processes in the cells are shut down and that the bromobimanes are not transported into the basal keratinocytes via active transport mechanisms. It is therefore unknown if the binding pattern for these compounds, with clusters of labeled basal keratinocytes, and labeling of keratin 5 and 14 would be reproducible in skin *in vivo*.
However, support for the *in vivo* relevance of hapten-keratin interactions was obtained in the ELISA experiment detecting anti-K14 antibodies in serum of hapten-exposed mice.

The knowledge of where in the skin, and with what proteins contact sensitizers reacts, will give valuable information for further studies of the immunological processes in ACD.

4.2 STUDIES OF CELL RESPONSE AND HAPTEN–MODIFIED INTRACELLULAR PROTEINS (PAPER II)

The next step was to apply the bromobimanes to cultured human epidermal keratinocytes to search for the same amino acid modification in a living system. Cells were exposed to the bromobimanes and visualized using epifluorescence and confocal microscopy (Figure 4.11). Uptake of the compounds takes place within seconds and the intracellular localization pattern was similar for mBBr and dBBr (Figure 4.11 b, e). Confocal microscopy images of cells exposed to mBBr revealed a filamentous binding pattern, stretching across the cell. The uniform fluorescence in the cytosol, nucleus and nucleolus can most likely be ascribed to the abundant intracellular protein glutathione (GSH), present in up to 10 mM inside cells (*41, 42*). The permanently fluorescent, non-reactive methylbimane was used as control and keratinocytes exposed to this compound showed virtually no uptake at all.

To investigate the effects of hapten exposure over a longer time period, the keratinocytes were exposed to the bromobimanes and imaged up to 24 h later. Most surprisingly, the cells started to expel micrometer-sized fluorescent membrane blebs (plasma membrane vesicles) at the cell surface after 1-2 h (Figure 4.12 a-c). The blebs then expanded to 5-10 µm after 10-15 min (Figure 4.12 d-g) and subsequently detached from the cells into the medium over time. The bleb response induced by the bidentate crosslinker dBBr was more prominent and was initiated at an earlier time point than the response induced by the monodentate mBBr. This observation was probably due to the crosslinking properties of dBBr as cytoskeleton crosslinking has previously been shown to provoke more extensive bleb formation (*69*).

A closer look of the cells revealed that membrane integrity of the cells was unchanged, and blebs were forming and detaching until the intracellular fluorescence decreased quickly as a sign of membrane breakdown. After ~ 5 h, the cells were found to be dead using trypan blue as an indicator (Figure 4.12 h). Interestingly, cells exposed to bromobimanes showed no changes in morphology even after membrane integrity breakdown, but blebs were still released over a time period of up to 24 h. None of the behaviors described above was detected for cells exposed to the control compound methylbimane.
Taken together, the cell responses are similar to those observed in necrosis. In this type of cell death, micrometer-sized blebs are known to form, which is in accordance with the results in this study. However, necrotic cell death is also characterized by cell swelling, disintegration and cytosol leakage (*70, 71*). None of these features were observed for the cells exposed to the bromobimanes and necrotic cell death was thus not likely to occur. For that reason, the cells were tested for apoptosis at two different time points (4 and 7.5 h) after exposure to bromobimanes using a test based on Annexin V and propidium iodide (PI). According to this test, apoptotic cells have externalized phosphatidylserine (PS) without simultaneous PI staining of DNA (which is a marker for loss of membrane integrity in necrotic cells). At the first time point (4 h), the cells were blebbing with intact cell membranes and no externalized PS could be identified. At the second time point (7 h), the majority of the cells had PI-stained nuclei which indicates necrotic cell membrane breakdown. Some of the PI-stained cells also showed externalized PS, thus ruling out apoptosis (Figure 4.12 i). Thus, some kind of modified apoptosis or necrosis is proposed to take place upon hapten exposure.

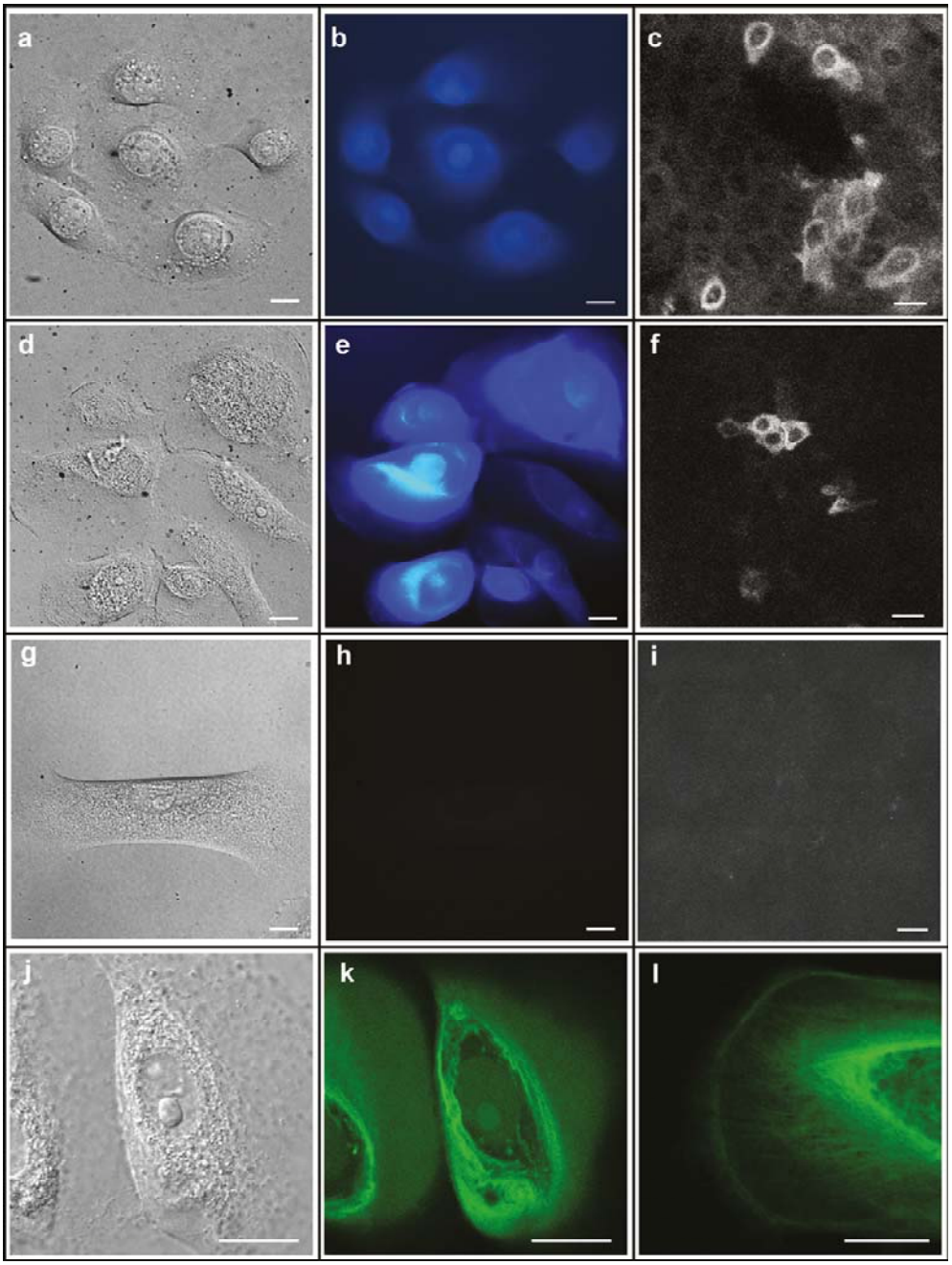


Figure 4.11. Microscopy images of bimane uptake in cultured keratinocytes and *ex vivo* skin. a, d and g, DIC (differential interference contrast) images of keratinocytes after exposure to mBBr, dBBr and methylbimane, respectively. **b, e and h,** epifluorescence images of mBBr, dBBr and methylbimane fluorescence 15 min after exposure (methylbimane 1 h after exposure). **c, f and i,** TPM images of the basal keratinocytes in excised skin tissue exposed to mBBr, dBBr, or methylbimane respectively. **j,** transmitted light image corresponding to **k**, a confocal image of mBBr reaction in keratinocytes and **l**. Scale bars = 20 µm.

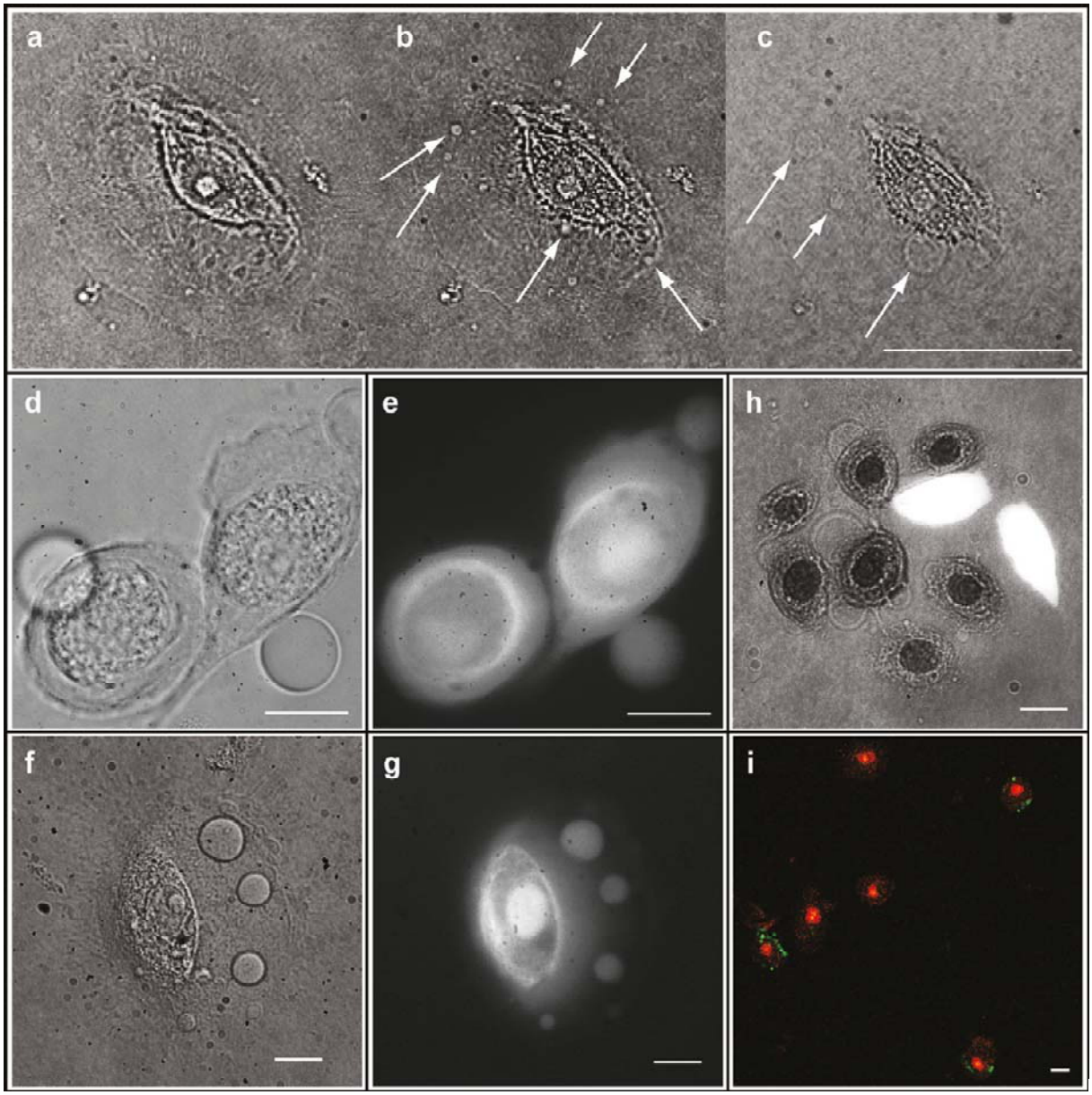


Figure 4.12. Microscopy images demonstrating blebbing responses and apoptosis staining of cultured keratinocytes after bromobimane exposure. a-c, DIC images showing bleb formation and expansion over a 2 h time period after exposure to mBBr. **d-e** DIC and epifluorescence image of blebs on keratinocytes 2 h after mBBr exposure. **f-g** DIC and epifluorescence images of blebs of keratinocytes 1 h after dBBr exposure. **h,** merged DIC/epifluorescence image of keratinocytes after approx. 4 h of mBBr exposure and subjected to trypan blue staining. Cells that have taken up trypan blue (dark cells) have lost the intracellular fluorescence and are releasing blebs. In the same colony, viable cells still exhibit bromobimane fluorescence (white due to overexposure). **i,** confocal image of cells showing PS externalization (green) and PI (red) uptake after 7 h of mBBr exposure. Scale bars = 20 µm.

As the intermediate filament proteins K5 and K14 had been identified as hapten targets in human skin tissue (section 4.1), the filamentous fluorescence labeling pattern in cultured cells suggested that these keratins may also be hapten targets in the fluorescent keratinocyte blebs.

Keratinocyte blebs formed 24 h after exposure to mBBr were separated from cells and collected for analysis. The blebs in medium were lyzed and the contents were desalted and concentrated, followed by analysis with Western blot. The blot confirmed the presence of K5 and K14 in the blebs (Figure 4.13). This was confirmed by LC-MS/MS of the corresponding fluorescent protein bands in the SDS gel.

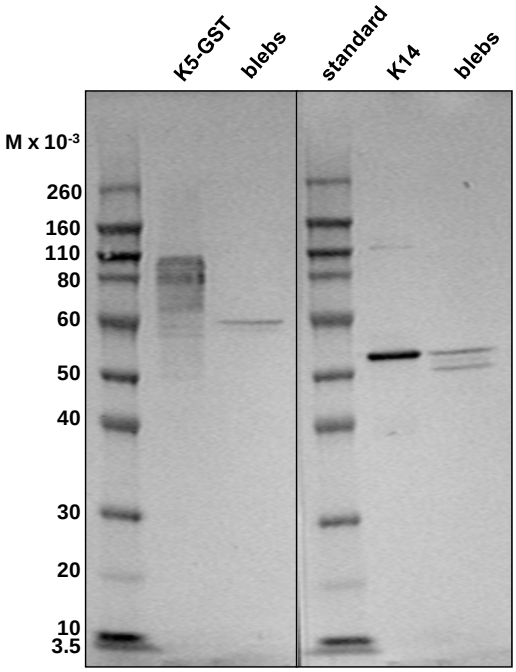


Fig 4.13.Western blot of mBBr-induced bleb content. Left panel: anti-K5. Right panel: anti-K14. The K5 positive control contains a glutathione S-transferase tag, explaining the higher molecular weight. The blebs contain both K5 and K14.

The sequence coverage of K5 in the MASCOT search of the MS data (Figure 4.14) revealed that the tryptic peptide containing C54 (V48–R62) was once again not found, suggesting that this peptide had been modified.

Keratin 5. Sequence Coverage: 51 %

(M)SRQSSVSFRS GGSR**SFSTAS AITPSVSRTS FTSVSRSGGG GGGGFGR**VSL
AGACGVGGYG SR**SLYNLGGS K**R**ISISTSGG SFR**NRFGAGA GGGYGFGGGA
GSGFGFGGGA GGGFGLGGGA GFGGGFGGPG FPVCPPGGIQ EVTVNQSLLT
PLNLQIDPSI QRVR**TEEREQ IK**TLNNK**FAS FIDK**VR**FLEQ QNK**VLDTK**WT**
LLQEQGTKTV R**QNLEPLFEQ YINNLRRQLD SIVGERGRLD SELRNMQDLV**
EDFKNKYEDE INKRTTAENE FVMLKKDVDA AYMNKVELEA KVDALMDEIN
FMKMFFDAEL SQMQTHVSDT SVVLSMDNNR **NLDLDSIIAE VKAQYEEIAN**
RSRTEAESWY QTKYEELQQT AGRHGDDLRN TK**HEISEMNR** MIQR**LRAEID**
NVKKQCANLQ NAIADAEQRG ELALKDARNK **LAELEEALQK** AKQDMARLLR
EYQELMNTKL ALDVEIATYR K**LLEGEECR**L SGEGVGPVNI SVVTSSVSSG
YGSGSGYGGG LGGGLGGGLG GGLAGGSSGS YYSSSSGGVG LGGGLSVGGS
GFSASSGR**GL GVGFGSGGGS SSSVKFVSTT SSSR**KSFKS

Figure 4.14. Sequence coverage of K5 in MASCOT search of MS data of mBBr-induced bleb content. Identified tryptic peptides are shown in bold.

The tryptic peptide corresponding to the peptide containing C54 modified by one mBBr (*m/z* 772.37) was, as in the case of human skin, found in the manual investigation of MS data. The fragmentation pattern in the MS2 spectra of this peptide confirmed that C54 was carrying one mBBr (Figure 4.15). This finding in living cells confirms the previous discovery of mBBr modification of C54 in *ex vivo* human skin tissue.

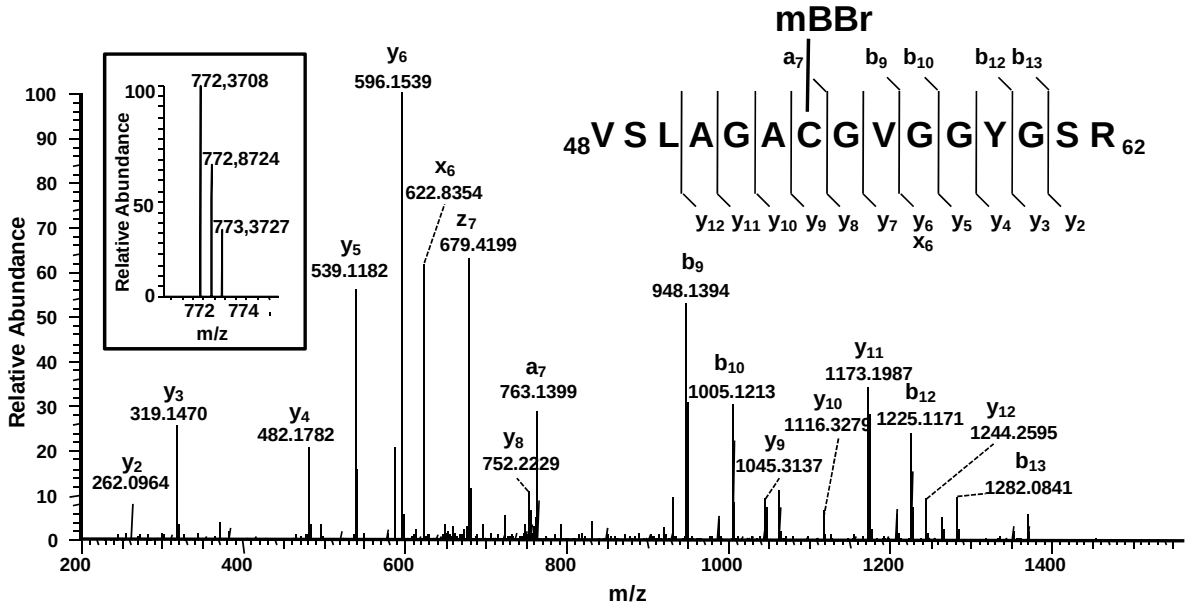


Figure 4.15. Tandem mass spectrometry (MS/MS) fragmentation spectrum. Tryptic fragment V48-R62 of K5 modified by mBBr at C54. Inset shows the M^{2+} ion with an m/z of 772.37. The mBBr-modification adds 190.0748 Da to the molecular mass of cysteine.

Normally, when cells enter apoptosis, keratins are cleaved by caspases to facilitate degradation (*72*). Interestingly, no signs of VEMD/A cleavage in K14 could be seen in the Western blot or the LC-MS/MS experiment. This confirms the finding that blebbing hapten-exposed keratinocytes do not enter regular apoptosis.

4.2.1 Concluding discussion

In this study, the same chemical tools as in Paper I (section 4.1) were used to investigate the consequences of hapten exposure in cultured undifferentiated normal keratinocytes. It was judged that normal keratinocytes had preferable properties, e.g. normal cell cycle time and metabolism, compared to immortalized keratinocyte cell lines, such as HaCaT. These properties make the chosen cell type resemble more closely the basal keratinocytes which were found to be hapten targets in the previous study.

The bleb response of the keratinocytes upon hapten exposure was unexpected and intriguing. Blebs are known to form from other cell types such as macrophages, monocytes and myoblasts upon exposure to e.g. formaldehyde and glyoxal (*73, 74*). In fact, blebs formed upon exposure to formaldehyde were first reported almost 100 years ago (*75*).

Blebs have also been shown to form in cultured human epidermal cells upon UV-irradiation and are suggested to form *in vivo* upon UV-irradiation (*76, 77*). It was also suggested that UV-induced bleb formation is connected to oxidative stress as application of the anti-oxidant vitamin E before UV-irradiation of human skin *in vivo* reduced the morphological changes of epidermis (*76*).

A common feature of the studies discussed above is that cell blebbing was seen as a sign of cell injury. This supports the findings in the present study, as the keratinocytes exposed to bromobimanes were entering cell death. However, whether the blebbing cells were apoptotic or necrotic could not be determined.

In a study by Pappinen *et a*l, irritants were shown to induce e.g. blebbing in a reconstructed rat epidermis model. In their study however, blebbing was only seen in cases where the skin model was severely damaged by irritants (*78*). This implies cell death by classical necrosis, which was not seen after exposure to the bromobimanes.

It was very satisfying to find that K5 present in blebs, induced by mBBr, was modified at the C54 residue. This finding supports the previous finding in *ex vivo* skin.

The keratinocyte blebs resemble e.g. civatte bodies, cytoid bodies and keratin bodies (*79-81*). These bodies then drop below the basement membrane in the skin where they are phagocytosed by macrophages and dendritic cells and their content subsequently presented to the immune system (*79, 82*). The finding that blebs contain modified K5 and K14, suggests that keratinocyte blebs may be involved in the presentation of modified epitopes previously not seen by the immune system, thereby establishing sensitization. It would be interesting to study if keratinocyte blebs are taken up by dendritic cells to confirm this hypothesis.

Since only thiol-reactive compounds were used in this study, one cannot say that keratinocyte bleb formation is a general phenomenon occurring upon hapten exposure. To confirm this theory, other haptens, with different reactivity, must be deployed. This subject is presented in Paper III.

5

Study toward a Sensitization Prediction *In Vitro* Assay (Paper III)

In this study, the generality of the bleb response found in Paper II (chapter 4) was studied. More specifically, it was investigated if haptens with different amino acid specificities could initiate keratinocyte blebbing. The potential of the blebbing response to form the basis of a sensitization prediction *in vitro* assay was also studied.

5.1 EUROPEAN UNION LEGISLATION AND ITS CONSEQUENCES

The importance of the development of an *in vitro* assay for the assessment of sensitizing potency of chemical compounds is discussed in the introduction of this thesis. Briefly, testing cosmetic products and their components on animals is prohibited in the EU (*23*). This is in line with the three Rs; aiming to refine, reduce and replace animal testing (*24*). Naturally, this legislation calls for rapid development of an *in vitro* skin sensitization prediction model.

The murine LLNA (described in section 3.2.1) is the recommended OECD guideline method for assessing contact sensitizing potency (*20*). This method provides graded results and classifies the sensitizing potencies of tested compounds into the four categories: extreme, strong, moderate, and weak sensitizers (discussed in section 3.2) (*21, 22*). This grading property is of great importance to manufacturers of cosmetic products, as it allows for assessment of safe concentration limits for the ingredients.

The European Union body ECVAM accepts method protocols for validation before OECD recommendation (http://ecvam.jrc.it). Today, there are four *in vitro* assays submitted to the ECVAM; the human cell line activation test (h-CLAT) (*83-85*), the direct peptide reactivity peptide assay (DPRA) (*86-88*), the myeloid U937 skin sensitization test (MUSST) (*89*) and KeratinoSens (*90-92*). These assays are all very promising and cleverly constructed; however, they cannot yet provide graded results.

5.2 KERATINOCYTE BLEB FORMATION UPON EXPOSURE TO DIFFERENT HAPTENS

To address the question if keratinocyte blebbing is a general response upon hapten exposure, a panel of compounds with different sensitizing potencies and preferred amino acid reactivity was set up (Table 3.1). The panel consisted of the extreme DNCB, the strong sensitizers mBBr, dBBr, glyoxal and formaldehyde, and the irritant non-sensitizers SDS and nonanoic acid. Nonanoic acid is structurally related to octanoic acid, a known non-sensitizing irritant (*38*).

5.2.1 Time and dose-response study

The first matters to be clarified were the time-frame and suitable compound concentrations to be used in the experiment, i.e. at what time point and concentration the bleb response reaches its maximum. Since the test panel only consisted of extreme and strong sensitizing compounds besides the non-sensitizers, the time and dose which gave the highest responses were of interest, as this allows for further testing with moderate and weak contact sensitizers.

A combined time and dose response study of DNCB and SDS was performed (Figure 5.1). SDS was included to allow for determination if an irritant gave rise to a blebbing response at any time point or concentration. Each compound was tested in three different concentrations (0.005 mM, 0.05 mM and 0.5 mM) with duplicates for each concentration. Eight images per well (two in each quarter of the well) were taken after 20 min, 3 h, 6 h, 24 h and 48 h. The blebbing response was assessed by manual counting of the frequency of blebbing cells in each image (Figure 5.2). Cells exposed to medium only or to vehicle (medium with 1 % dimethyl sulfoxide, DMSO) did not bleb at any time point (see Appendix 2 for images of non-affected cells and blebbing cells).
Interestingly, 0.05 mM, but not 0.5 mM or 0.005 mM, of the irritant non-sensitizer SDS induced quite high amounts of the keratinocytes to bleb after 24 hours of incubation (16 %). The response increased to 39 % after 48 h. SDS dissolved the cells at 0.5 mM (as it is a surfactant, designed to destroy membrane integrity of cells). This compound is a known false positive compound in the LLNA (*93*). Hence, the blebbing response at 0.05 mM was not overly discouraging.
DNCB induced the highest amount of blebbing cells after 24 h of incubation for both 0.05 mM and 0.5 mM (32 % and 65 %). Thus, 24 h was chosen as the end-point for the experiment. The lowest concentrations, 0.005 M and 0.05 mM, gave lower bleb responses than 0.5 mM at all time points. The highest concentration, 0.5 mM, was therefore selected for the screening test.

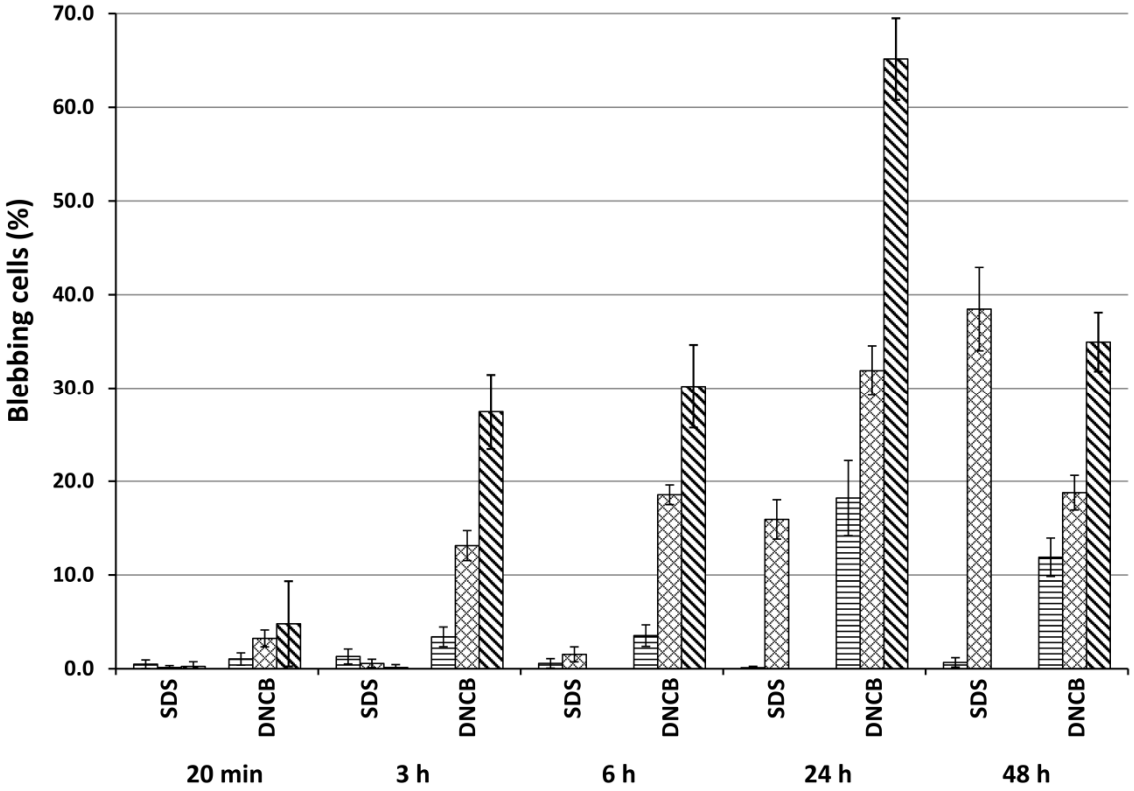


Figure 5.1. Amount of blebbing cells at five different time points. Horizontal stripes (=): 0.005 mM test compound. Crosses (x): 0.05 mM test compound. Diagonal stripes (\\): 0.5 mM test compound. Error bars indicate standard deviations.

5.2.2 Screening of test compounds

Cultured human keratinocytes were exposed to the panel compounds at 0.5 mM. All compounds were tested in duplicate. After 24 h, 16 images per well (four in each quarter) were taken and the frequency of blebbing cells assessed manually.

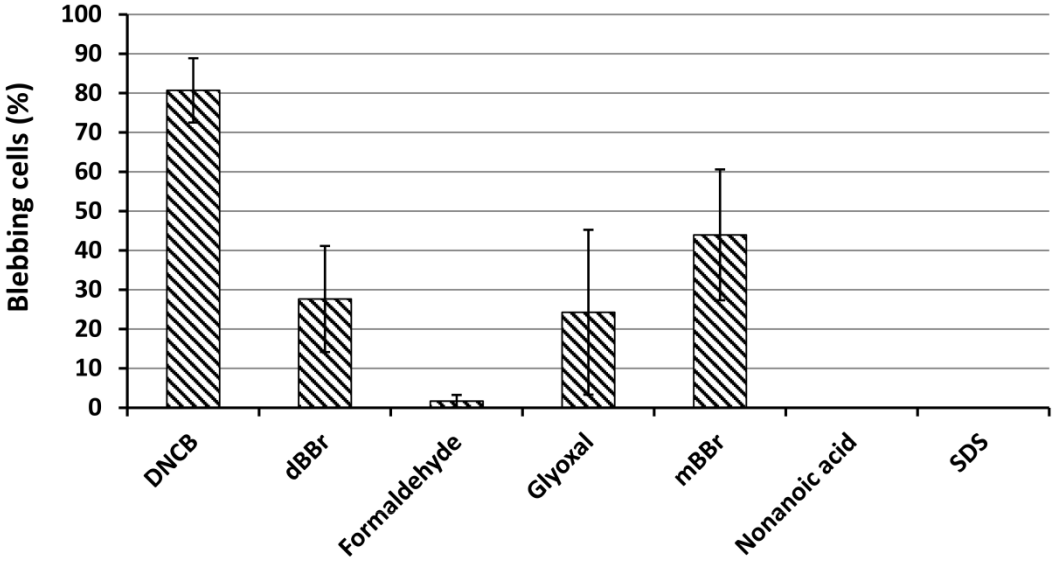


Figure 5.2. Amount of blebbing cells after 24 h of incubation with 0.5 mM test compounds. Control cells exposed to medium with 1 % DMSO or medium only were not blebbing. Error bars indicate standard deviations.

A first indication that the blebbing response could be linked to the sensitizing potency of the compound was seen. The extreme sensitizer DNCB induced the highest amount of blebbing cells (81 %) (Figure 5.2). The strong sensitizers mBBr, dBBr, formaldehyde and glyoxal induced less blebbing (44 %, 28 %, 2 % and 24 %, respectively).

Glyoxal and formaldehyde in particular gave rise to lower amounts of blebbing cells than the bromobimanes. These sensitizers are classified as strong when the EC3 values are reported in percent (0.7 % for both) (Table 3.1). In molar concentration however, these compounds have much higher EC3 values (233 mM and 127 mM, respectively) than other strong sensitizers (Table 3.1), i.e. are less sensitizing. Also, formaldehyde and glyoxal are in equilibrium with their non-reactive hydrate counterparts (Figure 5.3) (*94, 95*). Since these compounds can only be purchased in aqueous solutions and as all compounds in this screen were diluted in cell medium containing low amounts of DMSO, there are reasons to believe that the equilibriums are shifted toward the corresponding hydrate.

The non-sensitizing irritant nonanoic acid did not induce any blebbing. More irritants should therefore be tested before discussing if this test can distinguish non-sensitizing irritants from sensitizers.

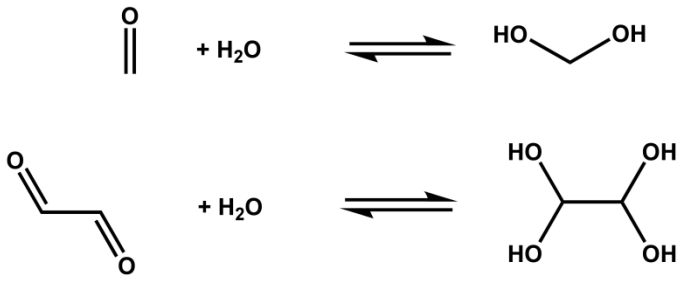


Figure 5.3. Formaldehyde and glyoxal equilibriums with corresponding non-reactive hydrates.

It was also noted that blebs formed after keratinocyte exposure to formaldehyde budded off faster into the medium than blebs induced by other haptens (not shown). Since only keratinocytes with blebs still attached to the cell surface were counted in this study, shed blebs induced by formaldehyde were not considered.

5.2.3 Blebs induced by different sensitizers all contain K5 and K14

The compounds mBBr, oxazolone and glyoxal cover the majority of hapten amino acid reactivities (cysteine, lysine and arginine). Thus, blebs induced by these compounds were subjected to proteomic studies to investigate the keratin content. To be able to perform such studies, relatively high amounts of blebs must be collected. Therefore, the extreme sensitizer oxazolone which produces high amounts of blebs, (Table 3.1) was used as an amine-reactive hapten instead of formaldehyde.
Western blot revealed that these blebs contained both K5 and K14 (Figure 5.4), as was shown for mBBr-induced blebs in paper II (chapter 4). Again, there were no signs of VEMD/A cleavage of K14 by caspases. These findings further indicate that the same cellular process, resulting in bleb formation but not apoptosis, occurs upon hapten exposure regardless of amino acid specificity of the hapten. The SDS-PAGE of the same samples showed that many other proteins also were present in blebs (not shown). Some of these proteins are discussed in the subsequent study.

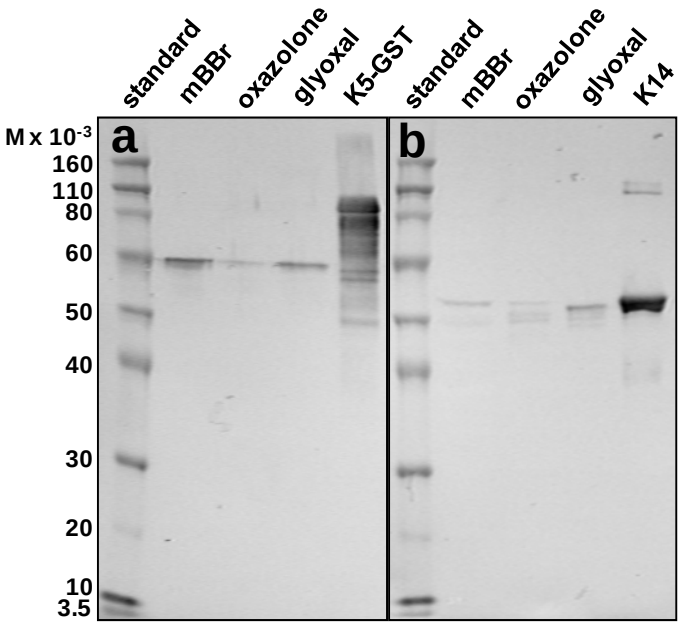


Fig 5.4.Western blot of bleb content. Left panel: anti-K5. Right panel: anti-K14. The K5 positive control carries a glutathione S-transferase tag, explaining the high molecular weight. See Appendix 2 for images of blebs induced by the compound used in the Western blot study.

5.3 CONCLUDING DISCUSSION

In this study, cultured human keratinocytes were exposed to different haptens and the amount of blebbing cells was used as a measurable end point. The first steps toward developing a bleb-based *in vitro* test for assessing the sensitizing potency of chemicals are presented.

Keratinocytes formed blebs upon exposure to low concentrations of SDS, but not upon exposure to nonanoic acid. As SDS is known to cause false positive results in the LLNA (*93*), the result was not too disappointing. Blebs in response to irritants have been shown in a rat epidermal skin model by Pappinen et al. However, blebs were only detected when the skin model was severely damaged by irritants (*78*). The cells in the present study have previously been shown to have unchanged membrane integrity while forming blebs (*96*). This suggest that the blebs detected by Pappinen and coworkers are not the same type as the ones found in the present study. Before claiming that the test method presented in this study can distinguish sensitizers from irritants, more irritants should be tested and evaluated.

As discussed earlier, formaldehyde and glyoxal are classified as strong sensitizers when the EC3 value is reported in % (w/v). In molar however, the EC values for these compounds are much higher than for other strong sensitizers. This is of course due to the low molecular weights of formaldehyde and glyoxal. These compounds induced lower amounts of blebbing cells than the other strong sensitizers and this behavior may be explained by this factor, in combination with the theory that these compounds exist more in the non-reactive hydrate forms in the test conditions used in this study.
Moreover, when the EC3 value of formaldehyde and glyoxal were determined using the LLNA, the compounds were dissolved in the AOO vehicle, consisting of acetone and olive oil

in a 4:1 ratio. This vehicle is relatively nonpolar and may shift the aldehyde-hydrate equilibriums toward the reactive aldehyde, resulting in the compounds being classified as strong sensitizers in the LLNA.
The very low bleb response for formaldehyde acquired in this study may be increased and optimized by using earlier time points for analysis, as it was noted that formaldehyde induced blebs budded off into medium earlier than for other test compounds. Using more concentrations in the screen may also aid in obtaining a correct classification.

Although no optimization of the parameters was performed in this study, this is still a promising pilot study. Screens with an extended panel of haptens covering moderate and weak sensitizers as well as additional non-sensitizers are currently in progress. The preliminary results have potential for a graded sensitization prediction model but are not discussed in this thesis.
An *in vitro* test based on the keratinocyte bleb response would be quick and easy. Test compounds are dissolved in cell medium with low amounts of DMSO and added to the cell culture. At certain time points, images of the wells are taken and analyzed. Of course, as with all cell based tests, the solubility of the compound in medium is a limitation. In this study, 1 % DMSO had to be added to the compound solution in medium to dissolve the compounds. Keratinocytes exposed to the vehicle did not form blebs at any time point; hence this relatively high DMSO concentration was regarded as non-toxic in this test.

This test also has the potential to detect prohaptens and prehaptens. If the prohapten is taken up by the keratinocytes, there is a possibility of metabolism of prohaptens into haptens in the cells. The formed haptens may then trigger a bleb response. Potential prehaptens can be air oxidized before they are added to the keratinocytes in this test. Although promising in theory, the ability of a bleb response-based *in vitro* test to detect pro- and prehaptens remains to be investigated.

Generally, it is not believed that there will be one single stand-alone *in vitro* method for assessing the sensitizing potency of chemicals. Probably, several methods will have to be combined to give reliable classifications that can be used in risk assessment of chemicals, and measuring keratinocyte blebbing in response to chemicals might be one of those methods.

6

Hapten Exposure and Autoimmunity (Paper IV)

The SDS-PAGE of keratinocyte bleb content revealed that not only were K5 and K14 present in blebs, but also many other proteins. The next goal was therefore to investigate the identity of these proteins. Also, as an earlier ELISA experiment (Paper I, chapter 4, Appendix 1) had shown that hapten-exposed mice had elevated levels of serum antibodies against K14, the presence of antibodies against other bleb proteins was investigated.

6.1 PROTEOMIC STUDY OF BLEB CONTENT

A focused panel of haptens, designed to include amine-reactive (oxazolone), thiol-reactive (mBBr and DNCB), arginine-reactive (glyoxal) and bidentate (and thiol-reactive, dBBr) haptens (Table 3.1), was set up.

To investigate the protein content of blebs, human cultured keratinocytes were exposed to mBBr, oxazolone and glyoxal according to the same methods used in Paper II (chapter 4). After 24 h, the blebs were collected, lyzed and desalted. The protein samples were run on a SDS-PAGE and the gel was subsequently coomassie-stained. The coomassie-stained gel showed comparable protein patterns for the tested compounds, suggesting that similar cellular processes occur after exposure to different haptens (Figure 6.1). Protein bands were cut from the gel, digested with trypsin and analyzed with nano-LC-MS/MS (Figure 6.1). The corresponding protein bands from the different lanes showed similar protein contents in blebs induced by the panel compounds (Table 6.1).

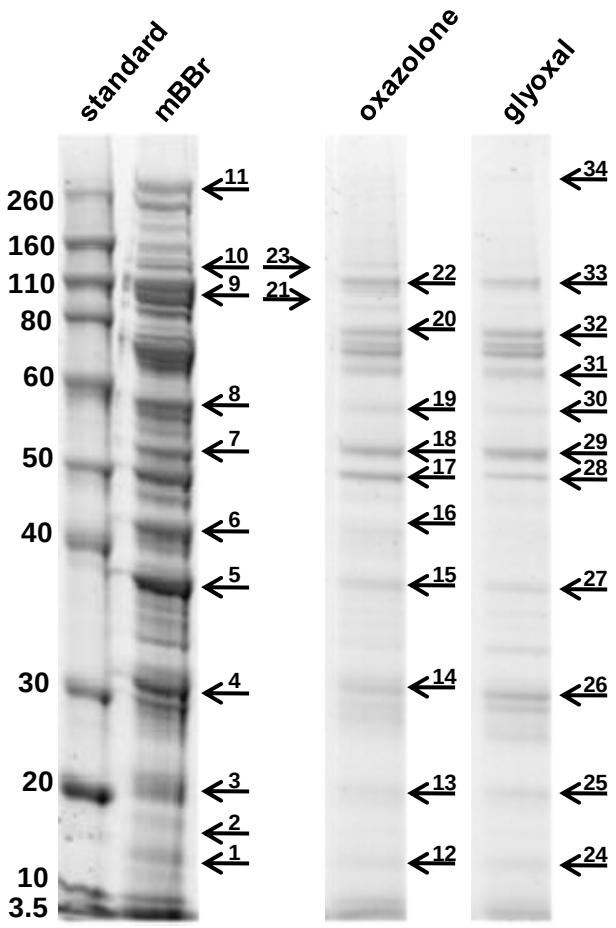


Figure 6.1. Coomassie stained SDS-PAGE of bleb content. Arrows and numbers indicate protein bands that were excised and analyzed. Examples of proteins identified in the numbered band are shown in Table 6.1.

Interestingly, many (but not all) of the identified proteins turned out to be autoantigens known in various autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA) and Sjögren's syndrome (SS) (Table 6.1 and references therein).

Table 6.1. Identification of proteins expelled in blebs with implications in autoimmune diseases

Protein name	mBBr	oxa	glyoxal	kDa	Band No.	Acc. No.	Autoimmune disease	Ref
Chaperone								
Calreticulin	+	+	+	48	7, 18, 29	P27797	COD, RA, SLE, SS	(*97-99*)
Endoplasmin	+	+	+	92	9, 22, 33	P14625	SLE	(*97*)
HSP 90	+	+	NA	83	9, 21	P08238	SLE	(*100*)
Cell adhesion								
Fibronectin	+	NA	+	278	11, 34	P02751	RA, SLE	(*101*)
Cytoskeleton								
Actin beta	+	+	NA	42	6, 16	P60709	CD, RA	(*102, 103*)
Actin gamma	+	–	NA	42	6	P63261	CD, RA	(*102, 103*)
Alpha actinin	+	+	+	103	9, 22, 33	P12814	ACAH	(*104*)
Cofilin 1	+	NA	NA	18	2	P23528	BD, PM/DM, RA	(*105*)
Ezrin	NA	+	+	69	20, 32	P15311	RA, SARP	(*106, 107*)
Myosin Ic	+	–	NA	122	10	O00159	RA	(*103*)
Plastin 3 (T-plastin)	NA	NA	+	71	31	P13797	SLE	(*108, 109*)
Membrane fusion								
Annexin 2	+	+	+	54	5, 15, 27	P07355	APS, RA	(*110*)
Metabolic enzyme								
Alpha enolase	NA	+	+	47	17, 28	P06733	CD, COD, PBC, RA, SLE	(*111*)
Glucose-6-phosphatase isomerase	+	+	+	63	8, 19, 30	P06744	RA	(*112, 113*)
Peroxiredoxin 1	+	+	+	22	3, 13, 25	Q06830	PVS, RA, SLE, SSc	(*114*)
Others								
60 kDa SSA/Ro ribonucleoprotein	+	–	–	61	8	P10155	SLE, SS	(*115, 116*)
Calmodulin	+	+	+	17	1, 12, 24	P62158	ACAH, PBC, SLE	(*117*)
Importin 5	+	+	NA	124	10, 23	O00410	T1D	(*118*)
Peptidyl-prolyl cis-trans isomerase A	+	+	+	18	1, 12, 24	P62937	SLE	(*119, 120*)
Protein disulfide isomerase	+	+	+	57	8, 19, 30	P07237	SLE, T1D	(*121, 122*)
Transitional endoplasmic reticulum ATPase	+	+	–	89	9, 22	P55072	AD, LBD, PBC ,PD	(*123, 124*)

Proteins with Mowse score > 35 were considered statistically significant ($p < 0.05$). ACAH, autoimmune chronic active hepatitis; AD, Alzheimer's disease; APS, anti-phospholipid syndrome; BD, Behçet's disease; CD, Crohn's disease; COD, celiac disease; DM, dermatomyositis; LBD, Lewy body dementia; PBC, primary biliary cirrhosis; PD, Parkinson' disease; PM, polymyositis; PVS, primary vasculitis syndrome; RA, rheumatoid arthritis; SARP, streptococcal-induced autoimmune response in psoriasis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic sclerosis; T1D, type I diabetes. More proteins (Mowse score >35) than the ones stated in the table were found, however, these were not regarded as significant from an autoimmune point of view, e.g. K14 and K5 in bands 8,19, 30 and 7, 18, 29, respectively. + indicates positive identification, – indicates no positive identification. NA, Not Available (corresponding protein band was not analyzed because of limitations in the LC-MS/MS analysis).

6.2 HAPTEN EXPOSURE INDUCE THE FORMATION OF AUTOANTIBODIES

Having established these very interesting results, the next step was to investigate if hapten exposure in general leads to the production of antibodies against some of the proteins found in the blebs.

Mice were therefore exposed to DNCB, dBBr, oxazolone and glyoxal for three consecutive days followed by a booster dose on day 10. Serum was collected on day 13. In this experiment, DNCB was used instead of mBBr because DNCB is a more commonly used thiol-reactive hapten in *in vitro* models and reactivity experiments. The bidentate hapten dBBr was also included to explore if crosslinking agents are able to induce autoantibodies against bleb proteins. Serum from untreated mice was used as negative control.

A small collection of the proteins in Table 6.1 was selected for antibody analysis; cofilin 1, annexin 2, calmodulin and peptidyl-prolyl cis-trans isomerase A (PPIA). The main criteria when choosing these proteins were high Mowse scores and implications in several autoimmune disorders. Eight-mers of the murine proteins were spotted in triplicates on a microarray chip, and serum samples were tested on the chip by LC Sciences (according to methods described in Paper IV).

The raw fluorescence data from the chip were first multiplied by the corresponding dilution factor of the serum. The data was subsequently normalized using the log2(foreground/background) transformation as recommended in Nahtman et al. (*125*). To assess the difference in antibody binding between treatment group and control group (non-exposed mice), two-sample t-tests were performed for each peptide, producing p-values along the amino acid sequences of the proteins. Differences with p-values of less than 0.05 were considered to be statistically significant. The top epitope hits (the epitope with the lowest p-value) for each protein and hapten versus control are listed in Table 6.2.

Table 6.2. Analysis of top epitope recognized by antibodies in serum from mice topically exposed to haptens compared to untreated mice

Protein	Oxazolone vs. control	DNCB vs. control	Glyoxal vs. control	dBBr vs. control
Annexin 2	NIETAVKT (41-48), 1.76E-07	QDTKGDYQ (313-320), 1.25E-03	–	GRRAEDGS (177-184), 9.20E-03
Calmodulin	LGTVMRSL (33-40), 4.61e-06	DFPEFLTM (65-72), 1.17E-02	FSLFDKDG (9-16), 9.49E-05	–
Cofilin 1	DRCTLAEK (145-152), 9.73E-05	DDPYTTFV (65-72), 4.26E-02	–	–
Peptidyl-prolyl cis-trans isomerase	FGKVKEGM (129-136), 2.15E-04	EKFEDENF (81-88), 1.38E-02	–	–

(Numbers) indicate amino acid residues. Numbers after comma (,) indicate the p-values Note that the secondary and tertiary structures of antibody epitopes are not taken into account, and that non-haptenized peptides were used.

The peptide array experiment showed that there are indeed autoantibodies against proteins released in blebs in serum from hapten-exposed mice. Interestingly, only exposure to oxazolone and DNCB induced antibodies against all the selected bleb proteins. This may be due to the extreme potencies of these sensitizers, as the weaker (but still strong) sensitizers glyoxal and dBBr only induced antibodies against one of the selected proteins (annexin 2 for dBBr and calmodulin for glyoxal) (Table 6.2). Another explanation for these differences may be that exposure to the crosslinker dBBr and the potential crosslinker glyoxal results in conformational epitopes being presented to the immune system. Since only linear peptides of the proteins were used in this study, antibodies against conformational epitopes were not detected.

The reason why a peptide array was performed instead of a simple ELISA is that the proteins (murine sequences) used on the chip are not commercially available. Also, the result from the array can be used for epitope mapping of the antibody response later on (Paper V, chapter 7).

Since antibodies against well-known autoantigens were detected, further signs of possible hapten-induced autoimmunity were examined. The serum from hapten-exposed mice was probed for the presence of antinuclear antibodies (ANA). ANA is directed against nuclear debris resulting from insufficient apoptotic clearance and its presence is commonly used clinically as a marker for autoimmunity in diseases such as SS, SLE and RA (*126*), and in drug-induced autoimmunity (*127, 128*). In the experiment, a commercially available ELISA kit detecting murine ANA was used. The results showed that serum from hapten-exposed mice indeed contained ANA whereas the untreated control showed no signs of this marker (Table 6.3). These findings indicate that nuclear debris is released upon hapten exposure, possibly via blebs. Interestingly, the bidentate, crosslinking hapten dBBr induced the highest amount of ANA, implying that crosslinking agents cause the skin cells to release more nuclear debris than monodentate haptens. Also, thiol-reactive (DNCB) and amine-reactive (oxazolone) haptens induced the production of similar amounts of ANA. This further corroborates the previously stated theory that similar cellular processes occur regardless of amino acid specificity of the haptens. The arginine-reactive hapten glyoxal induced the lowest amount of ANA.

Table 6.3. Analysis of sera from mice topically exposed to haptens

ELISA kit	dBBr	oxazolone	DNCB	glyoxal
Anti-Nuclear Antibodies (ANA)	129 μg/ml	78 μg/ml	78 μg/ml	37 μg/ml

Sera from untreated mice contained non-detectable levels of ANA (less than the lowest data point in the standard curve).

6.3 CONCLUDING DISCUSSION

As described in the introduction of this thesis (section 1.4), haptens have been proposed to induce autoimmune responses (*29, 30*). The intriguing findings that blebs contained known autoantigens and that hapten-exposed mice had circulating antibodies against a selection of these autoantigens support this hypothesis.

It is tempting to discuss that the detected autoantibodies may initiate or propagate autoimmune disorders. However, the potential clinical role of these autoantibodies remains to be investigated. The presence of ANA in hapten-exposed mice indicates that nuclear debris is released upon hapten exposure, possibly via blebs. However, whether blebs contain nuclear debris is not investigated in this study.

Bleb formation and release of autoantigens are not believed to be due to necrosis of keratinocytes induced by cytotoxicity, as the blebs did not show signs of classic necrosis *in vitro*. Instead, previous experiments presented in this thesis had shown that the cells have unperturbed membrane integrity while shedding blebs and did not swell before onset of blebbing (*96*).

Proteins expelled in blebs are likely modified by haptens and this might interfere with the processing of the proteins to peptides in antigen-presenting cells and new epitopes, previously hidden from the immune system, may theoretically be formed.

All together, the findings in this chapter are supported by the "waste disposal" theory of autoimmunity, i.e. that ineffective clearance of apoptotic bodies (possibly blebs) leads to break of self-tolerance (*127, 129-133*). Also, haptenization of intracellular self-proteins is one pathway that has been suggested to play a role in drug-induced autoimmunity (*127, 128*).

In conclusion, this study has identified autoantigens expelled in blebs upon hapten exposure, detected antibodies directed against these antigens *in vivo* and discovered elevated levels of the autoimmunity marker ANA *in vivo*. These findings provide new insights in how hapten-induced autoimmunity may occur.

7

Epitope Mapping of Keratin Antibodies in Contact Allergy (Paper V)

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8

General Discussion

In general, the lack of knowledge on which proteins haptens react with and where in the skin this reaction takes place was the driving force that initiated the studies presented in this thesis.

In Paper I, chemistry, biology, and physics were utilized together in a powerful combination to track the formation of HPCs in human skin tissue. Sophisticated microscopy and proteomic techniques revealed that keratins in the basal keratinocytes were targeted by bromobimanes. Moreover, the specific aa binding site for mBBr in K5 was determined. This represents, to the best of the author's knowledge, the first reported exact hapten target site derived from human skin. Gratifyingly, this study was noticed in the research community and received a three-page commentary by Lonsdorf and Enk in *Journal of Investigative Dermatology* (*134*).

It was very satisfying to find that K5 in keratinocyte blebs was modified by mBBr at the same aa residue (Paper II). The C54 residue of K5 is likely to be modified by mBBr to a large extent as the tryptic peptide containing this modification was found in the analysis of both skin and cultured keratinocytes. This particular cysteine residue is located in the N-terminal head region of K5. This region is involved in the connection with desmoplakin in the desmosomes (*63*), and disturbance of this connection through hapten modification may lead to disrupted cell-cell connections. In the epidermis, loss of this connection makes the cell perceive itself as "homeless", leading to anoikis, a form of apoptosis induced by loss of cell-cell connection, characterized by e.g. membrane blebbing (*135*). This suggests that hapten-exposure may induce keratinocyte blebbing by triggering anoikis *in vivo*. However, the absence of both VEMD/A cleavage of K14 in the blebs and signs of apoptosis indicate that a modified form of anoikis may occur.

Thus, in Paper I, a hypothesis were proposed where haptens induce keratinocyte blebbing, and that these blebs (containing hapten-modified proteins) are taken up by dendritic cells in the skin for further presentation to T-cells. The proposed theory may be investigated by transferring fluorescent blebs, formed by keratinocytes upon exposure to bromobimanes *in vitro*, to a culture of dendritic cells. Uptake of blebs by these immune cells may then be monitored by observing the uptake of fluorescence with e.g. laser scanning confocal microscopy.

One still unanswered question is if the bromobimane binding pattern found in human skin *ex vivo* would look the same in living skin. In cultured living keratinocytes the bromobimanes were rapidly taken up (within seconds) (Paper II). It is therefore possible that a broader uptake and binding of the bromobimane would be detected *in vivo* as transport mechanisms

into the cells would be active. Exposure of *in vivo* skin to bromobimanes with subsequent TPM analysis would indeed be very interesting.

In the third paper, the potential use of the bleb response in an *in vitro* sensitization prediction test was discussed. One thing that was not brought up for discussion in that study is the remarkable fact that in the REACH legislation (Registration, Evaluation, Authorisation and Restriction of Chemical substances) (EC 1907/2006), all compounds to be employed in products for human use are tested in the LLNA (*136*). In combination with the ban on testing cosmetic products and ingredients on animals in the EU, this means that even though the ingredients used in cosmetic products have already been safety assessed according to REACH, each ingredient must be retested and safety assessed in a non-animal based, *in vitro* assay to be permitted for use in cosmetic products (*23*). It seems as if the European Union makes the industry perform twice the amount of tests and not reduce the numbers of animals used.
It is of course important that cosmetic products are tested with reliable *in vitro* assays to avoid an increase in the prevalence of contact allergy. The cosmetics industry has heeded the call for alternative methods and is spending a lot of money and effort on research in this area to be able to offer their customers safe products. In addition, the European Union funded the project "Sens-it-iv" which made major research efforts to develop alternative methods during 2006-2010 (www.sens-it-iv.eu). These combined efforts have resulted in several clever assays, of which four have been submitted to ECVAM for prevalidation.
The finding that keratinocyte bleb formation may form the basis of an *in vitro* assay, discussed in this thesis, is a new contribution to this research field. It is the inventors' intention and hope that this method will allow for categorization of chemicals into sensitization potency classes. Another advantage of this proposed assay is that it will be easy to perform and the results simple to analyze. Ongoing development of an automatic counting system to assess the amount of blebbing cells will make the results even easier and significantly faster to analyze.
It is not to be expected that one single alternative method will be able to replace animal testing. Instead, combinations of several assays, e.g. a knowledge-based *in silico* method and a cell based assay, may provide sufficient information for reliable estimations of sensitizing and non-sensitizing compounds.

A general point worth making in this discussion is that the use of % (w/v) as concentration unit for EC3 values can be argued. It simply states how much of the compound in weight that is present in a volume. As the molecular weight of chemical compounds differ greatly, this unit does not give any valuable information to a chemist. Molar concentration clearly defines exactly how many molecules of the compound are present in solution. It may be so that % concentration may be of use in safety assessment of chemicals, but it is my belief that scientists should also always report the EC3 values in molar concentration.

In Paper IV, the focus was turned back to the content of the keratinocyte blebs. Besides K5 and K14, the blebs contained many proteins associated with autoimmune diseases. The presence of known autoantibodies and antibodies against some of these proteins suggest that hapten exposure, followed by bleb release, may play a role in the induction of autoimmunity. However, it is important to make a clear distinction between autoimmunity and clinical autoimmune disorders. Clinical symptoms are not general consequences of

formation of autoantibodies, probably due to different genetic predispositions in different individuals. Drug-induced autoimmunity (DIA) is characterized by temporary clinical effects, i.e. the symptoms are induced by the drug and improved or vanished when the drug is removed (*127, 128*). Whether the autoimmune effects of hapten exposure discovered in this study are temporary like DIA or if they are early signs of a chronic state is yet to be explored. When comparing with DIA, the effects seen are most likely to be transient.
According to Colipa, the European Cosmetics Association (www.colipa.eu), we use on average, six different cosmetic products before we even leave home in the morning. These products may all contain a variety of different contact sensitizers. In several studies, synergistic effects of combined contact allergens have been shown (*137-139*). If combinations of sensitizers also have synergistic consequences on the signs of autoimmunity, the massive daily exposure to haptens may potentially be troublesome for individuals with genetic predisposition for developing autoimmunity.

In the final study presented in this thesis, the antibody binding pattern against K5 and K14 was examined further. This epitope mapping indicated that it is possible to distinguish antibody patterns induced by exposure to cysteine, amine and arginine reactive haptens. For these groups, the antibody pattern overlapped at some parts of the sequences whereas other parts of the sequence showed significantly different patterns. One possible field of application of this new knowledge in the future is diagnosis of contact allergy. Analysis of a blood sample from the patient, together with investigation of what products the patient comes into contact with, may give good indications of what kind of compound the patient is allergic to. The patch test procedure (*140, 141*) may then be greatly reduced and refined, facilitating the working life for dermatologist and improving the experience for patients.

In conclusion, the studies presented in this thesis have contributed to the understanding of mechanisms behind contact allergy and to the development of alternative methods:

- Using a chemical biology approach caged fluorescent haptens were deployed to study the formation of HPCs. The first exact hapten targets in human skin and in cells were reported.

- The hapten-induced keratinocyte blebbing response was presented and used in a study toward developing a new *in vitro* test for assessing the sensitizing potency of chemicals.

- The bleb content was shown to have implications for autoimmunity.

- Finally, the antibody response against K5 and K14 was investigated in detail and its potential use to diagnose contact allergy discussed.

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Over and out
/Sofia

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Appendix 1: Supplementary Information to Chapter 4

[^{3}H]-thymidine incorporation and SI values for compounds tested in the LLNA

Table A1.1. LLNA data for mBBr. dBBr and methybimane.

Test compound and concentration (% w/v, mM)	[^{3}H]-thymidine incorporation (dpm/lymph node)	SI
mBBr		
control	1312.7	
0.01 %, 0.4 mM	1154.6	0.88
0.1 %, 3.7 mM	2887.5	2.20
1 %, 37 mM	14864.2	11.32
5 %, 184 mM	16149.0	12.30
10 %, 369 mM	16218.8	12.35
dBBr		
control	520.5	
0.001 %, 0.03 mM	593.8	1.14
0.01 %, 0.3 mM	647.3	1.24
0.1 %, 2.9 mM	602.5	1.16
1 %, 29 mM	13698.4	26.32
10 %, 286 mM	14089.3	27.07
methylbimane		
control	433.7	
0.001 %, 0.05 mM	530.6	1.22
0.01 %, 0.5 mM	353.5	0.82
0.1 %, 5.2 mM	340.0	0.78
1 %, 52 mM	514.1	1.19
2.5 %, 130 mM	405.0	0.93

All compounds were tested in DMSO vehicle.

ELISA experiment showing anti-K14 antibodies in serum of mice exposed to bromobimanes and SDS

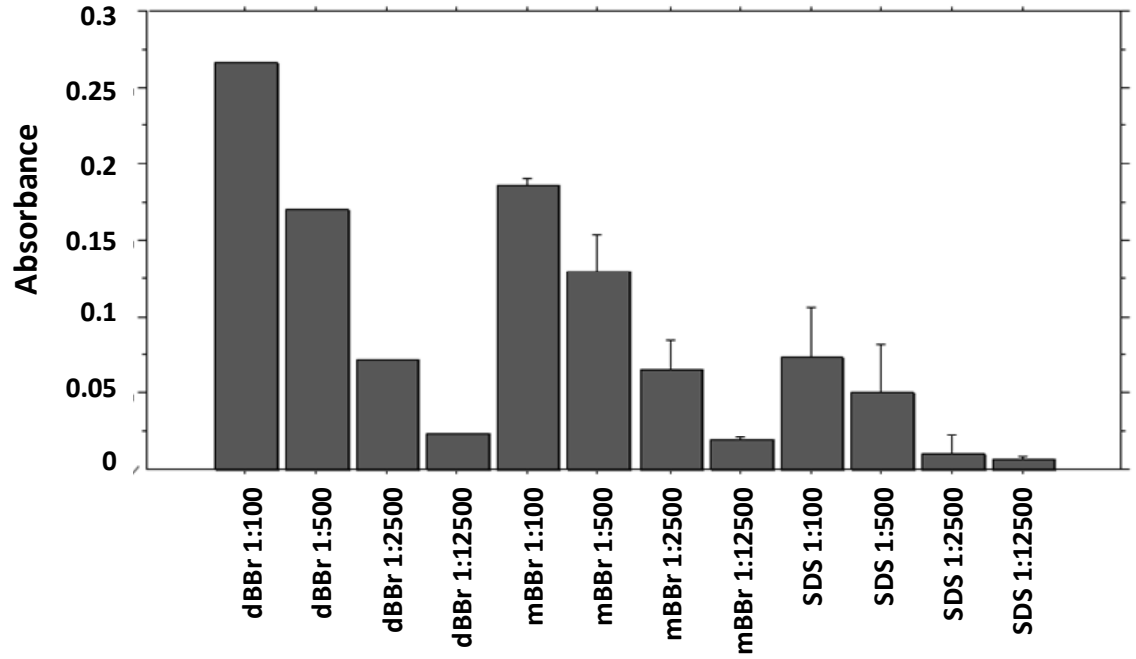

Figure A1.2. ELISA data showing the presence of K14 antibodies *in vivo.*
Anti-K14 antibodies (Ab) in mouse serum after topical exposure to dBBr, mBBr or irritant control (SDS) for 3 consecutive days, measured with a K14 ELISA. Sera were collected 3 days after final exposure. The assay detected anti-K14 Ab of the immunoglobulin (Ig) subclasses IgG, IgM and IgA. Pooled sera from each treatment group were diluted 1:100-1:12500 in PBS, as stated on the x-axis, each dilution analysed in triplicate. The serum pool for dBBr was analyzed once (data presented as mean of the triplicates) whereas the mBBr and SDS serum pools were analyzed twice (data shown as the mean of the triplicates from the two analyses +/- SD)

ELISA Method

96-well plates were coated with K14 (Genway, San Diego, CA, USA) (25 ng/well) at 4 °C over night. Plates were washed three times with buffer (Tris 0.05% Tween) and incubated with 150 µl/well blocking solution (0.5 % BSA, 0.05 M Tris, pH 7.4) for 1 h at room temperature. The plates were washed four times before addition of serum samples, antibodies (ab) or reaction buffer. Sera obtained from mice exposed to bimanes or SDS for three consecutive days were collected and pooled for each group (N = 3). Each serum sample was obtained three days after the final exposure, and serially diluted 1:100, 1:5, 1:5, 1:5 in sample buffer (0.05 M Tris 0,015 M NaCl, pH 7.4), corresponding to final dilutions of 1:100, 1:500, 1:2500 and 1:12500. Aliquots of 50 µl of the serum samples were added to the plates in triplicate. Mouse anti-K14 ab (Lifespan Biosciences) was used as a positive control and prepared in duplicate by serial dilution in sample buffer from 1:400 to 1:6400 on the plate. The plates were incubated at 37 °C for 2 hours. Fifty (50) µl biotinylated detection ab (1:1500 polyclonal goat anti-mouse IgG detecting IgG, IgM, IgA $F(ab)_2$ fragment) (Abcam) was added and further incubated at 37 °C for 2 hours. Fifty (50) µl streptavidin-peroxidase (1:2000) was added, and incubated for 1 hour at room temperature. ABTS solution (100 µl 1 mM in 70 mM citric buffer pH 4.2), containing 1 µl 30 % H_2O_2/ml ABTS was added. The plates were incubated in

the dark for 30-60 minutes. The absorbance was measured at 405 nm in a Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, US).

Appendix 2: Supplementary Information to Chapter 5

Keratinocytes exposed to medium only or medium with 1 % DMSO

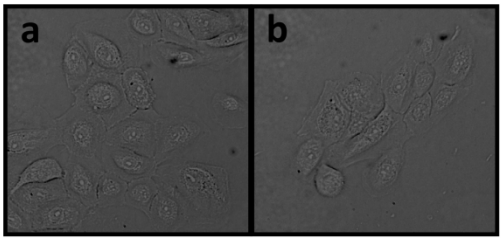

Figure A2.1. Images of untreated, non-affected keratinocytes. Untreated keratinocytes after 24 in medium, **e**. untreated keratinocytes after 24 h exposure to medium with 1 % DMSO.

Images of keratinocytes exposed to mBBr, oxazolone and glyoxal

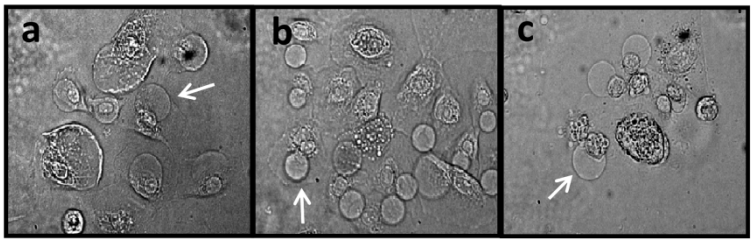

Figure A2.2. Blebs formed after 24 h of exposure to test compounds used in the western blot experiments (0.5 mM solution). **a**. mBBr, **b**. oxazolone, **c**. glyoxal, White arrows points at one bleb in each image of hapten-exposed cells (a-c). A human keratinocyte has a diameter of 20-30 μm.