IDENTIFICATION OF CATHEPSIN B AND L AS NOVEL UVA TARGETS
UPSTREAM OF CUTANEOUS LYSOSOMAL-AUTOPHAGIC DYSREGULATION

by

Sarah Diane Lamore

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SIGNED: Sarah Diane Lamore
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Thank you to all of my peers and colleagues for providing such a supportive and collaborative environment during my time here at the University of Arizona.
DEDICATION

To my family, for their constant support and encouragement. I never would have made it this far without you. To my husband Dave, who is the best husband in the world-thank you for all that you have done for me, especially over the last five years. I feel truly lucky to have you in my life. To my parents and brother, Jon, you have made me into the person I am today. Mom and Jon, thank you for always supporting me and being there for me without question. To my late father, you gave me an incredible foundation to succeed in all my endeavors and you are always with me.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>Ac</td>
<td>amidated carboxyl</td>
</tr>
<tr>
<td>AFC</td>
<td>amino-4-trifluoromethyl coumarin</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation endproduct</td>
</tr>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>activator protein</td>
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<td>Annexin V</td>
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<td>BCC</td>
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<tr>
<td>BCS</td>
<td>bovine calf serum</td>
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<tr>
<td>BFA</td>
<td>bafilomycin-A1</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
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<tr>
<td>CCL</td>
<td>chemokine (C-C motif) ligand</td>
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<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<tr>
<td>C.I.</td>
<td>confidence interval</td>
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<td>chaperone mediated autophagy</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic initiation factor alpha</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray-ionization-mass spectrometry</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FICZ</td>
<td>6-Formylindolo[3,2-b]carbazole</td>
</tr>
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LIST OF ABBREVIATIONS – Continued

FITC fluorescein isothiocyanate
FMK fluoromethylketone
GAPDH glyceraldehydes 3-phosphate dehydrogenase
HBSS Hank’s balanced salt solution
HEK human epidermal keratinocyte
HEMa human epidermal melanocyte (from adult)
HO-1 heme oxygenase 1
HPV16 human papilloma virus 16
4-HNE 4-hydroxy-2-nonenal
Hr hour
HRP horseradish peroxidase
Hsp heat shock protein
IEF isoelectric focusing
J joules
LAMP lysosomal associated membrane protein
LC3 Light Chain 3
LSD lysosomal storage disorder
m6p mannose-6-phosphate
MALDI-TOF-MS matrix assisted laser desorption-time of flight-mass spectrometry
MCA 7-methoxycoumarin-4-yl acetyl
MDA malondialdehyde
mg milligram
MHC Class II major histocompatibility complex class II
min minutes
mJ millijoules
mL milliliter
mM millimolar
MMP matrix metalloproteinase
mRNA messenger ribonucleic acid
MS mass spectrometry
NAC N-acetyl-L-cysteine
NAD+/NADH nicotinamide adenine dinucleotide
NAD(P)H nicotinamide adenine dinucleotide phosphate
NBF nitroblue diformazan
NBT nitroblue tetrazolium chloride
ng nanogram
NMSC nonmelanoma skin cancer
Nrf2 Nuclear factor (erythroid-derived 2)-like 2
n.s. not significant
8oxo-dG 8-Oxo-2'-deoxyguanosine
p62 sequestosome-1
LIST OF ABBREVIATIONS – Continued

PAGE  polyacrylamide gel electrophoresis
PBS   phosphate buffered saline
PI    propidium iodide
PUVA  psoralen + UVA
q.d.  each day (quaque die)
RB    Rose Bengal
RFU   relative fluorescence units
RNA   ribonucleic acid
RNase A ribonuclease A
RNO   p-nitrosodimethylaniline
RNS   reactive nitrogen species
ROS   reactive oxygen species
RT-PCR reverse transcriptase polymerase chain reaction
SCC   squamous cell carcinoma
SD    standard deviation
SDS   sodium dodecyl sulfate
sec   seconds
SEM   standard error of the mean
siRNA small interfering RNA
SOD   superoxide dismutase
SQSTM1 sequestosome-1
SNCA  α-synuclein
TBARS Thiobarbituric acid reactive substances
TBS   tris-buffered saline
TBST  tris-buffered saline, 0.1% tween 20
TEM   transmission electron microscopy
TGM2  transglutaminase 2
TMA   tissue microarray
TNF-α Tumor necrosis factor-alpha
TPA   12-O-tetradecanoyl-phorbol-13-acetate
µg    microgram
µL    microliter
µM    micromolar
UPR   unfolded protein response
UV    ultraviolet
UVA   ultraviolet A
UVB   ultraviolet B
UVC   ultraviolet C
ABSTRACT

Chronic exposure to solar UVA plays a causative role in skin photoaging and photocarcinogenesis. Guided by exploratory difference-in-gel-electrophoresis (DIGE)-proteomics, we identified the thiol-dependent cysteine-proteases cathepsin B and cathepsin L as novel UVA-targets undergoing photo-oxidative inactivation upstream of autophagic-lysosomal dysfunction. In human skin fibroblasts, exposure to noncytotoxic doses of chronic UVA (9.9 J/cm², twice a week, 3 weeks) caused pronounced photooxidative impairment of cathepsin B and L enzymatic activity suppressed by antioxidant intervention. Western blot analysis revealed extensive 4-hydroxy-2-trans-nonenal (4-HNE) modification of cathepsin B in UVA-exposed fibroblasts. Consistent with lysosomal impairment, accumulation of cellular autofluorescent material colocalizing with lysosomes was observed by confocal fluorescence microscopy, and extensive deposition of lipofuscin was detectable by transmission electron microscopy (TEM). Lysosomal expansion was further evidenced by increased immunodetection of lysosomal associated membrane protein-1 (Lamp-1) and Lysotracker-based flow cytometric analysis. While lysosomal membrane integrity remained intact, autophagic blockade was suggested by accumulation of cellular protein levels of LC3-II and p62 (sequestosome 1) in UVA-exposed fibroblasts. Furthermore, UVA-exposure modulated transcriptional levels of p62 (sequestosome 1, SQSTM1), α-synuclein (SNCA), and transglutaminase-2 (TGM2). Strikingly, pharmacological cathepsin B/L inhibition using CA074Me mimicked UVA-induced accumulation of lipofuscin and autophagic-
lysosomal proteins (Lamp-1, LC3-II, and p62), as well as changes at the transcriptional levels. In order to determine if UVA-induced lysosomal impairment requires single or dual inactivation of cathepsin B and/or L, we used a genetic approach (siRNA) to selectively downregulate enzymatic activity of these target cathepsins. Monitoring protein levels of Lamp-1, LC3-II, and p62, we observed that only dual genetic antagonism (targeting both CTSB and CTSL expression) could mimic UVA-induced autophagic-lysosomal alterations, whereas single knockdown (targeting CTSB or CTSL only) did not reproduce the UVA-induced phenotype. Similarly, TEM revealed massive accumulation of lipofuscin-containing lysosomal vesicles in fibroblasts only after CTSB/CTSL-double knockdown. Taken together, our data indicate for the first time that UVA impairs lysosomal function causing autophagic-lysosomal alterations downstream of cathepsin B/L enzymatic inactivation. This work provides evidence for a heretofore unrecognized ‘double-hit’ mechanism of UVA skin photodamage where primary photo-oxidative insult occurs simultaneously with impaired clearance of damaged molecules and organelles downstream of dual inactivation of cathepsin B and L.
CHAPTER 1:
INTRODUCTION

1.1 Physiology and Function of Human Skin

1.1.1 Major functions of human skin

Human skin is a complex organ functioning as an essential barrier, both preventing fluid loss and defending against environmental insults from physical, chemical, and microbial sources. Our skin provides us with vital protection not only by serving as a physical barrier, but also by actively participating in immune surveillance, regulating cell migration, and responding to changes in the environment such as temperature [3]. It is critical for heat regulation, sensation of exogenous factors, and it is designed to respond to mechanical forces with the capability of repairing itself when damaged [4]. Human skin is a stratified organ that is organized into three distinct layers: the outermost epidermal layer, the underlying dermis, and the lowermost hypodermis (or subcutis). The hypodermis is composed mainly of adipose tissue; it is important for temperature regulation and fat storage.

1.1.2 The Dermis

The dermis is the supporting matrix of the skin, consisting of connective tissue with a high capacity for water retention. The thickness of the dermis varies from 0.3 mm on the eyelid to 3.0 mm on the back [4, 5]. It is comprised of two layers: the papillary layer is the upper layer which is composed of thin, poorly organized collagen fibers, and the reticular layer is the deeper layer composed of thicker, more ordered collagen fibers.
Fibroblasts, the major cellular component of the dermis, produce the extracellular matrix proteins that compose the connective tissue, giving the skin its tensile strength, elasticity and resilience. Distinct populations of dermal fibroblasts have been identified in the two different layers of the dermis, as well as a third distinct population associated with hair-follicles [6]. The extracellular matrix is composed mainly of collagen (80-85% of the dry weight of the dermis), elastic tissue (2-4% of the dry weight of the dermis), glycosaminoglycans and proteoglycans (0.1-0.3% of the dry weight of the dermis) and other glycoproteins including fibronectins and integrins [4, 5]. The dermis is highly vascularized, which is important not only for nourishment of the skin, but also for temperature regulation through constriction and dilation. Sweat glands, hair follicles and sebaceous glands are all located within the reticular layer of the dermis, while the terminal networks of capillaries and tactile mechanoreceptors are located within the papillary layer [4, 5]. Resident dermal dendritic cells, mast cells and memory T cells all contribute to immune surveillance to actively defend the skin [7].

1.1.3 The Epidermis

The outermost layer of the skin, the epidermis, is attached to the dermis at the dermal-epidermal junction by the basement membrane, an intricate network of proteins that extend from inside basal keratinocytes into the papillary layer of the dermis. The basement membrane is absolutely critical for adhesion of the epidermis to the dermis, but also contributes to cell migration (i.e. during wound healing) and signaling events [4].

The thickness of the epidermis typically ranges from 0.05-0.1 mm, but can reach up to 1.5 mm; for instance, on the palms of the hand and soles of the feet [4, 5]. The
epidermis is avascular and nourished by diffusion from the dermis. The epidermis, which is formed from the continual division and outward migration of stem cells originating from the basal layer, is divided into four distinct layers: the stratum basale, the stratum spinosum, the stratum granulosum, and the stratum corneum. The innermost layer of the epidermis, the stratum basale, consists of a single row of columnar cells termed basal keratinocytes. The division of these cells gives rise to the stratum spinosum. In this layer, keratinocytes start producing an abundance of the insoluble protein keratin. These cells move outwards and progressively differentiate; forming the stratum granulosum where cells become flattened due to filaggrin, a filament aggregating protein and their cytoplasm appears granular. Differentiation-dependent caspase-14 activation leads to terminal differentiation [8], giving rise to anucleated corneocytes which have retained intracellular keratin and lipids and have a highly insoluble, cornified envelope formed by cross-linking of involucrin and loricrin. The stratum corneum, the outermost layer of the epidermis, is a durable and resilient barrier containing 15-20 layers of corneocytes that is continuously shed and restored from beneath, with skin turnover occurring about every four weeks [4, 5]. Keratinocytes constitute approximately 80% of epidermal cells; they provide structure and barrier formation, but are also involved in inflammatory signaling and angiogenesis [7, 9]. The epidermis also contains melanocytes that produce melanin, an endogenous sunscreen that gives skin its color, Langerhans cells (the resident antigen presenting cells of the epidermis involved in immune response) and Merkel cells, which have a putative role in mechanoreception.
1.2 Cutaneous ultraviolet exposure

1.2.1 Historical Perspective

In Western countries, prior to the early 20\textsuperscript{th} century, a light-skinned appearance was highly desirable whereas tanned skin was associated with lower class work that commonly involved the outdoors. In fact, many Asian, African and Middle Eastern societies still adopt the attitude that lighter skin is more attractive, and thus use of skin lightening agents among these populations is quite widespread \[10, 11\].

In the early 1900s several factors contributed to the Westerner’s public perception of tanned skin. In 1903, Niels Finsen won the Nobel Prize in Medicine for his contribution to the treatment of infectious diseases, most notably lupus vulgaris (a cutaneous tuberculosis infection) using phototherapy \[12\]. At the same time, it was found that exposure to ultraviolet light remedied rickets, a disease that reemerged in the industrialized, polluted cities of Europe and the northeastern United States due to lack of vitamin D \[13\]. In the 1923, after the multiple observations that ultraviolet light was beneficial in treating a range of diseases, photographs of a suntanned Coco Chanel vacationing in French Riviera were released. Tanned skin subsequently became fashionable and indicative of a luxurious lifestyle \[14\].

1.2.2 Human exposure to ultraviolet radiation

Ultraviolet (UV) light, literally meaning “beyond violet”, is electromagnetic radiation with a wavelength ranging from 100-400 nm; its spectrum is shorter than the violet end of the visible light, but longer than that of X-rays. The sun emits UV radiation in the ultraviolet C (UVC; 100-290 nm), ultraviolet B (UVB; 290-320 nm), and
Figure 1.1. Cutaneous ultraviolet penetration. Solar UVC (100-290 nm) is completely absorbed by the stratospheric ozone and therefore is of little biological relevance. Approximately 95% of solar UVB (290-320 nm) is absorbed by the ozone; UVB penetrates the superficial layers of the epidermis. Very little UVA (320-400nm) is absorbed by the ozone and it penetrates into the basal layer of the epidermis and deep into the dermis. Cutaneous effects of absorbed UV radiation include vitamin D synthesis, antimicrobial effects, impaired wound healing, photoaging, photocarcinogenesis and immunosuppression.
ultraviolet A (UVA; 320-400 nm) bands. The stratospheric ozone layer blocks 97-99% of solar UV radiation from penetrating through the atmosphere. UVC is completely blocked by the ozone layer and therefore has no impact on humans. Approximately 95% of solar UVB (290–320 nm) is absorbed by the ozone, whereas only a small percentage of UVA is absorbed by the ozone (Fig. 1.1) [15]. While solar UVB intensity varies enormously with time of year, time of day, cloud covering, latitude, and altitude, UVA radiation varies far less in intensity [16, 17]. Human skin is dually protected against UV radiation thereby minimizing absorption by DNA, protein and other constituents that consequently lead to cellular damage. First, a protein barrier concentrated in the stratum corneum absorbs and reflects UV light. Second, melanin produced by melanocytes serves to absorb UV light in the epidermis [4, 18].

The International Agency for Research on Cancer (IARC), a World Health Organization (WHO) agency, classifies solar UV radiation as a Group I carcinogen [19]. Despite growing public awareness of the hazardous effects of UV exposure, several reports indicate that a significant proportion of the general public are not protecting themselves from UV radiation. The 2007 Health Information National Trends Survey (HINTS) sponsored by the National Cancer Institute reported that only 3 out of every 10 adults routinely practices various sun-safe behaviors (including using SPF>15, wearing long sleeve shirts and/or protective clothing, and staying in the shade) [20]. A report which compiled data from several national surveys including the CDC’s Behavioral Risk Factor Surveillance System (BRFSS) and National Health Interview Survey (NHIS) indicated that 1 in 3 US adults reported being sunburned in the 2005, while one fifth of
white adults reported four or more sunburns within a single year. Sunburn prevalence is reportedly highest in young adults and lowest in older adults, and is more common among higher socioeconomic groups [20].

According to the 2008 NHIS, approximately 15% of adults reported using indoor tanning within the year and was most common in younger adults [20]. In 2009, the IARC added “use of tanning beds/sunlamps” to the list of Group I carcinogens [19]. The UV output and UVA/UVB dosage ratio of tanning devices varies considerably due to several factors including filter composition, power, and tube aging. The Food and Drug Administration (FDA) does not regulate the relative amounts of UVA and UVB in tanning devices, although it does limit acceptable amounts of UVC [21].

1.2.3 Cutaneous UVB effects

UVB (290-320 nm) photons penetrate into the superficial layers of the epidermis and are responsible for sunburn, recognized by erythema resulting from cutaneous vasodilatation mediated by several factors including nitric oxide and prostaglandins [22]. Sunburn is an acute inflammatory response putatively due to keratinocyte apoptosis, with maximal erythema and leukocyte infiltration 24-48 hours after exposure; it slowly resolves with subsequent desquamation or by keratinocyte-mediated phagocytosis [23, 24].

DNA is a major cellular UVB chromophore, thus giving UVB its mutagenic properties. Indeed, there is a strong correlation between chronic exposure to UV and incidence of skin cancers including melanoma (arising from melanocytes) and nonmelanoma skin cancers [NMSC arising from keratinocytes: basal cell carcinoma
(BCC) and squamous cell carcinoma (SCC)]. UVB is directly absorbed by the double bond in pyrimidine bases, and can result in pyrimidine dimers through the formation of a covalent bond between two neighboring pyrimidines. Several types of dimers can be formed through UV absorption; the most common are the cyclobutane pyrimidine dimer (CPD) and the 6-4 pyrimidine dimer ("6-4 photoproduct") [25]. UVB light produces signature TC→TT or CC→TT mutations, caused by mispairing of these photodimers during replication. Several studies have shown that these signature UVB mutations are frequently present in the p53 tumor suppressor gene in nonmelanoma skin cancers [26, 27].

In addition to its mutagenic properties, UVB radiation leads to local and systemic immunosuppression due to alterations of the cell-mediated immune response most likely initiated as a response to DNA damage [28, 29]. A multitude of factors contribute to photoimmunosuppression including induction of suppressor T cells, decreased numbers of Langerhans cells present in the skin, altered immunocompetency of Langerhans cells (reduced MHC Class II expression and antigen presentation capacity), and upregulation of immunosuppressive cytokines including interleukins (IL-1, IL-10, IL-12), Tumor Necrosis Factor α (TNF-α), and Tumor Growth Factor-β (TGF-β) [29-32]. UV-induced suppression of immune surveillance is thought to contribute to the emergence of skin cancers initiated by UV-induced cellular damage. This premise is strengthened by the observation that immunosuppressed individuals are at significantly increased risk of developing skin cancers, particularly SCC [23, 33].
Ultraviolet light is a unique carcinogen in that it does have beneficial effects. A very narrow band of UVB light (295-300 nm) converts 7-dehydrocholesterol, present in the epidermis, into vitamin D3 which, after two further hydroxylation steps, becomes the active hormone 1α, 25-dihydroxyvitamin D3 [13, 24].

1.2.4 Cutaneous UVA effects

More than 95% of the solar UV energy incident on human skin derives from the deeply penetrating UVA region (320-400 nm). Compared to UVB, UVA requires approximately 1000-fold higher levels of radiation to cause sunburn [34], so it was long considered irrelevant to skin damage. Unlike UVB which only penetrates the superficial layers of the epidermis, UVA photons penetrate deep into skin tissue, reaching both the basal layer of the epidermis where the actively diving keratinocytes and melanocytes reside, and the underlying dermis. Accumulating evidence strongly suggests that UVA is largely responsible for most of the chronic skin damage associated with photoaging and also contributes heavily to photocarcinogenesis [35-42]. Several research groups have demonstrated that UVA also contributes to immunosuppression, and there is most likely a synergistic interaction between UVA and UVB leading to the immunosuppressive effects that occur upon UV exposure [41, 43].

It is thought that UVA indirectly contributes to mutagenesis through oxidative DNA damage, particularly by means of the formation of 8-hydroxy-2′-deoxyguanine (8oxo-dG) adducts. This oxidative DNA lesion can cause G→T mutations via mispairing of 8oxo-dG with adenine during replication, or a T→G mutation due to incorporation of 8oxo-dGTP opposite adenine during replication [44]. These ‘signature’ T→G UVA
mutations are found much more frequently in NMSC than G→T mutations, although whether this ‘UVA signature mutation’ is formed solely from oxidative base damage remains unknown at this point [41, 45-48]. Interestingly, one study found that premalignant actinic keratosis (AK) lesions and SCC have ‘signature UVA’ p53 mutations at a higher incidence than the ‘signature UVB’ p53 mutation [46]. Data further supporting the involvement of UVA in mutagenesis and carcinogenesis come from numerous experiments including mouse studies demonstrating exposure to chronic UVA radiation alone induced skin cancers in a significant proportion of UVA-exposed mice [49, 50], and use of an organotypic skin model demonstrating UVA exposure alone induced p53 mutations [51]. One additional study revealed long-term UVA exposure (18 weeks) induced malignant transformation of the HaCaT keratinocyte cell line [52].

Significant differences exist between chronologically aged skin and photodamaged (“photoaged”) skin. The hallmarks of photoaged skin include a decrease in papillary dermal collagen with dense deposition of disorderly elastotic material in the reticular dermis [15, 53]; these changes in the extracellular matrix are thought to be responsible for the wrinkling and laxity of the photoaged skin. With abnormal deposition of basement membrane-like material surrounding the dermal vasculature [15], damaged dermal tissue provides less support to its vascularization, both causing a decrease in vessels along with a widening of existing vessels that become visible at the skin surface [3, 23]. The epidermis of photoaged skin is increased in thickness [54] and while the integrity of the stratum corneum is not compromised, there is a significant delay in barrier recovery upon stratum corneum insult [55]. Human photodamaged skin shows an
impaired capacity for wound healing, and animal models exposed to UVA alone showed a delay in wound healing [3, 23, 56]. ROS-dependent upregulation of several matrix metalloproteinases (MMP) at the mRNA and protein levels, including MMP-1, MMP-2, MMP-3 and MMP-9 is observed in photoaged skin [57-59].

1.2.5 The role of reactive oxygen species (ROS) in UVA-photodamage

Reactive oxygen species (ROS) are generated at low levels during normal cellular respiration and act as important signaling molecules [60]. However, elevated and uncontrolled levels of ROS lead to both oxidative damage of cellular biomolecules and redox dysregulation by directly altering signaling cascades or modulating cysteine-rich redox-sensitive proteins [61]. Indeed, excessive ROS has been implicated in numerous diseases including neurodegenerative disorders, chronic inflammation and cancer [60].

In contrast to the formation of mutagenic photoproducts through direct absorption of UVB radiation by skin cell DNA [27], UVA radiation results in little photoexcitation of DNA directly, and the cutaneous generation of ROS and organic free radicals is thought to be the major mechanisms of UVA-induced skin damage, contributing to both photoaging and photocarcinogenesis [35-40]. UVA radiation induces the formation of various ROS species, including singlet oxygen (\(^{1}\text{O}_2\)), superoxide radical (\(\text{O}_2^{-}\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and hydroxyl radical (\(^{\text{OH}}\)); of these, singlet oxygen is thought to be the predominant species [62]. Supporting this notion, UVA-induced cytotoxicity is greatly reduced when irradiations are performed in the absence of oxygen, whereas the presence of oxygen is inconsequential to UVC-induced cytotoxicity [63].
ROS generated by UVA exposure cause peroxidation of unsaturated lipids in cell membranes including phospholipids and cholesterol [40]. Oxidative modification of lipid membranes alters membrane permeability and fluidity and if severe enough, can lead to loss of membrane integrity [48]. The major end products of lipid peroxidation include 4-hydroxy-\textit{trans}-2-nonenal (4-HNE), malondialdehyde (MDA), 4-hydroxy-2\textit{E}-2-nonenal (4-HHE), and 4-oxo-2-nonenal (4-ONE) [64]. These reactive molecules are known to mediate signaling cascades thereby regulating cell proliferation, differentiation and apoptosis as well as cause damage to proteins and DNA by covalent modification [40, 48, 64, 65]. Indeed, numerous \textit{in vivo} and \textit{in vitro} studies have documented the formation of lipid peroxidation product-adducted proteins leading to functional impairment, and increased levels are associated with various disease states including alcoholic liver disease and diabetes [66-68].

Exposure of human skin or skin cells to moderate doses of UVA activates the transcription factors nuclear factor erythroid 2-related factor 2 (Nrf2), activator protein (AP)-1, and AP-2, all involved in endogenous defense mechanisms [69-71]. UVA also induces cutaneous upregulation of several antioxidant genes such as hemeoxygenase 1 (\textit{HMOX1}), superoxide dismutase 2 (\textit{SOD2}), glutathione peroxidase 1 (\textit{GPX1}), and methionine sulfoxide reductase A (\textit{MSRA}) [72-74]. These cellular modulations are most likely a mechanism for detoxifying UVA-induced ROS [75]. Several experiments have demonstrated suppression of UVA-induced damage, as evidenced by attenuation of DNA damage, modulation of specific genes, and/or cytotoxicity when UVA irradiations are performed in the presence of antioxidants [72, 76-78]. Additionally, overexpression of
catalase in an organotypic skin model abrogates UVA-induced oxidative DNA damage [79]. This combined data supports the central role of oxidative stress in UVA-induced photodamage.

Various sources of ROS are thought to contribute to the generation of cutaneous photooxidative stress including mitochondrial electron leakage, energy crisis and glycolytic blockade resulting from consumption of NAD during DNA repair, release of iron from intracellular stores leading to the generation of OH\(^-\) via the iron-catalyzed reduction of H\(_2\)O\(_2\) by O\(_2\)\(^-\), inflammatory signaling such as activation of NAD(P)H oxidase that generates O\(_2\)\(^-\), and endogenous photosensitizers discussed in 1.2.6 [80-88].

### 1.2.6 Cutaneous Photosensitization by endogenous chromophores

In addition to various cutaneous sources of UVA-induced ROS mentioned in 1.2.5 [80-88], light-driven electron and energy transfer reactions involving non-DNA skin chromophores as endogenous photosensitizers is thought to contribute to oxidative stress in UVA-exposed human skin [35, 37, 89, 90]. After initial formation of excited states of specific chromophores, photosensitization occurs as a consequence of their subsequent interaction with substrate molecules (type I photoreaction) or molecular oxygen (type II photoreaction) via energy and/or electron transfer (Fig. 1.2) [1]. In human skin, various chromophores including urocanic acid [91], riboflavin [92, 93], B\(_6\)-vitamers [94], melanin precursors [95], and advanced glycation endproducts [96, 97] have been proposed as endogenous UV-sensitizers, but molecular identity and causative involvement of relevant endogenous skin photosensitizers remain poorly understood [35, 37, 98-100].
Figure 1.2. Photosensitization reactions. Absorption of photon energy \((hv)\) by a sensitizer \((sen)\) results in formation of a reactive photoexcited state of the sensitizer \((sen^*)\). **Type I photosensitization reaction:** The excited sensitizer reacts directly with the substrate, in a one-electron transfer reaction, to produce a radical or radical ion in both the sensitizer and the substrate. A possible reaction is the direct transfer of the extra electron of \(Sen^-\) to oxygen thereby producing the superoxide radical anion \((O_2^-)\), and regenerating the original sensitizer \((sen)\). **Type II photosensitization reaction:** The excited sensitizer transfers its excess energy to ground-state molecular oxygen \((^3O_2)\), producing singlet oxygen \((^1O_2)\), and regenerating the ground-state sensitizer. Adapted from Foote, 1991 [1].
1.2.7 Cellular targets of UV and activation of signaling pathways

It is well documented that exposure to UV light triggers numerous signaling cascades; although exactly how these pathways become activated to transduce UV light exposure is not completely understood. Several upstream targets have been identified as putative cellular “receptors” involved in recognition of cellular exposure to UV radiation thereby activating signaling cascades; these include damaged DNA, RNA and organelles, as well as endogenous photoproducts including cis-urocanic acid (isomer of trans-urocanic acid produced during terminal differentiation and highly abundant in the stratum corneum) and 6-Formylindolo[3,2-b]carbazole (FICZ; photooxidation product of tryptophan) [101-104]. Additionally, UV-induced redox dysregulation is thought to play a role in the modulation of several signaling cascades (Fig. 1.3) [62, 70, 104-106]. Several experiments have shown UV-induced activation of the three MAPK signaling pathways [p38, C-Jun N-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERK-1 and -2)], epidermal growth factor receptor (EGFR), nuclear factor kappa-B (NF-κB), aryl hydrocarbon receptor (AhR), and nuclear factor erythroid 2-related factor 2 (Nrf2) [22, 70, 102-105]. It is important to note that while UVB-induced effects have received more consideration, exposure to UVA alone has been shown to activate many of these pathways including all three MAPK pathways and Nrf2 [62, 70]. It has also been observed that UVA-exposure leads to inactivation of calcineurin in whole skin explants and skin cell cultures [107] and inactivation of protein tyrosine phosphatase 1B [108]. Through these signaling cascades, a myriad of signaling molecules, enzymes, and transcription factors are modulated, including AP 1- and -2, adhesion molecules,
Figure 1.3. Major cellular targets of UV photons. Cellular UV targets include the (1) DNA (directly absorbed by UVB or indirectly damaged through UVA-induced ROS) (2) 28S subunit of ribosomal RNA (ribotoxic stress leading to altered translation) and ER (ER stress leading to UPR); (3) mitochondria (decreased cellular respiration and mitochondrial electron leakage); (4) cell membranes (altered membrane function and generation of lipid peroxidation products); (5) lysosomes (leading to release of proteases and labile iron which can give rise to the damaging Fenton reaction). These upstream targets, together with the generation of ROS, RNS, and endogenous photoproducts (cis-urocanic acid and FICZ play a role in UV-induced activation of signaling cascades. p53 is the major responder to UV-induced DNA damage leading to transcription of tyrosinase (in melanocytes only), as well as several other proteins that act in concert to repair DNA damage or trigger apoptosis. UV also induces activation of the three MAPK signaling pathways (p38 MAPKs, JNKs, and ERKs), EGFR, NF-κB, AhR, and Nrf2 leading to modulation of several proteins including AP 1- and -2, adhesion molecules, COX-2, prostaglandins, MMPs, TNF-α, and a multitude of other cytokines. These signals orchestrate UV-induced cellular responses that regulate immune response, cell cycle progression, and ultimately cell survival and death.
cyclooxygenase-2 (COX-2), prostaglandins, MMPs, TNF-α, and a multitude of other cytokines [69, 106, 109]. These signals orchestrate UV-induced cellular responses that regulate immune response, cell cycle progression, and ultimately cell survival and death [104].

The tumor suppressor p53 is recognized as the major responder to UV-induced DNA damage. It is p53 that is responsible for tyrosinase transcription (the enzyme that catalyzes the production of melanin), as well as determining cellular fate by “deciding” if cellular DNA is to be repaired or the cell shall undergo apoptosis [110, 111].

In addition to DNA, several other cellular targets of UV light have been identified (Fig. 1.3). Experimental evidence indicates that both UVA and UVB induce ribotoxic stress resulting from damage to the 28S subunit of ribosomal RNA, possibly contributing to activation of the MAPK signaling pathways [112, 113]. UV-induced mitochondrial respiratory dysfunction leads to decreased adenosine diphosphate phosphorylation and increased intracellular ROS as a result of mitochondrial electron leakage [114, 115]. Moreover, chronic UV (particularly UVA) exposure significantly increases levels of a 4,977-bp deletion of mitochondrial DNA (the so-called common deletion), which leads to decreased mitochondrial function [116, 117].

Endoplasmic reticulum (ER) stress, occurring as a result of accumulation of misfolded or unfolded proteins in the ER lumen, seems to occur in response to physiologically relevant doses of UVA and UVB as evidenced by the activation of the unfolded protein response (UPR). UVA exposure induces all three arms of the UPR (the ATF6, IRE1 and PERK pathways) in human dermal fibroblasts [118]. HaCaT
keratinocytes exposed to UVB demonstrated activation of the ATF6 and IRE1 pathways without activation of PERK [119].

Due to their high iron content and the free diffusion of hydrogen peroxide across membranes, lysosomes are particularly sensitive to oxidative stress by means of the iron catalyzed Fenton reaction [120, 121]. In cell culture models, UVA induces a rapid increase in labile iron content that results from damaged lysosomal membranes and this release of labile iron, particularly in the presence of UVA-induced ROS, can heavily contribute to cellular damage [87, 122]. Additionally, lysosomal membrane damage leads to the lysosomal release of proteases (i.e. cathepsins) into the cytosol, thus initiating programmed cell death through Bid cleavage followed by mitochondrial apoptosis [123, 124].

1.3 The lysosome

1.3.1 Lysosome structure and function

Lysosomes, discovered by Christian de Duve in 1949, are acidic (pH 4.0-5.0) organelles maintained by ATP-dependent proton pumps present in the lysosomal membrane. Approximately fifty hydrolytic enzymes are housed within the lysosome that collectively degrade damaged intracellular organelles, as well as intracellular and endocytosed macromolecules. While the most recognized function of the lysosome is degradation of damaged macromolecules and organelles through autophagy, lysosomes also play a pivotal role in several cellular functions, including intracellular transport of
certain vitamins, growth factor receptor recycling, growth factor degradation, antigen processing and presentation, and programmed cell death [125-127].

Lysosomes are formed from budding of the trans-Golgi network. Proteins destined for the lysosome are modified with mannose-6-phosphate (m6p) residues in the cis-Golgi; they are then recognized by m6p receptors in the trans-Golgi network and transported via transport vesicles to late endosomes, which arise from early endosomes containing endocytosed material. The late endosomes acidify and mature into lysosomes that lack m6p receptors [127]. Lysosomes continually fuse and divide, aiding in the uniform distribution of acid hydrolases within the lysosomal vacuoles [125, 128].

The importance of the lysosome in human physiology is highlighted by the group of more than fifty disorders caused by the genetic deficiency of specific lysosomal enzymes. Lysosomal Storage Diseases (LSDs) symptoms vary significantly, but have a common feature of deficient catabolism and accumulation of macromolecules in the lysosomes [129].

1.3.2 The lysosomal role in autophagy

The lysosome is the key executioner in the process of autophagy, which is a highly regulated catabolic process used to recycle cellular constituents. Autophagy is crucial in the digestion of damaged macromolecules and organelles as well as for protection during times of cellular stress, such as starvation. During periods of starvation, cytoplasmic proteins and lipids are digested to produce free amino acids and fatty acids that can be used to synthesize new proteins or oxidized by mitochondria [130]. It is important to note that over-induction of autophagy can lead to autophagic
programmed cell death in a manner that differs from classical apoptosis or necrosis [131, 132]. There are three different types of autophagy in mammalian cells based on the different pathways by which substrates are delivered to the lysosome: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy (Fig. 1.4).

Regardless of the mechanism by which the cargo is brought to the lysosome, the subsequent degradation of cellular components and recycling of monomeric molecules into the cytoplasm is a commonality between the different types of autophagy.

CMA is a selective process of lysosomal degradation that has been observed in response to nutritional starvation and mild stress induced by toxic compounds or oxidants [133, 134]. During CMA, Hsc70, recognizes cytosolic proteins containing a targeting motif of KFERQ (often exposed when the protein unfolds), and together with other chaperone proteins including hsp90, targets the substrate to the lysosome via lysosomal associated membrane protein 2A (LAMP2A) [135-137]. The target protein is subsequently translocated into the lysosome for its degradation. Therefore, CMA is unlike other forms of mammalian autophagy which require vesicle formation or major changes in the lysosomal membrane [138].

Microautophagy involves sequestration of cytoplasmic biomolecules through invagination or septation of the lysosomal or autophagosomal membrane, and is probably also involved in the turnover of lysosomes themselves. Little is known about the mechanism or regulation of microautophagy [139].
Figure 1.4. Key pathways dependent on lysosomal proteolysis. (1) Endocytosis; (2) Chaperone-mediated autophagy (CMA), characterized by its selectivity, is a process in which specific cytosolic proteins containing a targeting motif of KFERQ are recognized by hsc70 and targeted the lysosomal via LAMP2A; (3) Microautophagy involves the transfer of cytosolic materials into the lysosome by direct invagination of the autophagosomal or lysosomal membrane; (4) Macroautophagy involves formation of a double-membrane vacuole (autophagosome) which sequesters cytoplasmic proteins and organelles and then fuses with lysosomes to form an autophagolysosome where degradation of the autophagic substrates occurs. Regardless of the mechanism by which the cargo is brought to the lysosome, the subsequent degradation of cellular components and recycling of monomeric molecules into the cytoplasm is a commonality between the different types of autophagy.
Macroautophagy, often referred to simply as autophagy, is characterized by the formation of a double membrane vacuole which sequesters cytoplasmic proteins, aggresomes, dysfunctional mitochondria, proteasomes, and other organelles. The initially formed vacuole (autophagosome) then fuses with lysosomes resulting in the formation of an autophagolysosome (also called autolysosome), where degradation of the autophagic substrates occurs [130].

1.3.3 Lysosomal lipofuscin

Cellular aging is accompanied by a progressive increase of molecular damage. Lipofuscin, often referred to as “age pigment” is an intralysosomal heterogeneous mixture of oxidized and peroxidized macromolecules primarily of protein and lipid origin with a particularly high content of metal, especially iron [140]. Recognized by its broad spectrum of autofluorescence due to fluorophores originating from glycation end products (AGEs) and lipid peroxidation [141], lipofuscin is considered to be an outcome of incomplete digestion of lysosomal substrates [142]. Lipofuscin-loaded lysosomes appear as irregularly shaped osmiophilic granules of variable size and density when visualized by electron microscopy [141]. Lipofuscin is considered undegradable [141, 143] and accumulates in long-lived non-dividing cells such as neurons, cardiac myocytes, and retinal pigment epithelial cells [144-148] and in the aged dermis [149] evidently as a consequence of cumulative oxidative damage [145-148]. Indeed, a high amount of 4-HNE, MDA- and advanced glycation end-product (AGE)- modified proteins have been identified in lipofuscin of retinal-pigment epithelium cells [150]. Accumulation of
lipofuscin also occurs in various pathological conditions including macular degeneration and radiation-induced injury [125, 151], and is found abundantly in the skin of patients suffering from the lysosomal storage diseases ceroid lipofuscinosis and mucolipidosis, particularly within dermal fibroblasts [152-154].

Until recently, lipofuscin was considered as a mere hallmark of aging with little consequence on cellular function; however, mounting evidence suggests deleterious effects result from increased lipofuscin levels. Indeed, when identical types of postmitotic cells are compared, lipofuscin accumulation is rapid in short-lived species, and slow in long-lived species. That is, the rate of accumulation is inversely related to lifespan, signifying that lipofuscin accumulation most likely has deleterious effects on cellular function [145]. Experimental evidence indicates that accumulation of lipofuscin inhibits lysosomal [155, 156] and proteasome [157, 158] activity. Furthermore, dermal fibroblasts loaded with lipofuscin show increased cellular levels of ROS [159] and susceptibility to acute oxidative stress [160].

1.4 Cathepsins

1.4.1 Classification and processing of cathepsins

Cathepsins (derived from the Greek word kathépsein, meaning to digest) comprise one of the largest families of lysosomal enzymes. Because cathepsins are more stable in acidic environments and have broad substrate specificity, their major physiological role has been recognized as general protein turnover in the lysosome.
There are 15 human cathepsins, including 11 cysteine, 2 aspartic, and 2 serine proteases (Table 1.1).

As with many other protease families, all cathepsins are synthesized as catalytically inactive preproenzymes and require proteolytic removal of the amino-terminal prepro- and propeptide for their activity. The amino-terminal signal prepro-peptide targets the protein to the ER and is cotranslationally cleaved within the ER where N-linked glycosylation also occurs. Further modification with m6p residues occurs in the cis-Golgi apparatus, targeting the protein to the endosome/lysosome via binding to m6p receptors in the trans-Golgi apparatus. Upon reaching the acidic milieu of the late endosomes/lysosomes, cathepsins become active seemingly due to a weakened interaction between the propeptide and the catalytic subunit [161], and commence proteolytic processing. The proregion, which physically blocks the active site of the enzyme, is subsequently cleaved, resulting in a single chain active form of the enzyme. The single chain is further processed resulting in a double chain form consisting of a heavy and light chain held together by a disulfide bond. While in vitro data suggests that some cathepsins, including cathepsins B and L, can be autocatalytically processed [162, 163], it is not clear if the proteolytic processing is autocatalytic or dependent on other cathepsins or proteases; the cascade of activation remains rather unclear at this point. Irrespective of the exact enzymes involved, because of the distance between the active site and the cleavage sites, the process is most likely intermolecular rather than unimolecular [163, 164].
<table>
<thead>
<tr>
<th>Cathepsin</th>
<th>Gene Name</th>
<th>Gene Alias</th>
<th>Active site Residue</th>
<th>Protease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cathepsin A</td>
<td>CTSA</td>
<td>serine</td>
<td>carboxy-mono peptidase</td>
<td></td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>CTSB</td>
<td>cysteine</td>
<td>dipeptidyl carboxypeptidase/ endopeptidase</td>
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<tr>
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<td>cysteine</td>
<td>dipeptidyl aminopeptidase</td>
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<tr>
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<td>CLN10</td>
<td>aspartate</td>
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</tr>
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<td>aspartate</td>
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<tr>
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<td>cysteine</td>
<td>endopeptidase</td>
<td></td>
</tr>
<tr>
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<td>serine</td>
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<td>PKND</td>
<td>cysteine</td>
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<td>endopeptidase</td>
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</tr>
<tr>
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<td>CTSZ</td>
<td>CTSX, CTSZ</td>
<td>cysteine</td>
<td>carboxy-mono peptidase</td>
</tr>
</tbody>
</table>

Table 1.1. Human cathepsins, their active site and protease activity.
All eleven of the cysteine cathepsins belong to clan CA of the C1 (papain) family [165], and have been further divided into two groups designated cathepsin L-like (L, V, K, S, W, F and H) and cathepsin B-like (B, C, O, and X) based on sequence identities [166]. The mature domains of the all of the cysteine cathepsins are similar, ranging in size from 214-260 amino acids. On the other hand, the prodomains show very little similarity and differ substantially in length (41-209 amino acids) [165, 167]. Figure 1.5 depicts the major domains of cathepsin B and cathepsin L.

1.4.2 Catalytic Mechanism of Cysteine Cathepsins

The catalytic site of all cysteine cathepsins is formed by a nucleophilic cysteine residue, a histidine residue that acts as a general base in the hydrolysis of a target peptide bond, and an asparagine residue that stabilizes the ion pair formed by the Cys and His residues [165]. For example, Cys29, His199 and Asn219 form the catalytic triad of cathepsin B [168-170]. The catalytic site is located at the center of the substrate binding region, which has seven amino acid binding sites (subsites) that determine substrate specificity. However, only the S2 subsite forms a well-defined binding pocket accommodating the P2 residue of the substrate, thereby defining the primary specificity of the enzyme; the other subsites form shallow grooves and are much less structured [165]. For this reason, while some cysteine cathepsins have a preference for certain amino acids over others in the target sequence, they generally have broad specificity.

A unique feature of cathepsin B is its ability to act as both an endo- and exopeptidase; it is the only known cysteine cathepsin that has carboxy-terminus peptidyl-dipeptidase activity. The carboxydipeptidase activity of cathepsin B is due to its
Figure 1.5. **Cathepsin B and L.** Schematic structure of cathepsin B (A) and cathepsin L (B). Both proteins consist of an N-terminal pre signaling peptide (pre; light grey), the propeptide (pro, dark grey) and the light (light blue) and heavy chain (HC, blue). The position of the active site residues are shown in red and N-glycosylation sites are shown in green. The amino acid position outside the parentheses indicates that of the proenzyme, while the amino acid position in parentheses indicates that of the mature form of the enzyme (consisting of the heavy and light chains).
occluding loop, an insertion of approximately 20 amino acids that partially obstructs access to the active site cleft [2, 171].

1.4.3 Regulation of cathepsin activity

The means by which cathepsins are regulated are two- fold. First, the pH of their surrounding environment dictates their enzymatic activity. Indeed, many cathepsins have much shorter half lives at a neutral pH versus an acidic pH. For instance cathepsin B has a half-life of approximately 12 hours in acidic environments, whereas only a half-life of 30 minutes in neutral pH conditions [172, 173]. The one major exception to this is cathepsin S, which is more enzymatically active in a neutral environment compared to an acid environment [167]. The second mechanism in cathepsin regulation is protease inhibition. Serpins (serine proteases inhibitors), a group of about 40 proteins originally named for their activity against serine proteases but also inhibit cysteine proteases, covalently bind to the enzyme thereby inhibiting its proteolytic activity. Cystatins are endogenous reversible inhibitors of cysteine proteases. Other types of cysteine protease inhibitors in include stefins (also referred to as type I cystatins) and kinogens [174, 175].

1.4.4 Role of cathepsins in cutaneous homeostasis

While cathepsin have traditionally been recognized as key proteases involved in bulk lysosomal degradation, the fairly broad working pH spectrum and restricted distribution of some of the cathepsins suggest possible functionality beyond their lysosomal role. Recent studies have shown the important role cathepsins play in specific extra- and intracellular functions including hormone and enzyme maturation, antigen presentation, growth factor recycling, tissue remodeling, bone matrix resorption, and
programmed cell death [176-179]. Several experimental observations have shed light on the critical role many of the cathepsins play in maintaining skin homeostasis including wound repair, hair follicle morphogenesis, and epidermal differentiation and desquamation. Indeed, human diseases with cutaneous manifestations have been found to be the result of loss of function mutations in specific cathepsins. In 1996, mutations in the CTSK gene were identified as the cause of pycnodysostosis, characterized by short stature along with skin manifestations, including wrinkled skin and grooved nails [180, 181]. In 1999, it was discovered that a mutation in the CTSC gene is responsible for Papillon-Lefèvre syndrome, a disease with symptoms including severe palmoplantar hyperkeratosis and severe periodontitis resulting in the premature loss of the primary and secondary teeth [182, 183].

Recent research has demonstrated that cathepsin B is crucial for the process of wound repair. During induced migration of HaCaT keratinocytes by means of a scratch-wound assay, cathepsin B-containing lysosomes relocated from the perinuclear region to the cell periphery, followed by secretion of the mature form of the enzyme into the extracellular milieu. Furthermore, the amount of mature, enzymatically active cathepsin B associated with the cell surface was significantly higher during the initial stages of induced migration when compared with non-wounded controls and declined upon closure of the scratched area [184]. Regeneration of scratch-wounded monolayers of HaCaT or normal human epidermal keratinocytes (HEK) was markedly reduced when cell-impermeable cathepsin B inhibitors were added to the cell culture medium [184, 185].
A recent study suggests the importance of cathepsin K in the degradation of dermal extracellular matrix during scar remodeling. Normal skin exhibits low cathepsin K activity levels. However, within surgical scars, dermal fibroblasts show a marked increase in cytoplasmic cathepsin K expression that is most prominent in young scars and declines with age of the scar [186]. Yet another study has revealed involvement of reduced cysteine cathepsin activity as a possible explanation for the enlargement of the gingival connective tissue often seen in patients exposed to long-term systemic cyclosporine A (CsA) treatment. Exposure of human gingival fibroblasts to long-term CsA (6-8 passages) resulted in decreased cathepsin B and L activity and down-regulation of cathepsin B, H, and L mRNA synthesis as a consequence of impaired cAMP response element-binding (CREB)-DNA binding activity. As intracellular degradation of collagen is a recognized function of cathepsins, inhibition of cathepsin-mediated collagen degradation by CsA may be a contributing factor in development of cyclosporine A-induced gingival overgrowth [187, 188]. Another study found that hardening of the skin is a likely adverse effect of balicatib, a cathepsin K inhibitor. A small percentage of patients (~1.5 %) included in a clinical trial for the safety and efficacy of balicatib for the treatment of osteoporosis, developed morphea-like symptoms with resolution of symptoms within thirty months after cessation of the drug [189]. It cannot be determined if this side effect is due to cathepsin K inhibition alone since balicatib has off target effects, inhibiting enzymatic activity of other cysteine cathepsins [190-192]. These combined observations demonstrate the important role cathepsins play in extracellular matrix homeostasis and degradation.
Mice lacking cathepsin L have several cutaneous morphological characteristics, underlining the functional importance of cathepsin L in skin structure and function. Mice deficient in ctsl show delayed hair growth and develop periodic hair loss due to alterations of hair follicle morphogenesis and cycling [193, 194]. They also develop epidermal hyperplasia due to hyperproliferation of basal keratinocytes and gingival overgrowth [194-196]. Interestingly, in 2000, it was discovered that the phenotype of the furless mouse first described in 1954 [197] occurs due to a loss of function of ctsl as a consequence of a missense mutation of the gene [194]. Similarly, the nackt mouse mutation resulting in alopecia, first described in 1999 [198], is due to a 118 base pair deletion of ctsl resulting in loss of enzymatic activity [199].

Cathepsin L is described as a tumor suppressor due to its involvement in the recycling of growth factors, particularly epidermal growth factor (EGF). Experimental evidence suggests that keratinocyte hyperproliferation observed in ctsl -/- mice results from sustained growth stimulation due to increased recycling of internalized EGF from the endosome to the plasma membrane [200]. Comparing tumor progression in wild-type and ctsl -/- mice in a murine skin carcinogenesis model using topically applied 7,12-dimethyl-benz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), the number of DMBA/TPA-induced papillomas was significantly higher in cathepsin L deficient mice as compared to controls [201]. A similar study employing K14–HPV16 mice [expressing the early region genes of human papillomavirus type 16 (HPV16) under the control of the human keratin 14 (K14) promoter] showed increased tumor progression and metastasis in ctsl -/- mice compared with wild-type mice [202].
Additional studies have indicated the importance of several cathepsins in epidermal maintenance due to their functional contribution to keratinocyte terminal differentiation and desquamation. Desquamation is a tightly regulated, finely balanced process necessary for the shedding of skin. Corneodesmosomes, specialized desmosomes that are formed between the stratum granulosum and stratum corneum, are the major structures that must be degraded during desquamation. Corneodesmosin, an extracellular component of corneodesmosomes is hydrolyzed by several cathepsins, including cathepsin D and cathepsin V (originally called stratum corneum thiol protease until the discovery that it was identical to Cathepsin V in 2003 [203, 204]). Analysis of stratum corneum extract identified the presence of enzymatically active cathepsin V (24kDa). The authors also identified a novel cathepsin L-like protein with the capability of hydrolyzing corneodesmosomes that they termed stratum corneum cathepsin-L-like protease (SCCL) [204]. A similar study showed that enzymatically active cathepsin D is present in shed corneocytes, and immunoelectron microscopy revealed the colocalization of cathepsin D with corneodesmosomes [205].

Both cathepsin D and cathepsin E seem to have a functional role in keratinocyte differentiation thereby contributing to epidermal homeostasis. Mice deficient in cathepsin E have dryer skin, a thicker dermis, and significantly reduced subcutaneous tissue and hair follicles compared with those of wild-type littermates [206]. Primary keratinocytes isolated and cultured from ctse knockout mice show a reduction in TPA- induced transcriptional upregulation of differentiation induced keratin 1 and loricrin as compared to their wild-type litter mates [206]. Cathepsin D proteolytically activates
transglutaminase 1, an enzyme responsible for the cross-linking of involucrin and loricrin, the two major proteins that form the cornified envelope. Indeed, Cathepsin D knockout mice have reduced transglutaminase 1 activity and altered distribution of cornified envelope proteins as compared to wild-type mice [207]. Furthermore, ctse -/- mice spontaneously develop atopic dermatitis-like skin lesions by the age of 10 weeks, thought to be due to reduced degradation rates leading to systemic accumulation of IL-18 and IL-1β. Ctse-/- mice are also more sensitive to hapten-induced contact dermatitis than their litter mates, indicating the important role of cytokine degradation effected by cathepsin E [208].

Several cathepsins, including cathepsin B, H, K, L, and S, have been shown to cleave Bid in vitro, and incubation of full-length Bid with these cathepsins resulted in cytochrome c release from isolated mitochondria [209]. Much data collectively suggests that lysosomal cathepsins act upstream to cleave Bid which then activates Bax. One such study demonstrated that lysosomal cathepsins B and D are released into the cytosol of human melanocytes exposed to apoptogenic doses of UVA or UVB. Melanocytes pretreated with cysteine or aspartate cathepsin inhibitors (E64d or pepstatin, respectively) showed diminished UVA/B-induced translocation of Bax from the cytosol to the mitochondria and nuclear fragmentation. Conversely, microinjection of cathepsin B alone was sufficient to induce nuclear fragmentation [123].

1.4.5 Cathepsins in skin pathophysiology

Importantly, molecular changes affecting cathepsins have been identified as causative factors in various skin pathologies such as tumorigenesis and inflammatory
Dysregulation of cathepsins and/or their endogenous inhibitors (hurpins and cystatins) is associated with many inflammatory skin pathologies including psoriasis and atopic dermatitis. One study examining human skin tissue revealed the increased prevalence of the mature forms of cathepsin B, L and D in psoriatic lesions as compared to healthy tissue. Interestingly, after treatment with psoralen-UVA (PUVA) co-treatment (8-methoxypsoralen in combination with UVA) therapy that resolved psoriatic symptoms, the expression of cathepsins B, L and D was normalized [214]. This is in agreement with a more recent immunohistochemical study indicating a more abundant and diffuse staining pattern of cathepsin D in psoriatic skin tissue as compared with normal tissue. Similarly, PUVA treatment of psoriatic skin lesions resulted in a decreased, more granular cathepsin D staining that more closely resembled healthy skin staining pattern [215].

Recently, cathepsin S was discovered as an activator of proteinase-activated receptor-2 (PAR-2), a G-protein coupled receptor associated with nociception and itching, by means of hydrolyzing its amino-terminal tethered ligand [216]. While CTSS is not expressed in keratinocytes of nonpathological skin, several skin disorders including atopic dermatitis, actinic keratosis, and psoriasis exhibit increased cathepsin S staining in the dermis [213]. This same study further demonstrated that co-culture of HaCaT keratinocytes with T-cells or with TNF-α or IFN-γ (two major cytokines secreted by T-cells in psoriatic lesions) induces HaCaT cathepsin S protein expression. Interestingly, cathepsin S immunostaining is also detectable in psoriatic keratinocytes, and is accompanied by an upregulation of MHC Class II expression. Additionally, cathepsin S
is involved in MHC Class II expression and the processing of the MHC Class II-associated invariant chain (Ii) in IFN-γ stimulated HaCaT keratinocytes [213]. This is in agreement with an earlier study indicating upregulation of CTSS (mRNA level, protein level, and activity) in HaCaT or HEK cells that are stimulated with IFN-γ [217]. Furthermore, a recent study found that transgenic mice overexpressing human cathepsin S spontaneously developed chronic atopic dermatitis-like symptoms at 14-16 weeks of age, with macrophage and mast cell infiltration in both the epidermis and dermis. Interestingly, immunohistochemical staining revealed induction of PAR-2 expression in the epidermis and dermis of ctss-overexpressing mice compared to WT controls [218].

Many of the cathepsins, particularly cathepsins B, H, L, and K, play an essential role in ECM maintenance through both intracellular and extracellular ECM degradation [186, 219-221]. *In vitro* studies demonstrate that cathepsins B, H, and L can degrade fibronectin, laminin and collagen type IV at a physiological pH range [221] and melanoma cells have been shown to degrade collagen type IV intracellularly [219]. Because of their ability to degrade ECM proteins, cathepsins most likely contribute to tumor metastasis and invasion [167, 212]. Indeed, studies have shown that cathepsin B can degrade many ECM proteins with the same if not better efficiency than MMPs [222, 223].

Immunohistological observations that cathepsins B, D, L and K are present in higher abundance in melanoma [210, 212, 224], and analysis of over 50 melanoma patients (stages II-IV) found that melanoma patients had higher serum levels of cathepsin B compared to healthy individuals [225]. One particular study found that contact of
high-invasive, but not low-invasive, melanoma cells lines to collagen type I resulted in the release of mature cathepsin B [226]. Another recent investigation indicated an increase in extracellular and membrane-associated cathepsin B, but not cathepsin L or D, in cells isolated from metastatic melanoma as compared to primary cutaneous melanoma. Extracellular inhibition of cathepsin B by use of a cell-impermeable inhibitor, specific antibodies, or siRNA interference inhibited melanoma invasion as determined by an in vitro cell invasion assay. Furthermore, in vivo data from human melanoma xenografts showed that inhibition of cathepsin B by siRNA or systemic administration of a cathepsin B inhibitor (Ca074) slowed tumor growth and number of metastases [227]. Contradictory research indicates that extracellular cathepsin L rather than cathepsin B is important in the progression and invasion of melanoma. Inhibition of cathepsin L in human melanoma xenografts slowed tumor growth and angiogenesis and inhibition of cathepsin L inhibited invasion of metastatic melanoma cell lines in an in vitro cell invasion assay [228, 229]. Yet another study showed that pharmacological inhibition of intracellular cathepsin K inhibited invasion of metastatic melanoma cell lines in an in vitro cell invasion assay [212]. Immunohistological observations indicate cathepsins B, D, H, L and K are present in higher abundance in BCC [230, 231], and cathepsins B and D are higher in SCC [232] compared with normal skin tissue, although the role of cathepsins in NMSC progression has been far less studied than their role in the progression and invasion of melanoma.

1.4.6 Cathepsins as targets of solar skin damage
Recent research has focused on the mechanistic involvement of cathepsins in UV-induced cutaneous alterations and photodamage. The mechanistic involvement of the lysosomal cysteine protease cathepsin B in UV-induced cutaneous alterations and photodamage has attracted considerable research interest [87, 88, 122, 123, 233-235]. Earlier work has shown that in human fibroblasts exposed to cytotoxic doses of UVA, photooxidative rupture of lysosomal membranes was followed by cytoplasmic release of proteases including cathepsin B that degrade ferritin with mobilization of redox-active iron representing a key factor in UVA-induced fibroblast photodamage,[87, 88, 122] Similarly, lysosomal release of cathepsin B was identified as a causative factor involved in melanocyte cell death that occurred in response to exposure to apoptogenic doses of UVA/B significantly higher than the ones used in our model of chronic UVA exposure [123]. It should be mentioned that in another recent study performed in cultured presenescent fibroblasts downregulation of cathepsin B has been observed at the transcriptional and protein level in response to PUVA treatment used as a model of photoaging [234, 235]. Moreover, using a mixed UVA/B light source (30% UVA + 70 % UVB spectral output), loss of cathepsin B immunohistochemical staining was observed in human skin specimens after undergoing a six week exposure regimen, but no assessment of altered specific enzymatic activity was performed [235]. In addition, a role of cathepsin K in solar elastosis has been substantiated in human skin fibroblasts where an age-related decline in cathepsin K maturation was shown to compromise the process of orderly intracellular elastin degradation, leading to subsequent accumulation of elastin in the extracellular space [219]. Stimulation of cathepsin G production in response to
UVA was observed in dermal fibroblasts potentially contributing to the remodeling of elastotic areas in sun-damaged skin [236]. Moreover, UVA exposure causes alternate trafficking of cathepsin L in dermal fibroblasts leading to extracellular release, an effect that has been linked to the anti-fibrotic activity of repetitive cutaneous UVA exposure [237].

1.5 Two-dimensional difference gel electrophoresis (2D-DIGE) as a proteomic tool for the identification of novel UVA targets

Proteomic analysis using two-dimensional difference gel electrophoresis (2D-DIGE) in combination with mass spectrometry has been used successfully for the unbiased identification of novel molecular targets involved in various human pathologies including infectious diseases, cancer, neurodegeneration, inflammatory dysregulation, metabolic disease, and general chronological aging [238, 239]. DIGE is a variant of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), involving two or more separate protein samples that are covalently labeled with different fluorescent dyes and then mixed prior to PAGE analysis [240]. Using in-gel fluorescent imaging technology, 2D-DIGE enables proteomic detection of differences in protein abundance between samples, which is then followed by mass spectrometric identification of specific proteins displaying differential abundance between samples (Fig. 1.6).
Figure 1.6. Schematic of 2D-DIGE. (1) Total proteins are prepared from cells and protein concentrations are determined. Equal amounts of protein extract from each sample are labeled with different CyDye fluoros (size and charge matched). (2) Protein samples are mixed and simultaneously separated on a single 2-dimensional gel by isoelectric focusing (IEF) in the first dimension which separates proteins according to their isoelectric points followed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension which separated proteins according to their molecular weights. (3) After electrophoresis, differential fluorescence image analysis is performed. (4) The protein expression ratios between different samples are calculated in-gel. (5) Protein spots of interest are excised from the gel and identified by mass spectrometric analysis.
1.6 Hypothesis and specific aims

1.6.1 Central hypothesis:
Chronic exposure to UVA induces skin photodamage through alteration of specific protein targets causatively involved in skin aging and carcinogenesis.

1.6.2 Rationale
It is clear that exposure to ultraviolet A radiation has numerous detrimental cutaneous effects that ultimately lead to skin aging and carcinogenesis. Oxidative stress mediated by ROS is a significant factor in UVA-induced photodamage. However, the identity of protein targets causally involved in UVA-induced photoaging and photocarcinogenesis remains largely undefined at this point. Even if oxidative modification by UVA is established, the role as an upstream target or bystander is hard to define. Therefore, identification of critical molecular targets of UVA mechanistically involved in the causation of skin cell photodamage remains an important subject of ongoing research. The studies presented here aimed at identifying novel cutaneous molecular targets of chronic UVA delivered at environmentally relevant doses. These studies may further elucidate the mechanisms by which UVA induces skin aging and carcinogenesis.

1.6.3 Specific Aims

Specific Aim 1.
Target identification: Proteomic profiling of UVA targets in cultured human dermal fibroblasts

Rationale and approach:
In an attempt to identify novel UVA targets, UVA-induced proteomic changes were assessed in human dermal fibroblasts (Hs27 cells). These cells were chosen as a cell culture model because UVA penetrates deep into the dermis, and therefore UVA is the only source of ultraviolet radiation that dermal fibroblasts are exposed to. Fibroblasts were exposed to a non-cytotoxic regimen of chronic UVA (9.9 J/cm\(^2\) twice a week for 3 weeks total) or mock treated (Fig. 1.7B), and total protein extracts underwent CyDye-labeling followed by two-dimensional-difference-gel-electrophoresis (2D-DIGE)/mass spectrometric identification of differentially expressed proteins. Independent confirmation of expression changes was obtained by immunodetection, and follow-up experiments involved determination of enzymatic activity of cathepsin B, the lead candidate as a novel target of UVA photodamage.

From this initial Aim 1, we identified cathepsin B, a lysosomal cysteine protease as a potential UVA target. Further efforts herein aimed at validating cathepsin B as a UVA target in human dermal fibroblasts and exploring the functional consequences of UVA-induced modulation of cathepsin B.

**Specific Aim 2.**

**Target modulation: Explore the molecular mechanism underlying UVA-induced cathepsin B alteration**

*Rationale and approach:*

The underlying mechanism by which cathepsin B enzymatic activity is inactivated in response to UVA exposure was investigated. Transcriptional regulation of cathepsin B
was determined by measuring mRNA levels of *CTSB* and cutaneously expressed cystatins (*CSTA, CSTB, CSTC, and CST6*). Because ROS and redox dysregulation are important intermediaries of UVA-induced changes, the generation of ROS in response to acute and chronic UVA exposure in Hs27 fibroblasts was measured and modulation of genes involved in cellular stress was assessed in response to chronic UVA. Efficacy of antioxidant intervention targeting UVA-induced cathepsin B inhibition was also explored. Furthermore, post-translational oxidative modifications of cathepsin B were measured by mass spectrometric analysis and immunoblotting.

**Specific Aim 3:**

**Target validation:** Examine the causative involvement of cathepsin B inactivation in UVA-induced lysosomal-autophagic alterations.

**Rationale and approach:**

Because of earlier studies that reported UVA-induced lysosomal changes [87, 88] together with our initial observations that UVA exposure inhibits the lysosomal enzyme cathepsin B, we evaluated the effects of UVA on lysosomal structure and function. Lysosomal abundance and content was assessed by electron microscopy (EM), flow cytometric detection and confocal microscopy of lysosomotropic dyes and lipofuscin, and immunodetection of Lamp-1 and lysosomal substrates. Additionally, a lysosomal-autophagy-based array was employed to assess the impact of UVA on autophagic-lysosomal related genes.

Moreover, we explored the mechanistic involvement of cathepsin B inactivation in UVA-induced autophagic-lysosomal dysregulation by means of cathepsin B
pharmacological inhibition and genetic antagonism using siRNA approach. The impact of cathepsin B enzymatic inactivation on autophagic-lysosomal function was assessed by EM, flow cytometric detection and confocal microscopy of lysosomotropic dyes and lipofuscin, transcriptional modulation of autophagic-lysosomal genes, and immunodetection of Lamp-1 and lysosomal substrates. Prototype follow-up studies addressed the emerging role of UVA-induced cathepsin B inactivation on lysosomal clearance, endo-lysosomal dependent viral infection, and lipofuscin-dependent photosensitization.
Figure 1.7. UVA regimens employed for studies. ‘1 week’ UVA regimen: $5 \times 10^5$ Hs27 dermal fibroblasts were seeded on day 1. Cells were exposed to 9.9 J/cm$^2$ UVA on days 2, 3, 4, and 5 as detailed in materials and methods. ‘3 week’ UVA regimen: $5 \times 10^5$ Hs27 dermal fibroblasts were seeded on day 1. Cells were exposed to 9.9 J/cm$^2$ on days 2, 5, 9, 12, 16, and 19 as detailed in materials and methods.
CHAPTER 2:
MATERIALS AND METHODS

2.1 Chemicals. [L-3-trans-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA074Me) was purchased from Enzo Life Sciences (Plymouth Meeting, PA). L-3-trans-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline (Ca074) was purchased from EMD Chemicals (Billerica, MA). 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), DQ™ Green BSA, LysoTracker Yellow™, and LysoTracker Red™ were purchased from Invitrogen (Carlsbad, CA). Dulbecco’s phosphate-buffered saline (DPBS) and Hank’s balanced salt solution (HBSS) were purchased from MediaTech Inc. (Manassas, VA). Protected DHP-lysine [(2S)-Boc-2-amino-6-(3,5-diformyl-4-methyl-4H-pyridin-1-yl)-hexanoic acid t-butyl ester] was purchased from NeoMPS (San Diego, CA). Identity of the DHP-lysine preparation employed in sensitization experiments was confirmed by fluorescence spectroscopy and electrospray mass spectrometry [ESI-MS; m/z 437.56, (M+H)+] using a LCQ Classic quadrupole ion trap mass spectrometer from Thermo Finnigan (San Jose, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2 Cell Culture. Dermal neonatal foreskin Hs27 fibroblasts and human immortalized HaCaT keratinocytes from ATCC (Manassas, VA) were cultured in DMEM containing 10% bovine calf serum (BCS). Primary human epidermal keratinocytes (HEK; neonatal HEKn-APF, from Cascade Biologics, Portland, OR) were cultured using Epilife medium supplemented with EDGS growth supplement (Cascade Biologics). Primary human
melanocytes (HEMa; human epidermal melanocytes from lightly pigmented adults, from Cascade Biologics) were cultured using Medium 154 medium supplemented with HMGS2 growth supplement. Cells were maintained at 37°C in 5% CO₂, 95% air in a humidified chamber. HEK and HEMa were passaged using recombinant trypsin/EDTA and defined trypsin inhibitor.

2.3 Irradiation with solar UVA. A KW large area light source solar simulator, model 91293, from Oriel Corporation (Stratford, CT) was used, equipped with a 1000 W Xenon arc lamp power supply, model 68920, and a VIS-IR bandpass blocking filter plus UVB and C blocking filter (output 320–400 nm plus residual 650–800 nm, for UVA). The output was quantified using a dosimeter from International Light Inc. (Newburyport, MA), model IL1700, with a SED033 detector for UVA (range 315–390 nm, peak 365 nm), at a distance of 365 mm from the source, which was used for all experiments. Using UVB/C blocking filter, the dose at 365 mm from the source was 5.39 mJ cm⁻² sec⁻¹ UVA radiation with a residual UVB dose of 3.16 μJ cm⁻² sec⁻¹.

2.3.1 Chronic UV exposure regimens. For chronic UVA treatment, an exposure regimen was selected that delivered a physiologically relevant dose of UVA without causing compromised cell viability or altered proliferative rate after reseeding.

‘Three week’ UVA regimen (used for DIGE- and DIGE-related experimentation): Cells were exposed to 9.9 J/cm² UVA twice a week for a total of 18 days (59.4 J/cm² total UVA dose) as illustrated in Fig. 1.7B.

‘One week’ UVA regimen [used for exposure that required inclusion of pharmacological modulators (NAC and CA074Me) that would display cytotoxicity over
the length of the three week regimen]: Cells were exposed to 9.9 J/cm² UVA for four consecutive days (39.6 J/cm² total UVA dose) as illustrated in Fig. 1.7A.

For both regimens, cells were seeded at 5×10⁵ cells/ 100 mm dish and incubated overnight prior to UV exposure. Before each irradiation, cells were first washed with PBS and irradiated (10 mL PBS, 25 min). After irradiation, PBS was removed and fresh culture medium was added. For mock UV treatments, cells were washed with PBS, placed in 10 mL PBS and then incubated at room temperature in the dark for 25 min. For analysis, cells were harvested one hour after last UV exposure occurred.

2.3.2 UVA Photosensitization of DHP-lysine. Cells (1×10⁵) were seeded on 35 mm dishes and received photosensitization 24 hours later. For exposure of unirradiated cells to preirradiated test compound (‘preirradiation exposure’; Fig. 6.6D and 6.10C), cells were first washed with PBS and then incubated for 30 min with test compound in PBS that had been UVA-exposed immediately before.

2.4 Flow cytometric analysis of cell viability. Cell viability was determined using flow cytometric analysis of annexinV (AV)-propidium iodide (PI) stained cells using an apoptosis detection kit (APO-AF, Sigma, St. Louis, MO) according to the manufacturer’s specifications. Cells were rinsed with DPBS and resuspended in 300µL binding buffer. 1.5µL of FITC-conjugated AV and 3µL of PI was added to the cell suspension. Samples were mixed and incubated at room temperature with exclusion of light for 10 min to allow binding of AV to phosphotidyl serine located on the outer leaflet and penetration of PI through compromised cellular membranes. Flow cytometry analysis was performed on a FACScan analyzer (BD Biosciences, San Jose, CA). Results are displayed in a 4
quadrants in which AV⁻, PI⁻ (lower left quadrant) indicates viable cells, AV⁺, PI⁻ (lower right quadrant) indicates cells undergoing early apoptosis, and AV⁺, PI⁺ (upper right quadrant) indicates either late apoptotic or necrotic cells.

2.5 Senescence-associated β-galactosidase activity. Following the Campisi protocol, β-galactosidase activity at neutral pH was measured as a determination of cellular senescence [241]. One hour after last irradiation, cells were rinsed with DPBS and fixed for 5 min at room temperature in 2% formaldehyde–0.2% glutaraldehyde in PBS. Cells were then incubated in staining-solution (4.2 mM citric acid, 12.5 mM sodium-phosphate, 158 mM sodium chloride, 0.21 mM magnesium chloride, 2.21 mg/mL potassium ferrocyanid, 1.68 mg/mL potassium ferricyanid, 1 mg/mL X-Gal, pH 6.0) for 20 h at 37 °C. For control purposes, cells were also stained at pH 4.0. Cells were washed and placed in DPBS, and viewed using an Olympus IX70 microscope.

2.6 Proteomic Analyses. Analyses were performed at the University of Arizona Department of Chemistry Mass Spectrometry Facility, Arizona Proteomics Consortium (Tucson, AZ; protbase.org) and Applied Biomics (Hayward, CA)

2.6.1 Two dimensional (2D) gel electrophoresis followed by mass spectrometry. 2D gel electrophoresis was performed following standard procedures established at the SWEHS-Proteomics Core Facility available online (www.protbase.org). Briefly, after UVA exposure according to the ‘three week’ regimen, UVA-exposed and mock treated cells (1 x 10⁶ per group) were harvested by scraping and washed with PBS. Cells were directly extracted using isoelectric focusing sample buffer and 140 mg total cellular protein was loaded onto a 12.5% gel for two-dimensional electrophoresis (pH range 5–8).
After separation, proteins were visualized by silver staining and gel images are captured using the Investigator ProPic imager (Genomic Solutions).

### 2.6.2 Two-dimensional difference gel electrophoresis (2D-DIGE) followed by mass spectrometry.

**Sample preparation.** After UVA exposure according to the ‘three week’ regimen, UVA-exposed and mock treated cells (7 x 10⁶ per group) were harvested by scraping and washed with PBS. The pellet was frozen immediately on dry ice. For sample processing, 200 mL 2-D cell lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS) were added to the thawed samples followed by sonication at 4 °C. After centrifugation (14,000 rpm, 4 °C, 30 min) the supernatant was collected. Protein concentration was determined using the Bio-Rad assay and the lysate samples were diluted with the sample 2-D cell lysis buffer to the same protein concentration between 5 to 8 mg/mL. 2-D DIGE and mass spectrometric analysis were then performed in collaboration with Applied Biomics (Hayward, CA).

**Minimal CyDye labeling.** To 30 mg of cell lysate 1.0 mL of diluted CyDye (1:5 diluted with DMF; control: Cy3; UVA: Cy5; GE Health Care, Piscataway, NJ) was added and after vortexing the tube was kept on ice for 30 min. After addition of 1.0 mL of 10 mM L-lysine to each of the samples, samples were incubated on ice for an additional 15 min. After mixing Cy3 and Cy5 labeled samples and addition of 2x 2-D sample buffer (8 M urea, 4% CHAPS, 20 mg/mL DTT, 2% pharmalytes and trace amount of bromophenol blue), 100 mL destreak solution (GE Healthcare) and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/mL DTT, 1% pharmalytes and trace amount of
bromophenol blue) were added (250 mL final volume). After mixing and spinning the labeled samples were loaded into the strip holder [13 cm immobilized pH gradient (IPG) strip, Amersham BioSciences].

**IEF and SDS-PAGE.** After IEF according to the manufacturer’s protocol (Amersham BioSciences), the IPG strips were incubated in the freshly prepared equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue and 10 mg/mL DTT) for 15 min. The strips were rinsed in the fresh made equilibration buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue and 45 mg/mL iodosacetamide) for 10 min. The IPG strips were then rinsed once in the SDS-gel running buffer before transfer into the SDS-gel (12% SDS-gel prepared using low fluorescent glass plates) and sealed with 0.5% (w/v) agarose solution (in SDS-gel running buffer). The SDS-gel was run at 15 °C.

**Protein identification by mass spectrometry.** Spots of interest were picked using the Ettan Spot Picker (Amersham BioSciences) based on in-gel analysis and spot picking design performed using the DeCyder software. Gel spots were digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). The digested tryptic peptides were desalted by Zip-tip C18 (Millipore). Peptides were eluted from the Zip-tip with 0.5 µl of matrix solution (a-cyano-4-hydroxycinnamic acid; 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) and spotted on the MALDI plate (model ABI 01-192-6-AB). MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA).
MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

**Database search.** Both the resulting peptide mass and the associated fragmentation spectra were submitted to a GPS Explorer workstation equipped with MASCOT search engine (Matrix science) capabilities to search the database of the National Center for Biotechnology Information non-redundant (NCBInr). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage allowed in the search parameters. Candidates with either protein score C.I.% or Ion C.I.% greater than 95 were considered significant.

**2.6.3 Peptide Mass spectrometry (performed by the University of Arizona Department of Chemistry Mass Spectrometry Facility directed by Dr. Arpad Somogyi).** Mass spectrometry was performed using a Bruker Reflex III MALDI–TOF-Mass Spectrometer (MALDI-TOF-MS) equipped with a nitrogen laser (337 nm). Spectra were recorded in positive ion mode in linear configuration using α-cyano-4-hydroxycinnamic acid as matrix.

**2.7 Photodynamic treatment: generation of singlet oxygen by photosensitization of toluidine blue O.** Cells (5x10^5) were seeded on 100 mm dishes and received photosensitization 24 hours later. Prior to treatment, cells were washed with PBS. Cells
were incubated with toluidine blue O (XX μM in 10 mL DPBS) and exposed to visible light using a bank of cool white bulbs for 30 sec or 180 sec. After irradiation, cells were incubated at room temperature in the dark for an additional 20 min prior to harvesting for analysis.

2.8 Measurement of cathepsin B enzymatic activity. Cathepsin B activity was measured using the fluorometric cathepsin B assay kit from BioVision, Inc. (Milpitas, CA) according to manufacturer’s instructions. Cells (5x10⁵) were lysed in 0.5 mL of chilled lysis buffer. After 10 min incubation on ice, lysates were centrifuged at 10,000 g at 4 °C for 5 min and supernatant was retained for analysis. 50 μL of cell lysate was incubated with 50 μL of reaction buffer and cathepsin B substrate (Ac-Arg-Arg-AFC; 200 μM final concentration; 1 h at 37 °C). As a negative control, analysis was performed in the presence of the cathepsin B/L inhibitor Z-Phe-Phe-FMK (200 μM final concentration). The release of free amino-4-trifluoromethylcoumarin (AFC) was measured using a fluorescence plate reader (λex 400, λem 505; SpectraMax Gemini, Molecular Devices, Sunnyvale, CA). Additionally, protein concentration of cell lysates was determined using the BCA assay (detailed in 2.10) and cathepsin B activity was normalized to protein concentration per sample.

2.9 Measurement of Cathepsin L enzymatic activity. Cathepsin L activity was measured using the fluorometric cathepsin L activity assay kit from BioVision, Inc. according to manufacturer’s instructions with the following modifications. Cells (1x10⁶) were lysed in 0.5 mL of chilled lysis buffer. After 10 min incubation on ice, lysates were centrifuged at 10,000 g at 4 °C for 5 min and supernatant was retained for analysis.
μL of cell lysate was incubated with 50 μL of reaction buffer and Ca074 (1 μM final concentration) for 15 min at ambient temperature. Ca074 is added to irreversibly inhibit cathepsin B in order to eliminate interference from cathepsin B cleavage of the substrate. Cathepsin L substrate (Ac-Phe-Arg-AFC; 200 μM final concentration) was then added and mixture was incubated for 1 h at 37 °C. As a negative control, analysis was performed in the presence of the cathepsin B/L inhibitor Z-Phe-Phe-FMK (200 μM final concentration). The release of free amino-4-trifluoromethylcoumarin (AFC) was measured using a fluorescence plate reader (λex 400, λem 505; SpectraMax Gemini, Molecular Devices). Additionally, protein concentration of cell lysates was determined using the BCA assay (detailed in 2.10) and cathepsin L activity was normalized to protein concentration per sample.

2.10 Measurement of cathepsin D activity. Cathepsin D activity was measured using the fluorometric cathepsin D activity assay kit from BioVision, Inc. according to manufacturer’s instructions. Cells (1x10^6) were lysed in 0.2 mL of chilled lysis buffer. After 10 min incubation on ice, lysates were centrifuged at 10,000 g at 4 °C for 5 min and supernatant was retained for analysis. 50 μL of cell lysate was incubated with 50 μL of reaction buffer cathepsin D substrate (MCA-GKPIFFRLK(Dnp)-DR-NH2; 200 μM final concentration) was then added and mixture was incubated for 1 h at 37 °C. The release of free 7-methoxycoumarin-4-yl acetyl (MCA) was measured using a fluorescence plate reader (λex 328, λem 460; SpectraMax Gemini, Molecular Devices). Additionally, protein concentration of cell lysates was determined using the BCA assay.
(detailed in 2.10) and cathepsin D activity was normalized to protein concentration per sample.

2.11 Determination of Protein Concentration. Protein concentration of cellular lysate was determined using the Pierce® BCA Protein Assay Kit (ThermoScientific, Rockford, IL) according to the manufacturer’s instructions. Standards were generated by serial diluting (1:1) bovine serum albumin (BSA) to final concentrations ranging from 2.0 mg/mL to 0.03125 mg/mL. 25 µL of cell lysate or BSA standard was added to 200 µL of working reagent and incubated at 37°C for 30 min. Absorbance was measured (562 nm) using a BioTek® Synergy 2 microplate reader (Winooski, VT) and a standard curve was generated by plotting the average blank-corrected absorbance versus protein concentration for each standard. Protein concentration for each sample was calculated using curvilinear regression.

2.12 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity assay. UVA-induced alteration of GAPDH specific enzymatic activity was assessed in cytosolic cell extracts prepared from Hs27 fibroblasts (5 x 10^6 per sample) according to a published standard procedure measuring increase in absorbance at 340 nm (formation of NADH) in a reaction mixture containing 0.4 mM NAD^+, 50 mM sodium arsenate, 0.1 mM DTPA, 0.9 mM D,L-glyceraldehyde-3-phosphate, and 50 mM TrisHCl, pH 8.8. [242]. Reaction was started by the addition of cytosolic extract normalized for protein content using the BCA protein assay (detailed in 2.10). One unit of enzyme was defined as the amount forming 1 µmol/min NADH at 25°C.
2.13 Gene expression analysis by real time RT-PCR. One hour after last UVA exposure, total cellular RNA (5×10^6 cells) was prepared using the RNEasy kit from Qiagen (Valencia, California, USA). Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Roche Molecular Systems, Branchburg, NJ) and 200 ng of total RNA in a 50 µl reaction. Reverse transcription was primed with random hexamers and incubated at 25°C for 10 min followed by 48°C for 30 min, 95°C for 5 min, and a chill at 4°C. Each PCR reaction consisted of 3.75 µl of cDNA added to 12.5 µl of TaqMan Universal PCR Master Mix (Roche Molecular Systems), 1.25 µl of gene-specific primer/probe mix [Assays-by-Design; Applied Biosystems: CTSB (assay ID Hs00947433_m1), CTSL (assay ID HS00964650_m1), CSTA (assay ID HS00193257_m1), CSTB (assay ID Hs00164368_m1), CSTC (assay ID Hs00969174_m1), CST6 (assay ID Hs00154599_m1), LAMP1 (assay ID Hs00174766_m1), SQSTM1 (assay ID Hs00177654_m1), TGM2 (assay ID Hs00190278_m1), or GAPDH (assay ID Hs99999905_m1)] and 7.5 µl of PCR water. PCR conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s alternating with 60°C for 1 min (Applied Biosystems 7000 SDSGene-specific product was normalized to GAPDH and quantified using the comparative (ΔΔCt) Ct method described in the ABI Prism 7000 sequence detection system user guide. Expression values were averaged across three independent experiments (mean ± SD).

2.14 Gene expression array analysis. Total cellular RNA (5 × 10^6 cells) was prepared using the RNEasy kit from Qiagen (Valencia, California). Reverse transcription was performed using the RT2 First Strand kit (SA Biosciences, Frederick, MD) and 1 µg total
RNA. Expression array analysis using the Human Autophagy RT² Profiler™ PCR Expression Array (PAHS-084), the Human Oxidative Stress and Antioxidant Defense RT² Profiler™ PCR Expression Array (PAHS-065), or the Human Stress and Toxicity RT² Profiler™ PCR Expression Array (PAHS-003; SA Biosciences), each profiling the expression of 84 genes, was performed as published recently.[243] All reactions were run using the following PCR conditions: 95 °C for 10 min, followed by 40 cycles of 95°C for 15 s alternating with 60 °C for 1 min (Applied Biosystems 7000 SDS, Foster City, CA). Gene-specific product was normalized to GAPDH and quantified using the comparative (ΔΔCt) Ct method as described in the ABI Prism 7000 sequence detection system user guide.

2.15 Detection of intracellular oxidative stress by flow cytometric analysis. Induction of intracellular oxidative stress was analyzed by flow cytometry using 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) as a sensitive nonfluorescent precursor dye which upon peroxide-mediated oxidization generates the highly fluorescent molecule 2’,7’-dichlorofluorescein (DCF). A 5mg/mL stock of DCFH-DA was prepared in 100% ethanol. One hour after the last irradiation, DCFH-DA was added to the culture medium (5 μg/mL final concentration) and cells were incubated for 1 h in the dark (37°C, 5% CO2). Cells were harvested by trypsinization, washed with PBS, resuspended in 300 μL PBS and immediately analyzed by flow cytometry. To avoid direct photooxidation of the dye probe, cells were loaded with the indicator dye under light exclusion.

2.16 Flow cytometric quantification of cellular autofluorescence. One hour after the last irradiation, cells were harvested by trypsinization, washed with PBS, resuspended in
300 μL PBS, and immediately analyzed by flow cytometry (λex 488nm, λem 530 ± 15 nm).

2.17 Confocal Fluorescence Microscopy. One hour after last irradiation or CA074Me treatment, cells were trypsinized, reseeded on glass bottom 35 mm dishes (MatTek Corp., Ashland, MA) at 1x10⁵ cells per dish and cultured overnight. Prior to live imaging, cells were incubated in LysoTracker Red DND-99 (75 nM in growth medium) for 1 h at 37 °C/5% CO₂. Medium was removed and cells were incubated in DAPI (3 μM in HBSS) for 30 min. Cells were then washed several times and kept in HBSS for fluorescence microscopy. Using a SP5 spectral confocal microscopy system equipped with a Leica DMI6000 inverted microscope (Wetzlar, Germany), DAPI was detected between 450-550 nm with excitation at 405nm (UV laser source). Autofluorescence and LysoTracker Red were visualized using an argon laser (λex 488 nm), and a spectral scan (560-700 nm with 10 nm increments) was performed. Image analyses were performed using Leica Confocal Imaging software and distinction between fluorescent signals was accomplished by spectral separation.

2.18 Transmission Electron Microscopy. One hour after last UVA irradiation, cells were trypsinized, reseeded and cultured for 4h. Cells were fixed in situ with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH7.4), postfixed in 1% osmium tetroxide in cacodylate buffer, washed, scraped and pelleted. Cells were then stained in 2% aqueous uranyl acetate, dehydrated through a graded series (50, 70, 90 and 100%) of ethanol and infiltrated with Spurr’s resin, then allowed to polymerize overnight at 60 °C. Sections (50 nm) were cut, mounted onto uncoated 150 mesh copper grids, and stained with 2% lead
citrate. Sections were examined in a CM12 Transmission Electron Microscope (FEI, Hillsboro, OR) operated at 80 kV with digital image collection (AMT, Danvers, MA).

2.19 Immunoblot detection. One hour after last irradiation, cells were washed with PBS, lysed in 1x SDS-PAGE sample buffer (0.375 M Tris HCl pH 6.8, 50% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue) and heated for 3 min at 95°C. Samples were separated by 12% SDS-PAGE followed by transfer to nitrocellulose membranes (Optitran, Whatman, Piscataway, NJ). Membranes were incubated with primary antibody in 5% milk-TBST overnight at 4°C. HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Jackson Immunological Research, West Grove, PA) was used at 1:20,000 in 5% milk-TBST followed by visualization using enhanced chemiluminescence detection reagents. Equal protein loading was examined by β-actin-detection. The following primary antibodies were used: rabbit anti-cathepsin B polyclonal antibody, 1:200 (#3190-100, BioVision, Inc.); mouse anti-cathepsin L (#611084, BD biosciences, San Jose, CA); rabbit anti-nucleophosmin polyclonal antibody, 1:1,000 (#3542, Cell Signaling Technology, Danvers, MA); rabbit anti-HO-1 polyclonal antibody, 1:5,000 (#SPA-896, Stressgen Bioreagents, Ann Arbor, MI); rabbit anti-Hsp70 polyclonal antibody, 1:1,500 (#SPA-811; Stressgen Bioreagents); rabbit anti-Lamp-1 monoclonal antibody, 1:1,000 (#3243, Cell Signaling Technology); mouse anti-beclin 1 monoclonal antibody, 1:1,000 (#sc-48341, Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-sequestosome 1 (p62) monoclonal antibody, 1:200 (#sc-48402, Santa Cruz Biotechnology); rabbit anti-Nrf2 polyclonal antibody, 1:4000 (#sc-13032, Santa Cruz Biotechnology); rabbit anti-α-Synuclein monoclonal antibody, 1:1,000
(#4179, Cell Signaling Technology); rabbit anti-LC3 polyclonal antibody, 1:500 (#NB100-2331, Novus Biologics, Littleton, CO); rabbit anti-transglutaminase 2 monoclonal antibody, 1:2,000 (#3557, Cell Signaling Technology); rabbit anti-p38, 1:1000 (#9212, Cell Signaling Technology); rabbit anti-phospho-p38, 1:1000 (Thr180/Tyr182; #9211, Cell Signaling Technology); rabbit anti-phospho-eIF2α, 1:1000 (#9722, Cell Signaling Technology), rabbit anti-phospho-eIF2α, 1:1000 (Ser51; #3597, Cell Signaling Technology); rabbit anti-4-hydroxynonenal, 1:2,000 (#46545, Abcam, Cambridge, MA); mouse anti-actin monoclonal antibody, 1:1,500 (#A4700; Sigma).

Densitometry analysis was performed using image J software (rsbweb.nih.gov/ij).

2.20 Detection of 4-HNE adducted cathepsin B.

Cells (1x10^7) were lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and a cocktail of protease inhibitors (Roche, Indianapolis, IN). After pre-clearing cell lysate with Protein G Sepharose™ beads (GE Healthcare, Piscataway, NJ) to remove proteins that nonspecifically bind to the beads, protein concentrations were quantified by BCA assay (detailed in 2.10). 500µg cell lysate was incubated overnight with 5 µL anti–cathepsin B antibody (200 µg/mL; BioVision, Inc.). Protein G Sepharose™ beads (50 µL) were added and incubated for an additional 4 hr. After 4 washes with RIPA buffer, the immunoprecipititates were boiled in 1x SDS-PAGE sample buffer 5 min. Samples were separated by 12% SDS-PAGE followed by transfer to nitrocellulose membranes (Optitran, Whatman). Membranes were incubated with anti-4-HNE primary antibody (1:2,000, #46545, abcam) in 5% milk-TBST overnight at 4°C. HRP-conjugated goat anti-
rabbit secondary antibody (Jackson Immunological Research) was used at 1:20,000 in 5% milk-PBST followed by visualization using enhanced chemiluminescence detection reagents.

2.21 Degradation and clearance of DQ™-Green BSA. Lysosomal clearance was determined by measuring DQ™-Green BSA fluorescence by flow cytometry and confocal microscopy. DQ™-Green BSA, a fluorogenic substrate for proteases, is self-quenched red BODIPY dye conjugated to BSA (Molecular Probes, Eugene, OR) that requires enzymatic cleavage to generate a highly fluorescent product. Cells were incubated in DMEM media containing DQ-BSA (25 μg/mL) for 2 hours at 37 °C in 5% CO₂ and washed with DMEM. Cells were then cultured in complete growth medium in the presence or absence of CA074-Me (1 µM) for 28 hr at 37 °C in 5% CO₂. Cells were harvested, and fluorescence of DQ-BSA (λ<sub>ex</sub> 488nm / λ<sub>em</sub> 515 nm) was analyzed by flow cytometry [FACScan analyzer (BD Biosciences)] or fluorescence microscopy [Olympus BX50 and Spot (Model 2.3.0) camera].

2.22 HPV16 Virus and Infections. Experiments were performed in collaboration with the laboratory of Dr. Samuel Campos, Assistant Research Professor at BIO5 Institute, University of Arizona. Wild type HPV16 pseudovirions containing the pGL3 luciferase reporter plasmid were prepared as previously described [244]. HS27 and HaCaT cells were plated in 35 mm dishes and treated with UVA (‘1 week’ regimen’) or CA074Me (1 µM q.d, 4 consecutive days). Twelve hours after the last irradiation cells were infected with 1×10⁹ viral genomes per dish. At 24 hours post-infection, cells were washed once with PBS and each dish was lysed in 250 µl of 1X reporter lysis buffer (#E3971,
Promega). Luciferase activity, which was used as an indicator of HPV infectivity, was measured using luciferase assay reagent (#E1483, Promega) according to the manufacturer’s instructions. Infection levels were normalized to protein content by western blot for total GAPDH (#2118, Cell Signaling Technologies), and expressed as percent infection relative to untreated cells, which are set at 100%.

### 2.23 Small interfering RNA targeting CTSB and/or CTSL.

Hs27 fibroblasts were transiently transfected with a 50 nmol pool of four small interfering RNA (siRNA) oligonucleotides (oligos) targeting *CTSB* and/or *CTSL* or a 50 nmol pool of four nontargeting siRNA oligos using the DharmaFECT 1 transfection reagent (Dharmacon RNA Technologies, Lafayette, CO). The sequences of siGENOME CTSB SMARTpool (*CTSB* siRNA; GenBank: NM_147783) were GGCACAACUUCUACAACGU; GGAUGAGCGUGUCAACUAU; GGAACUUCUGGACAAGAAA; and GGAUCACUGUGGAAUGGA. The sequences of siGENOME CTSL SMARTpool (*CTSL* siRNA; GenBank: NM_001912) were CAGCUACUCUAAUUGGAA; UCCAGUAUGUUCAGGAAUA; GGAGAAACCAUUGUGGAAU; and CAGAUUUAUCGGCAUGAAU. The oligos were resuspended in the Dharmacon siRNA buffer and incubated in serum free media for 5 min. DharmaFECT 1 was also incubated in serum free media for 5 min before the addition of the siRNA oligos. The oligos were incubated with the transfection reagent for 20 min before cellular treatment. Complete media was added to the siRNA oligo mixture and the cells were incubated with the siRNAs in appropriate cell culture conditions for 48 h. Cells were then re-transfected with another 50 nmol pool of four siRNA oligonucleotides targeting CTSB and/or CTSL.
or a 50 nmol pool of four nontargeting siRNA oligonucleotides and incubated for an additional 48 h prior to analysis.

2.24 Immunohistochemical detection of cutaneous MDA- and DHP-lysine epitopes using a tissue microarray (TMA). Triplicates of a commercial human skin TMA (NS21-01-TMA, age grouped female normal skin tissue array; specimens of undisclosed anatomical location; twenty four array dots derived from 12 individual donors; Cybrdi, Rockville, MD) were processed for H&E staining, pan-MDA-immunohistochemistry, and DHP-lysine immunohistochemistry, respectively. Immunohistochemistry was performed using the Discovery XT automated staining platform (VMSI, Ventana Medical Systems, Tucson, AZ). Deparaffinization and antigen retrieval of cells and tissue were performed online. All steps were perform on this instrument using VMSI validated reagents, including deparaffinization, cell conditioning (antigen retrieval with a borate-EDTA buffer), primary antibody staining, detection and amplification using a biotinylated-streptavidin-HRP and DAB (3,3’-diaminobenzidine tetrahydrochloride) system, and hematoxylin counterstaining. DHP-lysine-adducted proteins were visualized using a validated murine primary monoclonal antibody (1F83, provided by Koji Uchida, Nagoya University, Japan; dilution: 1:100) [245]. MDA-adducted proteins were visualized using a polyclonal rabbit anti-MDA antiserum [(AP050), Biotrend-usa, Destin, FL; dilution: 1:50] [246]. Images were captured using an Olympus BX50 and Spot (Model 2.3.0) camera. Images were standardized for light intensity.

2.25 Assay for photosensitized inhibition of skin cell proliferation. Cells were seeded at 10,000 cells per dish on 35mm dishes. After 24 h, cells were washed, placed in PBS,
and exposed to the combined or isolated action of photodynamic test compound and irradiation (UVA). After irradiation cells were washed and fresh culture medium was added.

For exposure of unirradiated cells to preirradiated test compound (‘preirradiation exposure’; Fig. 8C), cells were first washed with PBS and then incubated for 30 min with DHP-lysine in PBS that had been UVA-exposed immediately before. Cell number was determined 72 hr later, and proliferation was compared to cells that received mock-irradiation in PBS.

2.26 Cell cycle analysis. Cells were seeded at 1 x 10^5 per dish on 35mm culture dishes (Sarstedt, USA) and left overnight to attach. After irradiation in the presence or absence of test compound, cells were washed with PBS and fresh culture medium was added. After 48 h cells were harvested by trypsinization, resuspended in 200 µl PBS, and placed on ice. After addition of 2 mL 70% (v/v) ethanol, 30% (v/v) PBS, cells were incubated for 30 min on ice. The fixed cells were pelleted by centrifugation, resuspended in 800 µl PBS, 100 µl ribonuclease A (1mg/mL PBS), and 100 µl propidium iodide (400 µg/mL PBS), and incubated for 30 min in the dark at 37°C. Cells were analyzed by flow cytometry and cellular DNA content was determined using the ModFit LT software, version 3.0 (Verity, Topsham, ME).

2.27 Caspase-3 activation assay. Treatment-induced caspase-3 activation was examined in Hs27 fibroblasts using a cleaved/activated caspase-3 (asp 175) antibody (Alexa Fluor 488 conjugate, Cell Signaling, Danvers, MA, USA) followed by flow cytometric analysis. Briefly, cells were harvested 24h after treatment, resuspended in PBS and fixed
in 1% formaldehyde. Cells were then permeabilized using 90% methanol and resuspended in incubation buffer (PBS, 0.5% BSA). After rinsing by centrifugation, cells were resuspended in incubation buffer (90 µl) and cleaved caspase-3 antibody (10 µl) was added. After incubation (40 min) followed by rinsing and centrifugation in incubation buffer, cells were resuspended in PBS and analyzed by flow cytometry.

2.28 Thiobarbituric acid reactive substances (TBARS) Assay. A standard procedure for the relative assessment of lipid peroxidation based on the photometric determination of thiobarbituric acid (TBA)-reactive material was followed using 1,3-diphenylthiobarbituric acid (DPTBA) for increased sensitivity. One hour after photosensitization by exposure to the combined action of UVA and DHP-lysine HaCaT keratinocytes were harvested by trypsinization. Untreated control cells were equally processed. A pellet of 10 x 10^6 cells was then resuspended in 1.25 mL sodium acetate (330 mM, pH 3.0), mixed with 100 µL 1,3-diphenylthiobarbituric acid (DPTBA, 120 mM in DMSO), and heated (95 °C, 30 min). After cooling, the mixture was extracted with ethylacetate (0.6 mL), and the organic layer was used for relative photometric determination of TBARS by measuring absorbance at 538 nm on a Shimadzu spectrophotometer (model RF-540).

2.29 Sensitization of protein photo-crosslinking and peptide photo-oxidation. Ribonuclease A (RNAse A, 10 mg/mL PBS) was irradiated (with UVA (9.9 J/cm^2)) in the absence or presence of DHP-lysine (50 µM, 200 µL total reaction volume). Protein oligomerization as a result of sensitized photo-crosslinking was visualized by 15% SDS-PAGE followed by Coomassie-staining. Melittin (1 mg/mL PBS) was irradiated with
UVA (9.9 J/cm²) in the absence or presence of DHP-lysine (50 µM, 200 µL total reaction volume) followed by mass spectrometry.

2.30 Quantification of DHP-lysine sensitized superoxide formation. DHP-lysine sensitized generation of superoxide anions was determined by nitroblue tetrazolium chloride (NBT) reduction and confirmed by scavenging of superoxide by SOD [96]. A 200 µL reaction volume containing 0.8 µL of a NBT stock solution (50 mg/mL) and various concentrations of DHP-lysine was irradiated in the presence or absence of SOD (3000 u) in triplicate on a 96 well microtiter plate. Replicate samples incubated in the dark were used as controls. Formation of nitroblue diformazan (NBF) as the photoreduction product was quantified measuring the absorbance at 560 nm on a Versamax microtiter plate reader (Molecular Devices, Sunnyvale, CA) using a nitroblue diformazan (NBF) standard curve.

2.31 Quantification of DHP-lysine sensitized hydrogen peroxide formation. DHP-lysine-sensitized-sensitized formation of peroxides was quantified according to a standard procedure with minor modifications [96]. Samples were prepared by UVA-irradiating DHP-lysine in PBS. Immediately after irradiation samples analyzed in triplicate on a 96 well microtiter plate. An aliquot (10 µl) was added to 95 µL H2SO4 (25 mM). After addition of 100 µl containing 0.5 mM ferrous ammonium sulfate, 200 µM xylenol orange, and 200 mM sorbitol in 25 mM H2SO4 the plate was incubated at room temperature for 30 min and the absorbance was determined at 570 nm on a Versamax microtiter plate reader (Molecular Devices, Sunnyvale, CA). A standard curve was generated using H2O2 as standard. Optionally, to ensure specificity of the assay, replicate
samples were irradiated in the presence of catalase (400 u/mL) and after protein removal using spin microdialysis the peroxide measurement suppressible by catalase was assigned to H₂O₂.

2.32 Assessment of singlet oxygen formation using the RNO-bleaching assay.

Following a published procedure for the detection of singlet oxygen in aqueous solution, p-nitrosodimethylaniline (RNO, 5 µM final concentration), Rose Bengal (RB, 1 µM, final concentration), and imidazole (8 mM, final concentration) were dissolved in PBS, pH 7.2 [247]. To 200 µL of this stock solution 50 µL of DHP-lysine or Rose Bengal dissolved in PBS at various concentrations (1-50 µM) were added and the final mixture was placed on a 96 well microtiter plate and subsequently exposed to increasing doses of UVA. In the assay solution, the reaction between imidazole and singlet oxygen results in the formation of a transannular peroxide that can bleach RNO. Loss of initial RNO absorbance due to singlet oxygen-dependent bleaching was monitored at 438 nm using a Versamax microtiter plate reader (Molecular Devices, Sunnyvale, CA). Duplicate samples were irradiated in the presence of the quencher NaN₃ (10 mM) in order to substantiate singlet oxygen involvement in DHP-lysine- or Rose Bengal-dependent RNO bleaching.

2.33 Spectroscopy. UV-spectra were recorded using a Cary 100 Bio UV-Visible Spectrophotometer from Varian, Inc. (Palo Alto, CA). Fluorescence spectra were recorded using a Spectramax Gemini XS (Molecular Devices, Sunnyvale, CA) 96 well-microtiter plate reader.
**2.34 Statistical analysis.** The results are presented as means (± SD) of at least three independent experiments. Where indicated, results are presented as means (±SEM). Data were analyzed employing either a two-sided Students T test or one-way analysis of variance (ANOVA) with Tukey’s *post hoc* test using the Prism 4.0 software. Differences were considered significant at $p < 0.05$ (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ for analysis by Students T test). For data analyzed by ANOVA, means with common letter differ ($p<0.05$).

**2.35 Figure illustrations.** Select figure illustrations were produced using Servier Medical Art. (www.servier.com)
CHAPTER 3:
PROTEOMIC IDENTIFICATION OF CATHEPSIN B AS A NOVEL UVA-TARGET IN HUMAN SKIN FIBROBLASTS

This chapter has been adapted from the following publication:

3.1 Introduction and Rationale

Although solar UVA radiation results in little photoexcitation of DNA directly, it is involved in cutaneous photoaging and photocarcinogenesis through induction of photooxidative stress mediated by reactive oxygen species (ROS).[35, 37, 39, 40, 82, 87, 88] Various molecular sources of ROS and redox dysregulation are thought to contribute to the generation of cutaneous photooxidative stress including NAD(P)H oxidase, endogenous photosensitizers, mitochondrial electron leakage, energy crisis and glycolytic blockade, lysosomal disruption upstream of iron dysregulation, and inflammatory signaling [80-88, 248]. In spite of the emerging role of UVA in cutaneous photooxidative stress, the identity of specific molecular targets modulated by UVA and causatively involved in solar skin cell damage remains largely undefined. Therefore, as an initial step to identify novel UVA targets, this study aimed at assessing proteomic changes in human dermal fibroblasts exposed to a chronic UVA regimen of 9.9 J/cm².
UVA twice a week for three weeks total (**Fig. 1.7B**). Initial attempts in our laboratory to identify chronic-UVA induced changed in protein abundance assessed by silver staining following 2D electrophoresis were unsuccessful. We therefore employed two-dimensional differential in-gel electrophoresis (2D-DIGE), a more accurate and sensitive method which allows simultaneous separation of proteins from 2 samples, combined with mass spectrometry as a discovery tool for the identification of novel UVA-targets causatively involved in UVA-induced photodamage.

### 3.2 Results

#### 3.2.1 Identification of differentially abundant proteins in Hs27 dermal fibroblasts exposed to a low-level chronic UVA regimen.

Proteomic changes that occur in human dermal fibroblasts in response to chronic UVA exposure were examined using 2D-DIGE technology (**Fig. 3.1B-C**). A regimen of chronic UVA exposure was selected that delivered a physiologically relevant dose without induction of cell death or inhibition of cellular proliferation. Viability was maintained throughout the duration of the experiment as confirmed by flow cytometric analysis of annexinV/ propidium iodide (AV/PI)-stained cells (**Fig. 3.1A**). Moreover, after completion of the irradiation regimen, proliferation rate based on cumulative population doublings monitored over ten days after reseeding [(control: 4.8 ± 0.1; UVA: 4.7 ± 0.3 (mean ± SD)] was unaltered and no experimental evidence of UV-induced cellular senescence was observed based on SA-β-galactosidase staining (data not shown).
Figure 3.1. UVA-induced proteome changes in human skin fibroblasts as analyzed by two-dimensional difference gel electrophoresis (2D-DIGE). (A) Cell viability of Hs27 fibroblasts after exposure to chronic UVA (‘3 week’ regimen) or mock treatment as determined by flow cytometric detection of annexin V-propidium iodide (AV/PI). (B) After chronic UVA exposure (9.9 J/cm², twice a week, three weeks, termed ‘three week regimen’) or mock treatment, total cell protein extracts were prepared followed by Cy3- (untreated control) and Cy5-labeling (chronic UVA exposure). After gel electrophoresis differential fluorescence image analysis was performed: Cy3-image (left panel) Cy5-image (middle panel), Cy3/Cy5-overlay (right panel). (C) Cy3/Cy5-overlay with molecular weight (Mw) and isoelectric point (pI) scales. Fluorescent spots displaying the highest UVA-induced expression differential [i.e. upregulation: spot #12 (red); downregulation: spot #16 (green)] were then excised for subsequent mass spectrometric analysis. A yellow reference spot displaying equal abundance in both protein samples is also indicated (spot #1).
To this end, after chronic UVA exposure (9.9 J/cm², twice a week, over three weeks; ‘3 week UVA regimen’) or mock treatment, total cell protein extracts were prepared followed by Cy3- (untreated control) and Cy5-labeling (chronic UVA exposure) and subjected to gel electrophoresis followed by differential fluorescence image analysis (Cy3/Cy5-overlay shown in Fig. 3.1B, right panel, enlarged in Fig. 3.1C) using DeCyder software (Fig. 3.2A and Fig. 3.3A).

**Table 3.1** lists the fluorescent spots exhibiting the highest UVA-induced expression differential that were excised and subsequently identified by mass spectrometric analysis (MS/LCS). Additionally, spot #1 displaying equal abundance in both protein samples was identified as β-actin [actin, cytoplasmic 1 (Mw: 42,000 Da; pI: 5.4; acc. no: gi|46397333; Fig. 3.1B]. Proteins identified with high confidence as being less abundant in the UVA-exposed fibroblasts compared to controls included thimet oligopeptidase (THOP), dihydropyrimidinase-like 2, cathepsin B, and autoantigen La while those proteins identified as being more abundant in UVA-exposed fibroblasts included nucleophosmin, heterogeneous nuclear ribonucleoprotein K (HNRPK), and microtubule-associated protein, RP/EB family, member 1 (MAPRE1).

Spot #12 displaying the most pronounced upregulation by 3.6 fold according to DeCyder analysis was identified as nucleophosmin (Mw: 38,000 Da; pI: 4.71; accession number: gi|825671) with protein score and total ion score confidence interval (C.I.) being 100% (Fig. 3.2A). Independent confirmation of UVA-induced nucleophosmin upregulation was obtained by immunodetection that indicated approximately 3.1 fold upregulation based on densitometric analysis of band intensity (Fig. 3.2B).
Table 3.1. UVA-induced protein abundance changes identified by 2D-DIGE followed by mass spectrometric analysis

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<th>Spot #</th>
<th>Top Ranked Protein Name(Species)</th>
<th>Fold change from control</th>
<th>Accession No. (gi)</th>
<th>Protein MW</th>
<th>Protein PI</th>
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</tr>
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**high confidence**

**low confidence**

**no confidence**
Figure 3.2. Nucleophosmin upregulation in UVA-exposed human skin fibroblasts as detected by 2D-DIGE mass spectrometry and immunoblot analysis. (A) Differential in-gel analysis (gel spot view: panels I and III; 3D-view: panels II and IV) was performed using the DeCyder software. The arrow indicates position of protein spot #12 from Fig. 1C (untreated: panels I and II; UVA-exposed: panels III and IV). Spot #12 was picked and identified as nucleophosmin using mass spectrometric analysis. (B) Upregulation of cellular nucleophosmin protein levels in response to UVA exposure was confirmed by Western blotting.
While thimet oligopeptidase (THOP), dihydropyrimidinase-like 2, cathepsin B, and autoantigen La all showed pronounced down-regulation of 7-10 fold upon UVA exposure, we chose to further study the lysosomal cysteine protease cathepsin B based on earlier reports of lysosomal destruction upon UVA exposure [87, 88]. Spot #16 displaying pronounced downregulation by almost 7.1 fold according to DeCyder quantitative analysis was identified as cathepsin B (Mw: 24,000 Da; pI: 5.44; accession number: gi|741376) with protein score and total ion score confidence intervals (C.I.) being 100% (Fig. 3.3A). Independent confirmation of UVA-induced cathepsin B downregulation was obtained by immunodetection [cathepsin B double chain (DC; 24 kDa) and single chain (SC; 29 kDa) forms] (Fig. 3.3B-C). Densitometric analysis indicated downregulation of the fully processed mature DC form in UVA-treated cells (59.4 J/cm² total dose; ‘3 week’ UVA regimen) by approximately 10.7 fold, a number in good agreement with DIGE analysis (Fig. 3.3A). Significant downregulation was also detectable in cells that received UVA exposure according to a shorter UVA regimen (39.6 J/cm² total dose; ‘1 week’ UVA regimen). Interestingly, an accumulation of the 29 kDa SC chain form concurrent with depletion of the DC 24 kDa form of the protein was observed in response to the ‘1 week’ or ‘3 week’ UVA regimens (Figs. 3B-C).

3.2.2 UVA-induced inhibition of specific Cathepsin B activity in human skin fibroblasts.

Based on the detection of UVA-induced changes in cathepsin B protein levels (Fig. 3.3A-C), we examined the possibility that chronic UVA-exposure impairs cathepsin
Figure 3.3. Cathepsin B downregulation in UVA-exposed human skin fibroblasts as detected by 2D-DIGE mass spectrometry and immunoblot analysis occurs with loss of specific enzymatic activity. (A) Differential in-gel analysis (gel spot view: panels I and III; 3D-view: panels II and IV) was performed using the DeCyder software. The arrow indicates position of protein spot #16 from Fig. 1B (untreated: panels I and II; UVA-exposed: panels III and IV). Spot #16 was picked and identified as cathepsin B using mass spectrometric analysis. (B) Immunoblot detection of cellular cathepsin B protein levels [double chain (DC) and single chain (SC) form] in UVA-treated (59.4 J/cm² total dose; ‘3 week’ UVA regimen) versus untreated control cells was performed as described in Experimental Procedures. (C) Immunoblot detection of cellular cathepsin B protein levels in UVA-treated (‘1 week’ UVA regimen) and control cells. (D) Loss of cathepsin B specific enzymatic activity in human skin fibroblasts exposed to UVA (1 day to 3 weeks) was detected using a fluorogenic enzyme substrate. Treatment with the cathepsin B inhibitor CA074Me (1µM, q.d., four consecutive days) served as a control (n=3, mean ± SEM; p<0.05).
B specific enzymatic activity in human skin fibroblasts (Fig. 3.3D). As a control, the cathepsin B inhibitor CA074Me (1µM, q.d., four consecutive days) was used causing complete loss of cathepsin B specific enzymatic activity without compromising cell viability or proliferation rate. After chronic UVA exposure of fibroblasts (‘3 week’ UVA regimen, identical to treatment used for 2D-DIGE analysis), specific enzymatic activity of cathepsin B in total cellular extracts was reduced by more than 60% as compared to mock-treated control. A significant loss of cathepsin B activity by approximately 10% was already detectable after a single exposure to UVA (9.9 J/cm²), and inhibition by approximately 50% was observed when UVA exposure occurred over the course of four consecutive days (‘1 week’ UVA regimen).

3.3 Discussion

2D-DIGE is a powerful analytical tool for the unbiased identification of protein targets that display altered expression levels in cutaneous cells exposed to cytotoxic stimuli and environmental stressors including UVB [249-251]. Here, using a proteomic approach that involved 2D-DIGE analysis followed by mass spectrometric identification and follow up confirmation by immunodetection, we have identified novel target proteins that display a pronounced expression differential in human dermal fibroblasts exposed to chronic UVA irradiation.

The protein displaying the highest differential upregulation in response to chronic UVA exposure was identified as nucleophosmin (nucleolar phosphoprotein B23, NPM1) (Figs. 3.1 and 3.2). Nucleophosmin is a nucleolar protein that undergoes stress-induced
nucleoplasmic relocalization displaying regulatory protein-protein interactions with important nuclear factors including cell cycle regulator checkpoint kinase 1 (Chk1), the tumor suppressor proteins p53, Rb, BRCA1, and the p53-antagonist MDM2 [252, 253]. Recently, it has been demonstrated that nucleophosmin is an UVC-damage response protein involved in attenuation of p53-dependent apoptosis that occurs in response to genotoxic stress in cultured human fetal WS-1 fibroblasts and U2OS osteosarcoma cells [252, 254]. Our data document for the first time the significant upregulation of nucleophosmin protein levels in human dermal fibroblasts exposed to chronic UVA, although functional implications of this UVA-induced expression differential were not explored in this dissertation.

The 24 kDa fragment of the mature cathepsin B double chain form (Figs. 3.1 and 3.3) was downregulated by approximately 7-fold in response to UVA. The lysosomal papain-like cysteine protease cathepsin B is known to undergo proteolytic maturation that encompasses cysteine protease-dependent processing of procathepsin B (41 kDa) to the proteolytically active single chain cathepsin B form (29 kDa) followed by further maturation to the cystine-linked double chain form (5 kDa and 24 kDa fragments) in the lysosomal compartment [255]. Figure 3.4 illustrates a three-dimensional model of cathepsin B. Loss of the cathepsin B 24 kDa fragment occurred as a result of both UVA treatment regimens (‘1 week’ and ‘3 week’ exposure) employed in this study, but a more pronounced effect was observed as a result of exposure according to the ‘3 week’ regimen (Fig. 3.3B and C).
Our data indicate for the first time that chronic UVA exposure causes alteration of cathepsin B maturation and dramatic loss of cathepsin B specific enzymatic activity in human dermal fibroblasts. Loss of cathepsin B activity was already detectable even after exposure to a single dose of UVA (9.9 J/cm²) (Fig. 3.3D).
Figure 3.4. 3D structure of human cathepsin B. (A) Model of the 29kDa mature form of human cathepsin B displaying the light chain (green) and heavy chain (red), the occluding loop (amino acid residues 106-124; shown in dark blue), the catalytic triad (Cys29-His199-Asn219), and the N-glycosylation site (Asn 113). (B) Close-up of catalytic site. (C) Close-up of catalytic site bound to Ca074, a specific cathepsin B inhibitor (CA074Me shown in light blue). Modeling performed by Dr. Vijay Gokhale, co-Director of the molecular modeling shared services (AZCC), based on the published crystal structure of CA030-inhibited cathepsin B [2].
CHAPTER 4: EVIDENCE FOR A PHOTOTOXIDATIVE MECHANISM OF UVA-INDUCED CATHEPSIN B INACTIVATION IN HUMAN SKIN FIBROBLASTS

Parts of this chapter have been adapted from the following publication:


4.1 Introduction and Rationale

In this study, a series of experiments were conducted to determine the molecular mechanism underlying UVA-induced cathepsin B enzymatic inactivation. As a first possibility, transcriptional modulation of cathepsin B and cutaneously expressed endogenous cysteine protease inhibitors was assessed. Indeed, transcriptional downregulation of cathepsin B has been observed in presenescent fibroblasts treated with PUVA [234, 235]. Moreover, transcriptional modulation of Hurpin (HaCaT UV repressed serpin), a member of the serpin family with selective inhibitory activity against cathepsin L, has been observed in HaCaT keratinocytes exposed to UVB [124, 256].

In addition to assessment of the above mentioned transcriptional changes, the mechanistic involvement of oxidative stress was investigated in UVA-induced inhibition of cathepsin B enzymatic activity. As mentioned in Chapter 1, it is well established that solar UVA radiation is involved in cutaneous photodamage through induction of
photooxidative stress [35, 37, 39, 40, 82, 87, 88]. This study therefore aimed at (1) confirming the generation of reactive oxygen species in human dermal fibroblasts exposed to the doses of UVA used in this study, (2) measuring changes in oxidative- and stress- related genes as a consequence of UVA-induce photooxidative stress, and (3) determining the mechanistic involvement of oxidative stress in UVA-induced cathepsin B enzymatic activity.

4.2 Results

4.2.1 UVA-induced cathepsin B alterations are not due to transcriptional changes of the cathepsin B or cystatin genes.

As evidenced by RT-PCR analysis of *CTSB*, the decreased specific cathepsin B enzymatic activity and diminished mature form of cathepsin B observed in human skin fibroblasts exposed to chronic UVA is not due transcriptional down-regulation of the *CTSB* gene (Fig. 4.1 and Table 4.1A). Additionally, the transcriptional levels of several cutaneously expressed cystatins, endogenous inhibitors of cysteine cathepsins, were measured in response to UVA. Cystatins A, B, C, and 6 (M/E), encoded by *CSTA*, *CSTB*, *CSTC*, and *CST6*, respectively, were not changed at the transcriptional level in UVA- exposed versus control fibroblasts (Fig. 4.1 and Table 4.1A). These data indicate that the decreased level of the 24 kDa form of cathepsin B observed in human skin fibroblasts exposed to chronic UVA (Chapter 3, Fig. 3.3) is not due to transcriptional modulation of the *CTSB* gene, or of the *CSTA*, *CSTB*, *CSTC*, and *CST6* genes encoding endogenous inhibitors of cathepsin B.
Figure 4.1. UVA-induced transcriptional changes of *CTSB* and cystatin genes in UVA-exposed human dermal fibroblasts. Differential gene expression of cathepsin B (*CTSB*) and cystatins A, B, C and 6 (*CSTA*, *CSTB*, *CSTC*, and *CST6*, respectively) relative to *GAPDH* in response to chronic UVA exposure (‘3 week’ UVA regimen) versus mock treatment (n=3; mean ±SD).
<table>
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<th>Gene Symbol</th>
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<tr>
<td><em>TXNRD1</em> (NM_003330)</td>
<td>3.2</td>
<td>0.021</td>
</tr>
<tr>
<td><em>CDKN1A</em> (NM_000389)</td>
<td>3.0</td>
<td>0.008</td>
</tr>
<tr>
<td><em>CCL5</em> (NM_002985)</td>
<td>-5.7</td>
<td>0.013</td>
</tr>
<tr>
<td><em>CCL4</em> (NM_002984)</td>
<td>-7.7</td>
<td>0.004</td>
</tr>
<tr>
<td><em>CXCL10</em> (NM_001565)</td>
<td>-11.4</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Table 4.1. Gene expression changes in human skin fibroblasts exposed to chronic UVA. (A) Cathepsin B and cystatin genes without significant expression changes in response to UVA exposure (p > 0.05). (B) Genes displaying UVA-induced (‘3 week’ UVA regimen) up- or downregulation of expression by at least threefold (p < 0.05) as detected using the RT² Human Stress and Toxicity Pathway Finder™ PCR Expression Array shown in Fig. 4.3A.
4.2.2 Generation of intracellular ROS in dermal fibroblasts exposed to UVA.

To examine whether the chosen regimen of UVA exposure is associated with induction of oxidative stress, generation of intracellular oxidizing species was examined after exposing Hs27 cells to UVA followed by loading with the redox-indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) which, when irreversibly oxidized, is converted to the fluorescent dye 2',7'-dichlorodihydrofluorescein (DCF; Fig. 4.2). Immediately after exposure of fibroblasts to a single dose of 9.9 J/cm² UVA, a significant increase (approximately 25%) in intracellular ROS levels was observed as indicated by an increase in DCF fluorescence. Cellular ROS levels returned to that of control approximately 12 hours afterward (Fig. 4.2A). Interestingly, an increased level of cellular ROS was observed at the end of the ‘1 week’ UVA regimen as compared to a single exposure of 9.9 J/cm² UVA (~80% increase versus ~25% increase compared to control, respectively; Fig. 4.2B). These data demonstrate UVA-induced formation of intracellular reactive species of sufficient longevity capable of oxidizing DCFH, such as protein peroxides [257], which is consistent with the occurrence of UVA-induced intracellular oxidative stress [37].

4.2.3 Gene expression profiling reveals an oxidative stress response in UVA-exposed human skin fibroblasts.

Modulation of gene expression in response to chronic UVA exposure (‘3 week’ UVA regimen) versus mock treatment was assessed using the RT² Human Stress and Toxicity Pathway Finder™ and Human Oxidative Stress and Antioxidant Defense
Figure 4.2. Induction of oxidative stress as measured by flow cytometric detection of DCF fluorescence in human dermal fibroblasts exposed to UVA. (A) Intracellular ROS levels immediately following UVA irradiation (left panel) and 12 hours after irradiation (right panel) of human dermal fibroblasts exposed to 9.9 J/cm$^2$ UVA. One representative histogram out of three similar repeats is shown. (B) Intracellular ROS levels immediately following UVA exposure [one single exposure (9.9 J/cm$^2$) and ‘1 week’ regimen; (n=3, mean ± SEM)].
RT² Profiler™ PCR Expression Arrays which each contain 84 genes (Fig. 4.3A and Table 4.1B).

UVA treatment upregulated expression levels of eight genes on the arrays by at least three-fold including genes encoding the heat shock proteins Hsp70B’ (HSPA6; 1042-fold), Hsp70 (HSPA1A; 11-fold), Hsp105 (HSPH1; 4-fold), the heat shock protein and antioxidant enzyme hemeoxygenase-1 (HMOX1; 18-fold), the antioxidant enzyme thioredoxin reductase 1 (TXNRD1; 3-fold), the cyclin dependent kinase inhibitor p21(WAF1) (CDKN1A; 3-fold), and the inflammatory transforming growth factor beta superfamily member GDF15 (GDF15; 9-fold). Of note, significant downregulation of chemokine encoding genes (CCL5, CCL4, CXCL10) was observed in response to chronic UVA exposure. UVA-induced upregulation of major stress response encoding genes (HSPA1A and HMOX1) was then confirmed at the protein level by immunoblot detection that revealed 4.7 fold (Hsp70) and 9.5 fold (HO-1) upregulation as determined by densitometric analysis (Fig. 4.3B).

In congruence with previous studies [70] and data from this current report indicating UVA-induced oxidative stress, increased levels of Nrf2 in response to chronic UVA treatment (‘1 week’ regimen) was observed as detected by immunoblot analysis (Fig. 4.3C). An elevated level of serine-51 phosphorylated eukaryotic initiation factor α (eIF2α) was observed in Hs27 fibroblasts exposed to chronic UVA treatment (‘1 week’ regimen; Fig. 4.3D), indicative of ER stress.
Figure 4.3. Stress response gene expression changes in UVA-exposed human dermal fibroblasts. (A) Differential gene expression in response to chronic UVA exposure (‘3 week’ regimen) or mock treatment was analyzed using the RT² Human Stress and Toxicity Pathway Finder™ and Human Oxidative Stress and Antioxidant Defense RT² Profiler™ PCR Expression Arrays. Three independent repeat experiments were analyzed using the two-sided Student’s t test as summarized in Table 4.1. Changes in cycle threshold (Ct) for genes of interest relative to ACTB for untreated control (x-axis) versus UVA-exposed (y-axis) cells are displayed as scatter blot. Upper and lower lines represent the cut-off indicating three fold up- or down-regulated expression, respectively. Arrows mark genes of specific interest. (B) Induction of Hsp70 and HO-1 protein expression in UVA-exposed fibroblasts from (A) was determined by Western blot analysis using β-actin detection as a loading control. (C) Upregulation of Nrf2 protein expression in whole cell lysates from UVA-exposed fibroblasts (‘3 week’ regimen) was determined by Western blot analysis using β-actin detection as a loading control. (D) Immunodetection of phosphorylated eIF2α (p-eIF2α) and total eIF2α in whole cell lysates from UVA-exposed fibroblasts (‘3 week’ regimen).
4.2.4 UVA-induced loss of cathepsin B enzymatic activity in human skin fibroblasts can be antagonized by antioxidant intervention.

Further evidence supporting the induction of cellular oxidative stress in response to chronic UVA treatment was obtained by quantitative detection of DCF-fluorescence after subjecting Hs27 fibroblasts to UVA treatment (‘1 week’ UVA regimen) performed in the presence or absence of the thiol-based antioxidant N-acetyl-L-cysteine (NAC, 10 mM) (Fig. 4.4). As expected, UVA exposure upregulated cellular DCF fluorescence intensity by almost 80% indicative of increased peroxide levels, an effect significantly suppressed by inclusion of NAC during UVA irradiation (Fig. 4.4A). In parallel, loss of cathepsin B enzymatic activity observed earlier in response to UVA exposure (Fig. 3.3D) was antagonized significantly if irradiation occurred in the presence of NAC (Fig. 4.4B). Similarly, loss of cathepsin B activity occurring upon a single exposure of 20 J/cm² was almost completely antagonized if irradiations were performed in the presence of dimethylcysteine (DMC, 10 mM), a potent antioxidant and carbonyl scavenger (Fig 4.4C).

4.2.5 Cathepsin B is inactivated in Hs27 fibroblasts exposed to singlet oxygen.

Cathepsin B inactivation was measured in response to increasing doses of acute UVA exposure. An exposure-response relationship was observed with increasing doses of UVA resulting in a more dramatic inhibition of cathepsin B enzymatic activity, indicating that UVA-induced cathepsin B inactivation is dose-dependent (Fig. 4.5A). In order to determine the susceptibility of cathepsin B to specific ROS, we measured
Figure 4.4. Antioxidant protection against UVA-induced cathepsin B enzymatic inactivation. (A) Modulation of cellular oxidative stress was examined in UVA-exposed human dermal fibroblasts irradiated (‘1 week’ UVA regimen) in the presence or absence of NAC (10 mM) by flow cytometric detection of DCF fluorescence. One representative histogram is shown. Bar graph depicts summarized data of three independent repeats (n=3, mean ± SEM; p<0.05). (B) UVA-induced changes in cathepsin B specific enzymatic activity were assessed in human dermal fibroblasts (‘1 week’ UVA regimen) irradiated in the presence or absence of NAC (10 mM) using a fluorogenic enzyme substrate (n=3, mean ± SEM). (C) UVA-induced changes in cathepsin B specific enzymatic activity were assessed in human dermal fibroblasts (20 J/cm² single exposure) irradiated in the presence or absence of DMC (10 mM) using a fluorogenic enzyme substrate (n=3, mean ± SEM).
Figure 4.5. Cathepsin B is inactivated in human dermal fibroblasts exposed to singlet oxygen. (A) Exposure-response relationship of UVA-induced (10, 20, or 30 J/cm² single exposure) changes in cathepsin B specific enzymatic activity in human skin fibroblasts as assessed using a fluorogenic enzyme substrate (n=3, mean ± SEM). (B) Cathepsin B specific enzymatic activity in human skin fibroblasts exposed to singlet oxygen [¹O₂; generated by photodynamic activation of toluidine blue O + visible light (TBL)] or H₂O₂ (100µM; 24 hr).
cathepsin B activity in Hs27 cells exposed to H$_2$O$_2$ and $^1$O$_2$ (Fig. 4.5B). Hs27 cells exposed singlet oxygen generated by the photodynamic treatment of cells with toluidine blue in the presence of visible light (TBL) [258], resulted in a dose-dependent inactivation of cathepsin B enzymatic activity [Fig. 4.5B; 79.1 % ± 3.6 residual protease activity after 30 sec TBL exposure versus 65.1 % ± 0.1 residual protease activity after 3 min TBL exposure (n=3; mean ± SD; p < 0.05)]. In contrast, cathepsin B activity was not diminished when cells were cultured in the presence of 100µM H$_2$O$_2$ for time periods up to 24 hr [Fig. 4.5B; 106.2 % ± 14.5 residual protease activity (n=3; mean ± SD; p > 0.05)].

4.2.6 Immunodetection of 4-hydroxy-2-trans-nonenal (4HNE)-modified cathepsin B in Hs27 exposed to chronic UVA.

To evaluate the potential modification of cathepsin B by the lipid peroxidation product 4-HNE, cathepsin B was immunoprecipitated from cell lysates of control or UVA-exposed Hs27 fibroblasts. Subsequent Western blot analysis using anti–4-HNE antibody showed increased modification of both the single chain and double chain of cathepsin B in Hs27 exposed to chronic UVA (‘3 week’ regimen; Fig. 4.6). Portions of the cell lysates were separated by SDS–PAGE for determination of cellular cathepsin B levels for the purpose of calculating the relative amount of modified protein for each of the forms of cathepsin B (single-chain and double-chain) (Fig. 4.6). Densometric analysis revealed the relative amount of the double-chain form of cathepsin B modified by 4-HNE was 1.5 ± 0.25 (mean ±SD, n=3) fold higher in UVA versus mock irradiated
Figure 4.6. HNE adduct formation of cathepsin B in human dermal fibroblasts exposed to chronic UVA. Human dermal fibroblasts were lysed following chronic UVA (‘3 week’ regimen) or mock treatment. Cathepsin B was immunoprecipitated from 500 µg cell lysate and subjected to 12% SDS-PAGE then probed for 4-HNE (top panel). As a loading control, Western blot analysis of cathepsin B [double chain (DC) and single chain (SC) form] was determined from equal portions (30 µg) of cell lysates used for immunoprecipitation (bottom panel).
fibroblasts. A more dramatic effect was seen in the single-chain form of cathepsin B, with an average of 4.2 ± 0.71 (mean ±SD) fold-increase in 4-HNE modified protein in UVA versus mock irradiated fibroblasts.

4.3 Discussion

Our data do not suggest the involvement of changes that occur at the transcriptional level affecting expression of genes encoding either cathepsin B itself (CTSB) or established peptide inhibitors of cathepsin B including cystatin A (CSTA), cystatin B (CSTB), cystatin C (CSTC), and cystatin 6 (cystatin M/E; CST6) (Fig. 4.1 and Table 4.1A) [259, 260]. In contrast, our experiments indicate a causative involvement of photooxidative mechanisms in UVA-induced cathepsin B alterations as evidenced by antioxidant suppression of cathepsin B inactivation (Fig. 4.4), susceptibility of cathepsin B enzymatic activity to singlet oxygen (Fig. 4.5) and detection of 4HNE-adducted cathepsin B (Fig 4.6).

UVA-dependent ROS formation (Fig. 4.2) and upregulation of several genes involved in the heat shock and oxidative stress response observed in Hs27 exposed to chronic UVA (‘3 week’ regimen; Fig. 4.3A and Table 4.1B) indicate oxidative stress in Hs27 exposed to chronic UVA. Furthermore, chronic UVA (‘3 week’ regimen) resulted in increased cellular levels of the transcription factor Nrf2 (Fig 4.3C), known to be upregulated in response to oxidative stress and induce a battery of detoxifying and antioxidant enzymes [261]. Phosphorylation of eIF2α in response to UVA (Fig. 3D) was also observed, indicating activation of the PERK-arm of ER stress in Hs27 fibroblasts.
exposed to chronic UVA. The transmembrane kinase PERK phosphorylates the α subunit of eIF2 in response to ER stress, a well documented mechanism to attenuate protein synthesis with the purpose of reducing further accumulation of unfolded proteins in the ER [118]. UVA-induced phosphorylation of eIF2α or the function downstream events such as translational attenuation was not further investigated in this dissertation.

With a clear indication of UVA-induced oxidative stress in Hs27 exposed to chronic UVA, we set out to determine the possible mechanistic involvement of ROS in UVA-induced cathepsin B inactivation. Indeed, antioxidant intervention with the general antioxidant NAC or the more potent carbonyl scavenger DMC protected against UVA-induced inhibition of cathepsin B (Fig. 4.4). Our data also suggests that singlet oxygen as generated by the photodynamic treatment of Hs27 fibroblasts with toluidine blue in the presence of visible light (TBL) [258] resulted in a dose-dependent inactivation of cathepsin B enzymatic activity (Fig. 4.5B). Regeneration of cathepsin B activity was not achieved under the reducing conditions of the enzymatic assay, indicating that oxidative cathepsin B inactivation is irreversible and does not result from active-site disulfide or sulfenic acid formation, or other reversible oxidative modifications.

4-HNE, one of the major lipid peroxidation products generated as a consequence UVA exposure as well as other sources of oxidative stress, can covalently adduct lysine, cysteine, and histidine residues of cellular proteins thereby impairing their function [262, 263]. Data in support of 4-HNE adduction of cathepsin B was obtained by Western blot analysis of immunoprecipitated cathepsin B protein from Hs27 exposed to chronic UVA (‘3 week’ regimen; Fig. 4.6) indicating that UVA treatment introduces 4-HNE epitopes.
Our data are compatible with previous reports demonstrating 4HNE covalent adduction of cathepsin B resulting in inhibition of enzymatic activity. In one report, treatment of either purified cathepsin B protein or mouse macrophages with 4-HNE resulted in a significant loss of cathepsin B activity. Immunodetection revealed 4-HNE modification of cathepsin B and further mass spectrometric analysis identified the active cite cysteine and histidine (Cys29 and His199, respectively) as the residues of cathepsin B covalently modified by 4-HNE [264]. Another study demonstrated a dose-dependent loss of both cathepsin B and L activity in human retinal epithelial cells treated with either 4-HNE or malondialdehyde (MDA), the two major lipid peroxidation end products. Mass spectrometry detected 4-HNE and MDA adducts of peptides within the active centers of both cathepsin B and L [265]. Figure 4.7 provides a model of cathepsin B active site Cys29- and His199-adducted 4-HNE.

Importantly, several earlier reports have documented the modulation of cathepsin B enzymatic activity in response to oxidative stress. Specifically, oxidative inactivation of cellular cathepsin B by Rose Bengal-dependent photosensitization and singlet oxygen generated from naphthalene endoperoxides has been reported [266, 267], and oxidative inhibition of cathepsin B by 5-aminoquinoline-8-ol through active site sulfenic acid formation has been demonstrated in vitro [268]. Furthermore, earlier studies have reported reversible photooxidative inactivation of active site cysteine residue-containing enzymes including protein tyrosine phosphatase 1B [242, 269, 270]. Therefore, further confirmation of 4-HNE-cathepsin B adducts by mass spectrometric analysis of cathepsin
B isolated from fibroblasts after chronic UVA exposure should be performed to unequivocally determine the nature and site of oxidative post-translational modification.
Figure 4.7 3D model of human cathepsin B with 4-HNE-modified Cys29 and His199. Three dimensional structure of human cathepsin B with the active site Cys29 and His199 modified by 4-HNE (4-HNE shown in light blue). Modeling performed by Dr. Vijay Gokhale, co-Director of the molecular modeling shared services (AZCC).
CHAPTER 5:
UVA INDUCES LYSOSOMAL ALTERATIONS MIMICKED BY DUAL INACTIVATION OF CATHEPSIN B AND L

Parts of this chapter have been adapted from the following publication:

5.1 Introduction and Rationale

Given our previous observations that UVA modulates cathepsin B activity, one of the major purposes of this study to investigate the effects of chronic UVA on other cysteine-dependent enzymes including cathepsin L and GAPDH, as well as the aspartate-dependent cathepsin D. To further elucidate the functional implication of chronic UVA on cellular proteolysis machinery, we also studied effects of chronic UVA on proteasome activity. Furthermore, we examined UVA-induced cathepsin B/L inactivation in other cutaneous cell types including keratinocytes and melanocytes.

Another important focus of this study was to investigate the effects of chronic UVA exposure on autophagic-lysosomal function in human dermal fibroblasts. Earlier reports have demonstrated lysosomal accumulation of autofluorescent material (lipofuscin) originating from lipid peroxidation damage in oxidatively stressed human cells [148, 271-274]. Our data demonstrating UVA-induced oxidative stress (Fig. 4.2)
led us to examine generation of cellular autofluorescence indicative of lipofuscin accumulation in response to UVA exposure.

Furthermore, given that cathepsin B is recognized as one of the major lysosomal housekeeping genes [178, 275], we tested the hypothesis that UVA-induced inhibition of cathepsin B enzymatic activity (Fig. 3.3D) may contribute to the generation of autofluorescent lipofuscin pigments in these cells through impairment of lysosomal clearance. To this end, Hs27 cells were exposed to the cathepsin B/L inhibitor CA074Me (1 µM, q.d., 4 consecutive days), and the effect on lysosomal structure and function was analyzed using flow cytometry, electron microscopy, gene array analysis and immunoblot detection. Additionally, confirmation of the findings was obtained through small interfering RNA (siRNA) gene knockdown of CTSB and CTSL in Hs27 fibroblasts.

5.2 Results

5.2.1 UVA inhibits enzymatic activity of both cysteine cathepsins B and L and the aspartate cathepsin D.

As indicated in chapters 3 and 4, cathepsin B enzymatic activity is diminished by approximately 60% in response to chronic UVA exposure in human dermal fibroblasts (Fig 3.3D). We decided to investigate the effects of UVA on other cathepsins known to be cutaneously expressed and important for skin function. In Hs27 fibroblasts exposed to chronic UVA (‘1 week’ regimen), specific enzymatic activity of cathepsin L (another cysteine cathepsin) was inhibited by approximately 40% compared with untreated controls [Fig. 5.1A; 60.7 % ± 13.0 residual protease activity after UVA exposure (n=3;
mean ± SD; p < 0.05]), while cathepsin D, an aspartate cathepsin was inhibited by approximately 20% [Fig. 5.1A; 79.2 % ± 8.6 residual protease activity after UVA exposure (n=3; mean ± SD; p < 0.05)]. Interestingly, loss of cysteine cathepsin enzymatic activities (both B and L) was antagonized significantly if irradiation occurred in the presence of the antioxidant NAC (10mM). In contrast, NAC co-irradiation did not affect the UVA-induced loss of cathepsin D enzymatic activity (Fig. 5.1A).

Enzymatic activity of GAPDH, an active site cysteine-containing glycolytic enzyme, known to be a common target of cytotoxic oxidative insult including Rose Bengal-dependent photosensitization [267], was not changed significantly in response to UVA exposure (‘1 week’ regimen) [Fig. 5.1A; control: 3.3 ± 0.5 versus UVA: 3.4 ± 0.4 specific enzymatic activity (u/mg cytosolic protein; n=3; mean ± SD]. These data suggest that UVA-induced changes affecting cathepsins B and L enzymatic activity may occur as a result of specific molecular mechanisms that do not cause the indiscriminate inactivation of other cysteine-dependent enzymes such as GAPDH.

After chronic UVA (‘1 week’ regimen), recovery of enzymatic activity occurred slowly over the course of 24-48 h for both cathepsin B and cathepsin L. Cathepsin B activity recovered to 80% of untreated cells 24 hours after UVA exposure and returned to that of control levels within 48 hrs (Fig. 5.1B). Enzymatic activity of cathepsin L returned to that of untreated controls within 24 hrs (Fig. 5.1C).

In order to determine if UVA-induced cathepsin B and L inhibition occurs in other cutaneous cell types or is a fibroblast specific phenomenon, we measured cathepsin B and L activities in human melanocytes and keratinocytes exposed to chronic UVA (‘1
Figure 5.1 Cathepsin B, cathepsin L, and cathepsin D activities of human skin fibroblasts exposed to UVA in the presence and absence of antioxidants. (A) UVA-induced changes in cathepsin B, cathepsin L, and cathepsin D enzymatic activities were assessed in human dermal fibroblasts irradiated ('1 week’ UVA regimen) in the presence or absence of NAC (10 mM) using specific fluorogenic enzyme substrates (n=3, mean ± SD). (B) Recovery of UVA-induced cathepsin B enzymatic inactivation 1 h, 6 h, 12 h, 24 h, and 48 h following chronic UVA exposure ('1 week’ regimen) as determined using a specific fluorogenic enzyme substrate (n=3, mean ± SD). (C) Recovery of UVA-induced cathepsin L enzymatic inactivation 1 h, 6 h, 12 h, 24 h, and 48 h following chronic UVA exposure ('1 week’ regimen) as determined using a specific fluorogenic enzyme substrate (n=3, mean ± SD).
week’ regimen) or mock treatment (Fig. 5.2). Importantly, as with human skin fibroblasts tested earlier, the ‘1 week’ UVA regimen did not affect the viability of these cells (Fig. 5.2A). Interestingly, HaCaT cells, an immortalized keratinocyte line, showed the highest sensitivity to UVA in the context of cathepsin B and cathepsin L inhibition (Fig. 5.2B; 21.4 % ± 0.9 residual CTSB protease activity and 44.6 % ± 4.0 residual CTSL protease activity after UVA exposure (n=3; mean ± SD; p < 0.05)). In contrast, primary human epidermal keratinocytes (HEK) exposed to UVA did not display significantly reduced cathepsin B or cathepsin L activity compared to controls (Fig. 5.2B; 92.0 % ± 9.8 residual CTSB protease activity and 84.4 % ± 14.5 residual CTSL protease activity after UVA exposure (n=3; mean ± SD; p > 0.05)). Cathepsin B and cathepsin L activities were reduced in primary human epidermal melanocytes (HEMa) upon UVA exposure (Fig. 5.2B; 72.3 % ± 5.7 residual CTSB protease activity and 76.3 % ± 8.9 residual CTSL protease activity after UVA exposure (n=3; mean ± SD; p < 0.05)).

5.2.2 Chronic UVA exposure enhances proteasome activity.

The two major intracellular proteolytic pathways that cells rely on for normal degradation and response to stress are the ubiquitin-proteasome system and autophagy. The catalytic centers of the proteasome complex have chymotrypsin-like, trypsin-like, and caspase-like/PGPH (peptidylglutamyl-peptide hydrolyzing) activities, cleaving peptide bonds after hydrophobic, basic, and acidic residues, respectively [276]. In order to determine if exposure to chronic UVA modulates proteasome activity, we measured
Figure 5.2. Cathepsin B and L activities of HEK, HaCaT and HEMa exposed to UVA. (A) After exposure to chronic UVA (‘1 week’ UVA regimen) cells were analyzed for viability as examined by flow cytometric analysis of annexin V-propidium iodide-stained cells. (B) UVA-induced changes in cathepsin B and cathepsin L enzymatic activities were assessed in HaCaT keratinocytes, primary human epidermal keratinocytes (HEK), and primary human epidermal melanocytes (HEMa) exposed to chronic UVA (‘1 week’ UVA regimen) using specific fluorogenic enzyme substrates (n=3, mean ± SD).
proteasome enzymatic activity in Hs27 cellular lysates using a luminescent protease assay that measures proteasome-dependent release of luciferin from specific precursor substrate peptides. As a positive control, the established proteasome inhibitor MG132 was used (10 µM, 6 h exposure time) (Fig. 5.3). In contrast to UVA-induced inhibition of cathepsin-mediated proteolytic activity, Hs27 exposed to chronic UVA ('1 week' regimen) showed enhanced proteasome activity with significant upregulation of chymotrypsin-, trypsin- and caspase-like/PGPH activities. In response to one single 9.9 J/cm² UVA exposure, an induction of caspase-like/PGPH activity was already observed in the absence of a significant upregulation of chymotrypsin- or trypsin-like activities.

5.2.3 Chronic UVA results in lysosomal expansion with accumulation of lipofuscin.

Visualization by confocal fluorescence microscopy revealed pronounced accumulation of cellular autofluorescent material (λex 488 nm/ λem 553-611 nm) displaying a punctate cytosolic staining pattern in human fibroblasts observed after chronic UVA exposure ('3 week' UVA regimen), but not in untreated control cells (Fig. 5.4A). Further studies employing Lysotracker Red™ and nuclear DAPI staining confirmed cellular colocalization of autofluorescent chromophores and lysosomal organelles (Fig. 5.4A, overlay panels III, VI, and IX). In addition, substantial accumulation of lysosomal vesicles as obvious from intense Lysotracker Red™ staining occurred in UVA-exposed fibroblasts (Fig. 5.4A, panel II versus panel V). An increase in the number and/or size of lysosomes in response to chronic UVA exposure was
Figure 5.3. Proteasome activity in human skin fibroblasts following UVA exposure. Luminescent analysis of chymotrypsin-like, trypsin-like, and caspase (PGPH)-like proteasome enzymatic activity in cells one hour after a single UVA treatment (9.9 J/cm²) or chronic UVA (‘1 week’ regimen). Treatment with MG132 (10 μM, 6 h) served as a positive control (n=3; mean ±SD).
confirmed independently by flow cytometric analysis of LysoTracker™ Yellow (Fig. 5.4G). Furthermore, accumulation of autofluorescent material in response to chronic UVA exposure as shown in Fig. 5.4A was confirmed by flow cytometric analysis of the same treatment groups that revealed a time-dependent increase in fluorescence intensity during the course of this three week experiment (Fig. 5.4B and 5.4F).

Lysosomal integrity seemed to be unaffected by UVA exposure as evidenced by maintenance of punctate staining of Lysotracker™ Red observed by confocal microscopy (Fig. 5.4A, panel V-VI) and confirmed independently by electron microscopy (Fig. 5.5A) that indicated extensive cytosolic accumulation of membraneous vesicles containing osmiophilic material commonly referred to as lipofuscin [140, 141]. Interestingly, other ultrastructural changes beyond vesicular accumulation of osmiophilic lipofuscin material were observed as a result of UVA exposure including mitochondrial shrinkage and loss of functional endoplasmic reticulum, structural changes with important implications for skin cell photodamage not addressed further in this study.

Accumulation of lysosomal-associated membrane protein 1 (Lamp-1) occurred in response to both UVA exposure regimens (‘1 week’ and ‘3 week’). Lamp-1 accumulation occurred at the level of the extensively glycosylated form of this lysosomal transmembrane glycoprotein detected at an apparent size range between 80 and 100 kDa, and the nonglycosylated form running at about 40 kDa was also observed (Fig. 5.5B). UVA treatment did not affect Lamp-1 expression at the transcriptional level as determined by quantitative RT-PCR assessing mRNA levels of LAMP1, the gene encoding Lamp-1 (Fig. 5.5C).
Figure 5.4. UVA-induced lysosomal changes in human Hs27 skin fibroblasts are mimicked by pharmacological inhibition of cathepsin B/L. (A) After exposure to chronic UVA (‘3 week’ regimen), CA074Me (1µM, q.d., 4 consecutive days), or mock treatment, cellular autofluorescence (panels I, IV, VII) and lysosomal staining (Lysotracker Red; panels II, V, VIII) were visualized by confocal microscopy. In addition, nuclei were stained using DAPI (panels II, V, VIII). Overlays of lysosomal staining and autofluorescence demonstrating colocalization are depicted in panels III, VI, and IX. [cont. on next page]
Figure 5.4. UVA-induced lysosomal changes in human Hs27 skin fibroblasts are mimicked by pharmacological inhibition of cathepsin B/L, cont. (B) The increase in UVA-induced autofluorescence intensity of was examined over the course of the ‘3 week’ irradiation period by flow cytometric analysis. The histograms depict fluorescence intensity of UVA- and mock-treated cells after treatment for four (19.8 J/cm² total dose; left panel), eleven (39.6 J/cm² total dose; middle panel), and eighteen days (59.4 J/cm² total dose; right panel). One representative set of histograms out of three similar repeats is shown. (C) After exposure to CA074Me (1µM, q.d., 4 consecutive days; structure shown on left-hand side) cells were analyzed for viability as examined by flow cytometric analysis of annexin V-propidium iodide-stained cells. (D) Changes in cathepsin B, cathepsin L, and cathepsin D enzymatic activities were assessed in cells exposed to CA074Me (1µM, q.d., 4 consecutive days) using specific fluorogenic enzyme substrates (n=3, mean ± SD). (E) Luminescent analysis of chymotrypsin-like, trypsin-like, and caspase (PGPH)-like proteasome enzymatic activity in cells exposed to CA074Me (1µM, q.d., 4 consecutive days; n=3; mean ±SD). (F) After exposure to chronic UVA (‘1 week’ regimen), CA074Me (1µM, q.d., 4 consecutive days), or mock treatment, cellular autofluorescence was measured by flow cytometric analysis (n≥3, mean ± SD). (G) After exposure to chronic UVA (‘1 week’ regimen), CA074Me (1µM, q.d., 4 consecutive days), or mock treatment, cellular lysosomal content as determined by flow cytometric analysis of Lysotracker yellow intensity (n≥3, mean ± SD).
Figure 5.5. Pharmacological inhibition of cathepsin B/L mimics accumulation of osmiophilic vesicles and LAMP-1 in human Hs27 skin fibroblasts. (A) After exposure to chronic UVA (‘3 week’ regimen), CA074Me (1µM, q.d., 4 consecutive days), or mock treatment, cells were examined by transmission electron microscopy (magnification: 8,800 x, top panel; 40,000 X, bottom panel): L (Osmiophilic vesicles indicative of lysosomal lipofuscin accumulation; M (mitochondrion); N (nucleus). (B) Cellular Lamp-1 protein levels in response to CA074Me (as specified in A) or UVA exposure (‘1 week’ and ‘3 week’ regimen) as examined by immunoblot analysis. (C) LAMP-1 mRNA levels in Hs27 cells exposed to UVA (‘3 week’ regimen) or CA074Me (1µM, q.d., 4 consecutive days) as determined by real time RT-PCR analysis (mean ± SD, n=3).
5.2.4 Pharmacological inhibition of Cathepsin B and L mimics UVA-induced lysosomal expansion and lipofuscin accumulation.

The cell-permeable epoxide based \([L\text{-}3\text{-}trans\text{-}(Propylcarbamoyl)oxirane\text{-}2\text{-}carbonyl}\text{-}L\text{-}isoleucyl\text{-}L\text{-}proline methyl ester\) (herein referred to as CA074Me) irreversibly inhibits cathepsin B by covalently binding to the active site cysteine 29 [2]. Because our data indicate UVA-induced inhibition of cathepsins B, D, and L, we wanted to stringently test the cathepsin B inhibitor employed in these studies for off-target effects against other cathepsins. To this end, we cultured dermal fibroblasts in the presence of CA074Me for four days (1 µM q.d.) prior to testing specific enzymatic activities of the above mentioned cathepsins. Importantly, treatment of fibroblasts with CA074Me did not affect cell viability (Fig. 5.4C). As expected, CA074Me results in nearly 100% inhibition of CTSB activity (Fig. 5.4D; 0.6 % ± 0.5 residual protease activity after UVA exposure (n=3; mean ± SD; p < 0.05)). Treatment with CA074Me also inhibited CTSL activity by 75% (Fig. 5.4D; 25.0 % ± 3.5 residual protease activity after UVA exposure (n=3; mean ± SD; p < 0.05)), consistent with the known promiscuity of cathepsin B and L substrate specificity [165]. In contrast, CA074Me treatment did not result in inhibition of CTSD activity (Fig. 5.4D; 99.7 % ± 7.1 residual protease activity after UVA exposure (n=3; mean ± SD; p > 0.05)). Of note, CA074Me treatment (1 µM, q.d., four consecutive days) also resulted in increased chymotrypsin-, trypsin- and caspase-like activities associated with the proteasome complex (Fig. 5.5E).

In order to address the hypothesis that UVA-induced inhibition of cathepsin B and/or cathepsin L enzymatic activity contributes to the generation of autofluorescent
lipofuscin pigments in these cells, Hs27 dermal fibroblasts were exposed to CA074Me (1 µM, q.d., 4 subsequent days), and autofluorescence was examined by confocal fluorescence microscopy (Fig. 5.4A, panel VII) and flow cytometric detection (Fig. 5.4F). CA074Me-treatment caused a marked increase in cellular autofluorescence displaying colocalization with lysosomes (Lysotracker Red™; Fig. 5.4A, panels VII-IX) with striking similarities to the UVA-induced autofluorescence pattern observed earlier (Fig. 5.4A, panels IV-VI). Similar to what was observed with UVA-exposed cells, Ca074Me treatment resulted in accumulation of a punctate pattern of intact Lysotracker Red™-positive vesicles, a finding also substantiated by flow cytometric detection of LysoTracker Yellow™ (Fig. 5.4G).

Accumulation of Lamp-1 that occurred in response to both UVA exposure regimens (‘1 week’ and ‘3 week’) also resulted from CA074Me treatment (Fig. 5.5B). As with UVA exposure, CA074Me treatment did not affect Lamp-1 expression at the transcriptional level as determined by quantitative RT-PCR assessing mRNA levels of LAMP1, the gene encoding Lamp-1 (Fig. 5.5C). Additionally, electron microscopy indicated accumulation of cytosolic membraneous vesicles containing osmiophilic material that occurred in the absence of lysosomal disintegration or membrane permeabilization in response to either UVA or pharmacological inhibition of cathepsin B (Fig. 5.5A).

5.2.5 Pharmacological inhibition of Cathepsin B/L mimics UVA-induced deficient cathepsin B maturation.
Figure 5.6. Deficient cathepsin B maturation as a result of UVA exposure or pharmacological inhibition of cathepsin B. Immunoblot detection of cellular cathepsin B protein levels [double chain (DC) and single chain (SC) form] in Hs27 fibroblasts exposed to chronic UVA (‘3 week’ UVA regimen) versus mock treated cells (left panel) or CA074Me-treated Hs27 fibroblasts (1µM, q.d., 4 consecutive days) versus untreated control cells (right panel).
After demonstrating CA074Me-induced accumulation of autofluorescent material that mimics UVA-associated changes, we tested the hypothesis that pharmacological inhibition of cathepsin B/L might also impact cathepsin B protein maturation in response to UVA as detected earlier by 2D-DIGE mass spectrometric analysis and immunoblot analysis (Fig. 3.3). Indeed, immunoblot analysis revealed the complete loss of the mature double chain (DC) form and a pronounced increase in the single chain (SC) form of cathepsin B in cells exposed to CA074Me (Fig. 5.6; 1 µM, q.d., 4 subsequent days).

### 5.2.6 UVA-induced transcriptional alteration of SQSTM1, PRKAA2, SNCA, and TGM2 expression are mimicked by Ca074Me treatment

To further explore the nature of UVA-induced lysosomal-autophagic alterations at the gene expression level we then employed the RT2 Profiler™ Autophagy PCR array platform (Table 5.1 and Fig. 5.7) that allows quantitative assessment of treatment-induced transcriptional changes of 84 autophagy-related genes. In human Hs27 dermal fibroblasts, chronic UVA exposure (‘3 week’ regimen) altered expression levels of eight genes on the array by at least two-fold. The gene displaying the most pronounced upregulation in response to UVA was identified as SQSTM1 (sequestosome 1; 3.5 fold) encoding p62, a multidomain scaffold/adaptor protein involved in autophagic degradation of ubiquitinated proteins and organelles that serves as a selective autophagic substrate itself [277, 278]. Moderate UVA-induction at the mRNA level was also observed with PRKAA2 (encoding the catalytic subunit of 5’ adenosine monophosphate-activated protein kinase, a sensor of cellular energy status) and SNCA (encoding α-synuclein, an...
Table 5.1. Human Autophagy™ Gene Expression Array analysis of human skin fibroblasts exposed to chronic UVA or pharmacological inhibition of cathepsin B. Differential gene expression in response to chronic UVA exposure (‘3 week’ regimen) or CA074Me treatment (1 µM, q.d., 4 consecutive days) versus mock treatment was analyzed using the RT² Human Autophagy™ PCR Array. Three independent repeat experiments were analyzed using the two-sided Student’s t test. (A) Genes equally down- or upregulated by at least twofold (*p < 0.05) in response to either treatment. (B) Genes down- or upregulated by at least twofold in only UVA or CA074Me treatment groups (*p < 0.05). (C) Genes not displaying significant expression changes in response to either treatment (*p > 0.05).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SQSTM1 (NM_003900)</td>
<td>Sequestosome 1</td>
<td>3.5* 4.6*</td>
</tr>
<tr>
<td>PRKAA2 (NM_006252)</td>
<td>Protein kinase, AMP-activated, alpha 2 catalytic subunit</td>
<td>2.2* 2.3*</td>
</tr>
<tr>
<td>SNCA (NM_000345)</td>
<td>Synuclein, alpha (non A4 component of amyloid precursor)</td>
<td>2.0* 6.7*</td>
</tr>
<tr>
<td>TGM2 (NM_004613)</td>
<td>Transglutaminase 2</td>
<td>-8.3* -4.4*</td>
</tr>
<tr>
<td><strong>(B)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP1LC3B (NM_022818)</td>
<td>Microtubule-associated protein 1 light chain 3 beta</td>
<td>2.4* 1.4</td>
</tr>
<tr>
<td>HSP90AA1 (NM_001017963)</td>
<td>Heat shock protein 90kDa alpha (cytosolic), class A member 1</td>
<td>2.1* 1.0</td>
</tr>
<tr>
<td>EIF2AK3 (NM_004836)</td>
<td>Eukaryotic translation initiation factor 2-alpha kinase 3</td>
<td>2.0* 1.2</td>
</tr>
<tr>
<td>HSPA8 (NM_006597)</td>
<td>Heat shock 70kDa protein 8</td>
<td>2.0* 1.0</td>
</tr>
<tr>
<td>CLN3 (NM_000886)</td>
<td>Ceroid-lipofuscinosis, neuronal 3</td>
<td>1.5 2.2*</td>
</tr>
<tr>
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<td>Cathepsin S</td>
<td>1.4 2.8*</td>
</tr>
<tr>
<td>IGFI (NM_006418)</td>
<td>Insulin-like growth factor 1 (somatomedin C)</td>
<td>-1.8 -2.6*</td>
</tr>
</tbody>
</table>

(A) Genes equally down- or upregulated by at least twofold (*p < 0.05) in response to either treatment.
(B) Genes down- or upregulated by at least twofold in only UVA or CA074Me treatment groups (*p < 0.05).
(C) Genes not displaying significant expression changes in response to either treatment (*p > 0.05).
Figure 5.7. Gene expression changes affecting the autophagic-lysosomal pathway in human skin fibroblasts induced by UVA exposure or pharmacological inhibition of cathepsin B/L. Scatter blot of differential gene expression in response to chronic UVA exposure (‘3 week’ regimen) or CA074Me treatment (1 µM, q.d., 4 consecutive days) versus mock treatment as analyzed using the RT² Human Autophagy™ PCR Expression Array (as summarized in Table 5.1). Upper and lower lines represent the cut-off indicating two fold up- or down-regulated expression, respectively. Arrows mark genes displaying similar expression changes in response to both treatments.
autophagic protein substrate and aggresome component) [279, 280]. Pronounced downregulation in response to chronic UVA occurred with \textit{TGM2} encoding transglutaminase 2, an essential enzymatic factor involved in autophagolysosome maturation [281, 282].

Unexpectedly, pharmacological inhibition of cathepsin B/L closely mimicked this specific pattern of UVA-induced expression changes affecting autophagy-related genes (Table 5.1 and Fig. 5.7). CA074Me-treatment (1 µM, q.d., 4 subsequent days) caused gene upregulation (\textit{SQSTM1, PRKAA2, SNCA}) and downregulation (\textit{TGM2}) as observed with UVA.

In order to further explore the unexpected similarities between expression changes caused by UVA and CA074Me we expanded the array of genes interrogated for transcriptional changes combining data derived from RT\textsuperscript{2} Human Autophagy\textsuperscript{TM} and Stress PathwayFinder\textsuperscript{TM} PCR Expression analysis (Fig. 5.3B). As indicated in chapter 4 (Fig. 4.3), chronic UVA exposure caused a pronounced upregulation of the cellular heat shock response, but these dramatic changes were not induced by CA074Me treatment. Comparative assessment of expression changes induced by UVA- versus CA074Me-treatment revealed statistically significant differences affecting heat shock protein-encoding genes including \textit{HSPA1A} (11 fold), \textit{HSPA6} (1041 fold), \textit{HSPA8} (2 fold), and \textit{HSPCA/HSP90AA1} (2.1 fold) all of which with the exception of \textit{HMOXI} (UVA: 18 fold versus CA074Me: 4.1 fold) were only upregulated in response to UVA (n=3, p<0.05; Fig. 5.8). Moreover, upregulated expression of \textit{EIF2AK3} (encoding eukaryotic translation initiation factor 2-alpha kinase 3, also called PERK, a regulator of the
unfolded protein response) and MAP1LC3B (encoding microtubule-associated protein 1 light chain 3 beta, also called LC3B, a protein involved in microtubule assembly and autophagosome formation) only occurred in UVA-exposed fibroblasts. In contrast, transcriptional changes exclusively observed in CA074Me-treated fibroblasts included CLN3 (2.2 fold upregulation) encoding the lysosomal protein CLN3 impaired in juvenile ceroid lipofuscinosis, CTSS (2.8 fold upregulation) encoding the lysosomal cysteine-protease cathepsin S, and IGF1 (2.6 fold downregulation) encoding insulin-like growth factor 1.

5.2.7 Immunoblot detection of Lamp-1, beclin 1, LC3-II, p62, α-synuclein, and transglutaminase 2 confirms UVA-induced autophagic-lysosomal dysregulation that is mimicked by pharmacological inhibition of cathepsin B/L

We further examined the occurrence of UVA- and CA074Me-induced alterations indicative of autophagic-lysosomal dysfunction at the protein level (Fig. 5.9). Protein levels of beclin1, a key initiator and established hallmark of autophagy known to be upregulated in response to various autophagy-inducing stimuli, remained unchanged in response to either treatment (Fig. 5.9A) [278, 283]. After detecting upregulation of the lysosomal glycoprotein Lamp-1 at the protein but not at the transcript level (Fig. 5.5B-C), we focused on immunodetection of LC3-II, a lipidated autophagosomal component and established indicator of autophagosome accumulation [278]. Indeed, pronounced accumulation of LC3-II occurred in response to both UVA and CA074Me treatment.
Figure 5.8. Comparative analysis of autophagic-lysosomal- and heat shock-related gene expression changes induced by UVA- versus CA074Me-treatment. Bar graph depicts selected data from combined RT² Human Autophagy™ and Stress and Toxicity PathwayFinder™ PCR Expression arrays (n=3, mean ± SD; only where applicable: * denotes statistically significant differences (p<0.05) between treated and untreated control; # denotes statistically significant differences (p<0.05) between UVA- and CA074Me-treated samples).
Autophagy is a dynamic process, and steady state levels of LC3-II are influenced by both synthesis and degradation of the protein. To determine if the UVA-induced increase in levels of LC3-II was due to enhanced autophagosome synthesis rather than reduced autophagolysosome degradation, we monitored the conversion of LC3-I to LC3-II in cells in the absence or presence of bafilomycin A1 (BFA), a potent inhibitor of vacuolar H+ ATPase (V-ATPase) thus inhibiting autophagolysosome degradation of LC3-II [284]. As Fig. 5.9B demonstrates, when LC3-II degradation was blocked by culturing cells with BFA for 4 or 8 h immediately after UVA treatment [(single 9.9 J/cm² UVA exposure (lanes 4-6) or ‘1 week’ UVA regimen (lanes 7-9)], levels of LC3-II were not increased over that of control cells (lanes 1-3) treated with BFA for 4 or 8 h. This established method of monitoring autophagosome formation [284, 285] suggests that LC3-II accumulation observed in dermal fibroblasts exposed to UVA is not due to an induction of autophagosome synthesis, but rather results from a blockade in autophagic degradation of LC3-II.

Further evidence in support of a degradation blockade over increased autophagic flux was obtained from immunodetection of p62. Pronounced accumulation of p62 (sequestosome 1, encoded by SQSTM1), a selective autophagy substrate known to undergo depletion during active autophagy such as amino acid starvation (Fig. 5.9C), occurred at the protein level in response to either treatment (Fig. 5.9A).[277, 278] Additional data indicating impaired autophagic-lysosomal protein turnover was obtained from immunodetection of α-synuclein (encoded by SNCA) (Fig. 5.9A). In response to either treatment we observed cellular accumulation of α-synuclein, a protein known to
Figure 5.9. Autophagic-lysosomal related protein changes in human skin fibroblasts induced by UVA exposure or pharmacological inhibition of cathepsin B. (A) After exposure to CA074Me (1µM, q.d., 4 consecutive days) or UVA (‘3 week’ regimen) protein levels were determined by Western blot analysis using β-actin detection as a loading control. Left panel: beclin 1, LC3-I, LC3-II; right panel: p62, α-synuclein, transglutaminase 2 (TGM2). (B) Western blot detection of LC3-I to LC3-II conversion in control (lanes 1-3), UVA-exposed [one single 9.9 J/cm² dose (lanes 4-6); ‘1 week’ regimen (lanes 7-9)], or starved (lanes 10-12) Hs27 cells in the absence of presence of 100nM BFA for 4 or 8 h. (C) Hs27 fibroblasts were cultured in HBSS and deprived of amino acids and serum for 1-24 hours. Protein levels of p62 were determined by Western blot analysis using β-actin detection as a loading control.
undergo autophagosomal degradation and primarily implicated in cytoplasmic inclusion body and aggresome formation [279, 280]. In contrast, a pronounced decrease of cellular levels of transglutaminase 2 (TG2 encoded by TGM2), an essential enzyme involved in autophagosome maturation, was observed (Fig 5.9A).[281, 282]

5.2.8 Functional implications of lysosomal impairment downstream of UVA-induced cathepsin B/L inactivation.

To further understand how UVA-induced changes influence lysosome-dependent cellular functions we employed prototype experiments to assess the impact of UVA exposure and UVA-induced cathepsin B/L inactivation on (I) lysosomal clearance, (II) endo-lysosomal dependent viral infection, and (III) lipofuscin-dependent photosensitization (Fig. 5.10).

Skin fibroblasts exposed to chronic UVA (‘3 week’ regimen) or cultured in the presence of CA074Me (1 µM, q.d., 4 consecutive days) displayed a delay in clearance of the self-quenched fluorescently labeled bovine serum albumin (BSA) as measured by fluorescence microscopy (control, chronic UVA and CA074Me treated) or flow cytometry (control versus UVA exposure only). DQ™-BSA is heavily labeled with BODIPY fluorescent dye, and upon proteolytic cleavage the single dye-labeled peptides will produce fluorescence. Hs27 cells were preloaded with DQ™-BSA for 2 hours to allow endocytosis and initial digestion of the protein, and the rate of disappearance of dequenched DQ™-BSA was monitored for 22 hours and 28 hours after initial loading. Fluorescence microscopy revealed a strong fluorescent staining in control cells as well as
cells exposed to chronic UVA or CA074Me. After 28 hr, the fluorescence was largely diminished in the control group, whereas treated cells (UVA or CA074Me) still displayed significant fluorescence intensity (Fig. 5.10A). Flow cytometric detection was conducted in order to quantify the DQ™-BSA fluorescence in chronic UVA (‘3 week’ regimen) versus mock irradiated Hs27 cells (Fig. 5.10B). Initial fluorescence after preloading cells with the dye (2 hr) was not statistically significant between the two groups (control: 98.4±5.8; UVA: 106.9±7.5 (raw fluorescence; mean ± SD, n=3; p>0.05). However, there was a significant difference in the remaining fluorescence between the two groups after a 22 hr incubation period [30.7% ± 2.1 fluorescence remained in control cells; 45.2% ± 1.9 remained in UVA-exposed fibroblasts (n=3; mean ± SD; p < 0.05)]. Similarly, after 28 hr incubation, there was a higher amount of fluorescence remaining in UVA-exposed fibroblasts versus mock irradiated cells [22.6% ± 1.8 fluorescence remained in control cells; 35.5% ± 1.7 remained in UVA-exposed fibroblasts (n=3; mean ± SD; p < 0.05)]. These results suggest that UVA exposure or CA074Me treatment has a diminished capacity to clear dequenched DQ™-BSA.

Because it has been shown that many human viruses are dependent on cathepsin B/L activity for infection of host cells [286-288], we used HPV16 as a model to study the effect of UVA-induced cathepsin B/L inhibition on viral infectivity. We observed a significant decrease in HPV16 infection in human skin fibroblasts or HaCaT keratinocytes exposed to chronic UVA (‘1 week’ regimen) or treated with CA074Me (1 µM, q.d., 4 consecutive days) compared to that of untreated controls (Fig. 5.10C-D).
Figure 5.10. Functional implications of UVA-induced cathepsin B/L inactivation on lysosomal clearance, endo-lysosomal viral infection, and lipofuscin-dependent photosensitization in human skin fibroblasts. (A-B) Following exposure to chronic UVA (‘3 week’ regimen), CA074Me (1µM, q.d., 4 consecutive days) or mock treatment, Hs27 cells were incubated in DMEM media containing DQ-BSA (25 µg/mL) for 2 h at 37 °C in 5% CO₂. Cells were washed and further incubated in normal growth media for an additional 28 h [for CA074Me group, CA074Me (1 µM) was added to growth media during this time], then harvested for analysis. Cells were analyzed by fluorescence microscopy (A) or flow cytometry (B). In (B), data are expressed as fluorescence clearance (% control). (C-D) Infectivity of HPV16 virus in HaCaT keratinocytes (C) or Hs27 fibroblasts (D) after chronic UVA exposure (‘1 week’ regimen) or CA074Me (1µM, q.d., 4 consecutive days) measured by an HPV16 encapsidated luciferase reporter assay. For CA074Me group, CA074Me (1 µM) was added to growth media during infection time. (E) Intracellular ROS levels determined by DCF fluorescence immediately following UVA irradiation (one single 9.9 J/cm² dose) in cells cultured in the presence or absence of CA074Me (1µM, q.d., 4 consecutive days) prior to irradiation (n=3; mean ± SD).
Lipofuscin contains fluorescent material that may act as a photosensitizer, thereby exacerbating UV damage [141, 150]. To examine whether the accumulation of lipofuscin resulting from cathepsin B/L inhibition has a functional implication in photosensitization, generation of intracellular oxidizing species was examined in Hs27 cells cultured in the presence or absence of CA074Me (1 µM, q.d., 4 consecutive days) prior to UVA exposure. Flow cytometric detection of DCF fluorescence revealed that fibroblasts cultured in the presence of Ca074Me (1 µM, q.d., 4 consecutive days) prior to a single exposure to UVA (9.9 J/cm²) display a significant increase in the cellular levels of ROS compared to cells exposed to UVA in the absence of the inhibitor (Fig. 5.10E). Exposure of Hs27 fibroblasts to UVA induced a small but significant increase (approximately 70%) over baseline fluorescence observed after loading the cells with the redox dye, which is consistent with UVA-induced intracellular oxidative stress [37]. There was an almost two-fold increase in the generation of intracellular ROS in Hs27 cells exposed to UVA after being cultured in the presence of CA074Me compared to cells exposed to UVA that were cultured in the absence of CA074Me [168.5% ± 12.1 above baseline in the absence of CA074Me versus 319.9% ± 47.2 above baseline in the presence of CA074Me (n≥3; mean ± SD; p < 0.05)]. Interestingly, cells cultured in the presence of CA074Me actually showed a significant decrease in baseline fluorescence (59.9% ± 5.8, p <0.05).

5.2.9 Dual genetic antagonism targeting both CTSB and CTSL expression mimics UVA induced autophagic-lysosomal alterations.
Previous data from this chapter demonstrates that UVA exposure results in inactivation of both cathepsin B and cathepsin L activities (Fig. 5.1) and that CA074Me treatment (1 µM, q.d., 4 consecutive days) mimics UVA induced changes. Considering that CA074Me inhibits both cathepsins B and L (fig. 5.4D), we used siRNA to selectively silence these specific cathepsin genes in order to test their relative contribution in mediating UVA-induced lysosomal impairment. The knockdown of cathepsin B, cathepsin L, or double knockdown of cathepsin B and L in Hs27 fibroblasts was confirmed by mRNA transcript levels (Fig. 5.11A), enzymatic activity (Fig. 5.11B), and immunodetection of the protein (Fig. 5.11C).

First, we examined the effect of siRNA-mediated knockdown of cathepsin B, cathepsin L, or double knockdown cathepsin B and L on protein levels observed to be modulated in response to UVA-exposure or CA074Me treatment (Fig. 5.9A). Slight accumulation of p62 occurred in human skin fibroblasts upon genetic silencing of either cathepsin B or cathepsin L, whereas the accumulation of p62 was much more pronounced in the case of dual genetic antagonism targeting both cathepsin B and cathepsin L (Fig. 5.12A). Equally, immunodetection of Lamp-1 and LC3-II revealed a marked accumulation of these two proteins only when both CTSB and CTSL were genetically targeted, whereas single knockdown of either CTSB or CTSL did not reproduce the UVA- or CA074Me-exposed phenotype (Fig. 5.12A). In agreement with these changes at the protein level, EM confirmed accumulation of cytosolic membraneous vesicles containing osmiophilic material only indicative of lipofuscin in fibroblasts after CTSB/CTSL-double knockdown (Fig. 5.12C). We then examined the effect of cathepsin B, cathepsin L, or
Figure 5.11. Confirmation of siRNA induced knockdown of cathepsin B, cathepsin L, or double knockdown of cathepsin B and L in Hs27 fibroblasts. (A) mRNA transcript levels of CTSB and CTSL in cells transfected with siControl or siRNA targeted to CTSB, CTSL, or CTSB and CTSL labeled as C, B, L, and B+L, respectively (n=3; mean ±SD). (B) Cathepsin B and cathepsin L enzymatic activity as determined by fluorogenic substrates for the same groups as indicated in (A) (n=3; mean ±SD). (C) Immunoblot detection of cellular cathepsin B and cathepsin L protein levels [double chain (DC) and single chain (SC) form] for the same groups as indicated in (A) using β-actin detection as a loading control.
Figure 5.12. Effect of cathepsin B and/or cathepsin L gene knockdown on autophagic-lysosomal related transcriptional and protein levels in human skin fibroblasts. (A) Protein levels of Lamp-1, LC3-I, LC3-II and p62 were determined by Western blot analysis using β-actin detection as a loading control in Hs27 fibroblasts 96 hours after transfection with siControl or siRNA targeted to CTSB, CTSL, or CTSB and CTSL. (B) Differential gene expression of LAMP1, SQSTM1, and SNCA for the same groups as indicated in (A) (n=3; mean±SD). (C) 96 hours after siControl, siCTSB, siCTSL, or siCTSB and CTSL knockdown, cells were examined by transmission electron microscopy (magnification: 25,000 X): L (Osmiophilic vesicles indicative of lysosomal lipofuscin accumulation; M (mitochondrion); N (nucleus).
cathepsin B and L gene knockdown on transcriptional changes observed in response to chronic UVA- and Ca074Me-treatment (Fig. 5.7) by individually assessing SQSTM1, SNCA, TGM2 and LAMP1 (Fig. 5.12B). Interestingly, in contrast to pharmacological inhibition of cathepsin B/L with Ca074Me, knockdown of cathepsin B and/or L did not result in the modulation of SQSTM1, SNCA, or TGM2 transcript levels.

5.3 Discussion

In this report we demonstrate that fibroblasts exposed to chronic UVA (‘1 week’ and ‘3 week’ regimens) display accumulation of lipofuscin-like autofluorescent material displaying lysosomal colocalization, a change suggestive of lysosomal impairment. Strong cellular autofluorescence measured by flow cytometry (Fig. 5.4F) was confirmed by confocal microscopy indicating the accumulation of lysosomal fluorescent pigments costaining with Lysotracker Red (Fig. 5.4A). Flow cytometric detection and confocal imaging of LysoTracker dyes revealed an expansion of lysosomal vesicles in fibroblasts exposed to chronic UVA (Figs. 5.4A and 5.4 G; ‘1 week’ and ‘3 week’ regimens), a finding in congruence with EM that indicates extensive cytosolic accumulation of membranous vesicles containing lipofuscin (Fig. 5.5A).

Remarkably, of the cutaneous cell types tested, HEMa and HaCaT keratinocytes, a spontaneously immortalized cell line [289], exhibited UVA-induced cathepsin B/L inhibition, while HEK did not show sensitivity to UVA in the context of cathepsin B/L inactivation (Fig. 5.2B). This could be due to their capability of detoxifying ROS.
Indeed, it has been demonstrated that HEK are less susceptible to UVA-induced oxidative stress and lipid peroxidation than dermal fibroblasts [290, 291].

It is important to note that earlier work has demonstrated the involvement of lysosomal disruption in UVA-induced fibroblast photodamage, involving photooxidative rupture of lysosomal membranes followed by cytoplasmic release of proteases including cathepsin B that degrade ferritin with mobilization of redox-active iron [87, 88]. However, typical UVA fluence (dose) and irradiance (intensity) employed in these studies were significantly higher than the ones used in our model of chronic UVA exposure. In our experiments, fibroblast viability, proliferative rate after reseeding, and integrity of lysosomal membranes as monitored by direct imaging involving confocal fluorescence (Fig. 5.4A) and transmission electron microscopy (Fig. 5.5A) were not impaired as a result of chronic UVA exposure, and other mechanisms different from lysosomal disintegration must therefore be involved in UVA-induced cathepsin B alterations.

The two major intracellular pathways that cells rely on for proteolysis of both normal and damaged proteins are the ubiquitin-proteasome system and autophagy. The proteasome is a large protein complex containing a proteolytically active 20S core particle with threonine protease activity. The catalytic centers have chymotrypsin-like, trypsin-like, and caspase-like/PGPH (peptidylglutamyl-peptide hydrolyzing) activities, cleaving peptide bonds after hydrophobic, basic, and acidic residues, respectively [276]. Earlier research has demonstrated that UVA exposure may also interfere with proteasomal protein degradation, another mechanism potentially underlying
pathologically altered protein turnover in the context of skin photodamage [292, 293]. Moreover, lipofuscin-dependent proteasomal inhibition has been demonstrated in human dermal fibroblasts representing another mechanism potentially exacerbating dysfunctional protein turnover under UV stress[157]. Interestingly, our experimentation indicates that UVA exposure leads to enhanced proteasome activity with significant upregulation chymotrypsin-, trypsin- and caspase-like/PGPH activities (Fig. 5.3) indicating that proteasome inhibition most likely does not contribute to the altered protein turnover observed in fibroblasts chronically exposed to UVA. The mechanism underlying upregulated proteasome activity and whether it is a compensatory mechanism for decreased lysosomal proteolytic capacity remains to be elucidated. Notably, it has recently been shown that proteasome activity is under Nrf2 transcriptional control and upregulation of proteasome activity is an important adaptation to increased oxidative stress [294].

Consistent with a causative involvement of UVA-induced impairment of cathepsin B/L function in the accumulation of lysosomal autofluorescence, the pharmacological cathepsin B/L inhibitor CA074Me that completely abolished cellular cathepsin B activity and inhibited ~75% cathepsin L activity without affecting cathepsin D activity (Fig. 5.4D) mimicked UVA-induced autofluorescence characteristics. Specifically, confocal microscopy revealed pronounced punctate cytosolic autofluorescence colocalizing with lysosomes in CA074Me-treated Hs27 fibroblasts (Fig. 5.4A), a finding confirmed by flow cytometric detection of cellular autofluorescence (Fig. 5.4F).
Earlier reports have demonstrated accumulation of autofluorescent lysosomal pigment (lipofuscin) originating from lipid peroxidation damage that was observed in oxidatively stressed human cells, including retinal pigment epithelial cells that accumulate the bisretinoid lipofuscin chromophore A2E under conditions of photooxidative stress [295], skin fibroblasts exposed to oxidative stress, and fibroblasts undergoing replicative senescence [148, 271-274]. The pronounced accumulation of autofluorescent material in lysosomal organelles that was observed as a consequence of either chronic UVA exposure (Fig. 5.4A, panels IV-VI) or pharmacological inhibition of cathepsin B/L (Fig. 5.4A, panels VII-IX) strongly suggests a causative role of cathepsin B and/or L structural and functional alteration in UVA-induced accumulation of autofluorescent pigments. This is consistent with the established role of cathepsin B and other lysosomal proteases in lysosomal maintenance and clearance of damaged proteins, further supported by recent experimental evidence that demonstrates the involvement of cathepsin B, L and D in removal of glycated AGE-modified proteins [296, 297]. Prior research has shown that lipofuscin may display activity as a photosensitizer. Specifically, retinal pigment epithelial cells loaded with lipofuscin demonstrate increased ROS levels and decreased viability when exposed to visible light [298, 299]. The increased generation of intracellular oxidizing observed in fibroblasts cultured in the presence of Ca074Me (1 µM, q.d., 4 consecutive days) prior UVA exposure compared to cells exposed to UVA in the absence of the inhibitor (Fig. 5.10E) suggests a functional role of lipofuscin generated as a result of cathepsin B/L inhibition as a photosensitizer. It is therefore tempting to speculate that UVA-induced cathepsin B/L inhibition may cause
accumulation of additional sensitizing epitopes, a vicious cycle of skin photooxidative stress to be substantiated by future experiments (Fig. 5.13).

In this study, we present evidence suggesting that UVA-induced inhibition of cathepsin B/L enzymatic activity is the causative factor operating upstream of lysosomal-autophagic impairment, a finding largely based on the identification of a similar pattern of changes affecting cellular phenotype and gene expression at the mRNA and protein level, observed in response to both UVA-treatment and pharmacological inhibition of cathepsin B/L. In our experiments, gene array analysis revealed UVA-induced expression changes affecting SQSTM1, PRKAA2, SNCA, TGM2, MAP1LC3B, HSP90AA1, EIF2AK3, and HSPA8 by at least two-fold (Table 5.1A and B; Fig. 5.7). Strikingly, comparative gene expression analysis indicated that the most pronounced changes (SQSTM1, PRKAA2, SNCA, TGM2) also occurred upon pharmacological inhibition of cathepsin B/L enzymatic activity using CA074Me, but no changes affecting CTSB expression occurred in response to either treatment (Table 5.1 and Fig. 5.7). Furthermore, a compelling similarity between the UVA- and CA074Me-induced expression pattern was detected at the protein level affecting Lamp-1, LC3-II, beclin-1, p62, α-synuclein, and transglutaminase-2 (Figs. 5.5B and 5.9A).

It is important to note that upregulation of Lamp-1 protein levels was not accompanied by changes at the transcriptional level (LAMP1) (Fig. 5.5B-C), yet expression changes affecting SQSTM1 (p62), SNCA (α-synuclein), and TGM2 (transglutaminase 2) at the mRNA level paralleled changes at the protein level (Table 5.1 and Figs. 5.7 and 5.9A). Interestingly, it has been demonstrated recently that SQSTM1 is
under transcriptional control of Nrf2, and that p62 activates Nrf2-dependent transcription by binding and inactivating the Nrf2-antagonistic factor Keap1, representing a positive feedback loop of p62-dependent transcriptional activation of SQSTM1 expression [300, 301].

In response to UVA exposure as well as CA074Me treatment, pronounced upregulation of the selective autophagy substrate p62 (sequestosome 1, encoded by SQSTM1), a cargo receptor for autophagic degradation of specific ubiquitinated target proteins, occurred at the mRNA and protein level (Table 5.1; Figs. 5.7 and 5.9A) [302]. The multidomain scaffold/adaptor protein p62 plays an important role in protein aggregate formation, cell survival, and apoptosis that involves binding of polyubiquitinated TRAF6 (involved in activation of the transcription factor NFκB) and caspase-8 (involved in apoptotic execution), respectively [278, 302, 303]. Importantly, p62 also binds the autophagy regulator Atg8/LC3 via its LIR region, and p62 has been proposed to regulate the packing and delivery of polyubiquitinated misfolded or aggregated proteins and dysfunctional organelles for clearance through autophagy [304]. It is now established that cellular levels of p62 are regulated through autophagy with p62 serving as a selective autophagy protein substrate [277, 278]. Indeed, increased autophagy is associated with reduction of cellular p62 levels (Fig. 5.9C), whereas inhibition of autophagy has been shown to upregulate cellular p62 levels, a finding further substantiated by the observation that p62 accumulates in autophagy-deficient mice [278, 305]. Therefore, pronounced upregulation of p62 protein levels observed equally in
response to UVA exposure or CA074Me treatment is consistent with the occurrence of autophagic blockade downstream of cathepsin B inhibition (Fig. 3B).

Consistent with impaired turnover of the autophagy substrate p62 and accumulation of the lysosomal membrane protein Lamp-1, pronounced upregulation of α-synuclein (encoded by SNCA), another autophagy substrate, occurred in response to either treatment in dermal fibroblasts (Table 5.1; Figs. 5.7 and 5.8). Indeed, recent research has shown that α-synuclein is degraded at least partly by chaperone-mediated autophagy involving cathepsins [279, 280]. Interestingly, α-synuclein constitutes the major protein in cytoplasmic Lewy bodies that accumulate in nigrostriatal neurons during progression of Parkinson’s disease where SNCA mutations may cause early-onset of this neurodegenerative disease [306]. Important functions of α-synuclein include modulation of lipid vesicle dynamics and mitochondrial energy production through complex I binding, but little is known about its specific function in cutaneous cells where expression in melanocytes has been documented [307].

Our study also identified downregulation of transglutaminase 2 (encoded by TGM2), an important factor in autophagosome maturation, at the mRNA and protein level (Table 5.1; Figs. 5.7 and 5.9) [281]. Specifically, it has been shown that genetic ablation of transglutaminase 2 resulted in accumulation of LC3-II, the lipidated autophagosomal membrane component, on pre-autophagic vesicles [282]. In contrast, subsequent formation of the acidic vesicular organelles in the same cells was suppressed, suggesting an impairment of the final maturation of autophagolysosomes associated with LC3-II accumulation that occurs in response to transglutaminase 2 downregulation.
Indeed, we observed pronounced formation of LC3-II in response to UVA and CA074Me exposure (Fig. 5.9A). LC3, the mammalian homologue of yeast Atg8 is an essential factor for autophagosome formation that relocates to and participates in the formation of the autophagosomal membrane after C-terminal proteolytic processing and posttranslational phospholipid-conjugation [278]. Therefore, after relocalization of LC3-I to newly-formed vesicles a more rapidly migrating lipidated form (LC3-II) is detectable by SDS-PAGE (Fig. 5.9A-B). Importantly, recent evidence suggests that lysosomal cathepsins including cathepsin B and D are involved in LC3-II turnover, and pharmacological inhibition of cathepsin B was shown to induce accumulation of LC3-II in macrophages [308, 309].

In the context of autophagic alterations, it is important to note that accumulation of LC3-II in response to UVA occurred in the absence of increased autophagosome synthesis as measured by the conversion of LC-I to LC3-II in the presence of BFA (Fig. 5.9B). With congruence, the above changes of autophagic-lysosomal components occurred in the absence of altered expression at the mRNA or protein level affecting beclin 1 (encoded by BECN1) (Table 5.1; Figs5.7 and 5.9). Indeed, beclin 1 is a critical component in the class III PI3 kinase complex (PI3KC3) involved in autophagosome formation [278, 283]. Due to its role in the initiation of autophagy, upregulation of beclin 1 is often observed during autophagic induction.

While our experimental evidence does not suggest that UVA exposure induces macroautophagy as evidenced by the synchronized accumulation of LC3-II in BFA-treated fibroblasts exposed to UVA or left untreated (Fig 5.9B), it is possible that other
forms of autophagy are being induced. In particular, it is quite plausible that CMA, a process known to be induced during times of increased cellular oxidative stress [133], is enhanced in Hs27 exposed to chronic low-level UVA. *HSP90AA1* (encoding heat shock protein 90kDa alpha, class A member 1) and *HSPA8* (encoding heat shock 70kDa protein 8; Hsc70), the two major chaperone proteins responsible for delivering substrates to LAMP2A, the lysosomal CMA receptor, were both upregulated in fibroblasts exposed to chronic UVA (*3 week regimen*; Figs. 5.7-5.8 and Table 5.1). It is well documented that increased cellular levels of Hsc70 and Hsp90 occur with increased CMA activity [310], although further research is needed to confirm induction of CMA in fibroblasts exposed to chronic low levels of UVA.

Because CA074Me inhibits both cathepsin B and L, we used siRNA to selectively target these specific cathepsin genes in order to test their relative contribution in mediating UVA-induced lysosomal impairment. Monitoring protein levels of Lamp-1, LC3-II, and p62, we observed that only dual genetic antagonism targeting both *CTSB* and *CTSL* expression could mimic UVA-induced autophagic-lysosomal alterations, whereas single knockdown targeting *CTSB* or *CTSL* only did not reproduce the UVA-induced phenotype (Fig. 5.12A). These findings were confirmed by electron microscopy that revealed accumulation of cytosolic membraneous vesicles containing osmiophilic material only in fibroblasts after *CTSB/CTSL*-double knockdown (Fig. 5.12C). Interestingly, siRNA-mediated knockdown of cathepsin B, cathepsin L, or cathepsin B and L did not result in the transcriptional modulation of *SQSTM1*, *SNCA*, or *TGM2* observed in response to UVA exposure of CA074Me treatment (Fig. 5.12B).
molecular mechanism underlying this disparity remains to be elucidated, but may originate from other UVA-modulated events, particularly in the context of oxidative stress signaling. For instance, SQSTM1 (encoding p62) has an ARE in its promoter region and is under transcriptional control of Nrf2 [300]; as we have shown that Nrf2 is upregulated in our model (Chapter 4, Fig. 4.3C), this may explain UVA-induced transcriptional upregulation of SQSTM1. While genetic antagonism of CTSB and CTSL did not induce transcriptional upregulation of SQSTM1, accumulation of p62 was observed indicating that the accumulation of p62 protein observed in UVA is most probably not due solely to transcriptional upregulation, and at least partially because of decreased turn-over of the protein. In the context of Nrf2 activation and autophagic impairment seen in UVA-treated fibroblasts, the cross-talk between autophagic impairment and Nrf2 activation may add a positive feedback loop.

Importantly, CA074Me treatment altered cathepsin B maturation causing the loss of the 24kDa mature form with accumulation of the 29 kDa single chain form (Fig. 5.6), a change of protein structure observed earlier in response to chronic UVA exposure (Figs. 3.3A-C). These data suggest that impaired maturation of the enzyme occurs from interference with autoproteolytic cleavage or proteolytic cleavage from other cysteine proteases. The impaired maturation of cathepsin B upon genetic knockdown of CTSL and impaired maturation of cathepsin L upon knockdown of CTSB demonstrates the likely importance for cathepsin B in the processing of cathepsin L and vice versa (Fig. 5.11C). Interestingly, complete inhibition of maturation is not observed with either protein, indicating that perhaps more than one enzyme is involved in the final maturation
step of both proteins.

The lysosome is an essential executioner involved in protein homeostasis. Taken together, our data demonstrate for the first time that chronic exposure to UVA induces autophagic-lysosomal dysregulation in human dermal fibroblasts. Moreover, we provide evidence that functional impairment of cathepsin B and L activity is a heretofore unrecognized causative factor in cutaneous UVA photodamage operating upstream of autophagic-lysosomal alterations as summarized in Fig. 5.13. This model proposes that inactivation of cathepsin B and L as a consequence of either UVA-induced photooxidative stress or direct pharmacological inhibition causes dynamic changes that are most consistent with a blockade of autophagic flux leading to lysosomal expansion with accumulation of lipofuscin-like material as evident from increases in autofluorescent (Fig. 1E), lysotracker-positive (Fig. 1G), and osmiophilic vesicles (Fig. 1F). Supporting this molecular mechanism, recent research has demonstrated that cathepsin B enzymatic activity is an important regulator of autophagic clearance [309]. Indeed, in macrophages derived from CTSB-knockout mice or wildtype macrophages exposed to CA074Me, autophagic flux was significantly impaired leading to the accumulation and stabilization of LC3-II containing autophagosomal and autophagolysosomal vesicles. Consistent with this finding, in cathepsin D-deficient and cathepsins B and L double-deficient mice, abnormal vacuolar structures resembling autophagosomes accumulate in neurons of the brain [308]. Additionally, it was recently described that MEF derived from ctsl-/ mice display increased number and size of LC3-positive acidic vesicles. While no significant
Figure 5.13. Dual inactivation of cathepsin B and cathepsin L as a causative factor in cutaneous UVA photodamage operating upstream of autophagic-lysosomal alterations. The model proposes that inactivation of cathepsin B and L as a consequence of either UVA-induced photooxidative stress (mediated by ROS), direct pharmacological antagonism (by CA074Me), or double knockdown of CTSB and CTSL causes autophagic-lysosomal dysregulation. Effects of chronic UVA exposure on phenotypic markers including cellular autofluorescence (not shown) and lysotracker Red staining (confocal microscopy images), protein expression pattern of the autophagic-lysosomal factors Lamp-1, LC3-II, beclin 1, p62, α-synuclein, and transglutaminase 2 are mimicked by CA074Me treatment indicating that UVA exposure causes autophagic-lysosomal dysregulation downstream of cathepsin B/L inactivation, a novel molecular mechanism potentially involved in UVA-induced skin photodamage. Differential stress response gene expression (including heat shock protein encoding genes) occurs in response to UVA but not CA074Me due to the causative involvement of ROS in UVA-induced cathepsin B/L inhibition that are not generated as a result of direct pharmacological antagonism by CA074Me.
impairment in the initiation of autophagy, formation of autophagosomes, or autophagosome-lysosome fusion was observed, large autophagolysosomes that colocalized with Lamp-1 were detected in ctsl-/ MEF indicating that degradation of autophagolysosomal content is impaired in the absence of cathepsin L [311]. It has also been shown that primary lysosomal dysfunction may impair autophagic flux as observed in genetic lysosomal storage disorders (LSD) [312, 313]. Moreover, in cells from LSD mice, an impairment of the autophagic pathway may also be associated with the inefficient degradation of aggregate-prone proteins such as mutated α-synuclein. It is therefore reasonable that a deficiency in lysosomal cathepsin B and L enzymatic activity caused by UVA-photooxidative inactivation is sufficient to cause major autophagic-lysosomal dysfunction.

The decreased capacity to clear dequenched DQ™-BSA (Fig. 5.10A-B) observed in skin fibroblasts exposed to chronic UVA or CA074Me indicates diminished lysosomal degradative capacity. Importantly, lysosomal proteolytic activity has a critical role in several cellular functions, including intracellular transport of certain vitamins, growth factor receptor recycling, growth factor degradation, antigen processing and presentation, and programmed cell death [125-127]. Furthermore, several studies have documented that many human viruses are dependent on cathepsins for binding, entry, and/or disassembly necessary for infection and replication [286, 287]. Indeed, our experimentation revealed Hs27 fibroblasts or HaCaT keratinocytes exposed to chronic UVA (‘1 week’ regimen) or treated with CA074Me (1 µM, q.d., 4 consecutive days; Fig. 5.10D) showed a decrease in HPV16 infection compared to that of untreated controls.
This is in agreement with a recent study indicating the important role of cathepsin B in HPV16 infection of human and murine cells [288]. In addition to further substantiating the emerging role of cathepsin B and L as novel targets in skin photodamage, it will be fascinating to further elucidate the functional implications of these changes for skin photocarcinogenesis and photoaging.
CHAPTER 6:
IDENTIFICATION OF THE MALONDIALDEHYDE-DERIVED PROTEIN EPITOPE DIHYDROPYRIDINE-LYSINE AS AN ENDOGENOUS SENSITIZER OF UVA-INDUCED PHOTOOXIDATIVE STRESS IN HUMAN SKIN CELLS

This chapter has been adapted from the following publication:

6.1 Introduction and Rationale
As mentioned in Chapter One, light-driven electron and energy transfer involving non-DNA skin chromophores as endogenous photosensitizers is thought to contribute to oxidative stress in UVA-exposed human skin, a process with relevance to photoaging and photocarcinogenesis [35, 37]. Malondialdehyde (MDA) is a reactive dialdehyde-intermediate resulting from lipid peroxidation chain reactions, and MDA has been involved in the pathogenesis of skin alterations associated with diabetes [314, 315], nonmelanoma and melanoma skin cancer [246], and photoaging [316]. Tissue damage by MDA involves covalent chemical adduction of protein-bound lysine residues potentially leading to fluorophore formation with protein crosslinking and further functional alterations [317-320]. Protein epitopes derived from MDA such as N\(^{\text{ε}}\)-(2-
propenal)lysine (N-propenal-Lys) and dihydropyridine (DHP)-type adducts including DHP-lysine [(S)-2-amino-6-(3,5-diformyl-4-methyl-4H-pyridin-1-yl)-hexanoic acid; Fig. 6.1] may accumulate under conditions of chronic oxidative stress in damaged human tissue [318, 321], and immunohistochemical analysis has identified DHP-lysine epitopes in cardiovascular atherosclerotic lesions [245]. Moreover, accumulation of MDA-derived epitopes with undefined chemical structure has been detected in cutaneous superficial spreading melanoma and squamous cell carcinoma [246]. These epitopes are also thought to be major fluorophores contained in the intracellular age pigment lipofuscin that accumulates in lysosomes as a consequence of cumulative oxidative damage [145-148]. Importantly, potential phototoxicity of lipofuscin has been demonstrated in the context of age-related macular degeneration where accumulation of the all-trans-retinal derived lipofuscin-fluorophore A2E induces blue light-dependent photooxidative stress in human retinal pigment epithelial cells [322]. In contrast, little is known about the potential phototoxicity of lipid peroxidation-derived protein epitopes in human skin.

In our own experiments, lipofuscin photosensitization activity was suggested in Hs27 fibroblasts exposed to UVA following pharmacological inhibition of cathepsin B/L (1 µM, q.d., 4 consecutive days) (Chapter 5, Fig. 5.10E). Here, DHP-lysine was used as an exploratory model to examine the consequences of accumulation of lipofuscin. The purpose of this study, was to (I) determine if MDA- and DHP-epitopes occur in healthy human skin and (II) determine if peptide-bound DHP-lysine is a potent sensitizer of
UVA-induced photooxidative stress in cultured human skin cells, substantiating a role of lipofuscin epitopes in UVA photosensitization.

6.2 Results

6.2.1 MDA- and DHP-epitopes occur in healthy human skin.

Cutaneous occurrence of lipid peroxidation-derived MDA- and DHP-epitopes was examined by immunohistochemical analysis of healthy human skin samples derived from a commercial cutaneous tissue microarray (Fig. 6.2; three representative specimens derived from 12 individual donors are depicted). Every specimen was analyzed by H&E staining, immunostaining for pan-MDA-derived epitopes, and specific immunodetection of DHP-lysine epitopes. Abundant immunoreactivity for MDA- and DHP-epitopes was detected throughout the epidermal and dermal layers with intra and extracellular localization. Similar immunostaining was observed with all 12 human skin specimens (data not shown). Immunostaining for MDA- and DHP-epitopes was detectable with highest intensity in the cellular epidermis, with somewhat attenuated staining throughout the dermis, and no immunoreactivity for either epitope was observed throughout the stratum corneum. Dermal staining for pan-MDA-epitopes occurred with higher intensity than for DHP-lysine epitopes.

6.2.2 DHP-lysine as a sensitizer of UVA-induced inhibition of skin cell proliferation.

The hypothesis that the DHP-lysine epitope displays activity as an UVA photosensitizer was then tested. To this end, we used Boc-protected DHP-lysine-tert.-
Figure 6.1. The lipid peroxidation-derived fluorophore dihydropyridine (DHP)-lysine. (A) Formation of DHP-epitopes on target lysine residues occurs by spontaneous malondialdehyde (MDA)-adduction. (B) Protected DHP-lysine as a model of peptide-bound DHP-lysine. (C) Fluorescence spectrum of protected DHP-lysine [excitation spectrum (λem at 485 nm; broken line, short dashes) and emission spectrum (λex at 395 nm; broken line, long dashes)]
Figure 6.2. Immunohistochemical detection of MDA- and DHP-epitopes in healthy human skin. A commercially available healthy human skin tissue microarray (NS21-01-TMA, Cybrdi) was processed for H&E staining (top row, specimens 1-3), pan-MDA-immunohistochemistry (middle row, specimens 1-3) using a polyclonal antibody (AP050), and DHP-immunohistochemistry (bottom row, specimens 1-3) using a monoclonal antibody (1F83). In all specimens, abundant staining for MDA- and DHP-epitopes occurs throughout the epidermal and dermal layers; Staining is most abundant in the cellular layers of the epidermis. Stratum corneum does not stain positive for either epitope. Three representative specimens are depicted.
butylester (termed ‘DHP-lysine’ throughout the results part), a synthetically accessible analogue of peptide-bound DHP-lysine with identical fluorophore structure as detailed in Fig. 6.1B-C and ‘Materials and methods’. Protected DHP-lysine [(2S)-Boc-2-amino-6-(3,5-diformyl-4-methyl-4H-pyridin-1-yl)-hexanoic acid t-butyl ester]. Identity of the preparation employed in sensitization experiments was confirmed by fluorescence spectroscopy (Fig. 6.1) and electrospray mass spectrometry [ESI-MS; m/z 437.56, (M+H)^+] using a LCQ Classic quadrupole ion trap mass spectrometer.

Consistent with a photodynamic activity associated with the fluorophore DHP-lysine, proliferation of human immortalized HaCaT keratinocytes was strongly suppressed upon combined exposure to a dose range of UVA-radiation in the presence of low micromolar concentrations of DHP-lysine, whereas exposure to the single action of DHP-lysine (up to 10 µM) or UVA (up to 9.9 J/cm²) did not inhibit cellular proliferation (Fig. 6.3A-B). Photodynamic effects on proliferation were observable at low concentrations of DHP-lysine (1 µM) requiring only a moderate dose of UVA (9.9 J/cm²; Fig. 6.3A). Similarly, proliferation was suppressed by more than 50 % when a low dose of UVA (1.65 J/cm²) was combined with DHP-lysine at a higher concentration (10 µM; Fig. 6.3B). Antiproliferative effects on HaCaT keratinocytes were confirmed by flow cytometric cell cycle analysis that revealed significant accumulation of HaCaT cells in the G2/M phase resulting from DHP-lysine photosensitization (4.95 J/cm² UVA; 10 µM DHP) but not from single treatment with UVA or DHP-lysine only (Fig. 6.3C). The pronounced accumulation of keratinocytes with 4n DNA content (27.7 ± 3.4 %, 48h after photosensitization; 13.2 ± 1.7 %, untreated controls) and depletion of cells with 2n DNA
Figure 6.3. DHP-lysine as a photosensitizer of UVA-induced inhibition of HaCaT keratinocyte cell proliferation. (A) Human immortalized HaCaT keratinocytes were exposed to UVA-irradiation (9.9 J/cm²) in the presence or absence of DHP-lysine (0.5-10 µM). Additionally, mock-irradiated cells were exposed to DHP (10 µM). Cells were washed with PBS, fresh growth medium was added, and cell number was determined 72 h later by cell counting. Proliferation was compared to untreated cells. (B) HaCaT keratinocytes were exposed to increasing doses of UVA-irradiation (up to 4.95 J/cm²) in the presence or absence of DHP-lysine (10 µM). Additionally, mock-irradiated cells were exposed to DHP (10 µM). After treatment, proliferation was assessed as detailed in panel A. For (a) and (B), means with common letter differ (p<0.05). (C) Cell cycle analysis by flow cytometric analysis of HaCaT keratinocytes stained with propidium iodide was performed 24h after photosensitization (DHP 10 µM; UVA 4.95 J/cm²). Histograms of a representative experiment are shown. The numbers summarize results (% of total gated cells; mean ± SD) from three independent experiments.
content (44.3 ± 2.0 %, 48h after photosensitization; 56.2 ± 3.2 %, untreated controls) is consistent with the sensitized induction of a G2/M block.

Similarly, proliferation of human skin fibroblasts (Hs27) was antagonized by UVA/DHP-lysine cotreatment, performed at UVA doses and compound concentrations that did not impair proliferation if delivered as single treatment (Fig. 6.4A-B). Photosensitized inhibition of Hs27 cell proliferation occurred in a similar range of DHP-lysine concentrations and UVA doses as observed with HaCaT keratinocytes.

6.2.3 DHP-lysine as a sensitizer of UVA-induced skin cell death.

Photodynamic induction of skin cell death by UVA/DHP-lysine cotreatment was then examined by flow cytometric analysis of annexinV/PI-stained cells performed 24 h after treatment (Fig. 6.5). Viability of HaCaT keratinocytes exposed to the combined action of high concentrations of DHP-lysine (50 µM) and UVA (9.9 J/cm²) was strongly reduced (Fig. 6.5A). An even more pronounced reduction in cell viability as a result of coexposure to DHP-lysine and UVA was observed in primary human epidermal keratinocytes (HEK) that displayed increased sensitivity to photodynamic induction of cell death as compared to HaCaT keratinocytes (Fig. 6.5D). Cell death of HaCaT keratinocytes occurred independent of caspase-activation since viability was not maintained if photodynamic treatment occurred after pre-exposure to zVADfmk, a potent pan-caspase inhibitor known to block caspase-dependent cell death (Fig. 6.5B) [323]. Interestingly, cell viability was completely unaffected if unirradiated cells were exposed
Figure 6.4. DHP-lysine as a photosensitizer of UVA-induced inhibition of human dermal fibroblast cell proliferation. (A) Human skin fibroblasts (Hs27) were exposed to UVA-irradiation (9.9 J/cm²) in the presence or absence of DHP-lysine (0.5-10 µM). Additionally, mock-irradiated cells were exposed to DHP (10 µM). Cells were washed with PBS, fresh growth medium was added, and cell number was determined 72 h later by cell counting. Proliferation was compared to untreated cells. (B) Hs27 fibroblasts were exposed to increasing doses of UVA-irradiation (up to 4.95 J/cm²) in the presence or absence of DHP-lysine (10 µM). Additionally, mock-irradiated cells were exposed to DHP (10 µM). After treatment, proliferation was assessed as detailed in panel A. For (A) and (B) means with common letter differ (p<0.05).
to DHP-lysine (50 µM) that had been pre-exposed to UVA (9.9 J/cm²) as specified in ‘Materials and methods’ suggesting that photodynamic induction of HaCaT cell death depends on the formation of a short-lived cytotoxic factor that is absent from the pre-irradiated DHP-lysine preparation (Fig. 6.5C).

Similarly, viability of human Hs27 skin fibroblasts was strongly impaired by UVA/DHP-lysine cotreatment performed at high UVA doses and compound concentrations that did not impair viability if delivered as single treatment (Fig. 6.6A). Upon combined exposure to DHP-lysine (50 µM) and UVA (9.9 J/cm²) no viable cell population was detectable by flow cytometry (Fig. 6.6A). Pronounced activation of caspase 3 as evident from flow cytometric detection of proteolytically cleaved procaspase 3 could be detected in Hs27 fibroblasts only in response to exposure to the combined action of DHP-lysine and UVA (Fig. 6.6C). However, as seen with HaCaT keratinocytes, no protection from photodynamic induction of cell death was achieved by pan-caspase inhibition (Fig. 6.6B) suggesting that necrotic cell death occurred independent of caspase activation. Indeed, upon microscopic inspection immediately after irradiation, cell morphology had changed dramatically displaying extensive rounding and vacuolarization consistent with necrosis. As observed with HaCaT keratinocytes, Hs27 cell viability was unaffected if unirradiated cells were exposed to preirradiated DHP-lysine (Fig. 6.6D).
Figure 6.5. DHP-lysine as a photosensitizer of UVA-induced keratinocyte cell death. (A-C) HaCaT keratinocytes were exposed to the combined action of UVA irradiation (9.9 J/cm$^2$) and DHP-lysine (50 µM). (A) Induction of cell death 24 h after exposure was examined by flow cytometric analysis of annexinV-PI stained cells. (B) Photosensitization was performed as in panel A in the presence of zVADfmk. (C) Unirradiated cells were exposed to DHP (50 µM) that was UVA-preirradiated (9.9 J/cm$^2$) and cell viability was examined 24 h after exposure. The numbers summarize results (% viable cells (lower left quadrant) of total gated cells; mean ± SD) from three independent experiments. (D) Primary human epidermal keratinocytes (HEK) were exposed to the combined action of UVA irradiation (9.9 J/cm$^2$) and DHP-lysine (50 µM). Induction of cell death 24 h after exposure was examined by flow cytometric analysis of annexinV-PI stained cells.
Figure 6.6. DHP-lysine as a photosensitizer of UVA-induced fibroblast cell death. Hs27 fibroblasts were exposed to the combined action of UVA irradiation (9.9 J/cm$^2$) and DHP-lysine (50 µM). (A) Induction of cell death 24 h after exposure was examined by flow cytometric analysis of annexinV-PI stained cells. (B) Photosensitization was performed as in panel A in the presence of zVADfmk. (C) Photosensitization was performed as in panel A, and caspase 3 activation was analyzed by flow cytometry using an Alexa 488-conjugated antibody directed against cleaved procaspase 3. (D) Unirradiated cells were exposed to DHP (50 µM) that was UVA-preirradiated (9.9 J/cm$^2$) and cell viability was examined 24 h after exposure. The numbers summarize results (% viable cells (lower left quadrant) of total gated cells; mean ± SD) from three independent experiments.
6.2.4 Skin cell oxidative stress resulting from DHP-lysine photosensitization.

To examine whether the observed cellular photosensitization by DHP-lysine is associated with induction of oxidative stress, generation of intracellular oxidizing species was examined after exposing HaCaT and Hs27 cells to UVA in the presence or absence of DHP-lysine followed by loading with the redox-indicator DCFH-DA (Fig. 6.7A, C) thought to be irreversibly oxidized and converted to the fluorescent dye DCF. Exposure of keratinocytes (Fig. 6.7A) and fibroblasts (Fig. 6.7C) to UVA in the absence of sensitizer induced a small but significant increase (approximately 50%) over baseline fluorescence observed after loading the cells with the redox dye, which is consistent with UVA-induced intracellular oxidative stress [37]. Exposure to DHP-lysine alone did not induce any enhancement of baseline fluorescence. When cells were exposed to the combined action of UVA and DHP-lysine, an up to 4-fold additional increase of DCF-fluorescence intensity was observed. These data demonstrate that photosensitization of skin cells by DHP-lysine enhances intracellular oxidative stress with formation of reactive species of sufficient longevity, such as protein peroxides [257], capable of oxidizing DCFH during cell loading after irradiation. Consistent with DHP-lysine-induced photooxidative stress, lipid peroxidation as assessed by formation of TBARS was elevated three-fold over control levels in photosensitized HaCaT keratinocytes (Fig. 6.7B).

Activation of mitogen activated protein (MAP) kinases by phosphorylation is an established cellular response to photooxidative stress [62, 324], and sensitizer-dependent potentiation of p38 activation by UVA has been used to assess photodynamic
effectiveness of therapeutic and endogenous photosensitizers [94, 325]. Using the combination of UVA and DHP-lysine, we investigated the photosensitized induction of p38-phosphorylation in cultured Hs27 skin cells (Fig. 6.7D). Cells were irradiated with increasing doses of UVA in the absence or presence of DHP-lysine and analyzed for p38-phosphorylation by Western blot analysis of protein cell extracts prepared 30 min after irradiation. In Hs27 fibroblasts, UVA-induction of dual phosphorylation of p38 (Thr180/Tyr182) was greatly potentiated by DHP-lysine at UVA doses ≥ 3.3 J/cm². In addition, upregulation of cellular heme oxygenase I (HO-1) protein levels was examined as another established marker of UVA-associated photooxidative stress in skin fibroblasts [326]. Indeed, DHP-lysine/UVA cotreatment strongly upregulated cellular levels of HO-1 as revealed by Western immunodetection (Fig. 6.7E).

6.2.5 Peptide oxidation resulting from DHP-lysine-photosensitization.

Cellular photosensitization is thought to cause photooxidation of biological macromolecules [257, 327]. Photosensitization of protein damage by DHP-lysine was examined using a ribonuclease A (RNAse A) photo-crosslinking assay. RNAse A was selected as a model target because it does not contain tryptophan residues, thereby excluding effects of this endogenous UV-sensitizer amino acid on photo-crosslinking [94]. When RNAse A (monomer, Mw 13,700 Da) was irradiated with UVA (9.9 J/cm²) in the presence or absence of DHP-lysine (50 µM) covalent protein-dimerization (28,000 Da) was detected using reducing SDS-PAGE analysis (Fig. 6.8A). RNAse-dimerization
Figure 6.7. Induction of oxidative stress in cultured human skin cells resulting from DHP-lysine photosensitization. (A) HaCaT keratinocytes were exposed to UVA-irradiation (9.9 J/cm²) in the presence or absence of DHP (50 µM) followed by loading with the intracellular redox dye DCFH-DA 1 h after irradiation. DCF-fluorescence intensity indicative of intracellular redox stress was then quantified by flow cytometric analysis. One representative histogram out of three similar repeats is shown. In the bar graph, means with common letter differ (n=3, mean ± SD; p<0.05). (B) Lipid peroxidation resulting from DHP-lysine photosensitization was examined in HaCaT keratinocytes exposed as specified in (A) followed by photometric detection of TBARS. (C) Hs27 fibroblasts were exposed and analyzed as specified in (A). (D) Photosensitized induction of p38 MAPkinase phosphorylation by the combined action of DHP-lysine and UVA was assessed in Hs27 fibroblasts. 30 min after photosensitization cells were lysed and analyzed by Western blotting. (E) Upregulation of cellular heme oxygenase-1 protein levels by the combined action of DHP-lysine and UVA was examined in Hs27 fibroblasts 24 h after photosensitization by Western blotting.
occurred with an approximate yield of 5% as measured by gel densitometry (data not shown).

Sensitization of macromolecular damage by DHP-lysine was studied in more detail examining peptide photooxidation by MALDI-TOF mass spectrometry. The peptide melittin (C_{131}H_{229}N_{39}O_{31}, Mw 2845.97, monoisotopic peak), previously used as a model target in studies of peptide oxidation and radiation damage [328, 329], was UVA-irradiated in the presence or absence of DHP-lysine (Fig 6.8B). UVA-irradiation of melittin in the absence of sensitizer did not induce the formation of any reaction products, but irradiation in the presence of DHP-lysine induced the formation of a reaction product in high yields. The detected mass increase of 32 u of the newly formed product [2877.76 u - 2845.80 u, monoisotopic peaks] provided clear evidence for DHP-lysine-sensitized introduction of molecular oxygen into the target peptide that could occur by type I or II photosensitization [257, 329, 330]. Remarkably, DHP-lysine dependent photooxidative modification of melittin was completely suppressed if UVA exposure occurred in the presence of the singlet oxygen quencher NaN₃. Consistent with mechanistic involvement of singlet oxygen in DHP-lysine dependent melittin photooxidation, the yield of photoproduct was increased by approximately 30% (based on mass spectrometric peak intensity) when sensitization was performed in PBS prepared in D₂O, another independent probe for the mechanistic involvement of singlet oxygen (data not shown) [96]. In contrast, hydroxyl radical scavenging using mannitol, iron ion chelation using deferoxamine mesylate, scavenging of H₂O₂ and superoxide radical anion using catalase
Figure 6.8. Induction and antioxidant modulation of protein and peptide photodamage sensitized by DHP-lysine. (A) Photosensitization of protein damage by DHP-lysine was assessed using an RNAse A photo-crosslinking assay. RNAse A (10 mg/mL PBS) was irradiated with UVA (9.9 J/cm²) in the absence or presence of DHP-lysine (50 µM) and a reaction aliquot was analyzed by 15% SDS-PAGE followed by Coomassie-staining [Migration positions of molecular weight standard (Mw), RNAse monomer, and RNAse dimer are indicated]. (B) Mass spectrometric analysis of peptide photooxidation sensitized by DHP-lysine. The peptide melittin (1 mg/mL PBS) was UVA-irradiated (9.9 J/cm² UVA) in the presence or absence of DHP-lysine (10 µM) followed by MALDI-TOF mass spectrometric analysis. In an additional group, exposure to UVA plus DHP-lysine occurred in the presence of NaN₃ (10 mM). Monoisotopic mass peaks are indicated.
or superoxide dismutase, respectively, did not interfere with DHP-lysine sensitization of melittin photooxidation (data not shown).

6.2.6 Photosensitization of ROS formation by DHP-lysine.

Next, direct chemical evidence for UVA-driven production of ROS from DHP-lysine was obtained. First, DHP-lysine-sensitized NBT reduction was examined as a function of DHP-lysine concentration (3.3 J/cm² UVA; Fig. 6.9A) or dose UVA (100 µM DHP; Fig.6.9B). In addition, the SOD-suppressible portion of photoreductively generated nitroblue diformazan (NBF) was determined serving as an established indicator of superoxide anion formation (Fig. 6.9B) [96]. At the highest UVA dose (3.3 J/cm²) NBT photoreduction by DHP-lysine occurred with approximately equimolar stoichiometry, e.g. UVA exposure of 80 µM DHP-lysine generated approximately 90 µM NBF (Fig.6.9A). Photoreductive generation of NBF was strongly inhibited in the presence of SOD, with more than 60% of NBF formation (100 µM DHP; 3.3 J/cm² UVA) blocked in the presence of SOD (15,000 u/mL).

Next, the dose response relationship of photosensitized H₂O₂ production was determined as a function of DHP-lysine concentration (Fig. 6.9C) and UVA dose (Fig. 6.9D). Significant production of H₂O₂ yielding concentrations up to 40 µM was observed only at high concentrations of DHP-lysine (50-800 µM) exposed to UVA (9.9 J/cm²) (Fig. 6.9C). At the highest concentration DHP (800 µM) significant production of H₂O₂ was observed at UVA doses ≥ 3.3 J/cm² (Fig. 6.9D).
Fig. 6.9. DHP-lysine-sensitized production of ROS (continued on next page).
Figure 6.9. DHP-lysine-sensitized production of ROS (cont.). Photosensitization of ROS formation upon exposure to UVA was examined over a wide concentration range of DHP-lysine (2.5 up to 800 µM). (A) DHP-lysine-sensitized NBT reduction measured by NBF generation as a function of DHP-lysine concentration (3.3 J/cm² UVA). (B) SOD-suppressible NBF formation indicative of superoxide formation was assessed by exposing DHP-lysine (100 µM) to increasing doses of UVA in the presence or absence of SOD (15000 u/mL). (C) Dose response of H₂O₂ production as a function of DHP-lysine concentration (9.9 J/cm² UVA). (D) Dose response of H₂O₂ production as a function of UVA dose (800 µM DHP-lysine). (E) Singlet oxygen formation as evidenced by the RNO bleaching assay. Loss of RNO absorbance resulting from photosensitization by Rose Bengal (RB, 1 µM) and DHP-lysine (20 µM) was examined as a function of UVA dose in the absence or presence of NaN₃ (10 mM). For all graphs (A-E), values represent the mean of three independent experiments ± SD.
Generation of singlet oxygen during DHP-lysine photosensitization was examined using the RNO bleaching assay as described earlier (Fig. 6.9E) [247]. Rose Bengal, an established photosensitizer for the efficient photodynamic production of singlet oxygen upon exposure to UVA and visible light, was employed as a positive control. Inhibition of RNO bleaching by the singlet oxygen quencher NaN₃ was assessed in order to substantiate involvement of singlet oxygen in DHP-lysine/UVA-induced RNO bleaching. RNO bleaching by DHP-lysine photosensitization occurred as a function of DHP-lysine concentration (data not shown) and UVA dose (3.3 to 9.9 J/cm²) and was strongly suppressed by inclusion of NaN₃ (Fig. 6.9D). The UVA dose response of RNO bleaching resulting from photosensitization by Rose Bengal (1 µM) was observed at a twenty fold higher concentration of DHP-lysine (20 µM), indicating an approximately twenty fold difference in the relative sensitizer potency of UVA-driven singlet oxygen production displayed by these two test compounds under the conditions employed.

6.2.7 Antioxidant suppression of DHP-lysine phototoxicity.

After demonstrating UVA-induced phototoxicity of DHP-lysine associated with induction of oxidative stress in cultured human skin cells and chemical detection of ROS, feasibility of antioxidant protection against DHP-lysine phototoxicity was examined in Hs27 skin fibroblasts (Fig. 6.10). First, photodynamic induction of cell death was examined in the absence or presence of various antioxidants (Fig. 6.10A). Among various antioxidant agents tested, complete cytoprotection against photosensitized induction of cell death was observed when UVA exposure occurred in the presence of
NaN₃. In contrast, no protection was observed upon cotreatment with catalase or SOD (data not shown). Similarly, inhibition of proliferation induced by combined treatment with UVA and DHP-lysine was strongly antagonized if photosensitization occurred in the presence of NaN₃, but catalase and NAC were not effective in protecting Hs27 cells (‘co-irradiation procedure’, Fig. 6.10B). However, when unirradiated cells were exposed to a high concentration of preirradiated DHP-lysine (50 µM; 9.9 J/cm² UVA; cells exposed immediately after UVA; ‘pre-irradiation exposure’), left on cells right after irradiation as detailed in ‘materials and methods’, significant inhibition of proliferation was observed. This antiproliferative effect could be antagonized by performing cellular exposure to preirradiated DHP-lysine in the presence of catalase or NAC, but not in the presence of NaN₃ (Fig. 6.10C).

Taken together with the results obtained from chemical ROS assays (Fig. 7), these data strongly suggest that short-lived singlet oxygen is the dominant ROS that mediates cytotoxic effects of DHP-lysine if UVA-exposure occurs in the direct presence of target cells. However, if DHP-lysine photoactivation occurs in the absence of target cells, singlet oxygen decay occurs before cells are exposed to the preirradiated material and only the chemically stable ROS H₂O₂ formed with low efficiency from UVA-exposed DHP-lysine (Fig. 6.9C-D) is capable of inducing antiproliferative effects in unirradiated target cells.
Figure 6.10. Antioxidant protection of human skin fibroblasts against DHP-lysine phototoxicity. (A) Human Hs27 fibroblasts were exposed to the combined action (‘co-irradiation exposure’) of UVA-irradiation (9.9 J/cm²) and DHP-lysine (50 µM) in the presence or absence of various antioxidants including NaN₃ (10 mM) and catalase (Cat, 400 u/mL). Viability was examined 24 h after exposure by flow cytometric analysis of annexinV-FITC/PI-stained cells. (B) Cells were exposed to the combined action of UVA-irradiation (4.95 J/cm²) and DHP-lysine (10 µM) in the presence or absence of various antioxidants including NaN₃ (10 mM), NAC (10 mM), and catalase (Cat, 400 u/mL). Proliferation was examined 72 h after exposure as specified in Materials and Methods. (C) DHP-lysine (10 µM) was exposed to UVA (9.9 J/cm²) (‘pre-irradiation exposure’) and then immediately added to un-irradiated cells in the presence or absence of various antioxidants (30 min exposure, followed by media change), and proliferation was examined 72 h later.
6.3 Discussion

UVA-sensitization by cutaneous chromophores is an important mechanism of skin cell photooxidative stress contributing to photoaging and carcinogenesis [37]. Here we demonstrate that the lipid peroxidation-derived protein epitope DHP-lysine is contained in human skin and present experimental evidence that DHP-lysine functions as a UVA-photosensitizer of cultured human skin cells.

Skin cell photosensitization by DHP-lysine resulted in dose dependent inhibition of proliferation, cell cycle arrest, and induction of cell death (Figs. 6.3 – 6.4). A causative involvement of oxidative mechanisms in skin cell phototoxicity of DHP-lysine was supported by detecting the sensitized formation of cellular peroxides and lipid peroxidation products (TBARS) (Fig. 6.7A-C). Moreover, UVA-induced upregulation of HO-1 expression and p38 MAPkinase-dependent stress signaling, established markers of photooxidative stress, were observed in skin fibroblasts (Fig. 6.7D-E) [62, 324, 331, 332]. Chemical analysis revealed the light-driven formation of singlet oxygen, superoxide anion radicals, and hydrogen peroxide resulting from UVA exposure of DHP-lysine (Fig. 6.9).

Formation of singlet oxygen was the dominant causative factor underlying skin cell phototoxicity of DHP-lysine as suggested by (I) the dependence of photodynamic efficacy on irradiation of cells in the direct presence of the sensitizer (Fig. 6.5C and 6.6D), (II) protective efficacy of the singlet oxygen quencher NaN₃, not observed with other antioxidants including catalase and NAC (Fig. 6.10A), and (III) indirect chemical detection of singlet oxygen by determination of NaN₃-suppressible RNO bleaching that
occurred at low concentrations of DHP-lysine (Fig. 6.9E). Moreover, DHP-lysine dependent melittin photooxidation was blocked by NaN₃ and enhanced by performing UVA exposure in D₂O (Fig. 6.8B). However, the mechanistic basis of DHP-lysine phototoxicity remains incompletely understood at this point, and the complex photochemistry involving type I and type II photosensitization mechanisms awaits further photochemical investigation. Future experiments will also examine the possibility that apart from UVA blue visible light may photoactivate DHP-lysine as suggested by its fluorescence excitation spectrum (Fig. 6.1C). Singlet oxygen-dependent modification of specific target proteins of cellular photosensitization has been described earlier [269, 333]. Although photosensitization of protein and peptide damage was demonstrated in simple chemical model systems in our experiments (Fig. 6.8), no specific protein targets involved in skin cell photosensitization by DHP-lysine have been identified at this point.

It is well established that lipid peroxidation-derived protein epitopes can originate from lysine adduction by reactive intermediates including MDA, 4-hydroxynonenal (4-HNE), and glyoxal that accumulate under conditions of oxidative stress in numerous damaged human tissues such as atherosclerotic lesions [245, 318, 334, 335]. Consistent with the causative involvement of photooxidative stress in macromolecular chemical damage during skin photoaging and carcinogenesis [37, 147, 336, 337], acrolein-, glyoxal, and 4-HNE-derived epitopes have been detected in photoaged skin [335, 338]. Cutaneous abundance in MDA-epitopes of unresolved chemical structure has been observed earlier with pathological accumulation of these epitopes in superficially spreading melanoma and nonmelanoma skin cancer [246].
Our immunohistochemical analysis demonstrates for the first time the abundant occurrence of DHP-lysine epitopes on unknown target proteins in healthy human epidermis and dermis (Fig. 6.2). In this prototype IHC study using an age-grouped human skin TMA with cutaneous specimens of unspecified anatomical location, no attempt was made to correlate epitope abundance with chronological age or molecular markers of photoaging expressed in the skin specimen, an analysis to be performed and published elsewhere. Future experimentation will also involve identification of specific cutaneous intra- and extracellular target proteins modified by DHP-lysine, since earlier work has already demonstrated that chemical posttranslational modification of skin proteins through oxidation, glycoxidation, and amino-carbonyl reactions targets specific proteins including keratin [336], elastin [338], collagen [339], and vimentin [340]. It will also be interesting to compare photodynamic activity of free DHP-lysine as performed in this study with DHP-lysine covalently incorporated into a defined peptide or protein structure.

DHP-lysine is an endogenous fluorophore associated with intracellular lipofuscin, a fluorescent age pigment known to accumulate in human fibroblasts under conditions of chronic oxidative stress [272, 319]. Together with earlier findings that demonstrate detrimental effects of lipofuscin accumulation on cellular lysosomal and proteasomal function and redox homeostasis [148, 159], our studies suggest a novel mechanistic role of lipofuscin-associated fluorophores as phototoxic mediators of skin photodamage as previously described for the lipofuscin-associated blue light fluorophore A2E involved in photooxidative deterioration of retinal pigment epithelial cells during progressive
macular degeneration [322]. Future mechanistic studies will therefore test the hypothesis that MDA-derived DHP-lysine epitopes in skin may play a similar role as sensitizers of UVA-induced cutaneous oxidative stress. Given the established role of photosensitization reactions in the initiation of cellular lipid peroxidation reactions [63], and based on TBAR formation in DHP-lysine/UVA exposed keratinocytes (Fig. 6.7B), it is also tempting to speculate that UVA-activated MDA-derived protein-epitopes may cause further lipid peroxidation and cutaneous accumulation of additional sensitizing epitopes, a vicious cycle of skin photooxidative stress to be substantiated by future experiments.
CHAPTER 7: SUMMARY OF STUDIES, SIGNIFICANCE, AND FUTURE DIRECTIONS

7.1 Summary of Studies

7.1.1 Identification of cathepsin B as a novel UVA target

The initial hypothesis of this dissertation states that chronic low level UVA exposure induces skin photodamage through alteration of specific molecular targets causatively involved in skin aging and carcinogenesis. 2D-DIGE is a powerful analytical tool for the unbiased identification of protein targets that display altered expression levels. To this end, we used a proteomic approach that involved 2D-DIGE analysis followed by mass spectrometric identification in order to identify prospective novel target proteins that display a pronounced expression differential in human dermal fibroblasts exposed to chronic UVA irradiation. The heavy chain (24 kDa) of the mature double chain form of cathepsin B was identified as one of the proteins displaying significant down-regulation in response to chronic UVA exposure. This loss of the 24kDa fragment was accompanied by an accumulation of the 29 kDa single chain form of the protein as detected by immunoblot analysis.

Our data indicate for the first time that chronic UVA exposure causes alteration of cathepsin B maturation and dramatic loss of cathepsin B specific enzymatic activity in human dermal fibroblasts. In additional to dermal fibroblasts, epidermal melanocytes and immortalized HaCaT keratinocytes also displayed sensitivity to UVA in the context
of cathepsin B inhibition, while UVA-induced cathepsin B inhibition was not observed in primary epidermal keratinocytes.

7.1.2 UVA induces oxidative stress: implications for cathepsin B and L enzymatic inactivation

The observed cathepsin B protein changes and loss of enzymatic activity occurred in the absence of transcriptional modulation of genes encoding either cathepsin B itself (CTSB) or established peptide inhibitors of cathepsin B including cystatin A (CSTA), cystatin B (CSTB), cystatin C (CSTC), and cystatin 6 (cystatin M/E; CST6) [259, 260].

Our experiments indicate a causative involvement of photooxidative mechanisms in UVA-induced cathepsin B alterations as evidenced by detection of UVA-dependent ROS formation, oxidative stress response gene expression profiling, and antioxidant suppression of cathepsin B inactivation. Western blot analysis of immunoprecipitated cathepsin B protein from Hs27 fibroblasts exposed to chronic UVA indicates that UVA treatment results in 4-HNE modification of cathepsin B, consistent with earlier reports that demonstrate inhibition of enzymatic activity resulting from 4-HNE adduction [264, 265].

7.1.3 UVA-induced lysosomal and proteasome changes

Here we show for the first time that chronic UVA causes a lysosomal blockade in human skin fibroblasts as evidenced by the accumulation of lysosomal lipofuscin, increased cellular levels of autophagic substrates such as p62, and lysosomal expansion substantiated by increased Lamp-1 protein levels.

In contrast to the observed blockade in lysosomal clearance, enhanced proteasome
activity with significant upregulation chymotrypsin-, trypsin- and caspase-like activities was observed in Hs27 fibroblasts exposed to chronic UVA. This indicates that there is not a complete blockade of cellular recycling mechanisms, but rather a specific inhibition of lysosomal-dependent digestion. Of note, skin dermal fibroblasts exposed to the cathepsin B/L inhibitor CA074Me also showed an increase in all three activities of the proteasome. The mechanism underlying upregulated proteasome activity and whether it is a compensatory mechanism for decreased lysosomal proteolytic capacity remains to be elucidated. Importantly, crosstalk between the autophagic-lysosomal and proteasome systems has been suggested [341, 342].

7.1.4 Cathepsins B and L as upstream UVA targets causing lysosomal blockade

Consistent with a causative involvement of UVA-induced impairment of cathepsin B structure and function in the accumulation of lipofuscin, treatment with the pharmacological cathepsin B/L inhibitor CA074Me mimicked UVA effects on cellular phenotype and gene expression. However, CA074Me is a pharmacological probe of limited stringency due to its inhibition of both cathepsin B and L activity. In order to elucidate the relative involvement of cathepsins B and L in the observed UVA-induced lysosomal dysfunction, we used a genetic approach (siRNA) to selectively downregulate enzymatic activity of these target cathepsins. We observed that only dual genetic antagonism (targeting both CTSB and CTSL expression) could mimic the UVA-induced cellular phenotype and autophagic-lysosomal related protein changes, whereas single knockdown (targeting CTSB or CTSL only) did not reproduce the UVA-induced phenotype. Indeed protein changes and lipofuscin accumulation that mimicked UVA-
induced changes were observed only in fibroblasts after CTSB and CTSL-double knockdown, but not in fibroblasts after knockdown of only CTSB or CTSL.

### 7.2 Significance and Future Studies

Taken together, our data demonstrate for the first time that chronic exposure to UVA impairs lysosomal function in human dermal fibroblasts. Moreover, we provide compelling evidence that inhibition of cathepsin B and L enzymatic activity is a heretofore unrecognized causative factor in cutaneous UVA photodamage operating upstream of autophagic-lysosomal alterations as illustrated in Fig. 7.1. This model proposes that inactivation of cathepsin B and L as a consequence of UVA-induced photooxidative stress causes dynamic changes that are most consistent with a blockade of lysosomal clearance. Of note, our studies indicate that antioxidant intervention can at least partially rescue UVA-induced cathepsin B/L enzymatic inactivation, providing evidence that protection from UVA-induced cathepsin B/L inhibition might represent a novel strategy for cellular photoprotection.

In the specific context of photoaging, our data provide evidence for a heretofore unrecognized ‘double-hit’ mechanism of UVA skin photodamage where primary photooxidative insult occurs simultaneously with impaired clearance of damaged molecules and organelles downstream of dual inactivation of cathepsin B and L (Fig. 7.1). This is in agreement with prior studies demonstrating massive accumulation of lipofuscin in dermal fibroblasts cultured under oxidative conditions in the presence of cysteine protease inhibitors, while only moderate lipofuscin accumulation was observed in
Figure 7.1. UVA-photodamage through a ‘double-hit’ mechanism. In addition to the established role of UVA as a source of cellular oxidative stress leading to damage of biomolecules, chronic UVA causes skin photodamage through impaired clearance of damaged molecules and organelles downstream of dual inactivation of cathepsin B and L, representing a novel ‘double-hit’ mechanism of UVA-induced photodamage. Under normal cellular conditions, biomolecules are brought to the lysosome (for instance, during macroautophagy as illustrated) where degradation and recycling occurs. However, upon exposure to chronic UVA, the lysosomal degradation pathway is impaired as a result in cathepsin B/L enzymatic inhibition. This results in accumulation of undigested lysosomal substrates and lipofuscin. Furthermore, UVA-induced formation of lipofuscin may have a functional role as a photosensitizer, thereby enhancing oxidative stress through a feedback loop, leading to a viscous cycle of cellular photo-oxidative stress (indicated by red arrow).
fibroblasts cultured under oxidative conditions or cysteine protease inhibitors alone [141]. Our findings may be of particular significance to cutaneous photobiology given the emerging role of several cathepsins in lysosomal removal of damaged and AGE-modified cellular proteins observed in dermal fibroblasts. Recent studies have identified cathepsins B, D, and L all as having the capability to degrade methylglyoxyl- and glyoxyl-modified albumin, while the proteasome completely lacked ability to degrade the AGE-modified albumin [297, 343, 344].

The lysosome is an integral part of several vital cellular housekeeping processes including all autophagic pathways (microautophagy, macroautophagy, and chaperone-mediated autophagy), growth factor receptor recycling, growth factor degradation, antigen processing and presentation, and programmed cell death [125-127]. Given the breadth of lysosomal-dependent cellular functions, the specific molecular consequences associated with UVA-induced lysosomal dysfunction downstream of cathepsin B inactivation remain to be elucidated. Our prototype follow-up studies that addressed the emerging role of UVA-induced cathepsin B/L inactivation in lysosomal clearance, endo-lysosomal dependent viral infection, and lipofuscin-dependent photosensitization (Chapter 5, Fig. 5.10) do in fact implicate this phenomenon in many cellular functions that may extend even beyond photoaging and photocarcinogenesis as discussed in the following sections. Indeed, depending on the cell type, the functional outcome of UVA-induced cathepsin B/L inhibition may vary significantly.

The studies presented in this dissertation were initiated by exploratory proteomic experimentation in an effort to identify novel molecular mechanisms underlying
cutaneous photodamage. Our findings have unveiled several new potential areas for scientific exploration with regard to UVA-induced cutaneous photodamage. The following future research objectives would provide further mechanistic insight into the emerging role of cathepsin B and L as a novel targets in skin photodamage and lysosomal dysfunction, and further elucidate the functional implications of these changes for skin photobiology.

7.2.1 **Confirm the specific mechanism underlying UVA-induced cathepsin B/L enzymatic inactivation.**

Evidence points to a photo-oxidative mechanism of UVA-induced cathepsin B and L inactivation. Further experimentation should therefore be performed aiming at unequivocally determining the detailed mechanism underlying UVA-induced alteration of cathepsin B and L function. Western blot analysis revealed increased levels of 4-HNE modified single chain and double chain cathepsin B in skin fibroblasts exposed to chronic UVA. Initial attempts in our laboratory to identify the specific oxidative post-translational modification leading to cathepsin B inactivation by mass-spectrometric analysis were unsuccessful. Further experimentation involving more rigorous mass spectrometric analysis of immunoprecipitated cathepsin B isolated from fibroblasts after chronic UVA exposure would therefore explicitly determine the chemical nature and exact residue(s) of post-translational modification of cathepsin B.

7.2.2 **Further validate UVA-induced cathepsin B/L inhibition.**

7.2.2A **Further substantiation of cathepsin B and L as UVA targets**
Rescue experiments involving transfection with *CTSB* and/or *CTSL* expression vectors following chronic UVA treatment would provide conclusive evidence that the phenotypic changes observed in response to chronic UVA are in fact due to inhibition of cathepsin B and L. Our studies reported here were conducted in cultured skin cells. Further experimentation should be performed that investigates the occurrence of these changes in whole skin tissue. To this end, studies to compare cathepsin B/L protein level and enzymatic activity, as well as autophagic substrate levels and lipofuscin accumulation in photoaged skin versus photo-protected skin could be conducted to determine UVA-induced cathepsin B/L changes in human skin. Similarly, organotypic skin constructs could be used for a more controlled exposure regimen.

### 7.2.2B Potential involvement of other cysteine cathepsins

Our data indicate that inhibition of cathepsin B and cathepsin L is sufficient to cause the phenotypic changes observed in skin fibroblasts exposed to chronic UVA exposure. Importantly, there are nine other cysteine cathepsins and it is quite reasonable that these cathepsins are also affected by chronic UVA exposure, whether it is due to direct photo-oxidation of the enzyme or through a more indirect mechanism that may involve impaired maturation/processing. Cathepsin K is expressed in dermal fibroblasts and keratinocytes and has been shown to have a pivotal role in collagen remodeling and wound repair [186, 219]. Indeed, an exploratory experiment performed in our laboratory indicates that cathepsin K activity is indeed inactivated by ~ 40% in response to chronic UVA [Appendix A (Fig. A1); ‘1 week’ regimen; 61.9 % ± 4.1 residual protease activity after ‘1 week’ UVA regimen (n=3; mean ± SD; p < 0.05)]. A comprehensive assessment
of cysteine cathepsin expression and their sensitivity to chronic UVA in skin cell types (keratinocytes, melanocytes and Langerhans cells) would provide a strong basis for further studies elucidating UVA-induced cathepsin alterations and their role in skin photodamage.

While cathepsins have been traditionally thought of as enzymes involved in bulk proteolysis, recent evidence suggests specific roles of many cathepsins in the context of cutaneous homeostasis and immune function [185, 186, 200, 345]. Therefore, in addition to impaired lysosomal clearance, many other cellular processes may be affected by UVA impairment of specific cathepsins, some of which are discussed in the following paragraphs.

7.2.3 Explore the role of UVA-induced lysosomal-autophagic dysfunction in skin biology.

7.2.3A Mechanistic involvement of lipofuscin accumulation

Our data indicate that UVA exposure not only causes cellular oxidative damage, but also compromises the lysosomal clearance of these damaged biomolecules through the inactivation of cathepsins, ultimately leading to increased cellular levels of lipofuscin. Earlier studies have shown that dermal fibroblasts loaded with lipofuscin show increased cellular levels of ROS [159] and susceptibility to acute oxidative stress [160]. Most importantly, lipofuscin has been shown to act as a photosensitizer; retinal pigment epithelial cells loaded with lipofuscin demonstrate increased ROS levels and decreased viability when exposed to visible light [298, 299]. Indeed, an exploratory experiment conducted in our hands indicates that UVA-induced cellular production of ROS is much
greater if cells are cultured in the presence of a cathepsin B/L inhibitor for several days prior to irradiation to allow build up of lipofuscin (Chapter 5, Fig 5.10E). Similarly, data from Chapter 6 indicates the functional role of DHP-lysine, one of the only known lipofuscin-associated fluorophores [245, 319], as a photosensitizer.

These combined data provide sufficient indication that UVA-induced formation of lipofuscin may have a functional implication where oxidative stress is enhanced through a feedback loop, leading to a viscous cycle of cellular photo-oxidative stress (illustrated in Fig. 7.1). This may explain, at least in part, why there is an observed increase in cellular ROS levels upon successive UVA exposures (Fig. 4.2B). Further studies should be conducted to (I) measure the accumulation of DHP-lysine epitopes in response to chronic UVA, and (II) further elucidate the functional role of lipofuscin as a photosensitizer.

**7.2.3B Consequence of p62 accumulation**

It is plausible that the resulting accumulation of endogenous p62 observed in chronically exposed UVA skin fibroblasts has functional significance with regard to carcinogenesis. Recent evidence indicates the importance of p62 dependent signaling in carcinogenesis and tumorigenesis, particularly through activation of NF-κB, with functional implications for the evasion of apoptosis [302, 346, 347]. Future experiments should therefore aim at studying the significance of UVA-induced p62 accumulation resulting from inefficient autophagic-lysosomal clearance. Specifically, experiments could be performed to measure NF-κB activation and transcription of downstream genes, including the anti-apoptotic target genes such as Bcl-xL. As dermal fibroblasts rarely
undergo carcinogenic events, this may be of particular relevance in keratinocytes and melanocytes, and experiments should therefore focus on these cell types. Lastly, as p62 has oncogenic properties and has been shown to be important in Ras-induced tumorigenesis [346], the accumulation of p62 may have a particular significance in promoting the clonal expansion of initiated skin cells leading to NMSC and melanoma.

7.2.3C Immunological consequences

The expression of many of the cathepsins has been documented in dendritic cells, one of the major antigen presenting cells [348]. Indeed, it has been shown that cathepsin K is essential for TLR-9 dependent signaling in dentritic cells [349] and cathepsins S and L are thought to be the key cathepsins involved in the processing of the MHC Class II invariant chain [345, 350]. However, the expression pattern of the cathepsins has not been investigated in Langerhans cells, specialized resident dendritic cells of the skin. Therefore UVA-induced inhibition of Langerhans cell cathepsin(s) represents a potential contributing factor in photo-immunosuppression. A series of experiments could be performed to (I) assess the expression pattern of the cathepsins in Langerhans cells (II) evaluate the susceptibility of these cells to UVA in the context of cathepsin inhibition and (III) measure the functional outcome with regards to UVA-induced effects on antigen presentation to T cells.

7.2.3D Effects on viral infectivity

Numerous reports have documented that many of the cathepsins are important for many steps involved in infectivity including binding, entry, and disassembly of many human viruses including HPV16 [288], Ebola virus [286] and influenza A [351].
Exploratory experimentation from our lab indicates that UVA-induced cathepsin B/L inhibition leads decreased HPV16 infectivity of Hs27 fibroblasts and HaCaT keratinocytes (Fig. 5.10C-D). Future research could focus on the effect of UVA on infectivity of other skin viruses. Perhaps of more physiological relevance, though, these data support the feasibility of assessing cathepsin inhibitors for the chemotherapeutic intervention targeting viral infection.

7.2.4 Explore the functional implication of UVA-induced inactivation of the tumor suppressor cathepsin L and lysosomal-autophagic dysfunction in photocarcinogenesis.

Several studies have indicated the importance of autophagy for cellular maintenance, and dysregulation of the autophagic process has been linked to numerous diseases including neurodegenerative disorders and cancer [352]. Besides the accumulation of p62 as discussed in 7.2.3B, UVA-induced inhibition of cathepsin L enzymatic activity may have functional outcomes with regard to carcinogenesis. Several studies have collectively defined cathepsin L as a tumor suppressor due to its essential role in the recycling of EGF [200-202]. It is therefore tempting to speculate that UVA-induced cathepsin L-inhibition may result in increased EGF levels and sustained EGF signaling, a hypothesis to be substantiated by future experimentation.

In addition, many of the cathepsins play an essential role in ECM degradation both intra-cellularly as well as in the extra-cellular environment [219, 220]. Indeed, studies have shown that cathepsin B can degrade many ECM proteins with the same if
not better efficiency than MMPs [222, 223]. This may have significance with regard to tumor invasion. Experiments investigating the effects chronic UVA exposure has on the ECM-degradative ability of cutaneous cells would provide further insight into the possible mechanistic involvement of UVA-induced cathepsin B/L inactivation in tumor invasion.
APPENDIX A:

UVA-INDUCED INHIBITION OF CATHEPSIN K ACTIVITY

A.1 Introduction and Rationale

Our studies have demonstrated that cathepsin B and cathepsin L, and to lesser extent, cathepsin D enzymatic activities are affected by chronic UVA exposure in human skin fibroblasts. The cathepsins are a large family of proteases, and the ability to determine the sensitivity of all of the 15 cathepsins to chronic UVA was beyond the scope of this dissertation. The purpose of this exploratory study was to determine the sensitivity of cathepsin K, a cutaneously expressed cysteine-dependent cathepsin that has been shown to have a pivotal role in collagen remodeling and wound repair [186, 219].

A.2 Materials and Methods

A2.1 Cell Culture and UVA irradiations. Dermal neonatal foreskin Hs27 fibroblasts from ATCC (Manassas, VA) were cultured in DMEM containing 10% bovine calf serum (BCS). Cells were maintained at 37°C in 5% CO₂, 95% air in a humidified chamber. UVA irradiations were performed according to the ‘1 week’ regimen as described in Chapter, section 2.3.1.

A2.2 Measurement of cathepsin K enzymatic activity. Cathepsin K activity was measured using the fluorometric cathepsin K assay kit from BioVision, Inc. (Milpitas, CA) according to manufacturer’s instructions. Cells (1x10⁵) were lysed in 0.5 mL of chilled lysis buffer. After 10 min incubation on ice, lysates were centrifuged at 10,000 g
at 4 °C for 5 min and supernatant was retained for analysis. 50 μL of cell lysate was incubated with 50 μL of reaction buffer and Ca074 (1 μM final concentration) for 15 min at ambient temperature. Ca074 is added to irreversibly inhibit cathepsin B in order to eliminate interference from cathepsin B cleavage of the substrate. Cathepsin K substrate was added (Ac-Lys-Arg-AFC; 200 μM final concentration; 1 h at 37 °C) and the release of free amino-4-trifluoromethylcoumarin (AFC) was measured using a fluorescence plate reader (λex 400, λem 505; SpectraMax Gemini, Molecular Devices, Sunnyvale, CA). Additionally, protein concentration of cell lysates was determined using the BCA assay (detailed in 2.10) and cathepsin K activity was normalized to protein concentration per sample.

A.3 Results

A3.1 Chronic UVA inhibits cathepsin K enzymatic activity

Cathepsin K activity was measured in human skin fibroblasts exposed to chronic UVA (‘1 week’ regimen) one hour after the final irradiation. Our results indicate that cathepsin K activity is inactivated by ~ 40% in response to chronic UVA [Fig. A.1; 61.9 % ± 4.1 residual protease activity after ‘1 week’ UVA regimen (n=3; mean ± SD; p < 0.05)].

A.4 Discussion

Our data indicate that inhibition of cathepsin B and cathepsin L is sufficient to cause the phenotypic changes observed in skin fibroblasts exposed to chronic UVA
exposure. Importantly, there are nine other cysteine cathepsins, and we therefore wanted to explore the possibility that other cysteine cathepsins are also affected by chronic UVA exposure. Indeed, cathepsin K activity is significantly diminished in response to chronic UVA (Fig. A.1). This exploratory study demonstrates the plausibility that several of the cathepsins are affected by chronic UVA exposure. A comprehensive study examining the cutaneous expression and sensitivity to UVA of all cathepsins would provide a solid basis for studying the functional implications of UVA-induced cathepsin inactivation.
Figure A.1. Cathepsin K activity of human skin fibroblasts exposed to chronic UVA. UVA-induced changes in cathepsin K enzymatic activity was assessed in human dermal fibroblasts exposed to chronic UVA (‘1 week’ UVA regimen; n=3, mean ± SD).
APPENDIX B:

CELLULAR ZINC IMBALANCE AND STRESS RESPONSE OF HUMAN SKIN CELLS AND ORGANOTYPIC SKIN EQUIVALENT MODELS EXPOSED TO ZINC PYRITHIONE

This chapter has been adapted from the following publications:


ABSTRACT. Zinc pyrithione (ZnPT) is an FDA-approved microbicidal agent used worldwide in clinical antiseptic products, over-the-counter (OTC) topical antimicrobials, and cosmetic consumer products including anti-dandruff shampoos. This study demonstrates that cultured primary human skin keratinocytes and melanocytes display an exquisite sensitivity to nanomolar ZnPT concentrations causing induction of heat shock response gene expression, impaired genomic integrity, and poly(ADP-ribose) polymerase (PARP)-dependent cell death. Rapid accumulation of intracellular zinc in primary keratinocytes exposed to ZnPT was observed by quantitative fluorescence microscopy and inductively coupled plasma mass spectrometry (ICP-MS), and PARP activation,
energy crisis, and genomic impairment are all antagonized by zinc chelation. In epidermal reconstructs (EpiDerm™) exposed to topical ZnPT (0.1 to 2% in Vanicream™), ICP-MS analysis revealed rapid zinc accumulation, and expression array analysis demonstrated upregulation of stress response genes encoding metallothionein-2A (MT2A), heat shock proteins (HSPA6, HSPA1A, HSPB5, HSPA1L, DNAJA1, HSPH1, HSPD1, HSPE1), antioxidants (SOD2, GSTM3, HMOX1), and the cell cycle inhibitor p21 (CDKN1A). IHC analysis of ZnPT-treated EpiDerm™ confirmed upregulation of Hsp70 and TUNEL-positivity. Taken together our data demonstrate that ZnPT impairs zinc ion homeostasis and upregulates stress response gene expression in primary keratinocytes and reconstructed human epidermis, activities that may underlie therapeutic and toxicological effects of this topical drug.

**Abbreviations**

3-ABA, 3-aminobenzamide; AV, annexinV; FITC, fluorescein isothiocyanate; DTPA, diethylenetriaminepentaacetic acid; EGR1, early growth response protein I; FPG, formamidopyrimidine-glycosylase; HEK, human epidermal keratinocyte; HEMa, human epidermal melanocyte; HO-1, heme oxygenase 1; HSP, heat shock protein; ICP-MS, inductively coupled plasma mass spectrometry; IHC, immunohistochemistry; MEF, mouse embryonal fibroblast; NAC, N-acetyl-L-cysteine; OTC, over-the-counter; PAR, poly(ADP-ribose) polymer; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine; TUNEL, terminal dUTP nick end labeling; ZnPT, zinc pyrithione.
B.1 Introduction and Rationale

Much research has focused on the identification and development of drug-like molecules for the prevention of skin damage from environmental insult, particularly photodamage from solar UV radiation [37, 353-356]. Research from various groups including our own has shown that small molecule inducers of the heat shock and nuclear factor-E2-related factor 2 (Nrf2) oxidative stress response pathways exert chemopreventive activity with suppression of skin cell photodamage, photooxidative stress, and photocarcinogenesis [357-359].

Zinc is an essential trace element with important cellular functions dependent on its activity as metal-cofactor of various enzymes and structural component of transcription factors and other zinc finger-containing DNA binding proteins [360, 361]. Zinc ion homeostasis plays an important role in cutaneous biology where it modulates epidermal barrier function [362], epithelial wound healing and tissue regeneration [363], and inflammatory and antimicrobial regulation [364].

Zinc is a prototype inducer of cytoprotective mechanisms including signaling through Nrf2 and upregulation of metallothionein and heat shock protein expression, all of which have been observed upon exposure to inorganic zinc salts (e.g. ZnSO₄) in the submillimolar (100-200 µM) concentration range [365-367]. Zinc-based compounds designed for topical delivery represent an important class of cutaneous therapeutics designed for photoprotective, antimicrobial, and anti-inflammatory intervention [368-370]. In an attempt to explore the potential cytoprotective activity of topical Zn compounds for skin protection against environmental insults this study focused on zinc
pyrithione (ZnPT; CAS# 13463-41-7), a 1:2 complex between a central zinc atom and the membrane permeable ionophore pyrithione (N-hydroxy-2-pyridinethione) [371].

ZnPT is an FDA-approved microbicidal agent used worldwide in clinical antiseptic products, over-the-counter (OTC) topical antimicrobials, and cosmetic consumer products including anti-dandruff shampoos where typical ZnPT concentrations are in the range of 1 to 2 % (w/v) [368, 372, 373]. Epidermal deposition and retention of the lipophilic metal chelate ZnPT following topical application has been demonstrated in human skin [374, 375], and percutaneous penetration of ZnPT through rat, rabbit, guinea pig, and rhesus monkey skin has been documented earlier [368, 376-378]. Topical safety and toxicity profile of this OTC-drug have been studied to some extent previously, and it has been established that ZnPT does not display a potential for primary irritation or sensitization of human skin [379, 380]. In the context of cutaneous pathology beyond antimicrobial intervention, ZnPT has been used as an investigational antipsoriatic drug and has also been examined as an experimental therapeutic for topical treatment of UVB-induced epidermal hyperplasia [381, 382].

Paradoxically, no studies have examined ZnPT cytotoxicity and genotoxicity in human skin cells as the likely target cells of topically administered ZnPT. Moreover, no valid studies with ZnPT are available to satisfy the data requirements for carcinogenicity assessment of this chemical as stated recently in the *AD Risk Assessment for the Reregistration Eligibility Decision (RED) Document on ZnPT* prepared by the US Environmental Protection Agency [383].

Given the photo-protective, therapeutic and toxicological potential of topical
ZnPT, the purpose of this study was to explore the potential cytoprotective activity of ZnPT while also characterizing the cytotoxicity profile of ZnPT in human primary skin cells including keratinocytes and melanocytes. Further studies were employed to characterize the molecular activity of ZnPT using a fully differentiated organotypic epidermal model with an intact stratum corneum.

B.2 Material and Methods

B2.1 Materials. All chemicals including ZnPT (CAS Number: 13463-41-7) were from Sigma Chemical Co. (St. Louis, MO, USA). A 1 mM stock solution of ZnPT was prepared by dissolving the compound in DMSO. The cell-permeable pan-caspase inhibitor Z-VAD-(OMe)-fmk was purchased from Calbiochem-Novabiochem (San Diego, CA). The PARP inhibitors 3-aminobenzamide and PJ-34 were from Sigma and Enzo Life Sciences Inc. (Farmingdale, NY), respectively.

B2.2 General cell culture. Primary human epidermal keratinocytes (neonatal HEKn-APF, from Cascade Biologics, Portland, OR) were cultured using Epilife medium supplemented with EDGS growth supplement. Primary human melanocytes (HEMa-LP, from Cascade Biologics) were cultured using Medium 154 medium supplemented with HMGS2 growth supplement. Both cell lines were passaged using recombinant trypsin/EDTA and defined trypsin inhibitor. Cells were maintained at 37 °C in 5% CO₂, 95% air in a humidified incubator.

B2.3 PARP-1/- mouse embryonic fibroblasts. Mouse embryonic fibroblasts (MEFs) derived from both PARP-1 wild-type (PARP-1+/+) and PARP-1/- mice generated by Z.
Q. Wang (Institute of Molecular Pathology, Vienna; [384]) were kindly provided by M.K. Jacobson, College of Pharmacy & Arizona Cancer Center, University of Arizona. MEFs were cultured at 37°C (5% CO₂) in DMEM supplemented with 10% BCS.

**B2.4 Cell proliferation assay.** Cells were seeded at 10,000 cells/dish on 35-mm dishes. After 24 h, cells were treated with test compound. Cell number at the time of compound addition and 72 h later were determined using a Z2 Analyzer (Beckman Coulter, Inc., Fullerton, CA, USA). Proliferation was compared with cells that received mock treatment. The same methodology was used to establish IC₅₀ values (drug concentration that induces 50% inhibition of proliferation of treated cells within 72 h exposure ± SD, n = 3).

**B2.5 Flow cytometric analysis of cell viability.** Cell viability was determined using flow cytometric analysis of annexinV (AV)-propidium iodide (PI) stained cells using an apoptosis detection kit (APO-AF, Sigma, St. Louis, MO) according to the manufacturer’s specifications. Cells were rinsed with DPBS and resuspended in 300µL binding buffer. 1.5µL of FITC-conjugated AV and 3µL of PI was added to the cell suspension. Samples we mixed and incubated at room temperature with exclusion of light for 10 min to allow binding of AV to phosphotidyl serine located on the outer leaflet and penetration of PI through compromised cellular membranes. Flow cytometry analysis was performed on a FACScan analyzer (BD Biosciences, San Jose, CA). Results are displayed in a 4 quadrants in which AV⁻, PI⁻ (lower left quadrant) indicates viable cells, AV⁺, PI⁻(lower right quadrant) indicates cells undergoing early apoptosis, and AV⁺, PI⁺ (upper right quadrant) indicates either late apoptotic or necrotic cells.
B2.6 Caspase-3 activation assay. Treatment-induced caspase-3 activation was examined in HEKs using a cleaved/activated caspase-3 (asp 175) antibody (Alexa Fluor 488 conjugate, Cell Signaling, Inc., Danvers, MA) according to the manufacturer's procedure. Briefly, after treatment, cells were harvested, rinsed with PBS and then fixed in 1% formaldehyde. Cells were then permeabilized using 90% methanol and resuspended in incubation buffer (PBS, 0.5% BSA). After rinsing by centrifugation, cells were resuspended in incubation buffer (90 μL) and cleaved caspase-3 antibody (10 μL) was added. After incubation (40 min) followed by rinsing and centrifugation in incubation buffer, cells were resuspended in PBS and analyzed by flow cytometry.

B2.7 Cellular ATP assay. Cells were seeded at 5,000 cells/well of an opaque 96-well plate. After 24 h, cells were treated with test compound. At various time points, ATP content per well was determined using the CellTiter-Glo luminescent assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Data are normalized to ATP content in untreated cells and expressed as means ± SD (n=3).

B2.8 Detection of intracellular oxidative stress by flow cytometric analysis. ZnPT-induced generation of intracellular oxidative stress was analyzed by flow cytometry using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a sensitive nonfluorescent precursor dye. A 5mg/mL stock of DCFH-DA was prepared in 100% ethanol. One hour after the last irradiation, DCFH-DA was added to the culture medium (5 μg/mL final concentration) and cells were incubated for 1 h in the dark (37°C, 5% CO₂). Cells were harvested by trypsinization, washed with PBS, resuspended in 300 μL PBS and
immediately analyzed by flow cytometry. To avoid direct photooxidation of the dye probe, cells were loaded with the indicator dye under light exclusion.

**B2.9 Determination of total cellular glutathione content.** Pharmacological modulation of intracellular glutathione content was analyzed using the photometric HT Glutathione Assay Kit (Trevigen, Gaithersburg, MD) based on the enzymatic recycling method involving glutathione reductase and DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman’s reagent). HEKs (1 x 10⁶) were exposed to a dose range of ZnPT (6 h exposure time) and harvested by trypsinization followed by sample processing according to the manufacturer’s instructions. Glutathione content of total cellular extracts was normalized to protein content determined using the BCA assay (Pierce, Rockford, IL).

**B2.10 EpiDerm™ skin equivalents: maintenance and sample preparation.** After shipment refrigerated EpiDerm™ inserts (EPI-200, 9 mm diameter; MatTek, Corp.) were equilibrated in 6-well format (5% CO₂, 37 °C, 1 h; 0.9 mL of EPI-200-ASY media per well) followed by change of medium. EpiDerm™ tissues were topically treated by applying ZnPT [0.1 - 2% (w/w) in Vanicream™, 90 mg total] or Vanicream™ only (90 mg) and cultured at 37° C for the indicated amount of time after which cream was gently removed using a cotton swab, and the apical side of the tissue was rinsed with PBS (500 µL, ten times).

The following sample preparation regimens were applied: (I) For zinc determination by ICP-MS, tissues were treated with ZnPT [2% (w/w); 3 h exposure time]. After cream removal, tissue was rinsed in PBS and then dissolved immediately in nitric acid (0.5 mL, 85 °C, 3h) followed by ICP-MS analysis. (II) For expression array
analysis, tissues were treated with ZnPT [2% (w/w)]. ZnPT exposure time was 24 h (or 1 h exposure time followed by removal of ZnPT and further incubation for 23 h at 37 °C, 5% CO₂). The tissue equivalent was then rinsed in PBS and homogenized (300 µL RNeasy lysis buffer). Homogenates were centrifuged (16,000 g, 10 min) to pellet tissue debris, and the supernatant was retained for RNA isolation using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. (III) For IHC and TUNEL analysis, tissues were treated with ZnPT [0.1% or 2% (w/w); 24 h exposure time). After cream removal tissues were fixed in neutral buffered formalin (10%, 24 h) and then placed in 70% ethanol followed by paraffin embedment.

**B2.11 Comet assay (alkaline single cell gel electrophoresis).** The alkaline Comet assay was performed on according to the manufacturer’s instructions (Trevigen, Gaithersburg, MD). Cells were seeded at 100,000 per 35mm dish 24 hours prior to treatment. Untreated cells were used as a negative control group. After treatment, cells were harvested by gently scraping, rinsed with ice-cold DPBS and suspended in 500 µL DPBS. 50 µL the cell suspension was mixed with 450 µL low-melting-point agarose and spread on pretreated microscope slides. Slides were allowed to dry protected from light, then immersed in ice cold lysis solution plus 10% DMSO and incubated at 4°C for 45 min. To allow DNA unwinding and expression of alkali-labile sites, slides were exposed to alkaline buffer (1 mmol/L EDTA and 300 mmol/L NaOH, pH >13) protected from light at room temperature for 45 min. Electrophoresis was conducted in the same alkaline buffer for 20 min at 300 mA. After electrophoresis, slides were rinsed three times in ddH2O then fixed in 70% ethanol for 5 min. Slides were dried for at least 1 hour at 32 °C.
Cells were then stained with SYBR® Green and analyzed with a fluorescence microscope (fluorescein filter) and analyzed using CASP software. At least 100 tail moments for each group were analyzed in order to calculate the mean ± S.D. for each group. The Fpg FLARE assay for assessment of Fpg-induced strand cleavage at oxidized purine bases was performed according to the manufacturer’s instructions (Trevigen). In brief, comet analysis was performed as described above, except that after lysis slides were washed with FLARE buffer three times and then incubated with Fpg enzyme solution (2 U Fpg/75 µl buffer/slide) or buffer only for 30 min at 37 °C.

**B2.12 Phospho-H2A.X detection by flow cytometry.** Treatment-induced accumulation of nuclear phosphorylated histone variant H2A.X (γ-H2A.X) was examined in HEKs using a phospho-histone H2A.X (Ser139) monoclonal antibody (Alexa Fluor 488 conjugate, Cell Signaling, Inc.) followed by flow cytometric analysis according to the manufacture's protocol. Briefly, after treatment, cells were harvested, rinsed with PBS and then fixed in 1% formaldehyde. Cells were then permeabilized using 90% methanol and resuspended in incubation buffer (PBS, 0.5% BSA). After rinsing by centrifugation, cells were resuspended in incubation buffer (90 µL) and cleaved caspase-3 antibody (10 µL) was added. After incubation (40 min) followed by rinsing and centrifugation in incubation buffer, cells were resuspended in PBS and analyzed by flow cytometry.

**B2.13 Zinquin fluorescence.** Zinquin ethyl ester (Sigma) was used as a probe for intracellular free Zn$^{2+}$ according to a published procedure [385]. HEKs (10,000) were seeded on microcover glasses (VWR) one day prior to experimentation. After rinsing with PBS, Zinquin ethyl ester (10 µM in PBS; Alexis) was added followed by incubation
in the dark (20 min, 37 °C, 5% CO₂). After exchange of PBS cells were then exposed to ZnPT for the indicated time, and rinsed coverslips were then mounted onto slides with glycerol. Formation of Zinquin-Zn²⁺ complexes was monitored by fluorescence microscopy (Olympus IX70 equipped with a 359/461 nm (λ_ex/λ_em) filter. Quantitative image analysis was performed using Image J software (rsbweb.nih.gov/ij).

**B2.14 Quantitative total zinc analysis of HEKs and EpiDerm™ tissue by inductively-coupled plasma mass spectrometry (ICP-MS).** Published ICP-MS protocols detecting total intracellular zinc were adapted as follows (Kondo et al. 2002; Rudolf and Cervinka 2010): After treatment, HEKs (1 x 10⁶) or EpiDerm™ tissue were rinsed with PBS and dried, followed by dissolution in concentrated nitric acid (0.5 mL, 85 °C, 3 h). Solutions were analyzed using an ELAN DRC-II ICP-MS (Perkin Elmer). Instrument parameters were as follows: RF power: 1450 W; dwell time: 50 ms; sweeps per replicate: 40; number of replicates: 3; acquisition mode: peak hopping; argon flow rate (L/min): nebulizer flow (0.95); coolant (15); auxiliary (1.3). Calibration standards were prepared from multi-element stock solutions purchased from AccuStandard. The stocks were diluted in 1% nitric acid to provide a working calibration curve of at least 5 points. Samples were also diluted with 1% nitric acid until their response was determined to be within the calibration range. Internal standards (Rh) were added to both standards and samples prior to analysis.

**B2.15 Human Stress and Toxicity PathwayFinder™ RT² Profiler™ PCR Expression Array.** After pharmacological exposure, total cellular RNA (5x10⁶ HEKs) was prepared using the RNeasy kit (Qiagen, Valencia, CA) according to the
manufacturer’s instructions. Reverse transcription was performed using the RT² First Strand kit (SA Biosciences, Frederick, MD, USA) and 1 µg total RNA. The Human Stress and Toxicity PathwayFinder™ RT² Profiler™ PCR Expression Array (SuperArray, Frederick, MD) profiling the expression of 84 stress- and toxicity-related genes was run as published recently [386], using the following PCR conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec alternating with 60 °C for 1 min (Applied Biosystems 7000 SDS, Foster City, CA). Gene-specific product was normalized to GAPDH and quantified using the comparative (ΔΔCt) Ct method as described in the ABI Prism 7000 sequence detection system user guide. Expression values were averaged across three independent array experiments, and standard deviation was calculated for graphing.

**B2.16 Immunoblot detection.** One hour after last irradiation, cells were washed with PBS, lysed in 1x SDS-PAGE sample buffer (0.375 M Tris HCl pH 6.8, 50% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue) and heated for 3 min at 95°C. Samples were separated by 4-15% SDS-PAGE (for PARP and PAR immunodetection) or 12% SDS-PAGE (for HO-1 and Hsp70 immunodetection) followed by transfer to nitrocellulose membranes (Optitran, Whatman, Piscataway, NJ, USA). Membranes were incubated with primary antibody in 5% milk-TBST overnight at 4°C. HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Jackson Immunological Research, West Grove, PA) was used at 1:20,000 in 5% milk-TBST followed by visualization using enhanced chemiluminescence detection reagents. Equal protein loading was examined by β-actin-detection. The following primary antibodies
were used: rabbit anti-HO-1 polyclonal antibody, 1:5000 (#SPA-896, Stressgen Bioreagents Stressgen Bioreagents, Ann Arbor, MI); rabbit anti-Hsp70 polyclonal antibody, 1:1,500 (#SPA-811, Stressgen Bioreagents); rabbit anti-PARP antibody, 1:1,000 (#9542, Cell Signaling, Danvers, MA); mouse anti-PAR monoclonal antibody, 1:1,000 (#4335-MC-100, Trevigen); mouse anti-actin monoclonal antibody, 1:1,500 (#A4700; Sigma).

**B2.17 HSPA6 ELISA.** The enzyme-linked immunosorbent assay for HSPA6 (also called Hsp70B’) was performed in 96 well format on cell lysates extracted from treated cells following kit instructions (‘Hsp70B prime’ ELISA Kit; Assay Designs). Briefly, 1x10^6 cells were seeded per T-75 flask one day before treatment. Cells (3x10^6 per group) were exposed to test compound for 24 h (37°C, 5% CO₂) and then harvested, washed with PBS, and lysed in 1x extraction reagent. After protein quantification using the BCA assay, samples were diluted to a range within the Hsp70B’ standard curve and processed according to the manufacturer’s instructions. Absorbance (450 nm) was determined on a microtiter plate reader (Versamax, Molecular Devices, Sunnyvale, CA). Data represent the average of three independent experiments.

**B2.18 Immunohistochemical detection of heat shock protein 70 (Hsp70).** Tissue sections (3 µm) from formalin fixed, paraffin embedded EpiDerm™ reconstructs were processed for standard hematoxylin and eosin (H&E) staining and Hsp70-immunohistochemistry. Immunohistochemistry was performed using the Discovery XT automated staining platform (VMSI, Ventana Medical Systems). All steps were performed using VMSI validated reagents, including deparaffinization, cell conditioning
(antigen retrieval with a borate-EDTA buffer), primary antibody staining, detection and amplification using a biotinylated-streptavidin-HRP and DAB (3,3′-diaminobenzidine tetrahydrochloride) system, and hematoxylin counterstaining. Hsp70 was detected using a murine primary monoclonal antibody (Assay Designs, C92F3A-5; dilution: 1:100) followed by an anti-mouse biotinylated secondary antibody. Images were captured using an Olympus BX50 microscope equipped with an Olympus Dp72 camera and CellSense Digital Image software. Images were standardized for light intensity.

**B2.19 In situ-Terminal dUTP Nick End Labeling (TUNEL) Assay.** Tissue sections (3µm) from formalin fixed, paraffin embedded EpiDerm™ reconstructs were collected onto slides, deparaffinized, rehydrated, and analyzed for DNA fragments using the DermaTACS™ in situ terminal deoxynucleotidyltransferase (TdT) kit (Trevigen) according to the manufacturer's instructions. Sections were treated with proteinase K and then incubated with TdT enzyme and brominated dNTP mixture (37 °C, 30 min). Afterwards, samples were labeled with biotinylated anti-BrdU antibody (37 °C, 30 min), followed by streptavidin-conjugated HRP and incubation with TACS Blue Label™ substrate and Red Counterstain C. Slides were dehydrated, clarified (ethanol, p-xylene), and mounted for viewing. Images were captured using an Olympus BX50 with an Olympus Dp72 camera and CellSense Digital Image software. The number of TUNEL-positive cells per viewing field (200x) was counted in six random fields, and percentage TUNEL positive cells was calculated.

**B2.20 Statistical Analysis.** Unless indicated differently, the results are presented as means ± S.D. of at least three independent experiments. Data were analyzed using the
two-sided Student’s $t$ test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) or by analysis of variance (ANOVA) with Tukey’s post hoc test using the Prism 4.0 software where means with the same letter are not significantly different.

B.3 Results

B3.1 ZnPT induces a massive heat shock response in human epidermal keratinocytes.

Modulation of stress response gene expression was examined in cultured human epidermal keratinocytes (HEKs) exposed to submicromolar concentrations of ZnPT using the RT$^2$ Human Stress and Toxicity Pathway Finder™ PCR Expression Array that allows assessment of 84 genes involved in the heat shock and antioxidant response. ZnPT treatment (500 nM, 24 h) altered expression levels of ten genes on the array by at least three-fold (Fig. B.1), including genes encoding the heat shock proteins HSPA6 ($HSPA6$; 129-fold), HSPA1A ($HSPA1A$; 35-fold), HSPA1L ($HSPA1L$; 7-fold), HSPB5 ($HSPB5$; 29-fold), and DNAJA1 ($DNAJA1$; 3 fold), the heat shock protein and antioxidant enzyme hemeoxygenase-1 ($HMOX1$; 7-fold), and the stress-responsive transcription factor early growth response protein 1 ($EGR1$; 6-fold).
Figure B.1. Gene expression array analysis of ZnPT-treated human skin keratinocytes. Differential gene expression in HEKs exposed to ZnPT (500 nM; 24 h) or left untreated was analyzed using the RT² Human Stress and Toxicity Pathway Finder™ PCR Expression Array performed in three independent repeat experiments and analyzed using the two-sided Student’s t test. Changes in cycle threshold (Ct) for genes of interest relative to GAPDH for untreated control (x-axis) versus ZnPT-treated (y-axis) cells are displayed as scatter blot. Upper and lower lines represent the cut-off indicating three fold up- or down-regulated expression, respectively. The arrows specify the ten genes with statistically significant (p < 0.05) ZnPT-induced up- or downregulation of expression by at least threefold as summarized in the Table.
ZnPT-induced upregulation of major target genes including \textit{HSPA6}, \textit{HSPA1A}, and \textit{HMOX1} was confirmed at the protein level (Fig. B.2). Indeed, HSPA6 (also called Hsp70B’), a protein not constitutively expressed and induced only under conditions of extreme cellular stress [387], was upregulated more than 250-fold in response to ZnPT (500 nM, 24 h) as assessed by ELISA analysis (Fig. B.2A). Consistent with the results obtained by HSPA6 analysis, pronounced upregulation of HSP70 was observed in HEKs exposed to ZnPT (Fig. B.2B-C), but not in cells exposed to ZnSO\textsubscript{4} (1 µM; data not shown). Cellular HSP70 levels were upregulated within 3 h exposure and maximal levels were observable at 6 h. Similarly, pronounced upregulation of cellular levels of HO-1, the protein encoded by \textit{HMOX1}, was observed after 3 h exposure to ZnPT (Fig. B.2B-C).

Taken together, these findings indicate that nanomolar concentrations of ZnPT induce a rapid stress response in HEKs characterized by massive heat shock response gene expression that is associated with upregulation of HSP70, HSPA6, and HO-1 protein levels.

\textbf{B3.2 ZnPT inhibits proliferation and induces caspase-independent cell death in human epidermal keratinocytes.}

Modulation of HEK proliferation and viability by exposure to ZnPT was assessed. Significant inhibition of cell proliferation was observed at low nanomolar concentrations of ZnPT (IC\textsubscript{50}: 256.8 ± 14.4 nM) (Fig. B.3). In contrast, no inhibition of proliferation was observed in response to ZnSO\textsubscript{4} exposure examined at similar concentrations. At
Figure B.2. Upregulation of HSPA6, HSP70, and HO-1 protein levels in ZnPT-treated human skin keratinocytes. (A) Induction of HSPA6 protein expression in ZnPT treated HEKs was determined by ELISA analysis at 24 h exposure. (B and C) Western blot analysis of ZnPT-induced HSP70 and HO-1 upregulation was performed in HEKs. (B) Time course of HSP70 and HO-1 upregulation induced by ZnPT. (C) Dose response of ZnPT-induced HSP70 and HO-1 upregulation analyzed at 24 h exposure.
Figure B.3. Anti-proliferative activity of ZnPT in human skin keratinocytes. Dose response relationship of ZnPT-induced inhibition of cell proliferation. After 72 h exposure to increasing concentrations of ZnPT and ZnSO₄ (1 µM), proliferation was examined by cell counting and expressed as % of untreated control (mean ± SD, n≥3). Representative light microscopy pictures were taken after 72 h exposure; panel I: control; panel II: ZnPT (250 nM); panel III: ZnPT (400 nM).
higher concentrations of ZnPT (≥ 500 nM) morphological changes consistent with induction of cell death including cell rounding and detachment were observed within 24 h exposure. We therefore examined the ability of ZnPT to induce cell death in HEKs using flow cytometric analysis of annexinV/propidium iodide (AV/PI) stained cells (Fig. B.4). A detailed dose response (100 to 1000 nM ZnPT, 24 h) analysis indicated that induction of cell death required a threshold concentration of approximately 500 nM, and no viable cells were detected after 24 h exposure to concentrations as low as 1000 nM (Fig. B.4A). Time course analysis showed that cell viability (AV-/PI-) was maintained for at least 6 h continuous exposure (500 nM ZnPT) (Fig. B.4B). Interestingly, at all time points and concentrations, cells with impaired viability stained mostly double-positive (AV+/PI+) indicative of cells in late apoptosis and/or necrosis, and only few cells were located in the lower right quadrant (AV+/PI-) indicative of early apoptosis (Fig. B.4A-C). Consistent with a caspase-independent mechanism of cell death, ZnPT-induced loss of cell viability was not influenced by the presence of the pan-caspase inhibitor zVAD-fmk (Fig. B.4C), and no proteolytic activation of caspase 3 occurred in response to ZnPT exposure as demonstrated by flow cytometric analysis using an Alexa 488-conjugated antibody that recognizes cleaved procaspase 3 (data not shown). In contrast, cotreatment with the cell impermeable zinc chelator diethylenetriaminepentaacetic acid (DTPA) completely protected from ZnPT-induced cell death (Fig. B.4C). Negligible cytotoxicity was observed upon exposure to ZnSO₄ (25 µM; Fig. B.4C). Interestingly, complete suppression of ZnPT cytotoxicity was also observed when exposure occurred in the presence of the thiol antioxidant N-acetyl-L-cysteine (NAC, Fig. B.4C). However,
Figure B.4. Cell death-inducing activity of ZnPT in human skin keratinocytes. (A) Dose response of ZnPT-induced cell death. Cells were exposed to ZnPT (24 h) or left untreated, and viability was assessed by flow cytometric analysis of AV-FITC/PI-stained cells. (B) Time course of ZnPT-induced cell death. Cells were exposed to ZnPT or left untreated and viability was assessed by flow cytometric analysis. (C) Induction of cell death upon extended exposure (24 h) to ZnSO₄ (25 µM) or ZnPT (1 µM) in the absence or presence of the pan-caspase inhibitor zVADfmk (42 µM), NAC (10 mM), or DTPA (60 µM) was assessed by flow cytometric analysis. The numbers indicate viable cells (AV+, PI-, lower left quadrant) in percent of total gated cells (mean ± SD, three independent experiments). Flow cytometric panels depict one representative experiment.
other antioxidant agents including superoxide dismutase (SOD), catalase, L-ascorbic acid, and the cell permeable SOD- and catalase-mimetic EUK-114 did not antagonize ZnPT cytotoxicity in HEKs (data not shown).

Taken together, these data suggest that ZnPT inhibits proliferation of HEKs at nanomolar concentrations and that loss of HEK viability observed at ZnPT concentrations equal or higher than 500 nM involves a caspase-independent mechanism of cell death.

**B3.3 ZnPT treatment induces rapid depletion of cellular ATP that is antagonized by pharmacological and genetic inhibition of PARP.**

In an attempt to identify functional alterations that precede ZnPT-induced loss of HEK viability, we examined changes in cellular energy status by monitoring ATP levels during exposure to ZnPT. Significant depletion of cellular ATP levels was detectable in HEKs within 1 h of exposure to ZnPT and exceeded 50 % after 6 h (Fig. B.5A). Importantly, these early changes in ATP levels occurred without loss of viability and impaired membrane integrity as verified by AV/PI-analysis (Fig. B.4B) and confirmed independently using the trypan blue exclusion assay (data not shown). In contrast, no depletion of ATP levels was observed with ZnSO$_4$ (50 µM; Fig B.5B), and cotreatment with DTPA (a cell impermeable zinc chelator) reduced ZnPT-induced depletion of ATP (Fig. B.5B), consistent with antagonism of ZnPT-induced cytotoxicity by this potent Zn-ion chelator as observed in Fig. B.4C. Similarly, cotreatment with TPEN, an intracellular zinc antagonist, also suppressed ZnPT-induced ATP depletion.
Figure B.5. PARP-dependent ATP depletion in ZnPT-treated human skin keratinocytes and mouse embryonal fibroblasts. (A) Time course of cellular ATP depletion in HEKs exposed to ZnPT in the absence or presence of PARP-inhibitors (3-ABA, 4 mM or PJ-34, 0.5 µM; added 1 h before ZnPT). (B) Cellular ATP depletion in HEKs exposed to ZnPT (1 µM, 6h) in the absence or presence of zinc chelators (DTPA, 60 µM or TPEN, 10 µM; added 1 h before ZnPT). In addition, cells exposed to ZnSO$_4$ (50 µM, 6h) or left untreated were analyzed (C) Cellular glutathione levels in HEKs exposed to a dose range of ZnPT (100-1000 nM, 6 h). Changes in cellular glutathione were not significant (n.s.). (D) Time course of cellular ATP depletion in PARP$^{-/-}$ versus wild-type MEFs exposed to ZnPT. All bar graphs represent mean ± SD, n=3 (E) Immunoblot detection of PARP-1 protein in PARP$^{-/-}$ versus wild-type MEFs. (F) Western blot analysis of ZnPT-induced HSP70 upregulation in PARP$^{-/-}$ versus wild-type MEFs as a function of exposure time. (G) Western blot analysis of ZnPT (500 nM)-induced HSP70 and HO-1 upregulation in HEKs as a function of exposure time and PJ-34 pretreatment (0.5 µM).
Cellular glutathione levels, an important indicator of cellular oxidative stress, did not change in response to ZnPT treatment (Fig. B.5C; 6 h exposure; total glutathione level of untreated controls: 74.4 ± 9.6 nmole/mg protein, n = 3) suggesting that oxidative stress was not involved in early induction of cytotoxicity and energy crisis in response to ZnPT treatment. Indeed, flow cytometric analysis using 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) as a peroxide probe and dihydroethidium (DHE) as a superoxide probe, a standard methodology for the assessment of cellular oxidative stress, did not reveal changes in cellular ROS levels in response to ZnPT treatment over the course of a 24 h observational range (data not shown).

In contrast, ZnPT-induced ATP depletion was prevented by co-administration of 3-aminobenzamide (3-ABA), an established inhibitor of poly(ADP-ribose) polymerase (PARP), an enzyme involved in induction of energy crisis in response to genotoxic stress (Fig. B.5A) [388-391]. This result was independently confirmed using the more potent and selective PARP inhibitor PJ-34 (Fig. B.5A) [392]. Moreover, using PARP-1 knockout (PARP-1−/−) and wildtype (PARP-1wt) mouse embryonic fibroblasts [384], it was observed that ATP depletion by ZnPT treatment was blocked in PARP-1−/− cells, providing compelling genetic evidence in support of PARP-1 involvement in ZnPT induced ATP depletion (Fig. B.5D-E). In contrast, ZnPT-induced upregulation of heat shock protein expression occurred independent of PARP expression in MEFs (Fig. B.5F); equally, HSP70 and HO-1 upregulation in HEKs exposed to ZnPT occurred irrespective of pharmacological PARP antagonism (PJ-34; Fig. B.5G). These data suggest that PARP activation is not required for the induction of heat shock protein
expression in response to ZnPT. Moreover, PARP-1<sup>-/-</sup> cells, but not PARP-1<sup>wt</sup> cells, displayed pronounced resistance to ZnPT-induced cytotoxicity, maintaining viability during extended exposure (Fig. B.6A). Independent confirmation of ZnPT-induced activation of PARP activity was obtained by immunodetection of poly(ADP-ribose) polymer (PAR)-modified cellular proteins (Fig. B.6B). Indeed, massive PAR-formation was observed in HEKs within 15 min exposure to ZnPT (5 µM) and disappeared within an hour indicating rapid polymer turnover. PAR-modification of proteins was strongly suppressed upon coadministration of DTPA and negligible PAR formation was detected as a result of exposure to ZnSO<sub>4</sub> (Fig. B.6C; 50 µM, 15 min exposure). As expected, ZnPT-induced PAR-formation was blocked by cotreatment with the PARP-1 inhibitor PJ34 (Fig. B.6C).

Taken together, these data provide pharmacological and genetic evidence in support of PARP-1-dependent ATP depletion as a crucial mechanism underlying ZnPT cytotoxicity in HEKs.

**B3.4 ZnPT treatment rapidly impairs genomic integrity in human epidermal keratinocytes.**

Pronounced induction of cellular stress gene expression (Figs. B.1 and B.2) and cytotoxicity observed at low concentrations of ZnPT (Figs. B.3 and B.4) combined with rapid ATP depletion that was prevented by PARP antagonism (Figs. B.5 and B.6) led us to examine the possibility that ZnPT induces DNA damage in HEKs. Using alkaline single cell gel electrophoresis (comet assay) as a sensitive genotoxicity assay [393-395],
Figure B.6. ZnPT-treatment induces formation of poly(ADP-ribose) polymer (PAR)-modified cellular proteins in human skin keratinocytes. (A) Induction of cell death in PARP\(^{-/-}\) versus wild-type MEFs upon exposure to ZnPT (24 h) as assessed in Fig. 1B-D. The numbers indicate viable cells (AV', PI', lower left quadrant) in percent of total gated cells (mean ± SD, three independent experiments). Flow cytometric panels depict one representative experiment. (B) Formation of poly(ADP-ribose) polymer (PAR)-modified cellular proteins as detected by immunoblotting of cellular extracts obtained from HEKs exposed to ZnPT (5 \(\mu\text{M}\)) as a function of exposure time. (left panel: Ponceau stained nitrocellulose membrane confirming equal protein loading; right panel: PAR-immunoblot). (C) Formation of PAR-modified cellular proteins as detected by immunoblotting of cellular extracts obtained from HEKs exposed to ZnPT (5 \(\mu\text{M}\); 15 min) in the absence or presence of DTPA (60 \(\mu\text{M}\)) or PJ-34 (2 \(\mu\text{M}\)). In addition, cells exposed to ZnSO\(_4\) (50 \(\mu\text{M}\), 15 min) or left untreated were analyzed.
Figure B.7. Impairment of genomic integrity in ZnPT-treated human skin keratinocytes. (A) Average comet tail moments. HEKs were exposed to ZnPT (100-500 nM; 1 to 12 h) and DNA damage was detected using the alkaline comet assay. As a positive control, cells were exposed to H$_2$O$_2$ (100 µM, 30 min). (B) Representative comets as visualized by fluorescence microscopy. C: untreated control; H$_2$O$_2$: positive control; right panels: ZnPT. (C) Induction of γ-H2A.X in ZnPT-treated HEKs (1 µM, 1 and 6 h exposure, left panel). As a positive control, γ-H2A.X was detected in UVB (240 mJ/cm$^2$) treated cells (1 and 6 h after UV, right panel). (D) Assessment of oxidative DNA damage using the Fpg-endonuclease modified comet assay. Cells were exposed to ZnPT (500 nM; 3 h) or H$_2$O$_2$ (100 µM; 20 min). Fold increase in mean tail moment of nuclei after incubation in buffer plus Fpg versus buffer only was calculated as specified in materials and methods.
the integrity of cellular DNA was examined in HEKs treated with ZnPT (100 and 500 nM, 1h up to 12 h exposure time). As a positive control, cells were exposed to H$_2$O$_2$, an established genotoxic agent. Exposure to submicromolar concentrations of ZnPT significantly impaired HEK genomic integrity within 1 h exposure time as evident from formation of nuclear comets (Fig. B.7A-B). ZnPT treatment (500 nM) induced comets with average tail moments that were increased approximately threefold over untreated controls within 1 h of exposure and exceeded control levels approximately fivefold within 12 h of exposure (Fig. B.7A).

In contrast, no comet formation was observed as a result of exposure to ZnSO$_4$ (1 µM, 1 h exposure time; Fig. B.8) and DTPA cotreatment completely suppressed ZnPT-dependent (500nM, 1h exposure time) increase in average tail moment (Fig. B.8). However, pharmacological PARP inhibition using PJ34 did not suppress the ZnPT-induced increase in average tail moment shown above (Fig. B.8), consistent with activation of PARP-1 occurring downstream of early impairment of genomic integrity.

It is important to note that loss of genomic integrity occurred in cells without compromised viability maintained over 6 h continuous exposure as demonstrated earlier (ZnPT 500 nM, Fig. B.4B). Remarkably, significant comet formation was even observed at very low ZnPT concentrations (100 nM, Fig. B.7A) that do not impair viability at any time point of observation (Fig. B.4A). In order to obtain independent experimental evidence in support of ZnPT-induced DNA damage, we used flow cytometric detection of the nuclear phosphorylated histone variant H2A.X (γ-H2A.X, Ser 139), a sensitive marker of DNA double-strand breaks and UVB-induced nucleotide
Figure B.8. DTPA-antagonism of ZnPT-induced impairment of genomic integrity. HEKs were exposed to ZnSO₄ (1 µM) or ZnPT (1 µM; 1 h) in the presence or absence of DTPA (60 µM) or PJ34 (2 µM), and DNA damage was detected using the alkaline comet assay. As a positive control, cells were exposed to H₂O₂ (100 µM, 30 min). Representative comets as visualized by fluorescence microscopy are displayed.
excision repair (Fig. B.7C) [396, 397]. Massive induction of γ-H2A.X was observed in response to UVB-exposure serving as a positive control. Importantly, significant upregulation of γ-H2A.X was observed in HEKs exposed to ZnPT (1 µM, 6h) and was detectable at time points as early as 1 h, suggesting ZnPT-induced early impairment of genomic integrity in HEKs. DNA comets formed from DNA unwinding under alkaline conditions are indicative of impaired genomic integrity resulting from single or double strand breaks, AP-site formation, or nucleotide excision repair [394]. Protection from ZnPT-induced cytotoxicity by the thiol antioxidant NAC (Fig. B.4C) therefore led us to examine the possibility that ZnPT associated comets in HEKs originate from oxidative DNA damage. To this end, we performed comet analysis under conditions that detect oxidative base damage [394], assessing formamidopyrimidine-glycosylase (Fpg)-induced strand cleavage at oxidized DNA purine bases (Fig. B.7D). Indeed, in H₂O₂ treated control cells, mean comet tail moment was increased after Fpg endonuclease treatment by approximately 70 % consistent with oxidative generation of Fpg-sensitive sites. However, mean comet tail moment remained unchanged in ZnPT exposed cells irrespective of Fpg treatment, providing experimental evidence against introduction of oxidative DNA damage by ZnPT exposure.

Based on these data, it was concluded that ZnPT-induced loss of genomic integrity in HEKs (I) occurs rapidly upon exposure to nanomolar concentrations before general impairment of cell viability and (II) occurs upstream of massive ATP depletion through PARP activation that does not originate from oxidative DNA damage.
B3.5 ZnPT impairs genomic integrity of human epidermal melanocytes with induction of energy crisis and heat shock protein upregulation.

In an attempt to further explore the effects of ZnPT exposure on other human epidermal skin cells, we examined primary human epidermal melanocytes (HEMa) as potential target cells. Indeed, antiproliferative effects on melanocytes were observed at doses even lower than those active against HEKs; almost complete inhibition of proliferation occurred at a threshold concentration of 100 nM ZnPT (Fig. B.9A). However, no induction of cell death was observed at this concentration and loss of viability was only observed upon exposure to much higher concentrations (2 µM; 24 h; data not shown). As observed with HEKs (Fig. B.2B-C), upregulation of HSP70 protein levels was detected in response to submicromolar concentrations ZnPT (Fig. B.9B). Moreover, massive ATP depletion by ZnPT occurred at early timepoints (1 h exposure) in HEMa (Fig. B.9C). This effect was again significantly antagonized by pharmacological PARP inhibition (3-ABA), a situation closely resembling PARP-dependent ATP depletion in response to ZnPT-induced genotoxic stress in HEKs (Fig. B.5A). Importantly, comet analysis demonstrated that ZnPT treatment rapidly impaired genomic integrity of HEMa, with approximately twenty-fold elevation of mean tail moment observed within one hour exposure (ZnPT 500nM; Fig. B.9D).

Taken together, these data demonstrate that ZnPT-induced inhibition of proliferation, impairment of genomic integrity, and rapid ATP depletion can not only be observed in HEKs, but also occurs in HEMa as another relevant cellular target in the human epidermis.
Figure B.9. ZnPT-induced impairment of proliferation, genomic integrity, and ATP homeostasis with upregulated HSP70 expression in human skin melanocytes. (A) Dose response relationship of ZnPT-induced inhibition of cell proliferation. After 72 h exposure to increasing concentrations of ZnPT, proliferation was determined as specified in Fig. 3. (B) Dose response of ZnPT-induced HSP70 protein upregulation by immunoblot analysis (24 h exposure). (C) Cellular ATP depletion induced by ZnPT (1 h) in the absence or presence of 3-ABA (4 mM, added 1 h before ZnPT). (D) Average comet tail moment induced by ZnPT (1 h exposure) and H₂O₂ (100 µM, 30 min exposure) performed as detailed in Fig 7A. Panels I-IV depict representative comets as visualized by fluorescence microscopy: I: untreated control; II: H₂O₂; III. ZnPT (100 nM); IV: ZnPT (500 nM).
B3.6 ZnPT induces rapid zinc overload in primary skin keratinocytes and reconstructed human epidermis.

To further substantiate the role of intracellular zinc dysregulation in ZnPT-induced impairment of HEK viability and function, we assessed the accumulation of free intracellular zinc ions in response to ZnPT exposure using Zinquin-based fluorometric analysis and total zinc ion quantification by ICP-MS (Fig. B.10). Quantitative analysis of Zinquin fluorescence revealed a rapid increase in intracellular free zinc concentrations by 2.5 fold in response to ZnPT treatment (1 µM, 5 min exposure), and elevated levels were maintained over the one hour observation period (Fig. B.10A). Further dose-response analysis indicated that at higher concentrations (5µM) ZnPT caused an almost 3 fold upregulation within 5 min exposure (Fig. B.10B). In order to independently confirm these data using an alternative analytical methodology and to avoid potential inaccuracies associated with a non-linear dose response relationship of zinquin fluorescence we then determined total cellular zinc content by ICP-MS analysis (Fig. B.10C). Consistent with the fluorescence data ZnPT exposure (1 µM, 1 h) caused a significant rise in total zinc content of HEKs that reached a maximum of 207.1 ± 7.1 ng per 10⁶ cells, an approximately twofold increase over untreated control cells. In addition, ICP-MS analysis indicated a significant elevation of intracellular zinc levels by almost 50% in response to submicromolar (500 nM) concentrations of ZnPT. Importantly, no increase in intracellular zinc levels in response to ZnSO₄ exposure was detected, even at a fifty-fold molar excess (50 µM) over ZnPT.
Figure B.10. Rapid induction of zinc dysregulation in ZnPT-treated in human skin keratinocytes. (A) Time course of increase in intracellular free zinc induced by ZnPT (1 µM) as detected by Zinquin fluorescence with representative fluorescence microscopy pictures: panel I: control; panel II: ZnPT (5 min); panel III: ZnPT (60 min). Quantitative analysis was performed as specified in Materials and Methods (mean + SD). (B) Dose-response analysis of ZnPT-induced changes in Zinquin-fluorescence (0.1-5 µM; 5 min exposure). (C) Total cellular zinc content as analyzed by ICP-MS after ZnPT (0.5 and 1 µM, 1 h) and ZnSO₄ (50 µM, 1 h) exposure (mean + SD; n=3).
Next, we tested the possibility that topical exposure of human skin to ZnPT would induce a rapid increase in epidermal zinc content. To this end, quantitative ICP-MS assessment of zinc accumulation in response to topical ZnPT exposure was performed in human reconstructed epidermis (EpiDerm™), an established organotypic model of full differentiated human epidermis (Fig. B.11A-C) [398, 399]. EpiDerm™ tissues were topically exposed to ZnPT [2% (w/w) in Vanicream™, 90 mg total; 3 h; Fig. B.11A] or Vanicream™ followed by ICP-MS (Fig. B.11C). An almost five-fold increase in EpiDerm zinc ion content [ZnPT-Vanicream™: 348.2 ± 34.0 versus Vanicream™ control: 72.2 ± 5.1 (ng Zn/ tissue reconstruct; mean ± SD; p < 0.01)] was measured in response to ZnPT topical application. In contrast, no statistically significant elevation of epidermal zinc content was observed following a control procedure where ICP-MS analysis was performed after the ZnPT-Vanicream preparation was topically applied followed by immediate removal and extensive rinsing (data not shown). This excludes artifactual ICP-MS detection of cutaneous zinc originating from residual ZnPT cream not removed during rinsing.

Taken together, these data indicate that exposure to ZnPT induces a rapid increase in intracellular zinc in primary keratinocytes. Furthermore, an organotypic epidermal model showed increase zinc accumulation upon topical exposure to ZnPT.
Figure B.11. ICP-MS and gene expression array analysis of ZnPT-treated human reconstructed epidermis (EpiDerm™). (A) Human reconstructed epidermis (EpiDerm™; 9 mm insert diameter) was treated topically and maintained in 6 well format. (B) H&E stained cross-section of formalin-fixed and paraffin-embedded terminally differentiated EpiDerm™ (SC: stratum corneum; E: viable epidermal keratinocytes). (C) ICP-MS analysis of zinc accumulation in EpiDerm™ in response to topical treatment {ZnPT [2% (w/w) in Vanicream™, 90 mg total] or Vanicream™ only; 3 h exposure time; mean ± SD}. (D) RT² Human Stress and Toxicity Pathway Finder™ PCR Expression Array analysis of differential gene expression in EpiDerm™ exposed to ZnPT [2% (w/w) in Vanicream™, 90 mg total; 24 h exposure] or Vanicream™ only. Changes in cycle threshold (Ct) for genes of interest relative to GAPDH for vehicle control (x-axis) versus ZnPT-treated (y-axis) EpiDerm™ are displayed as scatter blot. Upper and lower lines represent the cut-off indicating four fold up- or down-regulated expression, respectively. The arrows specify the genes with statistically significant upregulation greater than ten-fold (n=3; \( p < 0.05 \)). ZnPT-induced up- or downregulation of expression by at least twofold is summarized in Table A2.1.
B3.7 Gene array and IHC analyses demonstrate an upregulated stress response in reconstructed human epidermis exposed to topical ZnPT.

Next, modulation of stress response gene expression was examined in reconstructed human epidermis exposed to topical ZnPT. To this end, EpiDerm™ tissue equivalents were first treated with a ZnPT-Vanicream™ formulation (2%, 24 h continuous exposure) shown to be effective in elevating tissue zinc ion content (Fig. B.11C) and then subjected to expression profiling using the RT² Human Stress and Toxicity Pathway Finder™ PCR Expression Array technology (Fig. B.11D and Table B.1A). After 24 h continuous exposure of reconstructed epidermis to topical ZnPT, expression of the metallothionein encoding gene MT2A was dramatically upregulated by approximately 500-fold over Vanicream only-treated control, a finding consistent with cellular zinc ion overload and induction of a metal stress response [361, 400].

Indicative of a massive ZnPT-induced cellular heat shock response, numerous genes encoding heat shock-related proteins (including HSPA6, HSPH1, HSPA1A, HSPE1, DNAJB4, HSPD1, DNAJA1, HSPA1L, HSP90AA1, HSP90AB1, HSPA8, HMOX1, HSPA4, HSPA5, and HSF1, the gene encoding the transcriptional regulator heat shock factor-1) were significantly upregulated at the mRNA level. Moreover, ZnPT-induced expression changes in EpiDerm™ involved upregulation of genes controlling inflammatory and irritation-response signaling (TNF, NFKB1, LTA, GDF15, ANXA5), xenobiotic metabolism (CYP1A1, EPHX2, FMO1, FMO5, GSTM3), and oxidative stress response and redox signaling (SOD2, NOS2A, HMOX1, SOD1, GSR, GPX1, PRDX1). Importantly, ZnPT-induced upregulation also involved genes with major roles in
genotoxic stress response, DNA repair, and cell cycle progression (UNG, CDKN1A, DDIT3, MDM2, TP53, CHEK2, GADD45A, RAD50, DDB1, RAD23A).

A similar yet somewhat attenuated epidermal gene expression profile was observed when an alternative exposure regimen was followed where gene expression changes were assessed in response to short-term exposure to topical ZnPT-Vanicream preparation (Table B.1B; 2%, 1h exposure time followed by rinse and 23 h post-exposure incubation). Among various heat shock protein encoding genes upregulated by short term exposure to ZnPT, HSPA6 again displayed the most pronounced expression differential (173-fold upregulation), and massive induction of MT2A gene expression (150-fold upregulation) was observed. Interestingly, short term ZnPT exposure was not associated with transcriptional activation of DNA damage response gene expression, suggesting that ZnPT-associated impairment of epidermal genomic integrity only occurs under conditions of prolonged exposure.

These findings are consistent with earlier results indicating upregulation of gene expression in primary keratinocytes exposed to nanomolar concentrations of ZnPT (Fig. B.1). When comparing fold-induction of mRNA levels between ZnPT exposed HEKs and EpiDerm™ (Fig. B.12), a consistent pattern of heat shock response gene (HSPA1A, HSPA6, HMOX1) upregulation was observed. Remarkably, upregulation of MT2A and TNF was dramatically increased in ZnPT-treated EpiDerm™ over HEKs (MT2A: 499.4 ± 44.9 versus 5.7 ± 2.0 fold upregulation; TNF: 90.8 ± 29.3 versus 2.2 ± 0.7 fold upregulation; mean ± SD, n=3). A differential extent of ZnPT-induced upregulation of MT2A and TNF in EpiDerm™ over cultured HEKs may be related to molecular changes
Figure B.12. Comparison of gene expression changes observed in ZnPT-exposed cultured human keratinocytes versus ZnPT-topically treated EpiDerm™. Comparison of selected gene expression changes (mRNA fold increase from untreated control) in ZnPT-exposed cultured HEKs [500 nM, 24 h; Fig.B.1] versus ZnPT-exposed EpiDerm™ (Fig.B.11D).
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**Gene Symbol**
- **HSPA6 (NM_002155)**
- **MT2A (NM_005953)**
- **HSPA1A (NM_005345)**
- **DNAJB4 (NM_007034)**
- **LTA (NM_000595)**
- **HMOX1 (NM_002133)**
- **HSPH1 (NM_006644)**
- **CYP7A1 (NM_000780)**
- **HSP90AA1 (NM_005348)**

**Gene Name**
- heat shock 70kDa protein 6 (HSP70B*)
- metallothionein 2a
- heat shock 70kDa protein 1A
- DnaJ (Hsp40) homolog, subfamily B, member 4
- lymphotoxin alpha (TNF superfamily, member 1)
- heme oxygenase (decycling) 1
- heat shock 105kDa/110kDa protein 1
- cytochrome P450, family 7, subfamily A, polypeptide 1
- heat shock protein 90kDa alpha (cytosolic), class A member 1

**Fold Change**
- 173.0
- 150.2
- 17.9
- 17.4
- 10.1
- 9.6
- 9.6
- 7.4
- 5.4
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Table B.1. Quantitative analysis of gene expression changes in EpiDerm™ exposed to topical ZnPT-treatment. RT² Human Stress and Toxicity Pathway Finder™ PCR Expression Array analysis was performed as depicted in Fig. 4D (n=3; p < 0.05) (A) Numerical gene expression changes induced by topical ZnPT-treatment (24 h continuous exposure; 2% (w/w) in Vanicream™, 90 mg total). (B) Numerical gene expression changes induced by topical ZnPT-treatment (1 h continuous exposure followed by removal and subsequent 23 h incubation period; 2% (w/w) in Vanicream™, 90 mg total).
associated with terminal differentiation that only occurs in the organotypic model. Indeed, it has been shown earlier that stress gene expression and signaling is altered in human skin reconstructs compared to cultured skin cells [401].

Next, IHC tissue analysis of EpiDerm™ reconstructs after topical exposure to ZnPT was used to examine heat shock protein expression at the protein level (Fig. B.13A). Indeed, massive upregulation of cellular Hsp70 protein levels was observed in reconstructs that were exposed to topical ZnPT-Vanicream preparation (2%, 24h), a finding already suggested by gene expression array analysis that indicated upregulation of genes encoding numerous members of the Hsp70 protein family (Table B.1A). Vanicream™ treated control reconstructs did not stain positive for Hsp70, and a moderate upregulation of Hsp70 IHC staining was already observed in reconstructs exposed to a topical preparation containing considerably less ZnPT (0.1%, 24h; Fig. B.13A). Interestingly, Hsp70 upregulation in response to 2% ZnPT was most pronounced in keratinocytes residing in the basal layer of the reconstruct, consistent with an increased stress response in rapidly dividing cells that might not occur during later stages of terminal epidermal differentiation.

Based on pronounced induction of genotoxic stress observed in HEKs (Figs. 7 and 8) and in reconstructed epidermis (Table B.1), we assessed ZnPT-induced DNA fragmentation performing terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling in situ (TUNEL-IHC; Fig. B.13). Pronounced TUNEL-positivity (17.3 ± 4.4% positive cells) was detected in reconstructs that were exposed to topical ZnPT-Vanicream preparation (2%, 24h). Even upon exposure to the less concentrated ZnPT formulation
Figure B.13. Immunohistochemical assessment of Hsp70 induction and TUNEL positivity in EpiDerm™ exposed to topical ZnPT. (A) EpiDerm™ was exposed to topical ZnPT (24 h continuous exposure; 0.1 and 2% (w/w) in Vanicream™, 90 mg total) or Vanicream control only (n=3 per treatment group). Paraformaldehyde-fixed, paraffin-embedded 3 µm sections were then analyzed using Hsp70-IHC with hematoxylin counterstaining (left column). Alternatively, TUNEL-IHC was performed on duplicate tissue sections (right column). Per tissue section at least three high power fields were acquired; one representative photograph is shown (insert: 40 x magnification). (B) Bar graph displaying quantitative analysis of TUNEL-IHC (percentage TUNEL positive nuclei of total nuclei per high power field; mean + S.D.; n=3).
(0.1%, 24h) a considerable percentage of cells (3.6 ± 0.5 %) stained positive for fragmented DNA. However, no follow-up experimentation was performed that would allow to further define the mechanistic origin of ZnPT-induced epidermal DNA fragmentation.

Taken together, these data indicated that FDA-approved concentrations of ZnPT induce a stress response in an organotypic epidermal model associated with upregulation of several genes including MT2A and HSPA6, and TUNEL analysis also revealed topical application of ZnPT induces DNA fragmentation.

### B.4 Discussion

ZnPT is an important FDA-approved microbicidal OTC-drug, and annual production of ZnPT for FDA-regulated applications in skin products including creams, sprays, and shampoos now exceeds 450,000 kilograms in the US alone [368, 372, 373, 383]. In the context of cutaneous pathology beyond antimicrobial intervention, ZnPT has been used as an investigational antipsoriatic drug and has also been examined as an experimental therapeutic for topical treatment of UVB-induced epidermal hyperplasia [381, 382].

Toxicological concerns regarding ZnPT have been raised earlier based on cytotoxicity and genotoxicity observed in mouse lymphoma cells and embryotoxicity observed in fish [402, 403]. Paradoxically, no published experimental study has examined the potential cytotoxic and genotoxic activity of ZnPT on cultured primary human skin cells [383]. Furthermore, ZnPT and another antifungal Cu/Zn-chelator and
ionophore drug, clioquinol, have been shown to cause pathophysiologically relevant neuronal zinc disturbance that occurs through activation of TRPA1, a nociceptive ion channel and zinc-sensitive receptor for environmental irritants and oxidants [404]. As a consequence, clioquinol was withdrawn from the market when it was causatively linked to an epidemic of subacute myelo-optico-neuropathy [405].

Our data obtained in this study document for the first time the exquisite vulnerability of primary keratinocytes and melanocytes to nanomolar concentrations of ZnPT that rapidly induce a massive heat shock response and loss of genomic integrity. Upregulation of the cellular heat shock response in human epidermal cells occurred at submicromolar doses of ZnPT that also induced inhibition of cell proliferation and cell death (Figs. B.3 and B.4), therefore excluding feasibility of dissociating cytoprotective from cytotoxic effects of ZnPT as originally intended at the beginning of this study.

In addition to induction of heat shock response gene expression in HEKs, we observed that ZnPT treatment caused rapid depletion of cellular ATP levels (Fig. B.5). Both pharmacological inhibition of PARP-1 (Fig. B.5A) and genetic elimination using engineered PARP-1−/− MEFs (Fig. B.5D-E) antagonized ZnPT-induced early depletion of cellular ATP levels. These data provide compelling evidence for activation of PARP in response to ZnPT treatment, further confirmed by detection of PAR-modified cellular proteins within minutes of exposure to ZnPT (Fig. B.6B). In contrast, ZnPT-induced upregulation of heat shock protein expression occurred independent of PARP expression (Fig. B.5F-G), suggesting that PARP activation is not required for the induction of heat
shock protein expression and perhaps PARP activation and heat shock response are two separate cellular responses induced by ZnPT exposure.

It is well established that PARP activation occurs in response to genotoxic stress, leading to rapid cellular NAD and ATP depletion [388-392, 406]. Direct evidence for ZnPT-induced DNA damage was obtained by comet analysis (Fig. B.7A-B) and phosphorylation of histone H2A.X at serine 139 (γ-H2A.X; Fig. B.7C). Similar results were obtained in human primary melanocytes based on comet analysis (Fig. B.9D) and determination of PARP-dependent ATP depletion (Fig. B.9C).

Earlier experiments demonstrate that exposure of mammalian cells to various zinc salts (e.g. ZnSO₄, ZnCl₂, and Zn-acetate) is well tolerated at concentrations up to 200 µM without reduction of viability [365-367] as also observed in this study (Fig. B.3 and B.4A). However, potentiation of zinc effects and induction of cytotoxicity by zinc-specific ionophores including pyrithione has been observed earlier in noncutaneous mammalian cells [407, 408]. This study documents for the first time ZnPT-induced dysregulation of intracellular zinc ion homeostasis in primary keratinocytes as observed by quantitative fluorescence microscopy (Fig. B.10A-B) and ICP-MS (Fig. B.10C), building on earlier ICP-MS data that determined total zinc ion accumulation in cultured cancer cell lines exposed to ZnPT [409, 410]. ZnPT-induced impairment of viability (Fig. B.4), PARP activation (Fig. B.6B), energy crisis (Fig. B.5A), and genomic destabilization (Fig. B.7) were all antagonized by zinc chelation using either DTPA or TPEN, consistent with intracellular zinc ion accumulation as a causative factor in mediating the effects of nanomolar to low micromolar concentrations of ZnPT on
cultured keratinocytes. Indeed, cytotoxic effects of ZnPT on cultured mammalian cells of non-cutaneous origin have been associated with disruption of intracellular zinc homeostasis and activation of signaling cascades including the ras/ERK pathway [411, 412]. In our own experiments using HEKs, ZnPT induction of cell death occurred without involvement of caspases and was characterized by early PARP activation and rapid cellular ATP depletion, a molecular pathway reminiscent of many genotoxic agents known to induce caspase-independent cell death orchestrated by PARP activation [390, 392, 413].

The molecular mechanism of skin cell DNA damage caused by this widely used topical drug is currently unknown, and multiple mechanisms may underlie the detrimental effect of ZnPT on skin cell genomic integrity. For example, it has been shown earlier that disruption of intracellular zinc homeostasis is an important activator of endonucleases including DNase gamma [414]. Moreover, exposure to high levels of zinc ions can be associated with formation of reactive oxygen species that would provide another mechanistic basis for the genotoxic effects of ZnPT [415]. However, consistent with the absence of ZnPT-induced oxidative cellular stress as assessed by fluorescent redox probes and determination of cellular glutathione levels (Fig. B.5C), our experiments that detect Fpg endonuclease-sensitive oxidative lesions gave no indication for the generation of oxidative DNA damage as a consequence of exposure to nanomolar concentrations of ZnPT (Fig. B.7A-D). Lack of protection observed with all antioxidant treatments except NAC (Fig. B.4C) suggest that NAC-antagonism of ZnPT cytotoxicity
may rather originate from thiol-based chemical interference with ZnPT complex integrity rather than antioxidant activity [371].

The FDA code of federal regulations (21CFR § 358.710) specifies use of ZnPT as an active OTC-ingredient for the control of dandruff and seborrheic dermatitis. It is important to note that epidermal deposition and retention of ZnPT following topical application has been demonstrated in human skin [374, 375]. Skin permeation of radiolabeled ZnPT leading to urinary excretion of up to 0.2 % of topically applied starting material has been documented in rhesus monkeys [378], and ZnPT absorption was increased strongly when the integrity of the stratum corneum was disrupted by abrasion or repeated exposure to concentrated surfactant solutions. Taken together, earlier studies raise the possibility that topically applied ZnPT may indeed penetrate deep enough into the living human epidermis to impair human skin cell genomic integrity as observed here in cultured cells. Moreover, the known solubility of ZnPT in skin sebum and its retention in hair follicles observed previously suggest that topically applied ZnPT may also reach crucial stem cell populations in human skin hair follicles [368].

Remarkably, ZnPT activity against cultured HEKs was observed in the nano- to micromolar range, whereas topical OTC-products contain much higher levels (up to 2%) corresponding to millimolar concentrations. However, in intact human skin, a multi-layered differentiated epidermis with stratum corneum serves as an essential barrier against environmental insults including ultraviolet radiation and chemical exposure, thereby potentially minimizing cellular effects of topical ZnPT [355, 416]. We therefore examined molecular consequences of cutaneous ZnPT exposure in EpiDerm™, a 3-
dimensional organotypic human skin model that incorporates normal epidermal keratinocytes undergoing terminal differentiation with intact stratum corneum and barrier function used extensively for transdermal drug delivery, cutaneous absorption, and skin irritation studies [398, 399]. In this epidermal model, topical application of ZnPT induced zinc dysregulation as substantiated by ICP-MS-based detection of rapid zinc accumulation (Fig. B.11C). Expression array analysis indicated massive upregulation of the MT2A gene encoding metallothionein-2A (Fig. B.11D and Table B.1), shown earlier to be induced in the context of a cellular zinc stress response observed in Hep-2 cervical tumor cells exposed to ZnPT [409]. Further expression array analysis indicated upregulation of a wide range of stress response genes encoding heat shock proteins, antioxidants, and inflammatory factors, and IHC analysis revealed upregulation of Hsp70 protein levels (Fig. B.13A), confirming our earlier results obtained in cultured primary HEKs (Fig. B.2).

These findings are consistent with the established role of zinc-ions as potent inducers of metal stress, heat shock, and antioxidant stress response pathways involved in cytoprotective as well as cytotoxic activities [367, 417, 418]. Indeed, intracellular zinc ion availability has been shown to be involved in activation of stress response transcription factors including Nrf2 (electrophilic stress response), HSF (heat shock response), and MTF (heavy metal stress response) [365-367, 400, 419]. Moreover, a mechanistic involvement of intracellular zinc in lipopolysaccharide-induced TNF-α upregulation has recently been demonstrated in monocytes [420], and it is therefore tempting to speculate that TNF upregulation observed in ZnPT-exposed EpiDerm™ (that
only contains primary keratinocytes undergoing terminal differentiation) may equally be zinc-dependent, a hypothesis to be tested by future experimentation.

PCR array expression analysis of ZnPT-exposed EpiDerm™ also revealed a significant upregulation of multiple genes involved in cellular response to genotoxic stress (Table B.1). This finding is consistent with ZnPT-induced PARP activation downstream of early loss of genomic integrity as observed in cultured primary HEKs (Fig. B.8), where PARP activation, energy crisis, and genomic impairment were all antagonized by zinc chelation using DTPA and TPEN. Thus, TUNEL-positivity in ZnPT-treated EpiDerm™ (as shown in Fig. B.13) may indicate treatment-induced direct DNA fragmentation, but may also originate from DNA strand breaks introduced during later stages of apoptotic or necrotic cell death, a possibility that we have not further explored.

Taken together our data demonstrate for the first time ZnPT-induced impairment of zinc ion homeostasis and upregulation of stress response gene expression in primary keratinocytes and melanocytes as well as reconstructed human epidermis, molecular activities that may underlie therapeutic and toxicological effects of this topical drug.
APPENDIX C:

PEER REVIEWED PUBLICATIONS


REFERENCES


197. Green, E., **The genetics of a new hair deficiency, furless.** J. Hered., 1954. **45**: p. 115-118.


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