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Signal Transduction Pathways Induced by the Anti-Psoriatic Drug Anthralin in Cultured Human Keratinocytes

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1 INTRODUCTION

1.1 Psoriasis

1.1.1 Definition

Psoriasis is a common, genetically determined, inflammatory and proliferative disease of the skin with characteristic lesions that consist of chronic relapsing, sharply demarcated, erythematous, scaly papules and plaques preferentially localized to the extensor prominences and the scalp. In addition to the skin, psoriasis also affects nails, mucous membranes and joints.

1.1.2 Epidemiology of psoriasis

Prevalence rates of psoriasis vary among different regions and populations, with an estimated prevalence rate of 2 to 5% in Europe, and up to 6.6% in Australia, with variation in frequencies depending on the type of study and clinical examinations employed.[1-5] It is relatively infrequent among blacks, in the Japanese population, and in native North and South American populations.[6-9] The different prevalence rates in different countries have been, in part, attributed to differing solar radiation intensities which may act as a therapeutic environmental factor in these areas.[2;10;11]

In general, prevalence rates increase with age; evidence about an association of age at onset with disease severity is controversial.[12;13]

1.1.3 Etiology, pathogenesis and histopathology of psoriasis

Psoriasis exhibits a polygenic inheritance pattern with an estimated heritability as a multifactorial genetic disorder in the range of 60% to 90%.[7] Disease expression (i.e. disease onset and severity), however, is also dependent on environmental stimuli, such as stress, trauma, and infection.[14] As such, psoriasis is considered a complex trait, genetically. In several studies of familial psoriasis, strong associations with major histocompatibility complex

(MHC) alleles, including HLA-Cw6, HLA-B57, HLA-DR7, and HLA-Cw2 have been found.[15-18;18;19] Genetic linkage between psoriasis and MHC could be shown.[20;21]

It has been hypothesized that one of the major psoriasis genes resides in the MCH, on the short arm of chromosome 6, while several other psoriasis genes are distributed throughout the human genome. These other genes may be subdivided into two groups: those that are generally involved in several inflammatory and immune diseases apart from psoriasis and as such influence the severity of the disease ("severity genes") and in those that are more exclusively involved in the distinct psoriatic phenotype ("disease-specific genes").[22]

The pathogenesis of psoriasis involves four important cell populations: keratinocytes, lymphocytes, endothelial cells, and neutrophils. Psoriasis had previously been considered a dermatosis that was solely caused by benign epidermal hyperproliferation. However, in the mid 1970s, several immunological approaches were taken to study this condition in greater detail, particularly focusing on the inflammatory changes.

Current research indicates that the activation of T-lymphocytes is a central feature in the pathogenesis of the disease. Histopathologic examination of early psoriatic skin lesions demonstrates that the characteristic epidermal proliferation is preceded by infiltration of T-lymphocytes and macrophages into the skin. Only later does activation of endothelial cells occur that results in capillary leakiness and vascular alterations.[23]

It was found that psoriatic patients had elevated numbers of proliferating activated blood T- and B-lymphocytes and monocytic cells as compared to healthy controls, numbers correlating positively with disease severity.[24] Further investigations revealed that lesional psoriasis is rich in activated CD4+ and CD8+ T-cells[25] which release proinflammatory cytokines and lymphokines (such as IL-2, Interferon-γ, and TNF-α) that stimulate keratinocyte proliferation and induce abnormal epidermal maturation typical of psoriasis, such as the characteristic acanthotic epidermis.[26-31]

Evidence supporting the pivotal role of lymphoid cells in the pathogenesis of psoriasis could also be found in animal models: In mice, the psoriatic phenotype can be induced by naïve $CD4^+$ T-cells.[32] Wrone-Smith et al. used severe combined immunodeficiency (SCID) mice and transplanted normal-appearing skin obtained from psoriatic patients onto SCID animals. Subsequent subcutaneous injection of patients' activated peripheral blood mononuclear cells beneath grafted skin induced psoriatic skin lesions.[33]

In addition, the pivotal role of the T-lymphocyte was supported in clinical trials of psoriasis treatment. Clearing of psoriatic lesions was reported after treatment with immuno-modulating drugs, such as cyclosporine, methotrexate, and tacrolimus (FK 506), but also with anti-CD4+ monoclonal antibodies,[34-38] T-cell-selective toxins,[39] synthetic inhibitors of T-cell activation (SDZ 281-240).[40]

Apart from these findings, trials with monoclonal antibodies against T-lymphocyte derived cytokines, e.g. IL-2-receptor-antibody (anti-CD25, Basiliximab),[41] and most promising with TNFα-antibodies (for review, see La Duca, 2001[42]) showed improvement of clinical psoriasis symptoms and thus underscored the essential role of lymphocytes in the pathogenesis of psoriasis.

Nonetheless, the exact pathogenesis and relative roles of the different cell types in the development of psoriasis still remains controversial and awaits further research.[43]

1.2 Therapeutic approaches in the management of psoriasis

Various forms of treatment for psoriasis have been developed over the past several decades. These therapies are wide ranging and exhibit many differences in their chemistry, route of administration, and putative mode of action.

1.2.1 Systemic treatment

The systemic treatment approaches, mainly reserved for the more severe cases of psoriasis, include oral corticosteroids, retinoids, methotrexate, cyclosporine, and tacrolimus. Recently, the TNFα-receptor antibody infliximab has also been shown to decrease the clinical activity of psoriatic lesions.[44-47]
Most recently, selective inhibitors of epidermal growth factor receptor (EGFR) kinase activity, such as SU-5271 (Sugen Inc., CA), whose antiproliferatory effect on keratinocytes from psoriatic lesions had been shown before, are also being used in first clinical trials to investigate their effect on psoriatic lesions *in vivo*.[48]

Systemic corticosteroids exert their activity primarily through their direct "anti-inflammatory" properties as well as decreased vascular permeability that leads to a decrease in dermal edema and leukocyte transmigration into the skin. It is generally observed that, apart from the characteristic hypercorticoid side effects, the disease also tends to recur more severely after cessation of treatment. Therefore, prolonged oral corticosteroid use is no longer indicated in the treatment of psoriasis.
Oral retinoids, biological derivatives of vitamin A, are known to have profound effects on epithelial differentiation that provide benefits in treating psoriasis.[49] Retinoids also modulate pro-inflammatory responses observed in other dermatological diseases.
Methotrexate had long been reported to have activity as an antimetabolite of DNA synthesis and, thus, was considered to inhibit the hyperproliferation of psoriatic epidermis. However, more recent evidence indicates that it targets on psoriasis and improves disease activity through mononuclear cells, thus exerting an immunosuppressive effect.[50;51]
The immunomodulating substance cyclosporine exerts therapeutic effects through alteration of T-cell dependent cytokine secretion. The altered cytokine pattern leads to inhibition of T-cell activation and proliferation, but also diminished keratinocyte proliferation.[52]

1.2.2 Treatment with Ultraviolet Light

1.2.2.1 PHOTOTHERAPY

The beneficial effects of sunlight for various cutaneous disorders have been known for centuries. Specifically, the observation that many patients with psoriasis improve during the summer months due to increased sun exposure led to the investigation and use of artificial ultraviolet light in the therapy of this disease.

At present, various types of UVB radiation sources are in use, and the recent introduction of narrow-band (311 nm) irradiation is among the most effective.[53;54] Narrow-band UVB therapy suppresses lymphoproliferative responses and alters the functional antigen-presenting ability of epidermal cells which then leads to clearing of the psoriatic plaque.[55-57]

1.2.2.2 PHOTOCHEMOTHERAPY (PUVA)

Photochemotherapy, utilizing photodynamic properties of ultraviolet radiation, combines oral or topical exposure to a potent photosensitizer, psoralen, such as methoxsalen, with ultraviolet A phototherapy in the range of 320 to 400 nm. The drug is given prior to exposure to UVA, and the dosage of UVA is determined by the patient's skin type.[58] The proposed mechanisms of action of PUVA include DNA-interactions and adduct formation, inhibition of cell proliferation, induction of superoxide formation and apoptosis of immune cells within the skin and cell-mediated immuno-suppressive effects in involved skin.[59]

1.2.3 Topical treatments

The topical agents available for the treatment of psoriasis include emollients, keratolytics (e.g. salicylic acid), coal tar, topical corticosteroids, topical vitamin D_3 analogues, tazarotene, and anthralin.

1.3 Anthralin

1.3.1 Historical remarks

Anthralin (1,8-dihydroxy-9(10H)-anthracenone), also known as dithranol or Cignolin, has been in clinical use as a topical treatment for psoriasis for decades since its first description by Galewsky and Unna in 1916.[60;61] In 1876, Balmanno Squire, a British surgeon, had found chrysophanic acid, a natural product derived from Goa powder, effective in treating psoriasis. Goa powder (yellow araroba or chrysarobine) was extracted from the "tawny-colored" tree (Vouacopoua araroba) in southern Asia or South America. Subsequent research indicated that the active anti-psoriatic component in Goa powder was a reduced metabolite of chrysophanic acid, chrysarobine, which was later synthetically prepared as Cignolin (anthralin).[62;63]

1.3.2 Current Use of Anthralin

Nowadays, in many European and U.S. centers, anthralin is still the preferred topical treatment for psoriasis due to its high efficacy. Anthralin is applied to localized psoriatic plaques in concentrations of 0.1% to 10%.[64] The agent is applied as short-contact or overnight treatment.[65] The duration of remission after treatment with anthralin, ranging from 3.9 to 6 months, is longer than that of other topical treatments.[66-68] However, like most forms of current psoriasis therapy, the benefit of anthralin is limited by its undesirable side effects: inflammatory effects on the skin and poor acceptability because of staining of the skin and clothes which often times restricts its application to supervised, professional settings.

1.3.3 Chemical structure and generation of reactive oxygen species by anthralin

Anthralin is an odorless, yellow to orange-yellow microcrystalline powder. It is unsoluble in aqueous solution, but soluble in chloroform, ethanol and dimethyl sulfoxide (DMSO). The anthralin anion undergoes rapid light-catalyzed auto-oxidation to the anthralin radical via a free radical chain reaction, during which

molecular oxygen is reduced in a non-enzymatic, univalent reduction pathway that results in the generation of several reactive intermediates, including the initial superoxide ($O_2^{\bullet-}$) species.[69] Under physiological conditions, $O_2^{\bullet-}$ spontaneously dismutates to hydrogen peroxide (H_2O_2), but is catalytically enhanced by the superoxide dismutase (SOD) enzyme system. It can easily diffuse through biological membranes. Within the cell, H_2O_2 can be transformed to the highly reactive hydroxyl radical (•OH) in the iron-catalyzed Haber-Weiss reaction. (See *Figure 1.1* and *Figure 1.2*)

Once anthralin has undergone auto-oxidation to the anthralin radical, it becomes less active or inactive in its anti-psoriatic effect, and in its antiproliferative and antirespiratory potency.[70-74] The main oxidation products of the anthralin radical are danthron, anthralin dimer and anthraquinone dimer. The latter, in turn, is decomposed into polymeric degradation products (anthralin brown) through secondary radical species.[75] (See *Figure 1.3*) With higher grades of dimerization and polymerization, these oxidation products further lose their therapeutic properties and become more stable, less soluble and of darker color (brown to black).

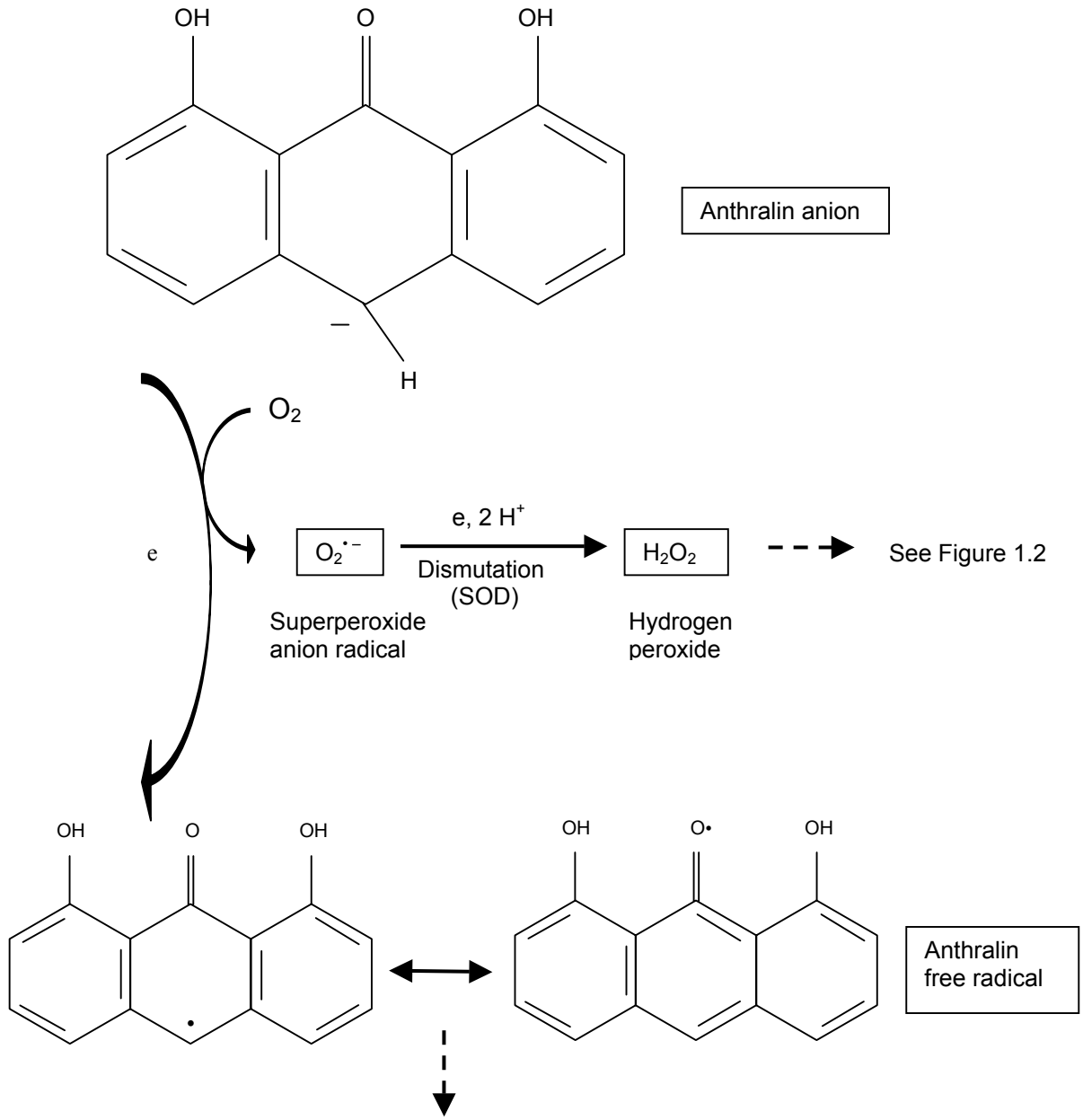

Figure 1.1:

Activation of anthralin to radical intermediates and concomitant generation of reactive oxygen species.[76]

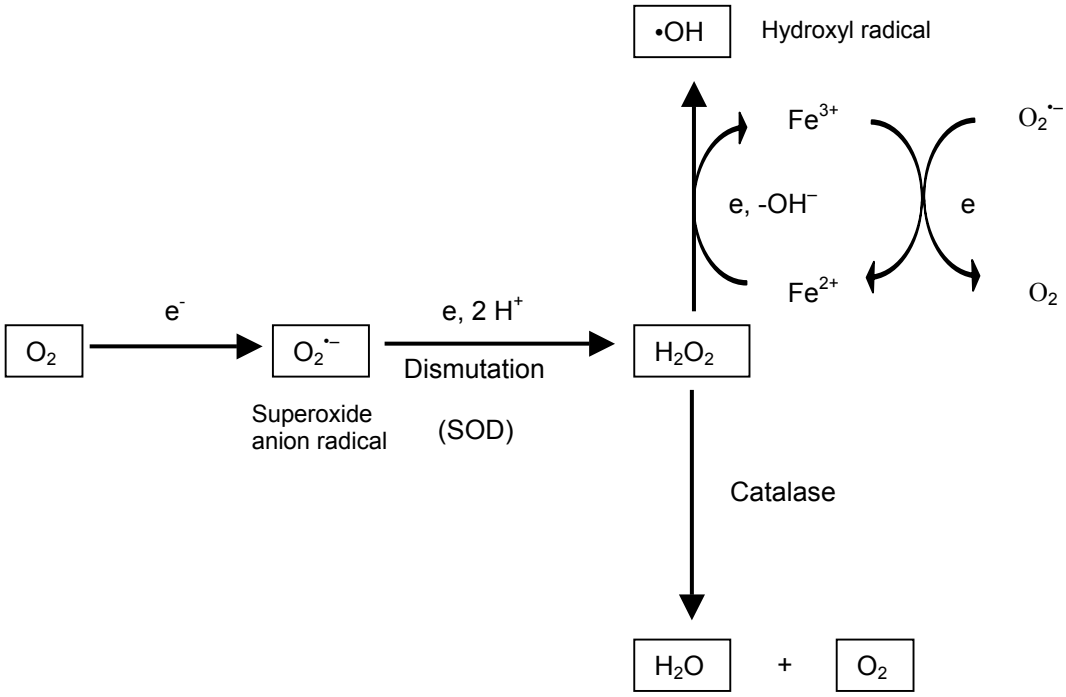

Figure 1.2:

Haber-Weiss-reaction[77]

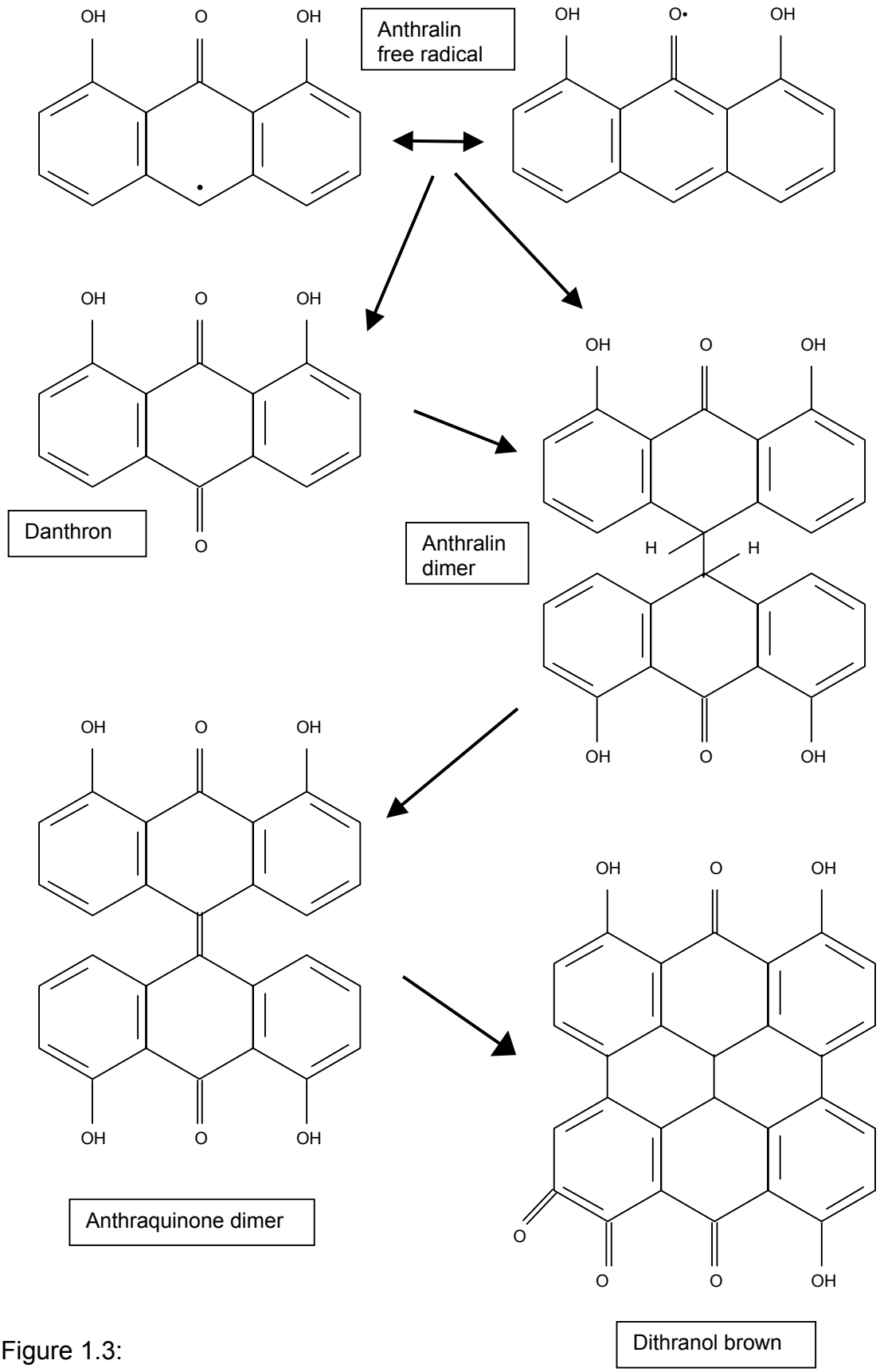

Figure 1.3:

Scheme of anthralin autooxidation (modified after Mahrle and Müller)[69;78]

Interactions of anthralin with a target tissue, such as skin, are required for its actions. Thus, the reactive oxygen species formed under physiological conditions may play a significant role in the mode of action, but also in the development of side effects, that include erythema and inflammation at the site of application of the drug.[79;80]

1.3.4 Mechanism of action of anthralin

Although anthralin has been in clinical use for more than eight decades, its mechanism of action still remains uncertain and results characterizing its activities are controversial.

Over years of research, it has been reported that anthralin inhibits glycolytic key enzymes such as glucose-phosphate dehydrogenase and glyceraldehydrogenase.[81;82] Other researchers have shown that under certain conditions anthralin may have antiproliferative, as well as antirespiratory, activity in a human-transformed epidermal keratinocyte line[71] or in skin fibroblasts in culture.[83;84] Hsieh et al. demonstrated cytotoxic effects of anthralin in rat keratinocytes,[85] whereas inhibition of DNA replication and repair synthesis and changes in keratinocytes differentiation have also been found as putative modes of action of anthralin.[86;87]

Alternatively, modulation of the cellular redox status or effects on mitochondria have been suggested to be the target of anthralin action.[85;88-90] Inflammatory and immune cells, such as neutrophils, macrophages, Langerhans cells, and lymphocytes, have been shown to be sensitive to this compound.[91-95] In addition, inhibition of leukotriene production, modulation of skin phagocyte activity and arachidonic acid metabolism, and suppression of immune responses have been reported following anthralin exposure.[26;91-93;96]

Additionally, it has been observed that anthralin treatment results in decreased binding capacities and cell-surface numbers of the epidermal growth factor (EGF) receptor and decreased expression of one of its cognate physiologic ligands, transforming growth factor-α (TGF-α).

It has been reported that reactive oxygen species (ROS, i.e. superoxide anion, hydroxyl radical, and singlet oxygen) are generated as reaction intermediates during anthralin oxidation.[69;69;85;97] Further research indicates

that anthralin exerts its main therapeutic effects by means of free-radical formation.[69;98]

In this study, the effects of anthralin on the generation of reactive oxygen species and signal transduction pathways will be examined, primarily in cultured human keratinocytes, and in peripheral blood mononuclear cells.

1.4 General cellular response mechanisms to environmental stresses

In order to survive and develop normally, eukaryotic cells must be able to respond to a diverse array of extracellular stimuli and adapt to adverse conditions. Often, cells adapt to environmental stresses and extracellular stimuli by modifying their responses to other stimuli, and by adjusting their gene expression programs. Depending on the nature of the extracellular stimulus, cell growth and division can be triggered, whereas other stimuli induce cell differentiation or even apoptosis, a specialized type of programmed cell death.

Typically, cells are able to activate various stress responses which serve to protect the homeostasis of the cell after exposure to environmental stresses. One common way to accomplish such reactions is by activating signal transduction cascades which biochemically transmit information from the cell surface to the transcriptional apparatus within the cell nucleus. Therein, the phenotypic response to cellular stresses will be generated and coordinated.

This sequential activation gives potential possibilities to vary the nuclear response, because of further interaction with other signaling pathways and also by differential activation states. Failure to activate these signal transduction pathways properly, however, can lead to dysfunctional cellular responses such as uncontrolled cell growth or improper regulation of energy metabolism.

A common motif in intracellular signaling are protein kinase cascades, wherein cascades of enzymes phosphorylate specific substrates, typically other kinases, modulate or alter their activational state and eventually lead to a cellular response.

One major family of protein kinases in eukaryotic cells that are involved in signal transduction are the mitogen activated protein kinases, or MAP kinases. The MAPK family consists of several subgroups of kinases that are highly conserved in their primary structure and their mode of activation by dual phosphorylation on serine/threonine residues.
In this study, the response to anthralin treatment of the c-Jun NH_2-terminal kinase (JNK) and the extracellular signal regulated kinase (ERK) subgroups of MAPK are investigated, and their relationship to upstream signaling proteins, such as the epidermal growth factor (EGF) receptor, is examined.

1.4.1 Lipid peroxidation and generation of reactive oxygen species (ROS)

During the last few years, increasing evidence has been obtained that reactive oxygen species (ROS) are involved in many physiologic and pathologic processes. ROS are derived from molecular oxygen and are highly reactive metabolites which can be generated by cellular or acellular mechanisms. They usually either have unpaired electrons (e.g. superoxide anion [$O_2^{\bullet -}$], hydroxyl radical [OH•]) or possess the ability to abstract electrons from other molecules (e.g. hydrogen peroxide [H_2O_2], hypochlorous acid [HOCl]).[99]
For years, ROS were solely considered as toxic metabolites of cell respiration which were principally able to react with all biological molecules. Oxidative stress is a condition of pro-oxidant/anti-oxidant disequilibrium, in which the generation of potentially harmful reactive oxygen species exceeds the ability of the tissue's antioxidant defense mechanisms to decompose them.
One particular reaction induced by ROS, called lipid peroxidation, targets membrane lipids, and propagates an autocatalytic free radical chain reaction which can lead to membrane damage. Damage to cell membranes through lipid peroxidation, but also damage to DNA, enzymes, proteins and carbohydrates are among the main resultant effects.[100;101]
More recent investigations have shown that ROS at low concentrations are also involved in many intracellular signaling processes, in cytokine, growth

factor, and hormone action and secretion, ion transport mechanisms, apoptosis, and transcriptional activity.[102;103]

In the context of immunological host defense, ROS are generated by macrophages and neutrophils and can act as anti-bacterial, anti-viral, and anit-tumor mediators.[104-106]

Since the skin is directly exposed to environmental hazards such as chemicals and irradiation, the above mentioned free radicals are of special importance, because they have been found to be induced by topical therapeutics, ischemia/reperfusion, or inflammatory processes.[98;100;107]

1.4.2 The epidermal growth factor (EGF) and its receptor

Epidermal growth factor (EGF) is a peptide hormone that functions as a mitogen to stimulate cell division of various cells of epithelial origin.

The receptor for EGF, one of four closesly related members of the epidermal growth factor receptor (EGFR) family, is a 170 kDa transmembrane glycoprotein, comprising an extra-cellular domain that binds EGF, a single membrane-spanning domain and a cytoplasmic domain that has intrinsic protein tyrosine kinase activity. The first evidence of the kinase activity of EGFR was identified in 1980 by Dr. Stanley Cohen and co-workers.[108] Later, it was among the first cell surface signaling protein and protooncogene products to be characterized by molecular genetic methods by Dr. Axel Ullrich and co-workers.[109] Cells containing the EGF receptor exist in most tissues of the body, with 20,000 to 200,000 receptor molecules per cell. Ligand binding by the extracellular domain results in homo- and heterodimerisation of the receptor and in subsequent autophosphorylation of the catalytic domain, enhancing the tyrosine kinase activity of the receptor. Ligands that bind the EGFR family members are the prototypic peptide hormone, EGF, as well as a growing family of EGF-like ligands, including TGF-α, amphiregulin, heparin-binding EGF (HB-EGF), epiregulin, betacellulin, epigen and viral produced mitogens, such as vaccinia virus growth factor.[110;111]

The EGFR has been identified as a major upstream element in different signaling pathways, such as the MAP-kinase-, Src-kinase and phosphatidinositol-3-(PI-3)-kinase pathway.[112;113] Among the multitude of

signaling pathways activated by the EGFR, the MAP kinase pathway is one of the best characterized: Upon autophosphorylation of the C-terminal region of the EGFR, the receptor contains multiple docking sites for signal transducers such as Grb2 and Shc.[114] These activate the small G-protein Ras which subsequently induces the serine-threonine kinase Raf and the dual specificity kinase MAPK/ERK kinase 1 (MEK1).[115] MEK1 activates the MAPKs ERK1 and ERK2 which ultimately leads to regulation of nuclear transcription factors, such as Elk-1 and c-fos.[116]

The EGFR family plays a critical role in the proliferation, migration, survival, and differentiation of target cells, and dysregulation of signaling by EGF receptors has been implicated in the pathogenesis and progression of human cancers and other hyperproliferative diseases, such as psoriasis.[117;118]

Normal human keratinocytes depend on activation of the EGFR for survival and cell growth. The EGFR system in epidermal keratinocytes is activated, in an autocrine manner, by ligands expressed by this cell type, including TGF-α, amphiregulin and HB-EGF. Blockade of the EGFR receptor in keratinocytes, leads to growth inhibition, cell cycle arrest, and terminal differentiation, suggesting a protective role of the EGFR for human keratinocytes.[119-121]

1.4.3 MAP kinases (MAPKs)

Extracellular signal-regulated kinases were first described by Dr. T. G. Boulton in 1990.[122] ERK1 and ERK2 are 42 kDa and 44 kDa serine/threonine kinases that belong to a family of enzymes that are activated by MEKs (MAPK/ERK kinases) through a phosphorylation cascade. The signal cascade is initiated by diverse extracellular signals, including growth factors (e.g. EGFR), hormones, osmolar shock, stress, and elevated temperature which are transmitted by growth factor-regulated receptor tyrosine kinase or G-protein coupled receptor (see above). Doubly phosphorylated ERK family members translocate from the cytoplasm to the nucleus, where they phosphorylate protein transcription factors. Thus ERKs transduce extracellular signals to genomic responses, such as proliferation or differentiation.

MAP kinases are regulated by their own phosphorylation status: Phosphorylated residues on ERKs can be removed by dual-specific phosphatases, resulting in inactivation of MAP kinases.[116;123-127]
The MEK/MAPK pathways have been reported to enhance the survival of epithelial cells in different settings.[128-130] Pharmacological blockade of MAPK phosphorylation led to reduced viability of cells.[119]They have also been found to be critical in the regulation of cell growth, differentiation or proliferation.[131;132]

1.4.4 C-Jun N-terminal kinase (JNK)

The c-Jun N-terminal kinase (JNK) family members were first identified in UV irradiated cells as protein kinases that phosphorylate the transcription factor c-Jun within its amino-terminal activation domain at Ser63 and Ser73. Phosphorylation, in turn, leads to an increase in transcriptional activity.[133] Further research revealed that the major JNK isoform is a 46 kD protein and is activated by dual phosphorylation at threonine and tyrosine sites. This kinase bears a distant relationship to the extracellular signal regulated kinase (ERK) family and, thus, represents a member of the MAPK family.[103] JNK is also called stress-activated protein kinase (SAPK) due to its activation by UV irradiation, ionizing irradiation, heat, DNA damaging agents, pro-inflammatory cytokines and environmental stresses, such as heat shock and hyperosmolarity.[134-140]
To date, it is known that JNKs are activated by a signaling cascade which involves two Ha-Ras related GTP-binding proteins of the Rho family, namely Rac and Cdc42Hs. These, in turn, activate diverse kinases MAPK/ERK kinases [MEK], mixed-lineage kinases [MLKs], TGF-β activated kinase-1 [TAK1], and others) which then phosphorylate downstream kinases, such as SEK1 (SAPK/ERK kinase-1). SEKs are the kinases that eventually catalyze the phosphorylation of JNKs. (For review, see Kyriakis.[141])

Due to its involvement in the response to cellular stresses, JNK has been proposed to serve as a major component in the pathway leading to programmed cell death or apoptosis.[142-145] However, additional research has

also shown an opposite effect, that is, JNK exerts a protective role that can be observed in some cell types, such as B-lymphocytes.[146] The JNK pathway also seems to be required for fetal development and embryonic viability in mice and for embryonic morphogenesis in mammals.[147] However, the actual role of JNK in specific cell types remains to be elucidated.

Activation of JNKs in response to both stress and mitogens (e.g. EGF)[103;148;149;150] has also been reported, so that ERK and JNK pathways, with their differential stimulation by various stimuli, can be considered as parallel and largely independent branches of the mitogen-activated protein kinase signal transduction.

1.5 Aims of the present study

Hypothesis: Anthralin induces free radicals and lipid peroxides in keratinocytes that trigger downstream signaling responses for cell survival.

To test the hypothesis, the following questions were addressed:

- Are reactive oxygen species produced in human keratinocytes after anthralin treatment?
- Is anthralin induced lipid peroxidation observed in human keratinocytes and peripheral blood mononuclear cells (PBMC)?
- Does anthralin treatment induce JNK activation in human keratinocytes?
- Is JNK induced in PBMC after anthralin treatment?
- Are reactive oxygen species involved in the activation process?
- Is anthralin induced lipid peroxidation involved in the JNK activation after anthralin treatment?
- Does anthralin induce EGFR autophosphorylation?
- Do reactive oxygen species contribute to EGFR phosphorylation?
- Does EGFR autophosphorylation after anthralin exposure lead to activation of the MAPK pathway?
- What is the role of reactive species in the phosphorylation of EGFR and MAPK?

2 MATERIALS AND METHODS

2.1 General remarks

Reagents were weighed on Sartorius 1202 NP scales. For weights below 1mg Mettler AE160 scales were used. Buffers and solutions were prepared with sterile water filtered in a Millipore Milli Q Water System. pH-adjustment was done at room-temperature with a Corning® pH-Meter 140. All chemicals, if not indicated otherwise, were purchased from Sigma® Chemicals, St. Louis, MO.

2.1.1 Materials

- 6-well-dishes (Cell Wells™, 6-Well with Lid, well diameter 35mm, Corning Inc., Corning, NY)
- Polystyrene, round-bottom tubes (without cap) 12x75mm style, (Falcon® 2052, Becton Dickinson Labware, Lincoln Park, NJ)
- Pipettes (costar® Stripette, 10ml, Corning Inc., Corning, NY)
- Pasteur Pipettes, 5 ¾ inches (VWR Scientific, West Chester, PA)
- Pipetman® (Gilson, Rainin Instrument Co., Inc., Woburn, MA)
- Biological ™ Brand Pipet Tips (Continental Lab Products, San Diego, CA)
- Combitips Plus, 2.5ml (Eppendorf®, Distributed by Brinkmann Instruments, Westbury, NY)

2.2 Cell Culture

2.2.1 Cultivation of human keratinocytes

2.2.1.1 Cell culture Media

Media base was an in house preparation of MCDB 153 medium. For preparation of **standard MCDB 153 media**, the following substances were added to the base:

- 0.1 mM Calcium
- 0.5 μM Hydrocortisone
- 0.1 mM Ethanolamine
- 0.1 mM Phosphoethanolamine
- Amino acids (L-Histidine, L-Isoleucine, L-Phenylalanine, L-Tryptophane, L-Tyrosine, L-Methionine)

Growth factor supplemented, **complete media**, was further supplemented with:

- 0.2% Bovine pituitary extract
- 10 ng/ml EGF
- 5 μg/ml Insulin

Penicillin and Streptomycin were added to cell media as antibiotics to prevent fungal and bacterial contamination of the cell cultures.

All cells were incubated at 37°C in humidified atmosphere containing 5% CO_2 and 95% air.

Cells were cultured in various tissue culture plastic ware, including:

- Corning®, 75cm^2 Straight Neck, (Tissue Culture Flask, Corning, Corning, NY)
- Tissue Culture Dish, 100mm, 60mm, 35mm diameter, (Falcon®, Becton Dickenson, Franklin Lakes, NJ)

2.2.1.2 Protocol

Normal human keratinocytes were isolated from neonatal foreskin specimens obtained fresh from the nursery of the hospital after routine postnatal

circumcision. Primary cultures were initiated and maintained in an undifferentiated, replicative state by growth and passage at subconfluence in complete, serum-free MCDB 153 medium in 75cm^2 tissue culture flasks.[151;152]

Keratinocytes from primary cultures were plated into secondary culture at 1-10x10^3 cells/cm^2 in tissue culture dishes of variable diameter, depending on the experimental protocol. Cells were grown to confluence and fed with standard medium (without growth factors) for at least 48 hours before experiments to achieve quiescence.[153] After the treatment, cells were harvested for further experimental procedures.

2.2.2 Isolation and cultivation of peripheral blood mononuclear cells (PBMC)

2.2.2.1 Cell culture Media

- RPMI 1640 (Gibco, Grand Island, NY)
- 2% fetal/normal calf serum (Summit Biotechnology, Fort Collins, CO)
- Penicillin/Streptomycin (200 I.E./ml)

2.2.2.2 Protocol

Heparinized blood from healthy blood donors was obtained from the blood bank of the hospital. After 1:1 dilution with RPMI 1640, blood was transferred onto Ficoll-Paque (Pharmacia, Piscataway, NJ) to isolate PBMC by density gradient centrifugation. Therefore, samples were centrifuged @ 600 g for 30 minutes at room temperature, and the lymphocyte-rich cell compartment was separated. Cells were washed twice in RPMI 1640 with 2% fetal/normal calf serum and Penicillin/Streptomycin (200 I.E./ml) and maintained in this medium until utilization. For experiments, only PBMC isolated within 3 hours were used.

2.2.3 Cultivation of HaCaT-cells

The HaCaT cell line is a spontaneously immortalized keratinocyte cell line from adult skin which maintains full epidermal differentiation capacity and therefore has been used as an in vitro carcinogenesis model.[154]

HaCaT cells were stably transfected with the pcDNA1/Neo + bcl-2cDNA vector, and cells transfected with an empty vector served as controls (neo). The parent cell line was a kind gift of Dr. N. Fusenig, German Cancer Research Center (DKFZ), Heidelberg, Germany.

2.2.3.1 Cell culture Media

- DMEM (Dulbecco's modified eagle's medium)
- 10% FBS (fetal bovine serum) (Summit Biotechnology, Fort Collins, CO)
- Penicillin (50 U/ml)/Streptomycin (50μg/ml)
- Geneticin (400 μl/ml)

2.2.3.2 Protocol

HaCaT cells were fed with 10% FBS in DMEM, supplemented with Penicillin/Streptomycin. The medium for the bcl-2 overexpressing clones and the neo-cells was additionally supplemented with 400 μl/ml Geneticin which is used to separate out newly growing clones of cells. For experiments, HaCaTs were grown to confluence and then treated according to experimental protocols.

2.3 Detection of Lipid Peroxidation

Lipid Peroxidation Assay

2.3.1 Cells

- Confluent human keratinoctyes
- Peripheral blood mononuclear cells (PBMC)

2.3.2 Reagents and Materials

- cPA (cis-parinaric acid) (Molecular Probes, Eugene, OR)
- BHT (Butylated hydroxytoluene)
- Anthralin (Biomol Research Laboratories, Plymouth Meeting, PA)
- DMSO (Dimethyl Sulfoxide)
- Fetal Bovine Serum (Heat inactivated, GibcoBRL, Life Technologies, Grand Island, NY)
- PBS pH 7.4 (Phosphate buffered saline solution)
- Standard medium (Complete, serum-free MCDB 153 medium)
- RPMI-1640 medium (Bio Whittaker, Watersville, MD) with 10% fetal calf serum, (Summit Biotechnology, Fort Collins, CO)
- FACS Star Plus® (Becton Dickinson, San Jose, CA)

2.3.3 Protocol

Anthralin was prepared in fresh DMSO immediately before use. Cis-parinaric acid (cPA) was dissolved in 97% ethanol which was purged with nitrogen to remove residual oxygen from the solution. Additionally, 1 μg BHT per ml was added as antioxidant before aliquots were stored at –20°C.

Primary normal human keratinocytes were grown to confluence in 6-well plates. Cells were then loaded with 10 μM cPA for 60 minutes at 37ºC. Cells were washed three times with PBS, and 2ml of standard medium was added. Subsequently, keratinocytes were treated with anthralin which was dissolved in DMSO for designated times. Exposure times were terminated by removal of medium, and cells were trypsinized for 8 minutes. The trypsinisation process was stopped by addition of 2ml of 8% fetal bovine serum. Keratinocytes were

transferred into round-bottom tubes, fixed with 0.4% paraformaldehyde in PBS, and placed on ice.

PMBC were loaded with 10μM cPA in RPMI 1640® containing 10% fetal calf serum and then treated accordingly.

10,000 cells of either cell type (HK and PBMC) were immediately analyzed by flow cytometry using a Becton-Dickenson FACS Star Plus® with a multi-line ultraviolet argon laser at 100 mW power excitation and emission wave lengths of 334-354 nm and 424 nm, respectively. The mean fluorescence for the untreated control cells (only cPA staining) was consistently set at 300 arbitrary units. By flow cytometric gating of cPA intensities, lymphocytes could be selectively detected for further analysis. Monocytes exhibit a bright fluorescence pattern, presumably because of ingestion of fluorochrome, and could thus be differentiated from lymphocytes in all experiments measuring loss of cPA by flow cytometry.

For both cell types, the untreated vs. DMSO-treated control showed no difference in lipid peroxidation.

2.4 Laser scanning confocal microscopy

2.4.1 Cells

- Confluent normal human keratinocytes

2.4.2 Reagents and Materials

- cPA (cis-parinaric acid) (Molecular Probes, Eugene, OR)
- Anthralin in DMSO (Biomol Research Laboratories, Plymouth Meeting, PA)

2.4.3 Principle of mode of action

In laser scanning confocal microscopy, the object is illuminated and observed in the focal plane as the in-focus image which is separated from the light of the out-of-focus planes. Imaging may be performed in the reflective or in the fluorescence mode. Confocal microscopy allows accurate and non-destructive optical sectioning in a plane perpendicular or parallel to the optical axis of the microscope. Further digital three-dimensional treatments of the data may be performed so as to visualize the specimen from a variety of angles.[155]

2.4.4 Protocol

For experiments, keratinocytes were cultured on glass coverslips, loaded with cPA for 60 minutes at 37ºC and treated with anthralin. Since keratinocytes are adherent to coverslips, images could be obtained representing the same microscopic field of keratinocytes. PBMC, in contrast, were loaded with cPA and treated afterwards using a Neubauer chamber. Because PMBC are non-adherent and migrate in the microscopic field, comparable, but not identical fields of cells are depicted.

Confocal images were obtained with an inverted laser scanning confocal microscope LSM410 (Zeiss, Deissenhofen, Germany) equipped with a coherent ultraviolet laser, Innova 300 (Santa Clara, CA). The emitted 100 mW laser light of 364 nm for excitation was deflected by a dichroic and E_m-filter, LP 395 and LP 397, respectively. Fluorescent intensities obtained with microscopy were converted into pseudocolors using the program Analyze® (Mayo Foundation, Rochester, MN).

2.5 Assay of extracellular hydrogen peroxide (H_2O_2) production

2.5.1 Cells

- Confluent normal human keratinocytes

2.5.2 Reagents and Materials

- A6550 (Amplex Red) (Molecular Probes, Eugene, OR)
- Horseradish peroxidase
- Anthralin in DMSO (Biomol Research Laboratories, Plymouth Meeting, PA)

Cytofluor II, fluorescence plate reader (Perspectives Biosystems, Framingham, MA)

2.5.3 Protocol

In order to measure extracellular H_2O_2 production after treatment with anthralin, Amplex Red (A6550) was used, a derivative of dehydroxyphenoxazine, which becomes highly fluorescent upon oxidation by H_2O_2 in the presence of horseradish peroxidase (HRP). A6550 was dissolved in DMSO, stocks were aliquoted and stored at -20ºC.

For the assay, normal human keratinocytes were grown to confluence in 24 well plates (Costar®, Corning, NY), and 100 μM Amplex was added. After treatment with anthralin, 1 U/ml HRP was added to the samples, and fluorescence intensity was detected using a microplate fluorometer (Cytofluor II, Perspectives Biosystems, Framingham, MA) with excitation/emission wavelength filters set at 590/615 nm. Fluorescence intensity of A6550 was measured within the linear region of the H_2O_2 dose-response curve.

Samples without cells, but containing the same substrates and exposed to the same treatment protocol represented controls. The fluorescent values of these controls and the background fluorescence of untreated, but Amplex exposed, cells were monitored over time and subtracted from the relative fluorescence intensity of treated samples. These values were converted into absolute pico-moles using an H_2O_2 standard curve.

2.6 Cell death/Cell viability assays

2.6.1 Propidium iodide and DAPI staining

2.6.1.1 Cells

- Normal human keratinocytes

2.6.1.2 Reagents and Materials

- Propidium iodide
- DAPI (Molecular Probes, Eugene, OR)
- Anthralin in DMSO (Biomol Research Laboratories, Plymouth Meeting, PA)
- PBS pH 7.4 (Phosphate buffered saline solution)

2.6.1.2.1 PROPIDIUM IODIDE

Propidium iodide (PI) is a water soluble, cell-impermeant, red fluorescent phenanthridinium intercalator. PI is thus generally excluded from viable cells whose membranes are intact. It can be excited with mercury- or xenon-arc lamps or with the argon-ion laser, making it suitable for use in fluorescence microscopy. PI binds with little or no sequence preference to DNA, and upon binding, its fluorescence is enhanced 20- to 30-fold, and excitation and emission maxima are shifted.

Due to its ability to bind DNA, PI can be used as a marker of cell damage and cell death, because only then is intracellular DNA exposed to the extracellular environment.[156;157]

2.6.1.2.2 DAPI (4',6-DIAMIDINO-2-PHENYLINDOLE)

DAPI is a blue fluorescent that associates with the minor groove of double-strand DNA. This binding produces an approximately 20-fold fluorescence enhancement that can be visualized with a mercury-arc lamp or with the UV lines of the argon-ion laser. Using DAPI it is possible to differentiate viable from dead cells in vitro.

2.6.1.3 Protocol

For the experiments, PI and DAPI were solved in dialyzed water at 1 mg/ml. Confluent normal human keratinocytes were treated with different concentrations of anthralin, before they were simultaneously stained with both dyes for 30 minutes at 2 μg/ml final concentration. After staining, cells were washed with PBS twice and then kept moist in PBS for further microscopic examination by fluorescent microscopy with the appropriate filters. (UV-filter/rhodamine-filter).

Using 40x magnification and an ocular grid, dead cells were counted in five representative fields per 35 mm dish. Dead cells were identified by their bright fluorescence. Untreated control dishes were fixed in methanol, then stained with PI and DAPI, to quantitate the numbers of dead cells and adjusting this value to 100%.

2.6.2 LIVE/DEAD® Viability/Cytotoxicity Assay

2.6.2.1 Cells

- Normal human keratinocytes

2.6.2.2 Reagents and Materials

- LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR)
- Anthralin in DMSO (Biomol Research Laboratories, Plymouth Meeting, PA)
- n-Propyl-Gallate (nPG)
- Solution A (pH 7.4)
- Dialyzed H_2O
- 10.0 mM Glucose
- 3.0 mM KCl
- 130.0 mM NaCl
- 1.0 mM Na_2HPO_4 anhydrous
- 0.0033mM Phenol Red
- 30.0 mM Hepes

2.6.2.3 Background

The LIVE/DEAD® kit provides a two-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells with two substances known to measure parameters of cell viability – intracellular esterase activity (by calcein) and plasma membrane integrity (by ethidium homodimer-1).

The polyanionic calcein is well retained in live cells and produces an intense uniform green fluorescence (excitation/emission wavelengths: ~495 nm/~515 nm). Ethidium homodimer-1 (EthD-1), in contrast, enters cells with damaged membranes and undergoes a multi-fold (~40x) fluorescence enhancement upon binding to nucleic acid, thereby producing a bright red fluorescence in dead cells (excitation/emission wavelengths: ~495 nm/~ 635 nm). EthD-1 is excluded by the intact plasma membrane of a living cell and serves thus as a marker of cell damage and cell death.

Background fluorescence levels are inherently low in this assay kit, because the dyes used are virtually nonfluorescent before interaction with cells.

2.6.2.4 Protocol

For the assay, human keratinocytes were grown to confluence in 24-well plates, then treated with n-propyl-gallate, a known effective antioxidant[158;159] and anthralin, and after that washed with Solution A once. Equal amounts of Solution A were then added to the samples, and cells were stained with 6 μM EthD-1 for 30 minutes at 37ºC with continuous rocking. Following this, fluorescence readings were obtained using the Cytofluor II reader.

For all experiments, control samples were included which consisted of live, untreated cells as a negative control and dead cells (after exposure to 70% methanol for 30 minutes) as positive control.

Samples were treated in triplets, which means that for all experiments, cells in three wells were exposed to the same treatment.

The percentage of dead cells was computed as follows:

$$\text{Dead cells} = \frac{(\text{sample} - \text{negative control})}{(\text{dead cells} - \text{negative control})} \times 100$$

2.6.3 Cell death assay for PBMC using Trypan Blue

2.6.3.1 Cells

- Peripheral blood mononuclear cells

2.6.3.2 Reagents and Materials

- Trypan Blue (Gibco, Grand Island, NY)
- RPMI-1640 medium, (Bio Whittaker, Watersville, MD) with 2% fetal calf serum, (Summit Biotechnology, Fort Collins, CO)
- Anthralin in DMSO (Biomol Research Laboratories, Plymouth Meeting, PA)

2.6.3.3 Background

Trypan Blue is one of several stains commonly used in dye exclusion procedures for viable cell counting. This method is based on the principle that live cells do not take up certain dyes, whereas dead (non-viable) cells do so due to membrane damage. Staining with Trypan Blue facilitates visualization of cell morphology.

2.6.3.4 Protocol

For the experiment, PBMC were incubated for 24 hours at 37ºC and 5% CO_2 in RPMI 1640 media with 2% fetal calf serum, before they underwent anthralin treatment. After the treatment, cells were carefully resuspended and were thereafter diluted on a 1:1 basis with 0.4% Trypan Blue (Gibco, Grand Island, NY). Immediately following this, viable and dead cells were counted using a Neubauer chamber. Viable cells could be distinguished due to their blue color, while living cells appeared transparent or white. For each probe, two separate sets of 16 quadrants were counted, which equals approximately 0.2 µl of the cell suspension.

Samples were set up in triplicates, indicating that three samples received the same treatment. An average was calculated from these triplicates, and the standard deviation of the mean was computed accordingly.

2.7 JNK kinase assay

2.7.1 Cells

Confluent human keratinocytes, fed without growth factors for 48 hours.

PBMC isolated within 3 hours before the experiment.

2.7.2 Reagents and Materials

- N-Acetylcysteine (NAC)
- Ascorbic acid 6-palmitate (AA6P)
- n-Propyl-Gallate (nPG)
- Anthralin in DMSO (Biomol Research Laboratories, Plymouth Meeting, PA)

2.7.3 Buffers

2.7.3.1 WCE-BUFFER

- Dialyzed H_2O
- 25 mM Hepes pH 7.6 (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
- 0.3 M NaCl
- 1.5 mM $MgCl_2$
- 0.2 mM EDTA ([Ethylenedinitrilo]tetraacetic acid)
- 0.1% Triton X-100 (t-Octylphenoxypolyethoxyethanol)
- 20 mM β-Glycerophosphate
- 0.1 mM Na-Vanadate
- 0.5% PMSF 34.8 mg/ml in 95% Ethanol (Phenylmethanesulfonyl fluoride)
- 0.1% Leupeptin 10mg/ml
- 1% Aprotinin 0.12 TIU/ml protein lyophilized powder from bovine lung

2.7.3.2 B-BUFFER

- Dialyzed H_2O
- 20 mM Hepes pH 7.6
- 2.5 mM $MgCl_2$
- 0.1 mM EDTA
- 0.05% Triton X-100

- 20 mM β-Glycerophosphate
- 0.1 mM Na-Vanadate
- 0.5% PMSF 34.8 mg/ml in 95% Ethanol
- 0.1% Leupeptin 10 mg/ml
- 1% Aprotinin 0.12 TIU/ml protein lyophilized powder from bovine lung

2.7.3.3 HEPES BALANCED BUFFER (HBB)

- Dialyzed H_2O
- 20 mM Hepes pH 7.6
- 50 mM NaCl
- 2.5 mM $MgCl_2$
- 0.4 mM EDTA
- 0.05% Triton X-100

2.7.3.4 KB-BUFFER

- Dialyzed H_2O
- 20 mM Hepes pH 7.6
- 20 mM $MgCl_2$
- 20 mM β-Glycerophosphate
- 1 mM Na-Vanadate
- 10 μM ATP (Adenosine 5'-Triphosphate)
- 2 mM $_{DL}$-DTT ($_{DL}$-Dithiothreitol)

2.7.3.5 SAMPLE BUFFER (2X)

- Dialyzed H_2O
- 20 mM Tris pH 8.0
- 20% Glycerol
- 10% 2-Mercaptoethanol
- 2% SDS (Sodium dodecylsulfate)
- 2 mM EDTA
- 0.01% Bromophenol Blue

The sample buffer was stored at –20ºC in the freezer.

pGEX GST c-Jun was gratefully provided by Dr. M. Karin, University of California, San Diego, CA.

2.7.4 Protocol

Normal human keratinocytes were grown to confluence in 10 cm dishes. To turn off growth factor-activated signaling events, cells were incubated in growth factor free starvation medium for 48 hours before treatment.
PBMC isolated within 3 hours before the experiment were maintained in RPMI-1640 medium with 10% fetal calf serum and Penicillin/Streptomycin.
After treatment, cells were placed on ice, lysed by adding 450 µl of WCE buffer supplemented with protease and phosphatase inhibitors, containing 1% Triton X-100. Crude cell lysates were transferred to 1.5 ml tubes and rocked for 30 min at 4ºC to ensure complete lysis of cells. Triton soluble and insoluble fractions were separated by centrifugation @ 14000 g for 15 min at 4ºC using a standard table-top microcentrifuge. The supernatants containing the Triton soluble fractions were transferred to 2 ml tubes. 1350 µl of B-buffer was added containing fresh protease and phosphatase inhibitors, and cells were incubated on ice for 10 minutes.
To measure JNK kinase activity, recombinant GST c-Jun (amino acids 1-223), the GST-tagged transcription factor and substrate for JNK, was expressed and purified from E. coli bacteria and coupled to GSH-agarose obtained from Sigma®, St. Louis, MO. The pGEX GST c-Jun construct was a kind gift from Dr. Michael Karin, University of Califonia, San Diego, CA. For the assay, GST c-Jun agarose beads were diluted in a 1:4 proportion with HBB-buffer and 60 µl were added to the samples and rocked for >30 min at 4ºC. After that, samples were washed thoroughly with HEPES balanced buffer (HBB) and the supernatant was removed down to the pellet of beads.
To perform the kinase assay, beads were incubated in 30 µl kinase buffer containing 5 µCi [γ-^{32}P]ATP (Dupont NEN, Boston, MA) in a 30ºC water bath for 20 minutes. To terminate the kinase reaction, 30 µl of 4x standard sample buffer was added, and all proteins were denaturated by heat at 100ºC for 5 minutes

before starting a 12.5% SDS-polyacrylamide gel electrophoresis (National Diagnostic, Atlanta, GA). After separation of protein, the gel was dried for 90 minutes at 80ºC and phosphorylated GST c-Jun was detected by autoradiography. (Kodak, New Haven, CT)

In order to compare activation responses in both cell types more specifically, total JNK protein was measured using a specific JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the number of cells used for the experiments was calculated accordingly.

2.8 Immunoprecipitation of the EGF receptor

2.8.1 Cells

- Confluent normal human keratinocytes

2.8.2 Reagents and Materials

- N-Acetylcysteine (NAC)
- n-Propyl-Gallate (nPG)
- Ascorbic acid 6-palmitate (AA6P)
- Ammonium Pyrrolidine Dithiocarbamate (PDTC)
- 3-tert-Butyl-4-Hydroxyanisole (BHA)
- PD153035 (Parke-Davis, Ann Arbor, MI)
- Anthralin in DMSO (Biomol Research Laboratories, Plymouth Meeting, PA)

2.8.3 Frackelton Buffer

- Dialyzed H_2O
- 20 mM Tris pH 7.4
- 30 mM $Na_4P_2O_7$ (Sodium pyrophosphate)
- 40 mM NaCl
- 50 mM NaF
- 5 mM EDTA
- 0.5% Triton X-100
- 5% Glycerol
- 0.1 mM Sodium Orthovanadate
- 0.12 TIU/ml Aprotinin 0.12 TIU/ml protein lyophilized powder from bovine lung
- 10 μg/ml Leupeptin
- 1 mM PMSF 34.8 mg/ml in 95% Ethanol
- 5 μg/ml Pepstatin in 95% Ethanol

2.8.4 Antibodies

- Anti-Human EGF Receptor, clone LA1, neutralizing, monoclonal (Upstate Biotechnology, Lake Placid, NY)

- HRP coupled phosphotyrosine antibody, monoclonal (PY20:HRPO, Transduction Laboratories, Lexington, KY)
- 15E11, monoclonal antibody (a kind gift from Dr. Nita Maihle, Mayo Clinic Rochester, MN, USA)

2.8.5 Protocol

Normal human keratinocytes were grown to confluence in 10 cm dishes. To turn off growth factor-activated signaling events, cells were incubated in growth factor free starvation medium for 48 hours before treatment.

Cell lysates were obtained by scraping keratinocytes into 1 ml of cold Frackelton buffer followed by 15 minutes of centrifugation @ 14000 g. 10 μg of LA1 EGFR monoclonal antibody and Protein G-Plus Agarose beads (Oncogene, Cambridge, MA) were added to the supernatant. Samples were incubated for 2-3 hours at 4ºC with continuous movement. Following this, immunoprecipitates were repeatedly washed in Frackelton buffer, and mixed with 20 μl Laemmli lysis buffer (2x). After heating the samples at 100ºC for five minutes, immunoprecipitates were separated electrophoretically by 10% SDS-polyacrylamide gel (National Diagnostic, Atlanta, GA). Proteins were then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with 2% bovine serum albumin, before they were probed with peroxidase coupled anti-phosphotyrosine antibody and visualized using the ECL™ detection system (Amersham Life Science, Arlington Heights, IL).

Immunoblotting with EGFR monoclonal antibody 15E11 was performed to reveal equal protein loading.

2.9 Detection of activated ERK1 and ERK2

2.9.1 Cells

- Confluent normal human keratinocytes

2.9.2 Reagents and Materials

- PD098059 (Parke-Davis, Ann Arbor, MI)
- SB203580 (Smith-Kline Beecham Pharmaceuticals, UK)
- N-Acetylcysteine (NAC)
- 3-tert-Butyl-4-Hydroxyanisole (BHA)
- Ammonium Pyrrolidine Dithiocarbamate (PDTC)
- Anthralin in DMSO (Biomol Research Laboratories, Plymouth Meeting, PA)

2.9.3 Buffers

2.9.3.1 TBS (TRIS BUFFERED SALINE PH 7.4)

- Dialyzed H_2O
- 50 mM Trizma® Base [Tris(hydroxymethyl)aminomethane]
- 50 mM Trizma® HCl
- 150 mM NaCl

2.9.3.2 SDS RUNNING BUFFER

- Dialyzed H_2O
- 6.06 g Trizma® Base [Tris(hydroxymethyl)aminomethane]
- 28.8 g Glycine
- 2.0 g SDS (Sodium dodecylsulfate)

2.9.3.3 TRANSFER BUFFER

- Dialyzed H_2O
- 6.06 g Trizma® Base [Tris(hydroxymethyl)aminomethane]
- 28.8 g Glycine

2.9.3.4 WASH BUFFER

- TBS (Tris buffered saline pH 7.4)
- 0.1% Tween™ 20 (Polyoxyethylenesorbitan Monolaurate)

2.9.3.5 BLOCKING BUFFER

- Wash Buffer
- 2% BSA (Bovine Serum Albumine)

2.9.3.6 LAEMMLI SAMPLE BUFFER (2X)

- Dialyzed H_2O
- 20 mM Tris pH 8.0
- 20% Glycerol
- 10% 2-Mercaptoethanol
- 2% SDS (Sodium dodecylsulfate)
- 2 mM EDTA
- 0.01% Bromophenol Blue

The sample buffer was stored at –20ºC in the freezer.

2.9.4 Antibodies

- anti-ACTIVE™ MAPK polyclonal rabbit antibody (Promega, Madison, WI)
- Anti-Rabbit IgG, horse radish peroxidase conjugate (Promega Corp., Madison, WI)

2.9.5 Protocol

Normal human keratinocytes were grown to confluence in 35 mm dishes. To turn off growth factor-activated signaling events, cells were incubated in growth factor free starvation medium for 48 hours before treatment.

After treatment, cells were extracted in lysis (sample) buffer supplemented with protease and phosphatase inhibitors. Samples were then heated twice at 100ºC for 5 minutes and mixed well. 20μg of total protein were resolved by electrophoresis through a 10% SDS-polyacrylamide gel (National Diagnostic, Atlanta, GA) and then electrotransferred to Immobilon-p (Millipore Corp., Bedford,

MA) membranes. After blocking, membranes were incubated for 90 minutes at room temperature with anti-ACTIVE™ MAPK polyclonal rabbit antibody that selectively recognizes the dually phosphorylated, active form of the MAPK enzymes ERK1/ERK2.[160]

After thorough washing, membranes were incubated with secondary antibody coupled to peroxidase (Anti-Rabbit IgG), and immunoreactive proteins were detected using an enhanced chemiluminescence detection system. (ECL™, Amersham Life Science, Arlington Heights, IL)

3 RESULTS

3.1 Effects of anthralin on lipid peroxidation in human keratinocytes (HK) and peripheral blood mononuclear cells (PBMC)

In order to characterize the initial rapid events that are induced by diffusable peroxides following anthralin exposure, we measured lipid peroxidation of cellular membranes as a representative determinant of damage to cellular constituents in human keratinocytes (HK) and in human peripheral blood mononuclear cells (PBMC).

The naturally fluorescent fatty acid cis-parinaric acid is structurally analogous to intrinsic membrane lipids[161] and is readily incorporated into cellular membranes.[162] Its extensive unsaturation makes it quite susceptible to oxidation, a property that can be exploited for detection of lipid peroxidation. Upon oxidation, it undergoes a stoichiometric loss in fluorescence that can be directly monitored by flow cytometry.[163]

The stable loss of cPA fluorescence measured by flow cytometry provides a quantitative measurement of lipid membrane damage that has been shown in different cell culture systems.[164;165]

HK and PBMC cells were loaded with cPA for 60 minutes, then washed and treated with anthralin.

3.1.1 Lipid peroxidation after anthralin treatment follows a dose-response curve that is specific for both cell types

Lipid peroxidation in PBMC decreased after much lower concentrations of anthralin than in HK. In PBMC, a decrease in fluorescence occurred at 100 nM anthralin and showed a dramatic decline after exposure to 0.3 μM anthralin over 30 minutes of treatment (see *Figure 3.1*). It reached a minimum at 3 μM anthralin and remained stable at higher concentrations.

In contrast, HK were much less sensitive and only showed significant decrease in fluorescence after treatment with 3 μM anthralin which continued to decrease with increasing concentrations, up to 100 μM anthralin for 30 minutes (see *Figure 3.1*).

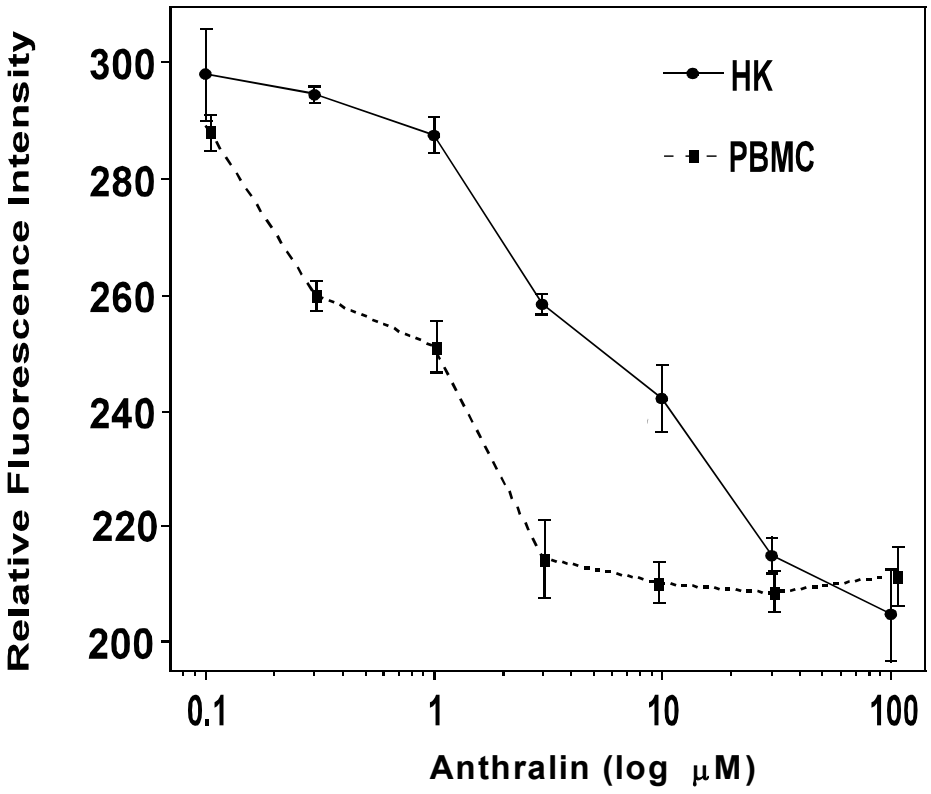

Figure 3.1:

The response to anthralin is dose-dependent in both cell types. PBMC are substantially more responsive to anthralin at lower doses than are HK. Cells were treated for 30 minutes. Results are representative of three independent experiments, with the error bars indicating the standard deviation of the mean.

3.1.2 Lipid peroxidation after anthralin treatment is time-dependent

Enhanced lipid peroxidation, as expressed by loss of cPA fluorescence intensity, was observed within 5 minutes after anthralin treatment in both cell types, and it further increased over 35 minutes of treatment (see *Figure 3.2*). The rapid onset and dramatic loss of fluorescence after anthralin exposure indicates that lipid peroxidation is a prominent early event in the cellular response to anthralin.

The specific concentrations used in the above experiments were based on the different sensitivities to lipid peroxidation of each cell type that were measured in experiments investigating the LPO response to different doses of anthralin.

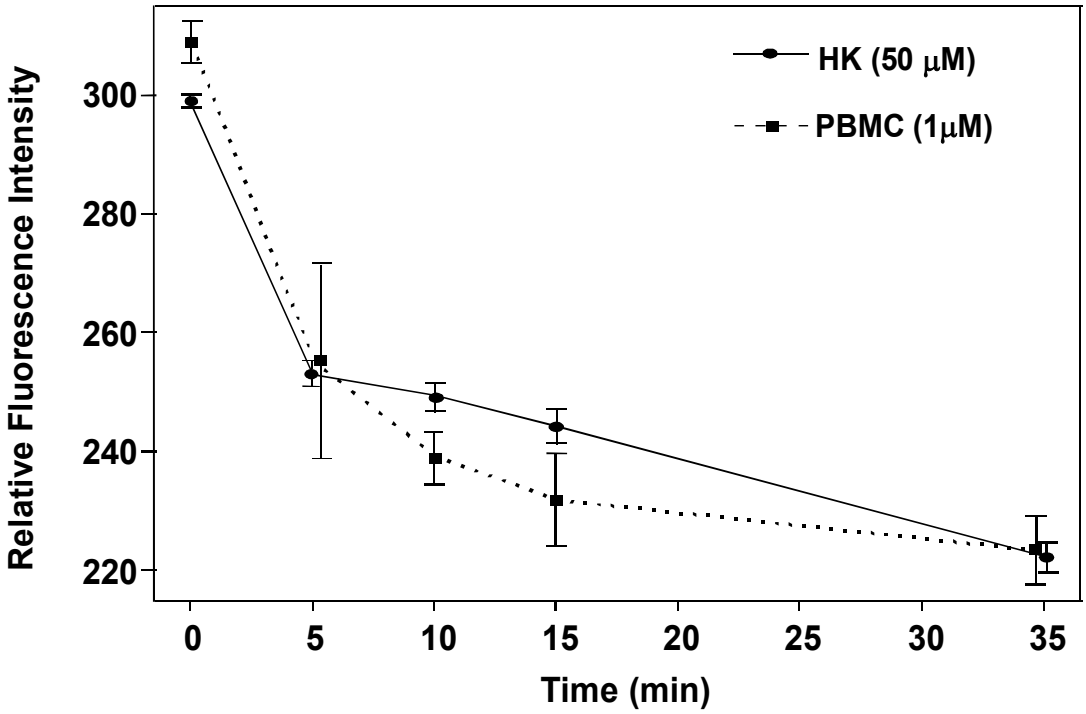

Figure 3.2:

Time-dependent decrease of cPA fluorescence intensity using 50μM anthralin for HK or 1μM anthralin for PBMC. (PBMC were specifically gated on lymphocytes during FACS analysis.) Results are representative of three independent experiments.

3.1.3 Laser scanning confocal microscopy of anthralin induced lipid peroxidation

The quantitative increase in lipid oxidation was visualized by laser-scanning confocal microscopy. Cells (HK and PBMC) pretreated with cPA were treated with anthralin using equi-dose concentrations of anthralin in HK and PBMC (i.e. doses that induce equal loss of fluorescence in both cell types) (see *Figure 3.1*). An increase in lipid peroxidation, as shown by loss of fluorescence, was observed within 15 minutes after anthralin treatment of PBMC (3 μM anthralin) and HK (100 μM anthralin) compared to untreated control cells. Fluorescence intensity of digitized images was converted into pseudocolors, where red color represents high fluorescence intensities versus blue color which indicates decreased fluorescence intensities after lipid peroxidation.

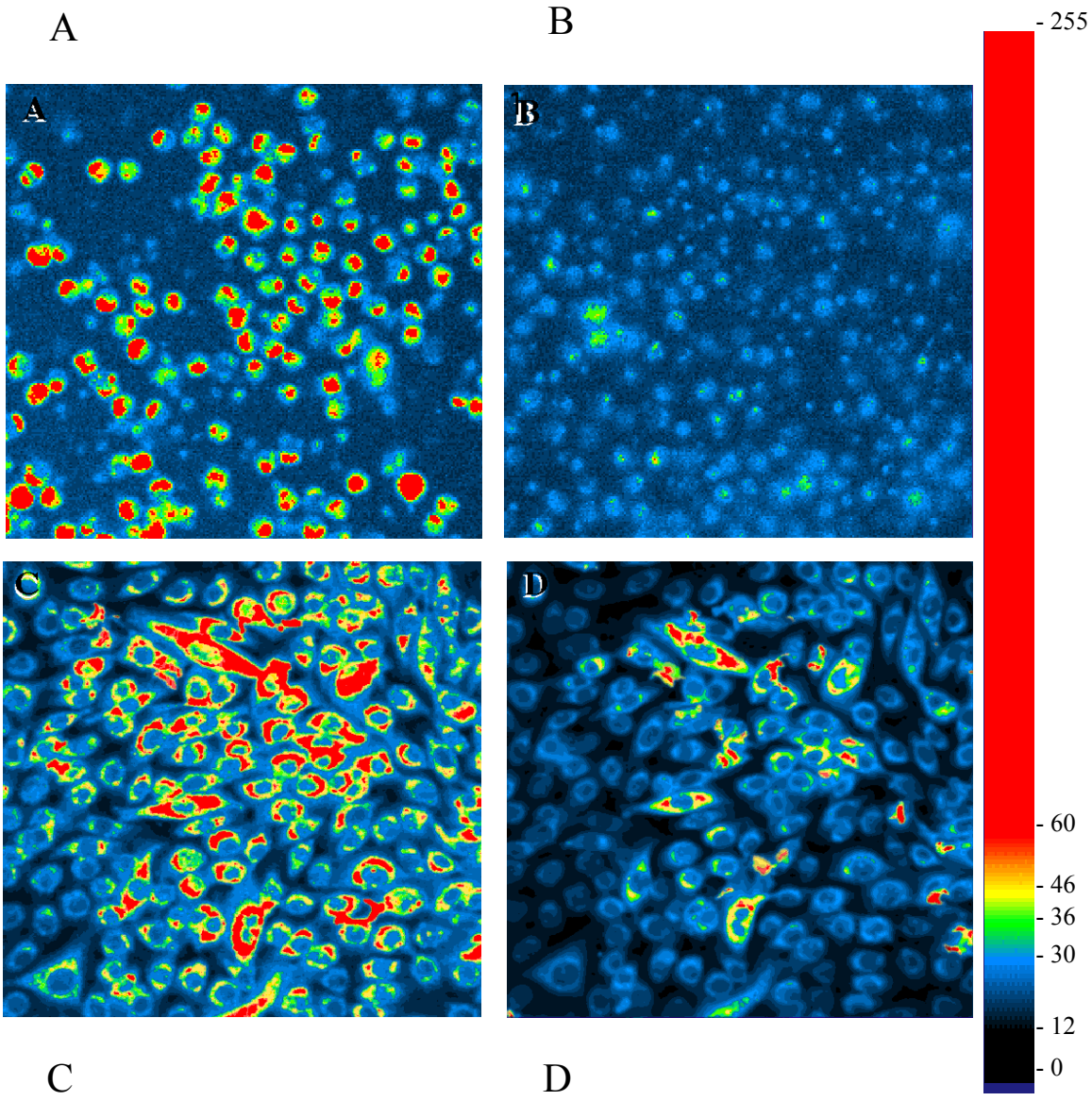

Figure 3.3:

Anthralin induces loss of fluorescence of cPA in keratinocytes and PBMC.

Increased lipid peroxidation indicated by loss of cPA fluorescence intensity is shown using laser scanning confocal microscopy. In panels A and B, different fields of PBMC in a Neubauer chamber are depicted. Panels C and D represent the same field of adherent keratinocytes. Images were obtained before (A and C) and after (B and D) treatment with anthralin for 15 minutes, and fluorescence intensities were converted into pseudocolors. Original magnifications: A and B: x 400, C and D: x 200.

3.2 Generation of extracellular H_2O_2 after Anthralin treatment, as measured with Amplex Red®

Even though anthralin has been in clinical use for many decades, its oxidizing capacity in epidermal keratinocytes has not yet been precisely characterized. To characterize anthralin induced signaling events and their sequence, the time course of anthralin induced H_2O_2 generation was initially established using an in situ assay. Amplex Red, a redox sensitive fluoroprobe, was used to measure the anthralin induced increase of H_2O_2 generation and to quantitate extracellular release of H_2O_2 in a time-dependent manner using a standard curve.

H_2O_2 generation was detected within minutes of anthralin exposure and increased during the first 120 minutes in a near linear fashion. (See *Figure 3.4*) In order to investigate if H_2O_2 generation is concentration-dependent, human keratinocytes were treated with different concentrations of anthralin for 60 minutes and a concentration-dependent pattern of H_2O_2 generation was detected. (See *Figure 3.5*)

These data demonstrate that H_2O_2 generation is rapidly induced after anthralin treatment of keratinocytes and may therefore be an important early, upstream event for the initiation of intracellular signaling.

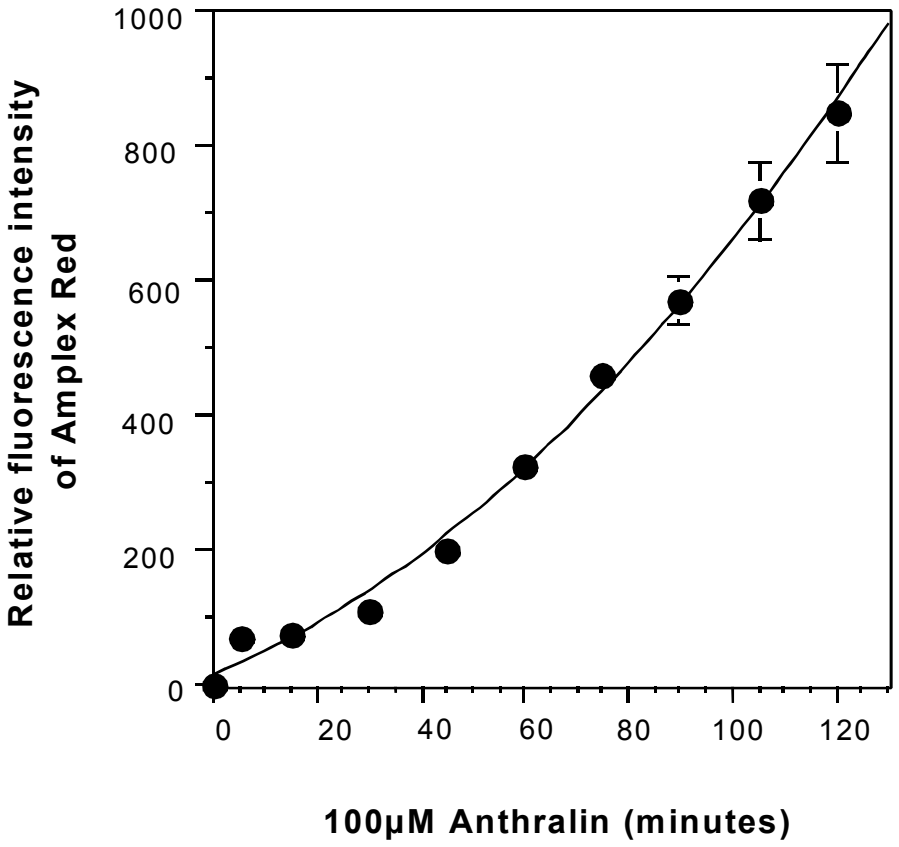

Figure 3.4:

H_2O_2 production as measured by Amplex Red is time-dependent in HK.

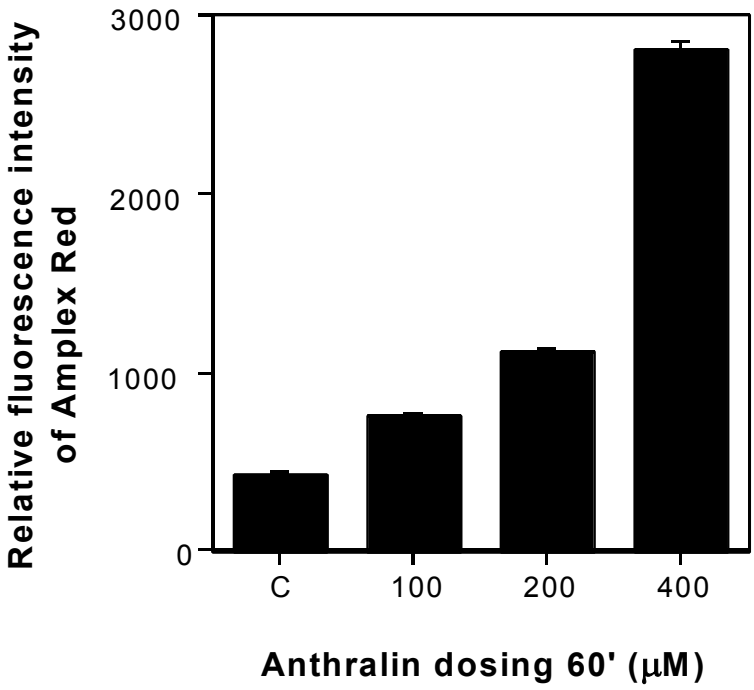

Figure 3.5:

H_2O_2 production as measured by Amplex Red is dose-dependent.

3.3 JNK-induction by Anthralin in human keratinocytes and PBMC

Anthralin is a known inducer of ROS[85], and ROS are involved in the activation of JNK in several cell types.[166-169] As described above, mononuclear cells play a pivotal role in the pathogenesis of psoriasis and are a major target in the treatment of the disease.[43;56;170]

Therefore, we examined whether anthralin induces JNK activation in human keratinocytes and PBMC. To more specifically compare activation responses in both cell types, total JNK protein was measured in keratinocytes and PBMC using a specific JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Based on the result of this measurement, the number of cells of either cell type for the following experiments was calculated accordingly. Cells were treated as described in detail in Materials and Methods.

3.3.1 Anthralin induces JNK in a dose-dependent manner

To identify the optimum concentrations of anthralin to be used in both cell types, JNK activation was investigated after different doses of anthralin at specified time points. In both cell types, JNK activation was dose-dependent: in keratinocytes, it was maximally induced by 200 μM anthralin for 90 minutes in the experiment shown here, whereas JNK in PBMC was strongly activated at considerably lower (20-40-fold lower) concentrations, with a strong signal at 5 μM of anthralin for 90 minutes and a maximum at 50 μM.

In control experiments, a strong signal of JNK induction could be observed at lower doses of 100 μM (data not shown). These variations may be due to the fact that the keratinocytes used in these experiments were derived from different, individual strains of HK and that these may also differ slightly in their sensitivity to anthralin.

These results indicate that HK are much more resistant to anthralin induced oxidative stress than PBMC and that PBMC could be the primary targets of anthralin therapy in psoriasis.

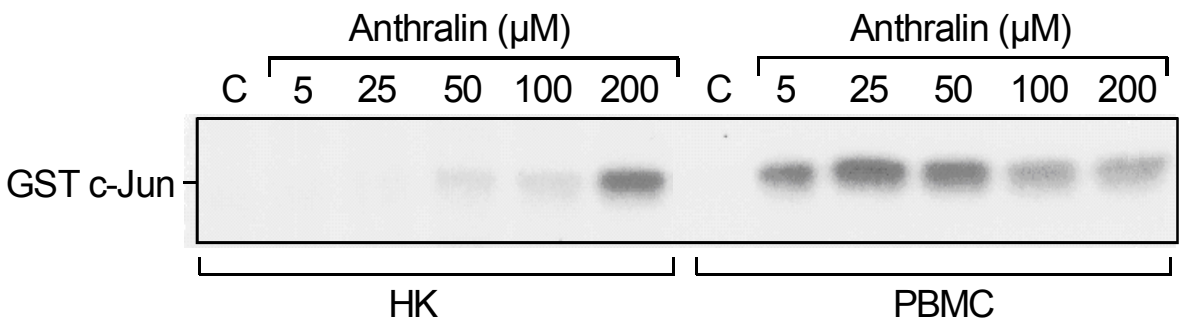

Figure 3.6:

JNK activation after anthralin treatment for 90 minutes in keratinocytes and PBMC follows a concentration-dependent course.

3.3.2 Time course of JNK activation after anthralin treatment

Confluent keratinocytes (deprived of growth factors for 48 hours) and freshly isolated PBMC were treated with equipotent doses of anthralin, 100 µM or 5 µM, respectively, based on previous experiments. JNK activation followed a similar time course in both cell types: it occurred within 30 minutes of exposure and reached maximum levels in keratinocytes after 120 minutes and in PBMC after 90 minutes.

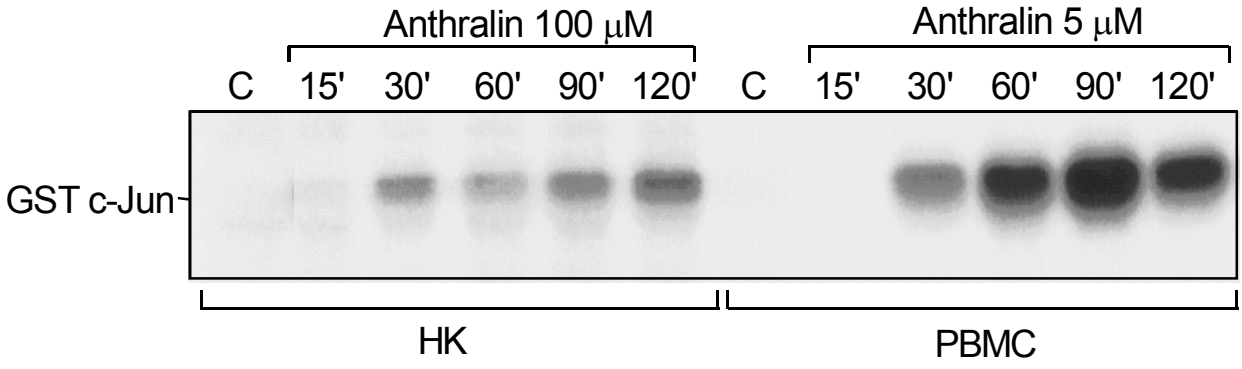

Figure 3.7:

Time course of JNK activation in human keratinocytes and PBMC after anthralin treatment at 100 µM or 5 µM concentration, respectively.

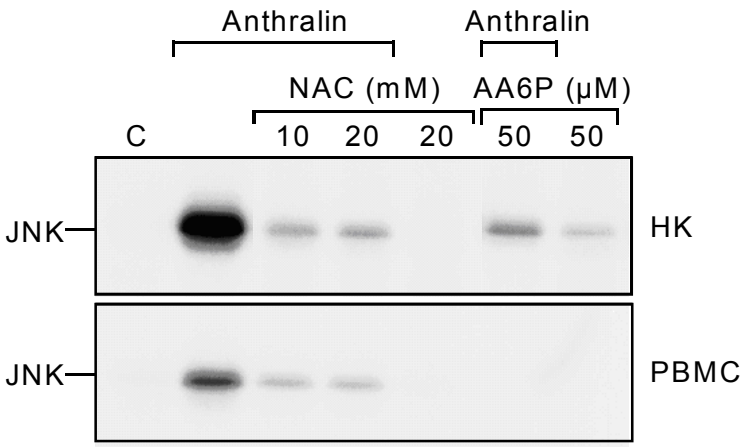

Figure 3.8:

Inhibition of anthralin induced JNK activity by NAC and AA6P pretreatment.

Inhibition of JNK activation by nPG differed in HK and PBMC. In HK, only at the higher dose of 200 µM of nPG a JNK-inhibition could be observed, whereas 100 µM did not show a significant effect. In PBMC, JNK was potently inhibited at both doses of nPG.

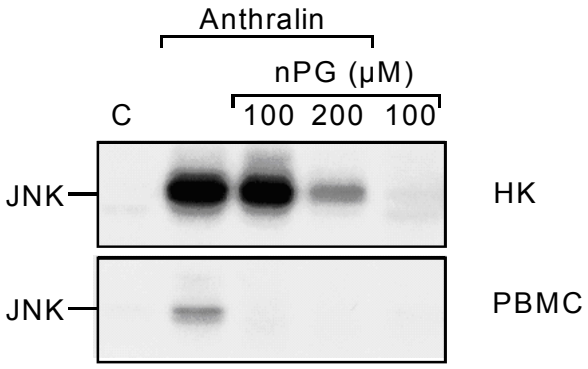

Figure 3.9:

Pretreatment with nPG inhibits JNK activation after anthralin treatment. HK and PBMC show different sensitivities to the antioxidant effects of nPG.

3.3.3 Bcl-2 overexpressing HaCaT cells show no JNK activation after anthralin treatment

HaCaT cells stably overexpressing Bcl-2 protein were used to examine the effect of this antiapoptotic protein on the effect of reactive oxygen species induced by anthralin treatment.

Bcl-2 is the first identified survial gene involved in the control of apoptosis, a highly organized, physiologic mechanism of programmed cell death.[171] The exact mechanism by which Bcl-2 functions to suppress apoptosis is still unclear, but phosphorylation may be required to regulate its function.[172-176] Bcl-2 has been localized to mitochondria, endoplasmic reticulum and nuclear membranes, sites where reactive oxygen species are generated. Bcl-2 does not appear to influence the generation of oxygen free radicals directly, but prevents oxidative damage to cellular constituents including lipid membranes.[177]

HaCaT cells were grown to confluence, and all three cell clones, the parental HaCaTs, the neo-control and the Bcl-2-overexpressing clone were treated with 100 μM anthralin for 60 minutes. JNK activity was assessed as described in Materials and Methods.

The control cells showed strong activation of JNK phosphorylation by anthralin, whereas JNK activity was completely inhibited in the Bcl-2 overexpressing clone suggesting that Bcl-2 plays a role in preventing cellular stress from reactive oxygen species.

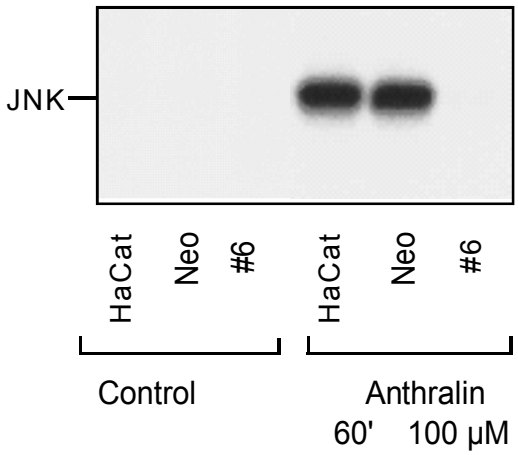

Figure 3.10:

JNK activity after treatment with anthralin (100 µM) for 60 minutes is inhibited in the Bcl-2 overexpressing HaCaT cell clone.

3.4 Effects of Anthralin on cell viability

3.4.1 Anthralin treatment results in dose-dependent cell death in human keratinocytes

Confluent keratinocytes were treated with a range of doses (0.3 μM – 100 μM) of anthralin for 45 minutes, and then stained with DAPI (4',6-diamidino-2-phenylindole) and propidium iodide (PI) dyes to visualize dead cells. Cells were counted utilizing an ocular grid, and an average of five representative visual fields was computed in each treated sample.

Results are shown as percentage of dead cells as compared to the untreated control which was set at zero percent cell death, with error bars indicating the standard deviation of the mean. The percentage of dead cells increased dramatically with higher concentrations of anthralin, reaching maximum levels of approximately 50% cell death at 100 μM anthralin for 45 minutes.

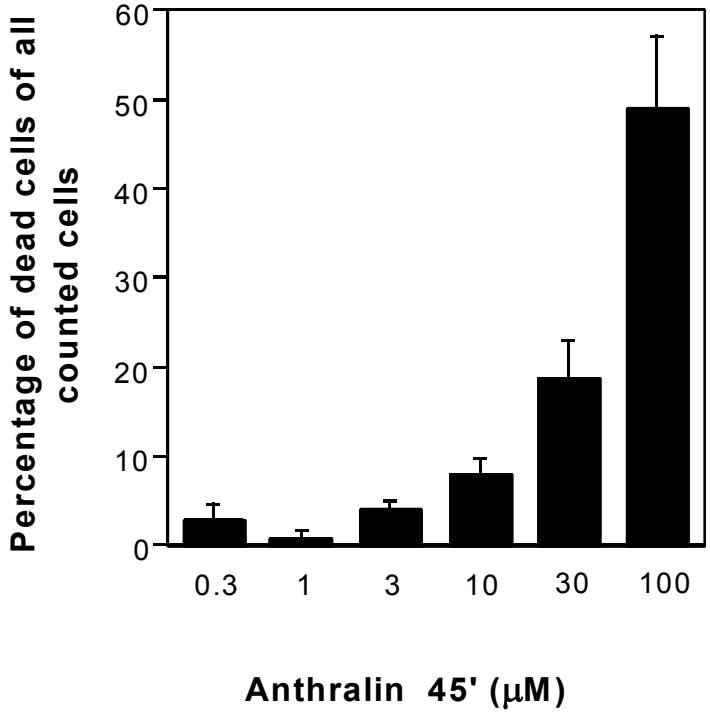

Figure 3.11:

Anthralin treatment for 45 minutes at designated concentrations results in increasing percentages of dead cells as assessed by DAPI and PI staining. Error bars show the standard deviation of the mean of five separate, representative visual fields.

3.4.2 Pretreatment with the antioxidant n-propyl-gallate (nPG) reduces the percentage of dead keratinocytes after anthralin treatment

Confluent normal keratinocytes grown in 24-well plates were pretreated with different doses of n-propyl-gallate (nPG) for 30 minutes, before 100 μM anthralin was added for 90 minutes. Cell death was assessed using the LIVE/DEAD® kit as described in Materials and Methods.

Cell death was measured as increase in fluorescence of ethidium homodimer (EthD-1). Pretreatment with nPG (50 μM, 100 μM, 200 μM) successfully reduced the cytotoxic effect of anthralin in a dose-dependent fashion as compared to anthralin treatment alone. It is remarkable that 200 μM nPG prior to anthralin is most effective, even though the control samples, treated with 200 μM nPG but no anthralin, showed a higher percentage of dead cells.

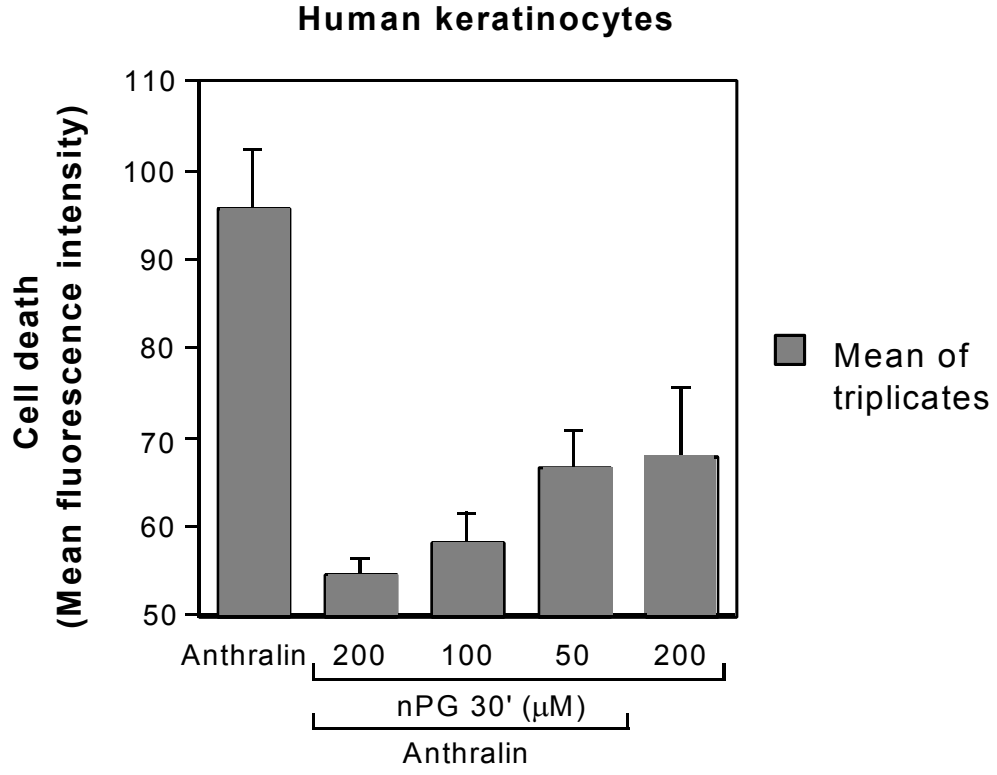

Figure 3.12:

Keratinocytes were pretreated with different concentrations of nPG for 30 minutes, before 100 μM anthralin was added. Subsequent cell death was measured as increase in mean fluorescence intensity of EthD-1.

3.4.3 Cell viability after treatment of PBMC with anthralin and n-propyl-gallate as assessed with Trypan Blue

PBMC were treated with 5 μM anthralin for 90 minutes before cell death was measured utilizing Trypan Blue staining. Additionally, samples were pretreated with different concentrations (1 μM, 20 μM, 200 μM) nPG for 30 minutes, before anthralin (5 μM) was added for 90 minutes and cell death was assessed.

Anthralin treatment resulted in an increase of dead cells compared to untreated controls from less than 5% to greater than 40%. Pretreatment with the antioxidative reagent nPG slightly decreased percentages of cell death at the lower concentrations of 1 μM and 20 μM, but not at 200 μM.

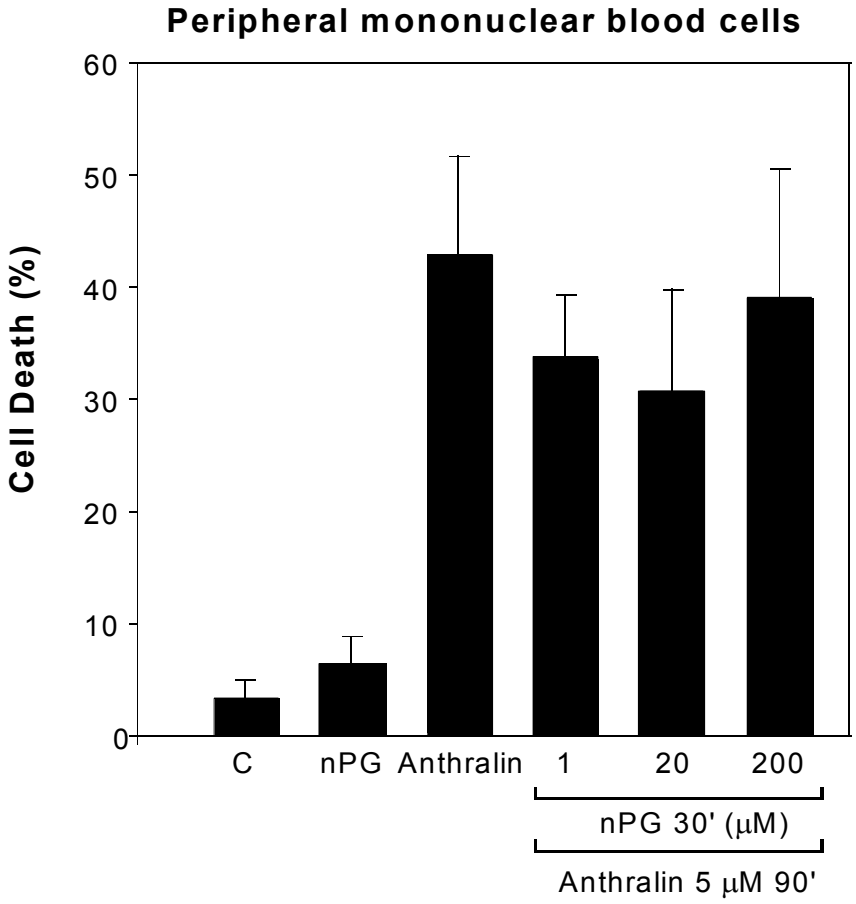

Figure 3.13:

Cell death after anthralin treatment alone rises to over 40% dead cells. Pretreatment with nPG for 30 minutes at designated doses decreases percentage of cell death slightly, but not significantly.

3.5 Effects of Anthralin on EGFR phosphorylation

After demonstrating the effect of anthralin treatment on JNK activity and stress related cellular responses in human keratinocytes, investigation of potential survival mechanisms was undertaken. Experiments were focused on the EGF receptor (EGFR) which is known to play an important role in keratinocyte differentiation and survival mechanisms.[119-121]

EGFR phosphorylation was assessed as described in Materials and Methods. Immunoblotting with EGFR monoclonal antibody 15E11 which detects total EGFR was performed to control for equal loading of protein in the described experiments.

3.5.1 Kinetics of anthralin induced EGFR phosphorylation

Confluent, resting human keratinocytes were treated with 100 μM anthralin for different time intervals, between 5 and 120 minutes. Within 15 minutes, phosphorylation of the EGFR was detected which reached maximum levels 60 minutes after anthralin exposure and plateaued at high levels afterwards.

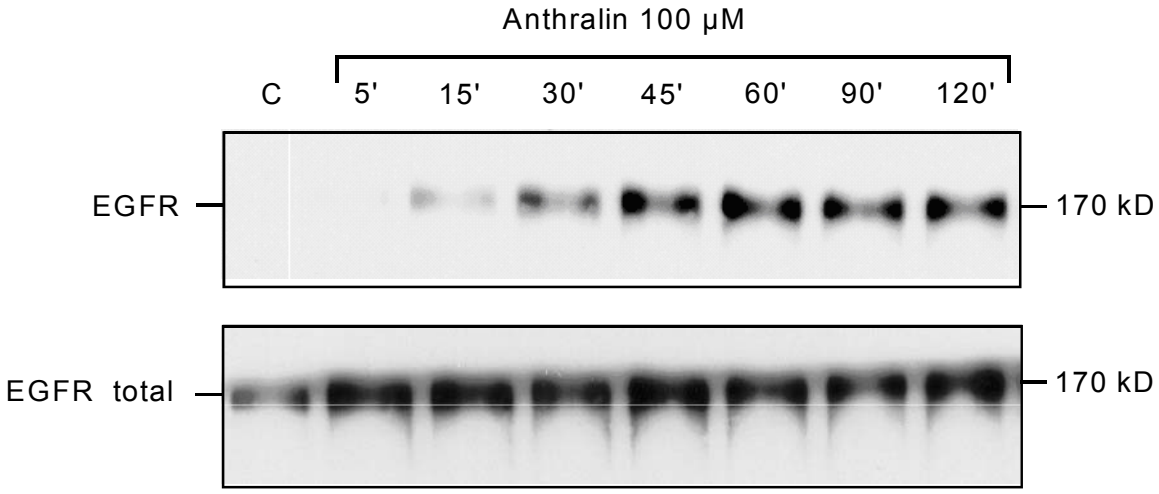

Figure 3.14:

EGFR phosphorylation after exposure to 100 μM anthralin is time-dependent. The lower blot depicts total EGFR after stripping PY20-antibody from the membrane and immunoblotting with 15E11 antibody.

3.5.2 EGFR-phosphorylation follows a concentration-dependent course

Keratinocytes were treated with different concentrations (range 10 – 300 μM) of anthralin for 30 minutes, before total cell protein was immunoprecipitated with monoclonal EGFR antibody. At concentrations as low as 10 μM, anthralin induced EGFR phosphorylation, and with increasing concentrations, EGFR phosphorylation also increased to reach maximum levels at 300 μM anthralin.

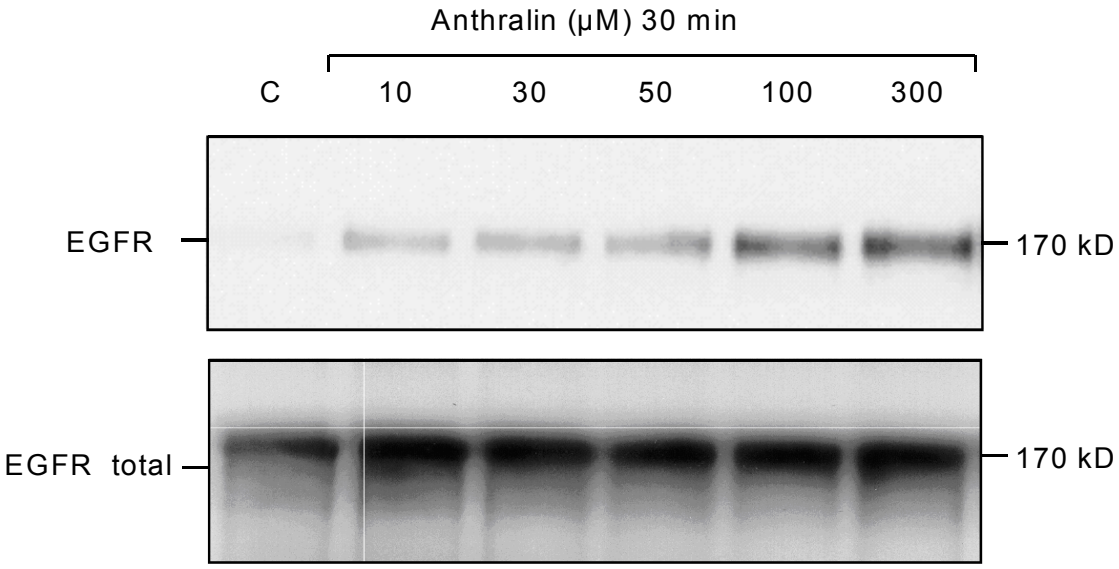

Figure 3.15:

Dose-dependent course of EGFR phosphorylation after treatment with anthralin for 30 minutes at designated concentrations.

3.5.3 The specific EGFR-inhibitor PD153035 is able to inhibit EGFR phosphorylation induced by anthralin

The synthetic compound PD153035 (Parke Davis, Ann Arbor, MI) has been found to be a potent and specific inhibitor of the EGF receptor tyrosine kinase that suppresses tyrosine phosphorylation of the EGF receptor at nanomolar concentrations in cell culture.[178] We therefore examined the effects of PD153035 on EGFR phosphorylation induced by anthralin treatment.

Confluent, resting keratinocytes were pretreated with 100 nM and 500 nM PD153035 for 30 minutes, before 100 μM anthralin was added for 60 minutes. Even at the lower dose, PD153035 potently inhibited anthralin induced EGFR phosphorylation. EGFR phosphorylation in the PD153035 treated condition also was decreased to below baseline levels.

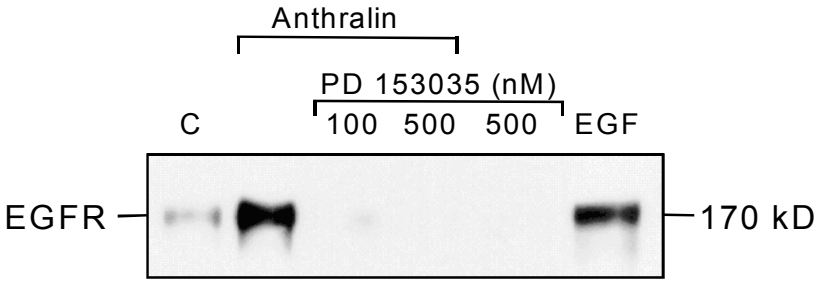

Figure 3.16:

Keratinocytes were pretreated with 100 nM or 500 nM PD153035, respectively, prior to exposure to 100 μM anthralin for 60 minutes.

3.5.4 Structurally unrelated antioxidants NAC, nPG, AA6P, PDTC, and BHA inhibit anthralin induced EGFR phosphorylation

To test whether reactive oxygen species are involved in EGFR phosphorylation induced by anthralin, cells were pretreated with diverse, structurally unrelated antioxidants.[179;180]

Confluent keratinocytes were fed with growth factor deprived media for 48 hours to down-regulate growth factor-activated signaling events. Cells were then pretreated for 30 minutes with PDTC (10 μM or 50 μM), BHA (10 μM or 50 μM), nPG (5 μM or 25 μM), AA6P (5 μM or 25 μM), or with NAC (10 mM or 20 mM) for eight hours. After pretreatment, they were exposed to 100 μM anthralin for 60 minutes. As control, cells were treated with EGF (10 ng/ml) for 15 minutes. In all experiments, keratinocytes were exposed to the antioxidative agent alone to exclude any receptor phosphorylation independent of anthralin treatment.

Inhibitory results vary among antioxidants used and also depend on the dose utilized:

- PDTC exerts moderate inhibition at the lower dose of 10 μM, but little effect at the higher dose of 50 μM pretreatment.
- Similarly, BHA strongly inhibits anthralin induced EGFR phosphorylation at 10 μM pretreatment, but fails to do so at the higher dose of 50 μM. (See *Figure 3.17*)

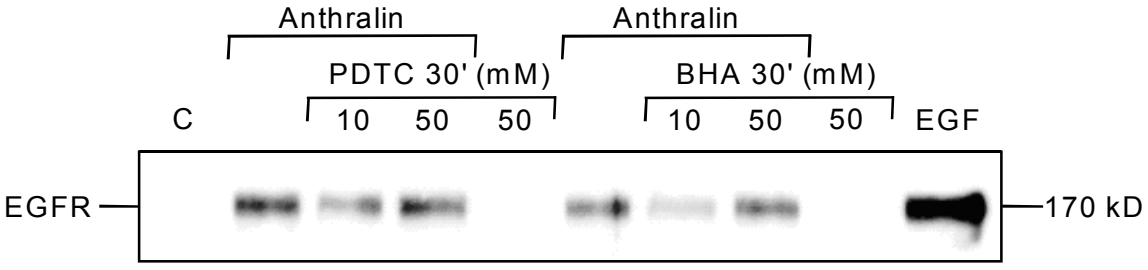

Figure 3.17:

Pretreatment with PDTC or BHA for 30 minutes prior to 100 μM anthralin treatment.

At the higher dose of 25 μM, nPG even intensifies the phosphorylation signal compared to baseline levels, whereas the lower dose of 5 μM is capable of moderate inhibition.

Pretreatment with 5 μM AA6P resulted in considerable inhibition of anthralin induced EGFR phosphorylation, but not the higher dose of 25 μM AA6P. (See *Figure 3.18)*

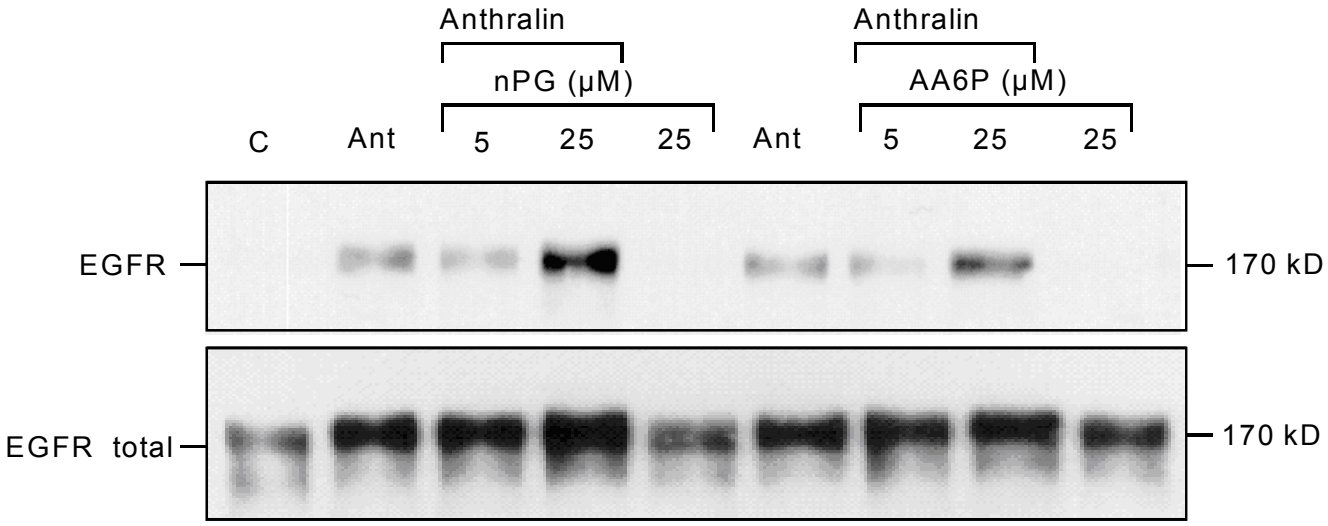

Figure 3.18:

Confluent, resting keratinocytes were exposed to nPG or AA6P for 30 minutes before 100 μM anthralin for 60 minutes.

Eight hour pretreatment with the radical scavenger NAC showed strong inhibition of EGFR phosphorylation at the lower dose of 10 mM and complete inhibition at 20 mM. (See *Figure 3.19*)

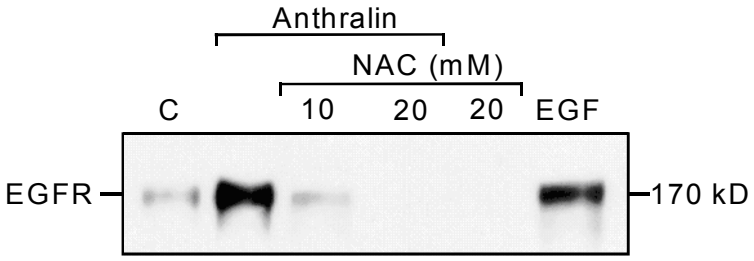

Figure 3.19:

Eight hour pretreatment with NAC, before cells were exposed to 100 μM anthralin for 60 minutes.

These results show a concentration-dependent effect of PDTC, BHA, nPG and AA6P which can be explained by known pro-oxidant versus anti-oxidant effects of these substances in cultured human keratinocytes.[181-183]

3.6 Effects of Anthralin on MAPK phosphorylation

The mitogen activated protein (MAP) kinases (MAPK), also referred to as extracellular signal-regulated kinases (ERK) are members of a family of enzymes that are regulated by many hormones and growth factors and whose activation can lead to changes in properties of cytoplasmic, membrane-associated, and nuclear proteins. MAPKs are widely involved in cellular proliferation and survival pathways.

We investigated if MAPKs in keratinocytes are also induced by anthralin treatment. Normal human keratinocytes were grown to confluence and maintained on growth factor deprived medium for 48 hours before experiments. Activation of ERK1 and ERK2 was determined using a phospho-antibody that selectively binds to phosphorylated, activated ERK1/2.

3.6.1 Anthralin activates ERK1/2 in a time-dependent manner

Cells were treated with 50 µM anthralin for designated times. ERK1/2 phosphorylation was induced after 5 minutes, and maximum activation was observed by 45 minutes following treatment. Maximum levels of activation did not increase further, but a second peak of activity appeared by 2 hours.

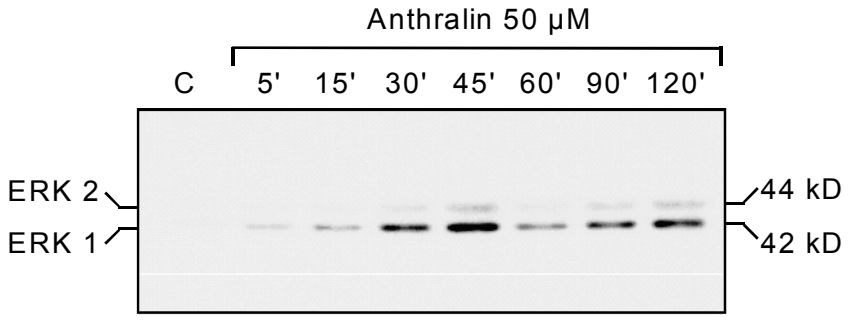

Figure 3.20:

Keratinocytes were monitored for 5 to 120 minutes after anthralin (50µM) exposure, before endogenous, activated ERK1/2 was detected by Western blotting.

3.6.2 ERK1/2 phosphorylation is induced by different concentrations of anthralin

Keratinocytes were incubated with 3 – 300 μM anthralin for 30 minutes. Concentration-dependent increase in kinase phosphorylation was observed which peaked at 60 – 100 μM anthralin, and declined at higher doses.

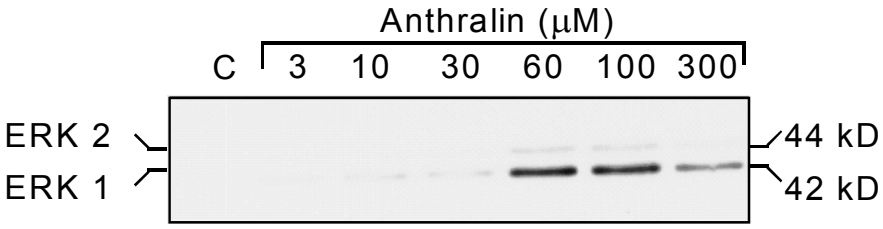

Figure 3.21:

Dose-dependent ERK1/2 activation by anthralin treatment with indicated doses, before immunoblotting with anti-active ERK1/2 antibody was performed.

3.6.3 PD098059 inhibits anthralin induced ERK1/2 phosphorylation

ERK1/2 are phosphorylated and activated by another enzyme, MEK1 (MAPK/ERK kinase), which can be specifically inhibited by the synthetic antagonist PD098059. PD098059 does not inhibit ERK1/2 activity itself, hence activated, phosphorylated ERK1/2 can be quantified.

Cells were treated with 3 μM, 10 μM, or 30 μM PD098059 for 30 minutes prior to anthralin exposure (50 μM for 30 minutes). ERK1/2 phosphorylation was strongly inhibited, and activation levels within cells treated with PD098059 were below baseline intensities.

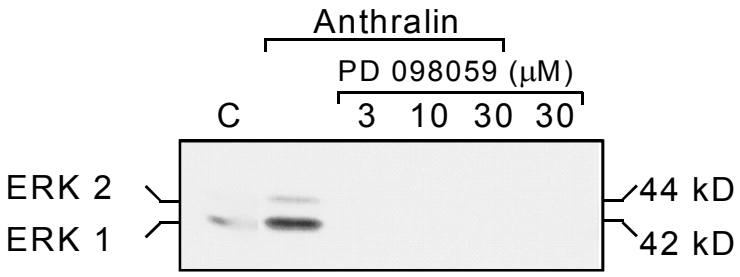

Figure 3.22:

Pretreatment with PD098059 results in complete inhibition of anthralin induced ERK1/2 phosphorylation.

3.6.4 PD153035 pretreatment inhibits anthralin induced ERK1/2 activation

In order to identify potential signaling pathways in keratinocytes treated with anthralin, the specific EGFR-inhibitor, PD153035, was used. Cells were pretreated with 20 nM, 100 nM, or 500 nM PD153035, respectively, for 30 minutes and then exposed to 50 μM anthralin for 30 minutes, a dose that is known to potently activate ERK1/2.

ERK1/2 phosphorylation was completely blocked by all concentrations of PD153035 used.

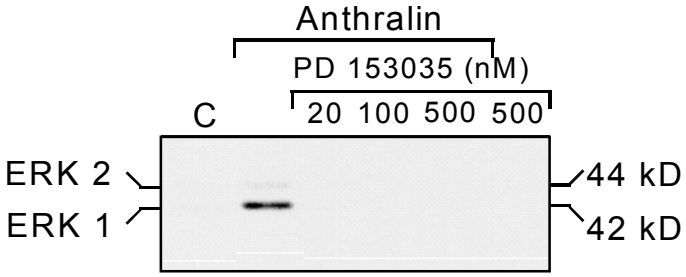

Figure 3.23:

Pretreatment with the EGFR-inhibitor PD153035 for 30 minutes results in inhibition of ERK1/2 activation by 50 μM anthralin for 30 minutes.

3.6.5 ERK1/2 phosphorylation is inhibited by SB203580

SB203580 is a synthetic antagonist that acts as a specific inhibitor of the MAP kinase family member p38/SAPK2.[184;185] We observed that pre-incubation of cells with SB203580 for 30 minutes also effectively inhibited anthralin induced ERK1/2 activation.

Confluent, resting keratinocytes were pre-incubated with different doses of SB203580 and then exposed to 50 μM anthralin for 30 minutes. 0.1 μM, 1 μM and 10 μM of SB203580 potently inhibited ERK1/2 phosphorylation. This is concordant with other studies that have also demonstrated that SB203580 is a less specific inhibitor than originally reported, but also exerts inhibitory effects on other protein kinases, such as LCK (lymphocyte kinase) or PKBα (protein kinase B).[186]

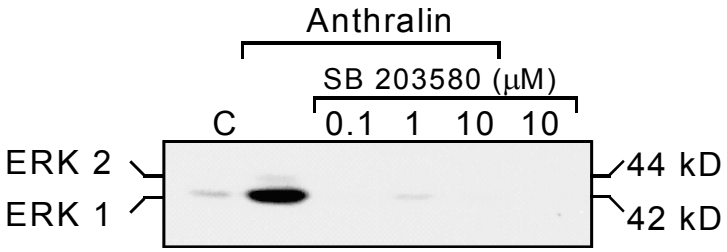

Figure 3.24:

Cells were treated with SB203580 for 30 minutes before 50 μM anthralin was added. SB203580 resulted in potent inhibition of ERK1/2 activation.

3.6.6 Structurally unrelated antioxidants modulate anthralin induced ERK1/2 activation

Human keratinocytes were pretreated with NAC, BHA, or PDTC, respectively, to further investigate the mechanisms involved in ERK1/2 phosphorylation after 50 μM anthralin treatment.

Eight hour pretreatment with 5 mM, 10 mM, or 20 mM NAC resulted in strong inhibition of ERK1/2 phosphorylation. It is noteworthy that inhibition was most marked at the lowest dose of 5 mM implicating a dose dependent mechanism of action of NAC.

In contrast, the lowest dose of BHA used, 3 μM for 30 minutes, was not sufficient to inhibit anthralin induced ERK1/2 activation whereas the higher concentrations of 10 μM and 50 μM showed clear inhibitory effects.

PDTC pretreatment for 30 minutes did not inhibit ERK1/2 phosphorylation at any of the concentrations used, but resulted in enhanced activation compared to the anthralin treated positive controls.

None of the antioxidants alone caused ERK1/2 activation.

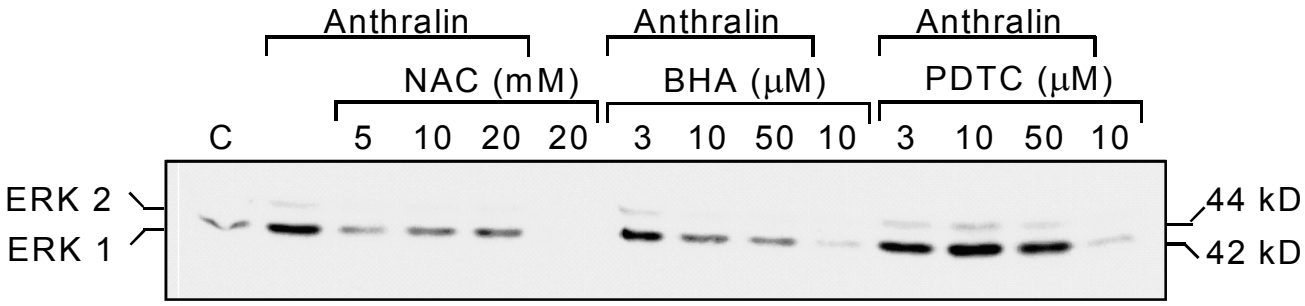

Figure 3.25:

Cells were pretreated with NAC for eight hours, or with BHA or PDTC for 30 minutes prior to exposure to 50 μM anthralin for 30 minutes.

4 DISCUSSION

4.1 Proposed Mechanism of Action of Anthralin

Despite decades of clinical use, the specific biochemical mechanisms targeted by anthralin, such as transmembrane receptor phosphorylation events in response to oxidative stress-inducing agents, have eluded discovery.

Several major targets of the therapeutic action of anthralin have been proposed: cellular factors involved in keratinocyte proliferation, keratinocyte differentiation, inflammatory processes, mitochondrial respiration, DNA synthesis, gene expression, as well as diverse effects on several enzyme systems.

Substantial research indicates that the generation of reactive oxygen species by oxidation of anthralin plays a pivotal role in its therapeutic effects on keratinocytes, but also presumably on peripheral blood mononuclear cells (PBMC).[70;187] Nonetheless, a direct link between generation of ROS and different biologic responses, such as antiproliferative[71] and proinflammatory actions,[73] or activation of signaling pathways,[188] remains to be more precisely characterized.

On the other hand, with regards to the understanding of psoriasis, the target cell for the primary defect of this common skin disease is still not clearly identified. Several candidate targets have been proposed, ranging from keratinocytes and fibroblasts to endothelial cells and lymphocytes. The most prominent pathologic features observed in psoriasis include infiltration of inflammatory components into the skin and abnormal differentiation and hyperproliferation of keratinocytes.

In the present study, specific cellular responses to anthralin treatment have therefore been examined, with special emphasis on effects of anthralin on signaling pathways in cultured human keratinocytes and in specific experiments in peripheral blood mononuclear cells.

4.2 Lipid Peroxidation in Human Keratinocytes and PBMC after Anthralin Treatment

Lipid peroxidation of cellular membranes in response to anthralin treatment was measured in keratinocytes and PBMC to characterize the initial rapid events and damage to cellular constituents. Loss of cPA fluorescence measured by flow cytometry has been used as a quantitative assessment of lipid membrane damage in different cellular systems.[161;162;165] In the present study, a similar time- and concentration-dependent increase in lipid peroxidation could be observed in both cell types, indicating that lipid peroxidation is a prominent early event in the cellular response to anthralin. At present, there are no data describing any more rapid effects triggering downstream signaling responses after anthralin treatment.

With regards to the anthralin concentrations required to induce this response, PBMC, specifically lymphocytes, are approximately 10-fold more sensitive to anthralin induced lipid peroxidation than keratinocytes. As a result of lipid peroxidation occurring in biological systems, alteration of protein and lipid structures and impairment of their function have been described.[189;190] These observations demonstrate that lipid peroxidation generated by anthralin is both an early and cell-type specific, membrane-associated event that may contribute to the pharmacological effects of this anti-psoriatic agent.

4.3 Generation of H_2O_2 after Anthralin treatment

There is sufficient evidence that reactive oxygen species (ROS) are generated after anthralin application in distinct cellular and acellular settings.[69;85;97;188;191]

The ROS characterized in many of these studies, however, comprise several different chemical entities, such as the superoxide anion, the hydroxyl radical (H_2O_2), and singlet oxygen. Inadequate specificity and quantification in previous studies, however, were reasons to address whether H_2O_2 is generated in human keratinocytes after anthralin exposure. This was

accomplished using the fluorescent probe technique with Amplex Red™. Zhou and Mohanty have shown earlier that this compound enzymatically detects extracellular H_2O_2 release with a high sensitivity and specificity.[192;193] In the present study, concentration-dependent anthralin induced H_2O_2 generation could be detected within five minutes of exposure.

4.4 The role of Anthralin induced ROS in lipid peroxidation

A link between the rapid generation of ROS and progressive lipid peroxidation of cellular membranes has been previously identified.[164;194]. Among ROS, hydroxyl radicals are considered the strongest initiators of lipid peroxidation,[195] but the precise mechanism of rapid initiation of lipid peroxidation is still unclear. In the present study, following anthralin exposure, a rapid induction of lipid peroxidation was observed after only five minutes, paralleling the rapid generation of H_2O_2.

Results from *in vivo* experiments suggest a connection between H_2O_2 generation and lipid peroxidation: Finnen et al. showed that application of free-radical scavengers after topical anthralin treatment resulted in a reduced inflammatory response. They conclude that anthralin induced free radicals cause lipid peroxidation which leads to skin inflammation.[73]

Taken together, these findings could underscore the link between lipid peroxidation and H_2O_2 production implicating H_2O_2 as an end product in anthralin induced lipid peroxidation. Other reactive oxygen species likely also play a causal role in lipid peroxidation and further studies will be required to examine the production of these oxygen intermediates by anthralin.

4.5 JNK pathway activation in human keratinocytes and PBMC after Anthralin treatment

The stress-activated protein kinase cascade (SAPK) or JNK pathway is activated by diverse stress-related stimuli and has been implicated in the regulation of cellular proliferation and apoptosis.[103;196]

Among these stress-related stimuli, reactive oxygen species have been shown to play a role in JNK activation in different cell types.[166;197] Since anthralin is known to induce ROS, the activation of JNK after anthralin treatment was investigated in both keratinocytes and PBMC. In both cell types, anthralin activates JNK in a similar time- and dose-dependent fashion, but the threshold response in PBMC occurs at about 40-fold lower concentrations of anthralin compared to keratinocytes.

The finding that JNK is activated by ROS is concordant with findings by Benhar et al. who could show that elevation of ROS levels led to potentiated JNK activation in NIH 3T3 cells.[198] However, Liu et al. did not find JNK activation after H_2O_2 treatment in a human hepatoma cell model, suggesting differential sensitivities to ROS in different cell types.[199]

To further assess the relevance of ROS as putative second messengers in the activation of JNK by anthralin, the inhibition of endogenous ROS by pretreatment with different antioxidants was examined. JNK activation was potently inhibited by antioxidants in keratinocytes and PBMC.

The finding that selected antioxidants and inhibitors of lipid peroxidation, such as NAC and AA6P, suppress anthralin-mediated JNK activation in keratinocytes and PBMC establish lipid peroxidation as a novel and critical upstream event in the cascade of JNK activation following anthralin treatment. This is in agreement with earlier findings in UVB-irradiated human keratinocytes that lipid peroxidation precedes JNK activation and can be inhibited by antioxidants.[200] Similarly, Kim et al. showed JNK activation after H_2O_2 treatment in Chinese hamster lung fibroblast cells. In this model, JNK activation could also be potently inhibited by pretreatment with antioxidants.[201]

The temporal order of events, lipid peroxidation → generation of ROS → JNK activation, implicates both ROS and lipid peroxides as important early mediators of JNK activation by anthralin.

4.6 Effects of Anthralin on keratinocyte and PBMC viability

Henseleit et al. found that anthralin at concentrations of 1 nM to 10 μM caused necrotic cell death in HaCaT cells supposedly due to damage to plasma membranes and mitochondria.[202] Signs of necrosis and apoptosis in keratinocytes of healthy human skin after application of anthralin were also described by Kanerva.[203]

In the study presented here, anthralin exposure caused dose-dependent cell death both in keratinocytes and PBMC. Discrimination between necrotic and apoptotic cell death was not highly reproducible with the methods used. Nonetheless, in order to investigate if reactive oxygen species play a role in diminished cell viability, the effect of n-propyl-gallate (nPG) on cell death was assessed. In both cell types, nPG could reduce the cytotoxic effects of anthralin suggesting that part of the therapeutic action of anthralin is due to ROS-induced cell death of keratinocytes and PBMC. Interestingly, it was observed that nPG-treatment alone resulted in increased cell death as compared to keratinocytes treated with nPG and anthralin. This result is in accordance with findings that nPG can exert pro-oxidant effects in different experimental settings.[204-206]

4.7 Different sensitivities of keratinocytes and PBMC to anthralin

As discussed above, keratinocytes and PBMC differ in their sensitivity towards anthralin treatment. Lipid peroxidation was induced in PBMC at much lower concentrations of anthralin than in keratinocytes. Similarly, anthralin induced JNK activation occurred in PBMC at about 40-fold lower

concentrations of anthralin as compared to human keratinocytes (HK). These results indicate that HK are more resistant to anthralin induced stress and ROS-production than PBMC.

These results are similar to findings suggesting that anthralin primarily inhibits granulocyte function and DNA replication. Chang et al. investigated the intraepidermal accumulation of polymorphonuclear leukocytes after anthralin application and found a significant reduction of leukocyte numbers.[207] Van der Vleuten et al. found that application of anthralin in a micro-encapsulated carrier system significantly decreased dermal accumulation of polymorphonuclear leukocytes and T-lymphocytes in humans. They did not observe any substantial effect on epidermal growth and differentiation parameters.[208]. Contrary results were published by Gottlieb et al.[209] They compared the effect of similar concentrations of anthralin on cell growth in keratinocytes versus lymphocytes and found a far more inhibitory effect on keratinocytes than on lymphocytes.

The observed sensitivity of PBMC to anthralin treatment may also be reflected in the psoriatic pathology of infiltrating lymphocytes. Johnson et al. have reported a 50-fold higher sensitivity of PBMC or T-lymphocytes in comparison with keratinocytes towards the cytotoxic effects of psoralen plus ultraviolet A radiation (PUVA).[210] In an *in vitro* model of methotrexate (MTX) treatment, Jeffes et al.[211] demonstrated that proliferating lymphocytes were more than 100 times more sensitive to MTX treatment than keratinocytes, thus implying them as a likely major cellular target of MTX.

Even though anthralin induced cell death was shown in both keratinocytes and PBMC in the present study, due to methodical constraints, the results cannot be compared directly. Thus, these experiments are not suitable to investigate distinct sensitivities towards anthralin of both cell types that may eventually be leading to cell death at differing doses. Further research should therefore also focus on cell death as an investigative end point.

Taking the results together, there is strong evidence that anthralin exerts differential cytotoxic effects that preferentially target PBMC and that the therapeutic effects of anthralin are due to elimination of lesional lymphocytes from the psoriatic plaque.

4.8 The role of ROS as signal transmitters

A growing body of evidence indicates a vital role for ROS in mediating cellular responses by various extracellular ligands. Among other functions, such as ion transport, transcriptional activity, neuromodulation and apoptosis, ROS have been associated with the regulation of cytokine, growth factor, and hormone function and secretion.[102;103;106;134;212-219]

The biological mechanism of action of ROS still remains unclear: Several reports have indicated an increase in intracellular levels of ROS upon binding of ligand to its receptor, while in other cases reactive oxygen species initiate cellular responses themselves. In vascular smooth muscle cells, for example, it was shown that addition of PDGF resulted in increased intracellular levels of H_2O_2. Addition of free radical scavengers not only prevented the increase in H_2O_2 levels, but also blocked the physiological function of PDGF on its receptor. The mechanisms by which reactive oxygen species eventually cause downstream signaling events is discussed controversially: ROS may inactivate protein tyrosine phosphatases which leads to increased protein tyrosine phosphorylation.[212] Another explanation implies that ROS directly activate critical signaling molecules and thus trigger downstream events.[220]

4.9 The role of the EGF receptor

In psoriasis, several studies have indicated a dysregulation of the keratinocyte growth factor receptor-ligand system.[117;118;221] In affected skin, [^{125}I]EGF binding was increased in the upper strata of the epidermis, and the distribution of immunoreactive EGF receptors was altered as compared to normal epidermis of comparable thickness.

Transforming growth factor-α (TGF-α), amphiregulin and the EGF receptor are overexpressed in epidermal hyperplasia of psoriatic epidermis.[222-224] In addition, the insulin-like growth factor-1 (IGF-1) receptor is expressed at high levels in psoriasis. In cultured keratinocytes, activation of IGF-1 receptor leads

to increased numbers of EGF receptors on the cell surface and promotes keratinocyte proliferation in synergy with EGF/TGF-α.[225;226]

Blockade of the EGFR kinase by the synthetic receptor inhibitor PD153035 induced a dose-dependent growth inhibition and cell cycle arrest in keratinocytes from psoriatic tissue.[121]

Ligand independent phosphorylation of a wide range of transmembrane receptor families, including epidermal growth factor receptor,[227] platelet-derived growth factor receptor,[212] fibroblast growth factor receptor,[228] interleukin-1 receptor, tumor necrosis factor-α receptor,[229] T cell receptor-coupled tyrosine kinases[230] is increasingly recognized as an important and widely occurring event in cellular physiology.

As discussed above, it has been suggested that ROS function as intracellular second messengers that may be involved in the activation of intracellular signaling pathways[231;232] and therefore, are likely candidates for mediating ligand independent phosphorylation of cell surface receptors.

In human keratinocytes, UVB-induced H_2O_2 was previously identified as an important second messenger required for EGFR phosphorylation.[227;233] It was also shown that ROS are important mediators of ERK1/2 activation after UVB irradition in normal human keratinocytes.[227]

In view of these findings, the role of reactive oxygen species generated by anthralin on the phosphorylation of the EGFR was further investigated.

Anthralin induced EGFR-phosphorylation was observed in a concentration- and time-dependent fashion in human keratinocytes. These results are contrary to findings by Richter et al. who report that anthralin does not cause EGFR phosphorylation in a squamous carcinoma cell line overexpressing EGFR.[234] Hence, the effects of anthralin and its metabolites may differ depending on the cell line investigated.

Several lines of evidence show that anthralin-induced EGFR phosphorylation is mediated by ROS, and more specifically by H_2O_2.

First, the time-course of EGFR-phosphorylation after anthralin treatment parallels the generation of H_2O_2. Second, pretreatment with PD153035 potently inhibits EGFR phosphorylation induced by anthralin. The third line of pharmacologic evidence is the inhibitory effect of the structurally different

antioxidants, such as nPG, NAC, AA6P, PDTC, and BHA. This observation is consistent with previous reports about inhibitory effects of these antioxidants in UVB-induced EGFR phosphorylation.[227]

However, it must be conceded, that neither the time-dependent correlation nor antioxidants that are not specific for H_2O_2 are sufficient evidence to prove that H_2O_2 is an important mediator required for anthralin-induced EGFR phosphorylation. Clear distinction between the requirement for H_2O_2 in this process and H_2O_2 generation simply as a compensatory cellular response, as well as identification of H_2O_2 among other ROS potentially involved in EGFR phosphorylation, including hydroxyl radicals and superoxide anions, can be made only by decreasing endogenous levels of H_2O_2 in proximity to its production. This could be achieved by using a specific scavenger of H_2O_2 such as the enzyme catalase. Using this approach in the future, more specific investigation of ligand-independent EGRF phosphorylation after anthralin treatment of human keratinocytes would be possible.

Gamou et al. investigated distinct differences in specific phosphorylation sites of EGFR following addition of EGF versus exposure to hydrogen peroxide.[235] It was found that in an EGFR overproducing cell line H_2O_2 exposure preferentially led to phosphorylation of specific tyrosine sites of the EGFR. In contrast, EGF treated cells and cell membranes exhibited a different pattern of phosphorylation sites (predominantly serine, threonine and tyrosine sites). These results underscore that the apparent similarity of ligand-induced and ligand-independent receptor phosphorylation awaits further elucidation. Likewise, the relative activity and biological potency of anthralin/H_2O_2 induced EGFR-phosphorylation still remain to be determined, though in the present study it could be shown that it enhances ERK1/2 activation.

4.10 Anthralin induced ERK1/2 activation

The MEK/MAPK pathways have been reported to contribute to the survival of epithelial cells in different settings.[128-130]

In human keratinocytes, MEK (=MAPK/extracellular signal-regulated kinase kinase) activity depends on EGFR activation. Conversely, disruption of EGF receptor signaling impacted the MEK/MAPK pathway, to the extent that keratinocytes become more prone to apoptosis.[119]

These findings could be confirmed in the present study: specific blockade of anthralin induced EGFR autophosphorylation by PD153035 completely abrogated ERK activation in human cultured keratinocytes.

Extracellular signal-regulated kinase (ERK) is one of the three major MAPK that is predominantly activated by growth factors.[236;237] However, there is growing evidence that the MAP kinase pathway can also be activated by ROS.[102;197]

In UVB-irradiated keratinocytes, ligand-independent ERK-activation, mediated by ROS, has already been shown.[238] These results are consistent with the findings of the present study that anthralin induces ERK activation through generation of oxidative stress. Nonetheless, further research is required to more precisely delineate the mechanisms of stress-induced MAPK activation.

4.11 Clinical relevance of the presented *in vitro* experiments

Concentrations of 0.1% (4.4 mM) to 1% (44 mM) anthralin are commonly used for topical therapy of psoriasis,[64] and, depending on the formulation, application procedure and water content of the skin, up to 12% of the topically applied have been measured in the epidermis.[239] Thus it can be calculated that concentrations used in the present study are well within the range used *in vivo*. In addition, the effect of anthralin was investigated in primary human keratinocytes and human peripheral blood mononuclear cells which represent major cell types involved in the pathogenesis of psoriasis. Additional studies on psoriatic tissue or on a model of psoriasis will further elucidate the actual mechanism of action of anthralin.

4.12 Conclusion

It has been suggested that 9-anthrones or ROS produced during the oxidation process mediate biologic responses to anthralin.[188] In the present study, it was demonstrated that lipid peroxidation is involved in the activation of c-jun-*N*-terminal kinase after anthralin treatment in human keratinocytes (HK) and peripheral blood mononuclear cells (PBMC). Lipid peroxidation and JNK activation were observed at several-fold lower doses in PBMC than in HK making PBMC a likely primary target for anthralin's therapeutic efficacy.
Meves et al. showed that lipid peroxidation is a prominent upstream event required for UVB radiation or arachidonic acid-induced JNK activation in keratinocytes.[240] Since end products of lipid peroxidation were recently found to induce ROS,[241] it is possible that products of lipid peroxidation may also be involved in the EGFR/ERK1/2 signaling pathway activation response induced by anthralin. Alternatively, the inhibition of glutathione-sensitive, membrane-bound protein tyrosine phosphatases as shown after UV radiation and H_2O_2 exposure may be an important target to modulate EGFR phosphorylation.[242] Thus, several different mediators or mechanisms may be involved in anthralin-induced signal transduction and await further investigations.

ERK is one of the three major MAPKs that are predominantly activated by growth factors.[236] However, in the present study, it was shown that ERK1/2 is activated by the oxidative stress-inducing agent anthralin, which is consistent with previous findings that ROS mediate UVB-induced ERK activation.[238] More specifically, H_2O_2 was the mediator identified in this process, that was found to be functionally relevant for cell survival in keratinocytes.[233] Even though ligand-independent, anthralin-induced phosphorylation of EGFR appears to be similar to ligand-induced autophosphorylation of EGFR,[153] distinct differences in specific sites of phosphorylation of EGFR induced by H_2O_2 versus the peptide ligand EGF have been demonstrated.[235] The relative kinase activity and biologic potency of phosphorylated EGFR induced by EGF versus anthralin remain to be determined. The difference between ligand-induced and ligand-independent

EGFR phosphorylation was reason to investigate this process and its downstream signaling effects using the specific EGFR-inhibitor PD153035. PD153035 blocked anthralin-induced EGFR autophosphorylation, and interestingly, also completely abrogated ERK1/2 activation following anthralin exposure. These results suggest that ligand-independent EGFR autophosphorylation induced by anthralin is required for ERK1/2 activation.

Lesional skin of psoriasis is characterized by epidermal hyperproliferation and perturbed maturation of the epidermis as well as vascular alterations.[243] Anthralin decreases keratinocyte TGF-α expression, inhibits cellular binding of ^{125}I-labeled EGF, and selectively inhibits keratinocyte growth.[209] One study found that anthralin inhibited not only the mitogenic effect of EGF but also the phosphorylation of EGFR.[234] However, in the present study, anthralin induced phosphorylation of EGFR in primary human keratinocytes in the present study, a finding contrary to that report.
How can the known antiproliferative effect of anthralin[234] be reconciled with the present finding that anthralin induces EGFR phosphorylation, an event that is commonly associated with proliferation? In response to injury and stress, growth factor or cytokine receptors[244-246] as well as ERKs[247] of various cell types have been shown to be critical for cell survival. Peus et al. showed that UVB-induced EGFR/ERK pathway activation plays a protective role important for cell survival in keratinocytes.[233] Similarly, it can be hypothesized that anthralin-induced EGFR phosphorylation in keratinocytes of the psoriatic plaque that overexpress EGFR[117] might be part of a cellular defense mechanism that could explain the higher resistance to anthralin-induced erythema, compared with perilesional skin.[73]

How could a potential defense mechanism in keratinocytes that protects cells against anthralin toxicity contribute to the therapeutic mode of action? A pathogenic role has been suggested for the dermal and epidermal inflammatory infiltrates within lesions of psoriasis that are rich in lymphocytes and release proinflammatory cytokines.[28;56] In support of these findings, in the present study, PBMC were more sensitive to anthralin than keratinocytes and thus may represent the most likely therapeutic target.[248] In view of these findings, it is proposed that treatment-induced growth factor receptor,

specifically EGFR, phosphorylation may contribute to the increased resistance of keratinocytes to anthralin, compared to the greater sensitivity of lymphocytes, and may, therefore, play a critical role in the mode of action of anthralin. However, cellular death of human keratinocytes and PBMC following anthralin exposure could not be compared directly due to methodical constraints in the present study. In order to further elucidate the biologic actions of anthralin, future research should focus on this aspect. In addition, investigations using keratinocytes and mononuclear cells derived from psoriatic tissue should be performed to validate findings from unaffected cells (as presented here) in affected skin.

It can be concluded that the balance between pro-oxidative and antioxidative mechanisms of the keratinocyte determines the activities of oxidative stress-induced EGFR phosphorylation and likely that of other transmembrane receptors. The present study identifies anthralin-induced ROS and more specifically H_2O_2 as an important early mediator required for ligand-independent EGFR phosphorylation and further downstream signaling.

5 SUMMARY

Over years of research, it has been reported that anthralin, one of the most efficacious topical psoriasis treatments, inhibits key cellular enzymes,[81;82] exerts antiproliferative and antirespiratory activity,[71;83;84] demonstrates cytotoxic capacity and shows effects on mitochondria,[85;88-90] inhibits DNA replication and repair synthesis and changes keratinocyte differentiation.[86;87] In addition, modulation of skin phagocyte activity and suppression of immune responses have been reported following anthralin exposure.[26;91-93;96] Furthermore, it has been observed that anthralin treatment results in altered cell-surface receptor functioning.[117-121]

To further elucidate anthralin's effects and biochemical mechanisms of action, signal transduction pathways were investigated in human keratinocytes (HK) and in specific experiments in peripheral blood mononuclear cells (PBMC) after anthralin treatment.

It was demonstrated that anthralin exerts potent effects on keratinocytes and mononuclear cells through strong induction of lipid peroxidation and c-jun-N-terminal protein kinase (JNK) activation, a stress-induced signal transduction pathway. Lipid peroxidation was observed rapidly within minutes, and half-maximal levels of lipid peroxidation were reached at a 10-fold lower concentration of anthralin for PBMC versus normal keratinocytes. JNK activation was detected in PBMC at an approximately 40-fold lower anthralin dose compared to keratinocytes. In both cell types, selected inhibitors of lipid peroxidation prevented JNK activation. These findings identify anthralin as a novel and potent inducer of JNK activation and demonstrate that JNK activation is mediated, at least in part, by lipid peroxidation which is among the earliest and most proximate, membrane-related responses to anthralin yet described.

To further investigate signal transduction mechanisms involved after anthralin treatment, the epidermal growth factor receptor/extracellular regulated kinase1/2 (EGFR/ERK1/2) pathway that plays a critical role in cellular proliferation and cell survival was examined.

Anthralin treatment induced time- and concentration-dependent phosphorylation of EGFR and ERK1/2 activation in primary human keratinocytes. Several lines of evidence show that this process is mediated by reactive oxygen species. First, anthralin induces time-dependent generation of H_2O_2 as assessed by a specific *in vitro* assay. Second, a correlation was found between time-dependent increase in anthralin-induced EGFR phosphorylation and H_2O_2 generation. Third, several structurally different antioxidants inhibited EGFR phosphorylation induced by anthralin. The epidermal growth factor receptor-specific tyrosine kinase inhibitor PD153035 abrogated anthralin-induced EGFR phosphorylation and activation of ERK1/2. These findings establish the following sequence of events: 1) H_2O_2 generation, 2) epidermal growth factor receptor phosphorylation, and 3) extracellular-regulated kinase activation. These data identify anthralin-induced reactive oxygen species, and more specifically H_2O_2, as an important upstream mediator required for ligand-independent epidermal growth factor receptor phosphorylation and downstream signaling.

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Signal Transduction Pathways Induced by the Anti-Psoriatic Drug Anthralin in Cultured Human Keratinocytes

Abstract

Der genaue zelluläre Wirkmechanismus des Anthralins, eines der wirksamsten topischen Antipsoriatika, ist trotz zahlreicher wissenschaftlicher Untersuchungen in den letzten Jahrzehnten noch nicht vollständig aufgeklärt. In der vorliegenden Arbeit wurden daher in der humanen Keratinozytenkultur Signaltransduktionswege nach Anthralin-Exposition untersucht; bei spezifischen Fragestellungen außerdem der Einfluss von Anthralin auf periphere mononukleäre Blutzellen (PBMC).

Es konnte gezeigt werden, dass Anthralin deutliche Effekte auf humane Keratinozyten (HK) und PBMC ausübt, indem es eine starke Induktion der Lipidperoxidation (LPO) bewirkt und zeit- und konzentrationsabhängig die *c-jun-N-terminal-Protein-Kinase*-Signal-Kaskade (JNK) aktiviert, einen v.a. durch Stress induzierten Signaltransduktionsweg. Die Induktion der LPO wurde innerhalb weniger Minuten beobachtet; halb-maximale LPO-Messwerte wurden in PBMC mit 10-fach niedrigeren Anthralin-Konzentrationen als in HK erreicht (0,3 µM *versus* 3 µM). Im Vergleich zu HK wurde eine JNK-Aktivierung in PBMC schon bei einer ungefähr 40-fach niedrigeren Anthralin-Dosis demonstriert (5 µM *versus* 200 µM). In beiden Zelltypen wurde die JNK-Aktivierung durch ausgewählte Inhibitoren der LPO (N-Acetylcystein, Ascorbinsäure-6-Palmitat, n-Propylgallat) verhindert. Die vorliegenden Ergebnisse identifizieren Anthralin damit als einen starken Induktor der JNK-Aktivierung in HK und PBMC und zeigen weiterhin, dass die JNK-Aktivierung durch LPO vermittelt wird. Dies ist eine der ersten und sehr membrannah liegenden zellulären Antworten, die bisher im Zusammenhang mit Anthralin nachgewiesen wurden. Die höhere Sensitivität der PBMC für Anthralin deutet weiterhin darauf hin, dass die Wirkung des Anthralins, zumindest teilweise, durch stress-induzierende Effekte auf PBMC vermittelt wird.

Als weitere Signaltransduktionsmechanismen, die eine wichtige Rolle im Rahmen zellulärer Proliferation und des Zellüberlebens spielen, wurden die Phosphorylierung des Epidermalen Wachstumsfaktor Rezeptors (EGFR) sowie die Aktivierung der extrazellulär regulierten Kinasen (ERK1 und ERK2) in HK nach Anthralin-Behandlung untersucht und eine zeit- und konzentrationsabhängige Aktivierung nachgewiesen. Es konnte gezeigt werden, dass dieser Vorgang durch reaktive Sauerstoff-Spezies als wichtige membrannah liegende Mediatoren vermittelt wird: 1) Mittels eines spezifischen *in vitro* Messansatzes wurde eine zeitabhängige Bildung von Wasserstoffperoxid (H_2O_2) nachgewiesen. 2) Es besteht eine Korrelation zwischen zeitabhängigem Anstieg der Anthralin-induzierten EGFR-Phosphorylierung und der H_2O_2-Bildung. 3) Strukturell voneinander unabhängige Antioxidantien inhibieren die Anthralin-induzierte ligandenunabhängige EGFR-Phosphorylierung. Diese Ergebnisse identifizieren H_2O_2 somit als wichtigen Mediator für die ligandenunabhängige Phosphorylierung des EGFR und die Induktion nachfolgender Signaltransduktionskaskaden nach Anthralin-Behandlung.