Retinoic Acid Metabolism Blocking Agents and the Skin

In vivo and in vitro Studies of the Effects on Normal and Diseased Human Epidermis

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Abstract

Retinoic Acid Metabolism Blocking Agents (RAMBAs) increase the endogenous levels of all-trans retinoic acid (RA) by inhibiting CYP26 enzymes. Thus they are believed to mimic the effects of retinoid treatment. Their mechanism of action and effects on vitamin A metabolism in keratinocytes are however uncertain. To explore this and the function of CYP26 in human skin was the main purpose of the project.

The effects of two RAMBAs (talarozole and liarozole) on the expression of retinoid biomarkers in epidermis were studied in vivo and in vitro. Normal human skin (n=16) exposed to topical talarozole for 9 days showed similar response as previously reported for topical RA, even though no skin inflammation occurred. Lamellar ichthyosis patients (n=11) treated systemically with liarozole showed variable clinical improvement after 4 weeks with only mild effects on the retinoid biomarkers and the expression did not always correlate at the protein and mRNA levels. In these studies the proinflammatory transcripts IL-1α and TNFα were down-regulated by RAMBAs. In vitro, using an organotypic epidermis model we first studied how the RA metabolism was affected by adding RA and/or RAMBAs. We next examined the effects of the same agents on the expression of vitamin A metabolising enzymes in monolayer cultures of proliferating and differentiating keratinocytes. The results show among other things that CYP26 A1 and B1 are both involved in the catabolism of RA, and that talarozole potently increases the level of endogenous RA, primarily by inhibiting CYP26B1. However the drug’s biological effects cannot be solely attributed to increased RA levels.

In conclusion, RAMBAs are promising new drugs for treatment of skin disorders, but further studies on their mechanism of action are needed.

Keywords: CYP, CYP26, retinoids, vitamin A, RAMBA, metabolism, keratinocyte, epidermis, retinoid regulated genes

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urn:nbn:se:uu:diva-9325 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-9325)
I dedicate this work to former generations who did not have the same opportunities to a good education and could not pursue their dreams.
The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them

Sir William Bragg
This thesis is based on the following studies, which are referred to by their Roman numerals:


III **Pavez Loriè E**, Chamcheu J-C, Vahlquist A, Törmä H. Both all-trans retinoic acid and cytochrome (CYP) 26 inhibitors affect the expression of vitamin A metabolising enzymes and retinoid biomarkers in human organotypic epidermis, *manuscript*.

IV **Pavez Loriè E**, Li H, Vahlquist A, Törmä H. Retinoid signaling in human epidermal keratinocytes: The importance of cytochrome P450 (CYP) 26 for retinoic acid metabolism, *manuscript*.

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Abbreviations

ADH  Alcohol dehydrogenase
ARAT  Acyl-CoA:retinol acyltransferase
CRABPI  Cellular retinoic acid binding protein I
CRABPII  Cellular retinoic acid binding protein II
CRBPI  Cellular retinol binding protein I
CRBPII  Cellular retinol binding protein II
CYP  Cytochrome P450
CYP26  Cytochrome P450 26
CYP2S1  Cytochrome P450 2S1
ER  Endoplasmic reticulum
HB-EGF  Heparin-binding EGF-like growth factor
ICHYN  Ichthyin gene
IL-1α  Interleukin-1α
KRT  Keratin
LB  Lamellar bodies
LI  Lamellar ichthyosis
LRAT  Lecithin:retinol acyltransferase
NADPH  Nicotinamide adenine dinucleotide phosphatase-oxidase
RA  All-trans retinoic acid
RAL  Retinal
RalDH2  Retinal dehydrogenase 2
RAMBA  Retinoic acid metabolism blocking agent
RAR  Retinoic acid receptor
RARE  Retinoic acid response element
RBP  Retinol binding protein
RDH16  Retinol dehydrogenase 16
RDHE2  Epidermal retinol dehydrogenase 2
RE  Retinyl ester
ROH  Retinol
RXR  Retinoid X receptor
SDR  Short-chain dehydrogenase/reductase
TGase-1  Transglutaminase type 1
TGM1  Transglutaminase type 1 gene
TNFα  Tumour necrosis factor α
Numerous dietary factors, including vitamin A, are essential for the homeostasis of the skin. Based on this fundamental knowledge, vitamin A and its natural and synthetic metabolites (retinoids) have been used as treatment for different skin disorders. Some retinoids have stood the test of time and are still in use for treating keratinisation disorders, such as psoriasis and ichthyosis, even though their systemic use is associated with a risk of severe side-effects, such as teratogenicity. This has prompted a search for new remedies which might mimic the effects of retinoids, although with a more targeted action and with lesser side-effects. One such approach is to block the degradation of endogenous retinoic acid by using substances called Retinoic Acid Metabolism Blocking Agents (RAMBA). This thesis expands over the field of vitamin A in the skin and puts the present knowledge of its metabolism and catabolism to the test, particularly the effects of substances, which block the catabolising enzymes in skin cells.

Elizabeth Pavez Loriè, Uppsala, 30 September 2008
Introduction

The skin

As the largest organ in the body and functioning as the body’s interface with the outside milieu, the skin’s most important function is as a barrier protecting the body from the external environment. Other important functions of the skin include water diffusion barrier, participation in the immune system, temperature regulator and involvement in the biosynthesis of vitamins D.

The skin comprises two major tissue layers, the inner layer, dermis, and the outer layer, epidermis, making it a complex and dynamic organ. Beneath the dermis the subcutaneous fat can be found (Fig. 1). The dermis consists of a dense connective tissue, giving skin characteristics of durability and elasticity. It also contains blood and lymph vessels, nerve endings and adnexal structures such as sweat glands, sebaceous glands and hair follicles. The dermis regulates the body temperature and supplies the epidermis with nutrients via the capillary network. Much of the body’s water supply is stored within the dermis. A basal membrane connects the dermis with the epidermis (1).
The skin consists of two main layers: epidermis and dermis. Underlying the dermis is the subcutis. Dermis contains not only of blood and lymph vessels, but also nerves, sebaceous glands and hair follicles. The predominating cells in the epidermis are keratinocytes.

The epidermis is non-vascularised and represents stratified cornified epithelium, which provides protection against harmful mechanical, microbial, chemical and physical factors from the outer milieu. This constantly renewing skin layer is thinnest on the eyelids (0.05 mm) and thickest on the palms and soles (1.5 mm). It mostly consists of layers of cells called keratinocytes that produce keratins, a family of structural proteins. In a process called terminal differentiation (maturation) these skin cells move towards the surface where they undergo desquamation, a process where the outer layers of the skin are shed. The different stages of keratinocyte differentiation are represented in the different epidermal layers (strata) that can be seen microscopically. These layers, from the bottom upwards, are stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Fig. 2) (1).
Figure 2. Epidermis consists of four layers: stratum basale, spinosum, granulosum and corneum. Here the keratinocytes undergo a process of terminal differentiation, which includes changing the expression of cytokeratins. This process demands equilibrium between cell-proliferation, differentiation and desquamation processes.

Stratum basale consists of keratinocyte stem cells and transit amplifying keratinocytes, which have a column-shaped structure. This layer also harbours pigment-producing cells (melanocytes), which protect the skin against UV-light. In this epidermal layer the keratinocytes express keratins (KRT) 5 and 14, which are part of the cytoskeleton. As keratinocytes divide they push already formed cells (committed to differentiation) upwards to the next layer. Stratum spinosum consists of keratinocytes that have limited dividing capacity and have a polyhedral shape. This layer also harbours Langerhans' cells that are bone marrow derived and act as antigen presenting cells. From stratum spinosum and upwards, keratinocytes express keratins 1 and 10, which are regarded as markers of keratinocyte differentiation.

Other differentiation markers that are synthesised here is involucrin, a component of the cornified envelope, and the enzyme trans-
glutaminase 1 (TGase-1). Stratum granulosum consists of non-dividing, flattened keratinocytes. These cells produce keratohyalin granules that contain profilaggrin (the precursor of filaggrin) and enzymes that degrade the cell nucleus and its organelles (1). In this layer the cornified cell envelope is formed beneath the cell membrane, its formation is triggered by a rise in intracellular calcium levels (2). The cornified envelope is build up from the cross-linking of different proteins such as loricrin and involucrin, a process that is catalysed by TGase-1 and 3. Importantly, lipids stored in intracellular structures called lamellar bodies (LB) are released into the extracellular environment and attach to the cell envelope (3). Parallel to this, the keratinocytes’ nuclei degrade and many other intracellular structures enter an apoptotic process leading to “dead” corneocytes.

The stratum corneum consists of flat skin cells in which keratin filaments are aggregated and cross-linked under the influence of filaggrin. Together with the cell envelope and lipids, they provide the epidermis a mechanical and water-permeability barrier function (1).

The entire maturation process of keratinocytes takes 5-8 weeks and at steady-state the process of desquamation, when corneocytes are shed from the skin surface, counterbalances regeneration. The whole process is controlled by the action of agents such as cytokines, growth factors, calcium, vitamin A, vitamin D and steroid hormones. They control proliferation and/or differentiation of keratinocytes and thus maintain epidermal homeostasis (1).

An increased proliferation or decreased removal of keratinocytes leads to a thicker horny layer, also called hyperkeratosis, which is a hallmark of keratinisation disorders, such as ichthyosis.

Ichthyosis

The descriptive name ichthyosis is used for a heterogeneous group of genetically derived keratinisation disorders. The word is derived from the Greek word “ichthys” (fish) highlighting one of the main features of this group of diseases: visible scales all over the body (4, 5). Ichthyosis can be a debilitating disease, causes social isolation, require life-long treatment and may severely impair the patient’s quality of life (6). On the basis of aetiology and clinical symptoms, ichthyosis can be divided in four major groups (Table 1).
Table 1. The four major groups of ichthyosis (4, 5, 7, 8).

<table>
<thead>
<tr>
<th></th>
<th>Autosomal dominant ichthyosis vulgaris (ADIV)</th>
<th>X-linked recessive ichthyosis (XRI)</th>
<th>Lamellar ichthyosis (LI) or Autosomal recessive congenital ichthyosis (ARCI)</th>
<th>Bullous ichthyosis (BI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>1/250</td>
<td>1/2000-6000 in the male population</td>
<td>1/100,000-300,000</td>
<td>1/300,000</td>
</tr>
<tr>
<td>Appearance</td>
<td>First months or years of life</td>
<td>First week of life</td>
<td>Present at birth (collodion baby)</td>
<td>Present at birth</td>
</tr>
<tr>
<td>Inheritance</td>
<td>Autosomal dominant</td>
<td>Recessive X-linked</td>
<td>Autosomal recessive</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>Symptoms</td>
<td>Hyperkeratosis on extremities</td>
<td>Brown scales on the neck, extremities and trunk</td>
<td>Generalised dry thick skin, erythema, alopecia, ectropion, lack of sweating</td>
<td>Blisters, erythema and hyperkeratosis</td>
</tr>
<tr>
<td>Aetiology</td>
<td>Mutations in filaggrin</td>
<td>Deficiency of steroid sulphatase</td>
<td>Mutations in TGM1, ICHYN, ABCA12, ALOXE3, ALOX12B, CYP4F2</td>
<td>Keratin mutations</td>
</tr>
</tbody>
</table>

At present the treatment of ichthyosis is mainly focused on reducing hyperkeratosis and other symptoms such as skin dryness, erythema, fissures and itching. The choice of treatment relies on the experience of the physicians and the symptoms and preferences of the patients. Different keratolytics, such as salicylic acid and urea (5), hydrating agents, such as propylene glycol (9) and lubricating creams are used as treatment. Gånemo et al (10) showed in a pilot study that a combination of lactic acid and propylene glycol in a cream base had synergistic effects on the ichthyotic skin without causing too much irritation.

Other drugs used to treat ichthyosis are vitamin D (11) and retinoids, i.e. vitamin A derivates (tretinoin, isotretionin, acitretin, tazarotene) (12). These substances modulate the expression of genes involved in keratinocyte growth and differentiation.

Retinoids have been used topically to treat mild forms of the disease and orally to treat more severe manifestations. However this treatment can cause significant skin irritation (12) and, in the case of systemic treatment, carries a risk of teratogenicity, liver toxicity, skel-
A new approach is to increase the cellular levels of endogenously produced all-trans retinoic acid. This can be achieved by blocking the catabolism of this molecule with agents called Retinoic Acid Metabolism Blocking Agents (RAMBA). This thesis focuses on the effects of specific RAMBAs on the skin and compares their properties with those of retinoids.

Vitamin A and retinoids

History

The word vitamin was originally derived from the term "vital amine." In 1912, the pioneer Dr. Casimir Funk referred to these substances "vita amines" based on the belief that these accessory factors were chemical amines, similar to thiamine, the vitamin involved in the deficiency disorder, beriberi. A year after, two groups simultaneously and independently discovered vitamin A, then known as "fat-soluble-A" because it was found in lipid-rich food and gave growth arrest in developing animals when deprived from this type of food. Soon the term "fat-soluble A" was combined with Funk’s designation to become "vitamin A" (14). Later Karrer et al isolated and determined the chemical structure of retinol and Wald et al determined retinol’s role in visual function. These two major breakthroughs in vitamin research were awarded with the Nobel Prize in 1937 and 1967 respectively (14).

The importance of vitamin A in the body is illustrated by the symptoms that follow vitamin A deficiency such as night blindness, growth arrest, skin dryness and higher vulnerability to infections (14). Two pioneers in the field of vitamin A research, Wolbach and Howe, described as early as in 1925 that vitamin A-deficient mice exhibited a hyperkeratotic epidermis (15). Frazier and Hu, in 1931, also described the relationship of hypovitaminosis and follicular hyperkeratosis of the skin (16). In the 1940s, all-trans retinoic acid (RA) was synthesised and it was found to be more potent than retinol (ROH) in reversing the skin symptoms of vitamin A deficiency (17). After these fundamental observations, several studies explored the therapeutic usefulness of vitamin A in dermatology. A major breakthrough was the efficient treatment of acne with RA in 1962 (18). Since then, many new synthetic retinoids have been developed and tested in dermatology (19).
Uptake, processing and transport in the body

Vitamin A (retinol) is supplied via the diet as preformed retinyl esters (RE) from animal or is formed from certain vegetable-derived carotenoids, which serve as vitamin A precursors (the most potent being β-carotene). Ingested RE is hydrolysed to ROH in the intestine lumen and absorbed as free ROH by the intestinal cells where it forms complexes with cellular retinol binding protein II (CRBPII) (19). β-carotene enters the enterocytes and is converted into two retinal (RAL) molecules, which are protected by CRBPII from oxidating into RA. These two molecules are reduced to ROH by a retinal reductase. Whatever its source is, ROH serves as a substrate for the enzyme lecithin:retinol acyltransferase (LRAT), which re-esterifies ROH to RE. On leaving the intestinal wall, RE is incorporated into chylomicrons, i.e. lipoproteins, and transported via the general circulation to the liver (19). In the liver vitamin A can be stored as RE, mainly in fat storing cells (20). From 50 to 80% of the total body ROH concentration is stored as RE in these cells and depending on the plasma concentration of ROH, it is released as ROH bound to its plasma carrier, retinol binding protein (RBP) (19). RA also exists in plasma and other body fluids at about 100-fold lower concentration than ROH (21). The ROH-RBP complex is presented to the target cells for vitamin A in the body (19). In addition to its many important functions such as vision, spermatogenesis and bone growth, vitamin A is essential in the proliferation and differentiation of epidermal keratinocytes and thus essential in the homeostasis of the skin (19, 22), where not only ROH, but also β-carotene, RE, RAL, RA and 3,4-didehydro retinoids can be found (23). Most of the biological activities of vitamin A in the skin are carried out by two of its metabolites, i.e. RA and 9-cis retinoic acid (9-cis RA). The principal ways of vitamin A metabolism in the cell are shown below (Fig. 3).
Figure 3. Metabolism of vitamin A in keratinocytes.
The uptake and processing of vitamin A in the skin

The uptake of vitamin A by epidermal keratinocytes probably involves an RBP receptor expressed on the keratinocyte surface (24-26). Intracellular retinol binds to cellular retinol binding protein I (CRBP1), which functions as a transport protein as well as a stabiliser of this lipid soluble molecule. Noy et al have suggested that the level of free CRBP1 in the cell determines the amount of ROH that is taken into the cell (27).

CRBP1-bound retinol is either metabolised into RE, a process catalysed by LRAT or acyl-CoA:retinol acyltransferase (ARAT) (28, 29) or converted into RAL depending on the ratio between free CRBP1 and bound CRBP1 (22). The conversion to RAL is a reversible oxidation/reduction, which can be catalysed by three enzyme families: the alcohol dehydrogenases (ADH), some cytochrome P450 (CYP) enzymes and short-chain dehydrogenases/reductases (SDRs) (22). In the latter family, two enzymes have been identified in epidermis RDH16 (RoDH4/hRDH-E) (30, 31) and RDHE2 (32), where RDH16 is known to oxidate ROH or reduce RAL (33).

The next step is an irreversible conversion of RAL to RA, which is catalysed by retinal dehydrogenases, where retinal dehydrogenase 2 (RalDH2/ALDH1A2) is known to be expressed in epidermis (34). It is also believed that some CYPs are capable of catalysing RAL to RA metabolism, but the physiological relevance of this metabolism is still unclear (35).

Once formed, RA binds to another group of intracellular binding proteins, cellular retinoic acid binding protein (CRABP) I and II, and either enter the nucleus or is directed to degradation by oxidation performed by cytochrome P450 dependent enzymes (CYPs), e.g. the CYP26 family and CYP2S1 (19) (36).

CRABPI and CRABPII

Generally, retinoid binding proteins solubilise and stabilise their hydrophobic and labile ligands. They are also believed to be part of the transport, metabolism and action of their ligand (37).

The two CRABPs are highly homologous and bind RA with high affinity (38); CRABPI has the lowest dissociation constant of the two (0.4nM vs. 2nM) (39). Other natural retinoids, e.g. 4-hydroxy-RA, 4-oxo-RA and 18-hydroxy-RA, also bind to these proteins (40). By contrast, CRABPs have low affinity for 9-cis-RA and no affinity for 13-cis-RA (38). CRABPs also have a distinct tissue specific expression pattern; CRABPI is expressed in more tissues than CRABPII (38), but both are present in the cytosol and in the cell nuclei (41).
Although the exact functions and roles of CRABPI and CRABPII are not completely understood (42), differences in their tissue expression and affinity for RA can give us an idea about their cellular function. It seems that CRABPI is widely expressed in adult tissue (22) and it has also been demonstrated that CRABPI does not protect RA from elimination (40), but encourages its degradation (43). Dong et al (37) later described that CRABPI could regulate RA degradation. On the other hand cells that synthesis RA express CRABPII, which seems to be found in the same place as CRBP (22).

Examples of the differences between the two RA binding proteins can be found in the skin. Here CRABPI can be detected in dermis and epidermis in basal keratinocytes (44) and melanocytes (45), whereas CRABPII is mostly expressed in epidermis in suprabasal keratinocytes (44, 46, 47). Both CRABPII and CRBP gene transcription are up regulated by retinoids in the skin (48, 49). As mentioned before, both CRABPs have been found to be present in the nucleus, but only CRABPII has shown to affect the RA-mediated gene activation (37, 50, 51). CRABPII is also induced by keratinocyte differentiation in vitro, which leads to increased cellular concentration of RA and probably a retention of newly synthesised RA in the cells (52). Interestingly Siegenthaler et al (53, 54) and Karlsson et al (44) have shown that in hyperproliferative skin diseases, the pattern of CRABPs is altered.

**CYP26 and CYP2S1**

The cytochrome P450 dependent enzymes (CYP, P450) consist of heme-containing proteins found in all domains of life. These enzymes catalyse lipid-soluble substrates, a variety of xenobiotics and environmental toxins (55). The most common reaction catalysed by cytochrome P450 is a monooxygenase reaction, e.g. insertion of one atom of oxygen into an organic substrate (XH) while the other oxygen atom is reduced to water:

\[ XH + O_2 + 2e^- \rightarrow XOH + H_2O \]

The term P450 derives from the observation that this cytochrome has an absorption peak at 450 nm, provided that the iron atom of the heme group is reduced and complexed to carbon monoxide (56).

Most CYPs are membrane-bound proteins found in the endoplasmic reticulum (ER). The protein that donates electrons, needed in
the reaction, to P450s in the ER is called NADPH cytochrome P450 reductase, which is also a membrane bound protein (56).

The CYP enzymes are generally expressed in the liver, but have also been found in other tissues, e.g. the skin, where some of the enzymes play an important role in RA homeostasis. RA is catabolised to 18-OH-RA, 5,6-epoxy-RA or 4-OH-RA, that is further oxidised to 4-oxo-RA. All these metabolites are further metabolised to more polar metabolites (57). These metabolites have been found to be more or less biologically active (58, 59). As early as in 1979, Roberts et al (60) hypothesised that the oxidation of RA could depend on CYPs because they occurred in microsomes. Later it was shown that by adding RA to the system the 4-hydroxylation is increased, suggesting an RA stimulation of the enzyme(s) (61).

One family of CYPs, called CYP26, is very specific towards RA. The first member of this family to be characterised is CYP26A1 (P450RA1) (62). This enzyme is expressed in many different tissues and is induced by RA both in vivo and in vitro (63-65), suggesting that RA catabolism includes feed forward loops. In man, the CYP26 family is composed of not only CYP26A1, but also by two other members, CYP26B1 and C1 (66, 67). Although the members of the CYP26 family are similar in their sequences and have a high specificity towards RA, they show distinct expression patterns. CYP26B1 is for example more widely expressed in the adult brain tissue as compared to CYP26A1 (66). Furthermore, CYP26B1 appears to play a role in hair follicle development because it is found in the developing hair follicles in mouse embryos (68). It has also been reported that CYP26C1 oxidise 9-cis RA. So far this enzyme has only been detected in low levels in various human tissues (67). Another member of the CYP26 family, CYP26D1, has been characterised in zebrafish and showed inducibility to RA, but its role in RA catabolism has yet to be discovered (69, 70). Therefore, from here on CYP26D1 will not be included when referring to CYP26 in the text.

The mechanisms and effects of this group of enzymes are not well characterised. Some reports have suggested that each of the CYP26 family members have individual roles in the catabolism of RA because they do not have overlapping expression in the developing embryo (68), but other groups have shown that a certain cooperation or collaboration between these subtypes exists in developing mouse embryos (71, 72). Perhaps their function depends more on the amount of RA that is to be catabolised, shifting the expression of CYP26 subtypes towards either a more individual function or collaboration between the different CYP26 members.
Another CYP enzyme that has been identified in human skin is CYP2S1. This enzyme has shown the capacity to catabolise RA into 4-OH-RA and 5,6-OH-RA but not into 4-oxo-RA. CYP2S1 also shows a two-fold induction by topical exposure to RA (73). The same report stated that other agents, such as coal tar, also induce CYP2S1. More recently another group showed that CYP26A1 is expressed in the basal layer of epidermis (74) and that it was not affected by coal tar, suggesting that CYP2S1 and CYP26A1 are differently regulated in the skin.

Biological effects of retinoids in skin

Most of the biological activities of retinoids are carried out through binding to specific retinoid receptors, which belong to the superfamily of nuclear receptors including steroid, vitamin D (VDR), thyroid (TR), the peroxisome proliferator-activated (PPAR) and a number of other receptors (75). Retinoic acid receptors (RAR) possess high affinity for RA, which thus serves as a potent activator of these ligand-dependent transcriptions factors. Another receptor family, retinoid X receptors (RXR), has 9-cis-RA as its natural ligand (76). Usually RAR family members( α, β, γ) and the RXR family members (α, β, γ) form heterodimer complexes (76).

In the skin the predominant RAR is RARγ, whereas RARα is expressed at minimal levels (77). The levels of RXRs are higher than those of RARs, RXRα being the most abundant retinoid receptor (77). The most common receptor complex in the human skin appears to be RARγ - RXRα (77). Retinoid receptors have six functional domains of which two are important to achieve gene transcription: the ligand-binding domain and the DNA-binding domain by which the receptor binds to the target genes (78). The retinoid receptor dimers are localised in the nucleus accompanied by co-repressor molecules where they bind (in its apo-form) to specific DNA regulatory sequences called retinoic acid response elements (RAREs). These elements are usually situated in the upstream region of target genes (75). Heterodimers bind more efficiently to these response elements than homodimers. The binding of RA to RAR induces a conformational change in RAR, which not only dissociates the co-repressors and permits the binding of co-activators to the complex, but also gives RXR the opportunity to bind its ligand, which could further induce the RAR-mediated transcription of the retinoid-regulated gene (79, 80).

Among epidermal genes that are retinoid regulated are several cytokeratins (KRT5, KRT6, KRT14 and KRT17) and CRABPII (81, 82). Studies performed by Steijlen et al (83, 84) and later by Virtanen et al...
have shown that also KRT2 and KRT4 are retinoid regulated in human epidermis, although no RAREs have so far been identified in these genes. Furthermore, the CYP26A1 gene, involved in retinoid metabolism, possesses two RAREs \((87)\). The other CYP26 subtypes and CYP2S1 are affected by RA, but so far no RARE have been identified \((66, 67, 73)\).

Another mechanism by which RA and its receptors regulate the differentiation and proliferation of epidermal keratinocytes is by acting as an antagonist of activating protein-1 (AP-1) \((88)\), which is important for keratinocyte differentiation \((89)\). This mechanism does not involve RAREs in the affected genes.

### Retinoid therapy of skin disorders

The term “retinoid” has changed over the years to now include the naturally occurring vitamin A metabolites and synthetic compounds that show biological activities, which are characteristic for vitamin A \((90)\). They can be divided in three groups, which are outlined in Table 2.

<table>
<thead>
<tr>
<th>Group I, non-aromatic</th>
<th>Group II, mono-aromatic</th>
<th>Group III, poly-aromatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>all-trans retinoic acid (tretinoin)</td>
<td>etretinate</td>
<td>tazarotene</td>
</tr>
<tr>
<td>13-cis retinoic acid (isotretinoin)</td>
<td>acitretin</td>
<td>adapalene</td>
</tr>
</tbody>
</table>

The first generation includes RA and 13-cis-RA. The second generation includes substances where aromatic substitution of the beta ionone ring structure has been performed. The third generation contain substances that interact with certain retinoid receptor and have more than one aromatic group.

Among the diseases known to respond to retinoids are various types of genetic disorders of keratinisation, such as psoriasis (a polygenetic disorder) and ichthyosis \((17, 91, 92)\). Of the first generation retinoids, tretinoin (RA) and isotretinoin \(13\)-cis-RA) are still used in the treatment of acne, but their skin irritating effects \((93)\) limit their use. \((94)\). Acitretin and tazarotene are used in the treatment of psoriasis \((91)\). Etretinate and acitretin, a metabolite of etretinate, are equally effective in the systemic treatment of psoriasis and their side-effects are similar \((95, 96)\). Due to the teratogenic effects of these drugs, EU
regulations demands that women of childbearing age have to continue with contraception for two years, after finishing the treatment (97). Topical tazarotene are in clinical use in the treatment of psoriasis, where its main side-effects are skin irritation, desquamation and dry skin (98, 99). The Food and Drug Administration (FDA) in the U.S. has still not approved oral treatment with this substance due to lack of safety data.

The use of retinoids in ichthyosis depends on the severity of skin manifestations and the patients’ general health, sex, age, etc. Mild forms of ichthyosis can be treated with topical retinoids such as RA (tretinoin) and tazarotene. Severe forms of ichthyosis are often continuously treated with oral acitretin. Lamellar ichthyosis responds to etretinate, acitretin and isotretinoin. Even in some severe cases of collodion baby systemic retinoid treatment has been used to facilitate membrane shedding (100). Although retinoid treatment dramatically reduces hyperkeratosis it can lead to a wide range of adverse effects (see page 18) (16, 100).
Retinoic acid metabolism blocking agents (RAMBAs)

To achieve a better response and to reduce the risk of side-effects caused by retinoid treatment, new indirectly acting substances have been developed. One approach is to use RAMBAs to prevent the \textit{in vivo} catabolism of endogenous RA and augmenting its tissue level by blocking the CYP26-dependent 4-hydroxylation of RA (36). Once treatment is stopped these agents are quickly eliminated from the body (101). The proposed mechanism of action for these substances is shown below (Fig. 4).

Figure 4. Retinoic acid metabolism blocking agents, proposed mechanism of action in the cell.

The first azole with CYP inhibitory effects was the anti-fungal substance ketoconazole. This agent showed the capacity to inhibit CYP mediated catabolism in rodents (102), but is not specific for CYP26 (103). A second generation CYP inhibitor is liarozole (R75251; liarozole fumarate R85246) (103) (for chemical structure see Fig. 5). This imidazole derivate lacks the anti-fungal effects of ketoconazole (103), but enhances the endogenous plasma RA levels and reduces the elimination rate of injected RA in plasma (104, 105). It is a more specific inhibitor of CYP26 than ketoconazole (106, 107), but still inhibits aromatase, important in sexual development and adrenal steroid metabolising enzymes (101).
Liarozole is the most studied RAMBA, showing promising effects in different cancer forms (108) and skin disorders, such as psoriasis and ichthyosis (96, 109-115).

More recently, a highly selective and active retinoic acid 4-hydroxylase inhibitor was synthesised, named talarozole (Rambazole™, R115866) (for chemical structure see Fig. 5). Compared to liarozole it exhibits increased efficacy both in vitro and in animal experiments (116) and has low effects on the biosynthesis of steroids (101). Homology models of the interaction between CYP26A1, B1 and talarozole have also added more evidence that it is a specific RAMBA indeed (117, 118). This drug is also quickly eliminated from the body (119). It is also worth mentioning that the plasma levels of RA have not increased above physiological levels in patients treated with talarozole or liarozole.

To date, three clinical studies with oral talarozole have been published (120-122). Bovenschen et al (122) assessed the expression of T-cell markers and NK-cell receptors with and without treatment of talarozole. These biomarkers tended to be reduced after treatment, suggesting that this agent might have anti-inflammatory effects, which could be looked at in psoriasis inflammation studies in the future.

**Figure 5.** Chemical structures of liarozole and talarozole

Only two reports have been published on the biological effects of liarozole on retinoid-regulated genes in humans (123, 124) and little has been reported about talarozole from this perspective. New knowledge about both the CYP26 family of enzymes and CYP2S1 has en-
abled a more detailed study of the role of these enzymes in human skin. This knowledge and further clinical trials will lead to novel therapies for patients suffering from different skin disorders, such as ichthyosis.
Aim of the research

The objective of this study was to gain a better understanding of the biological effects of RAMBAs (CYP26 inhibitors) on normal and diseased human skin in vivo and to further explore the mechanisms of action of these compounds in vitro in human keratinocytes, focusing on the role of vitamin A metabolism.

Specific aims:

- To study the effects of topically administered talarozole on the expression of biomarkers of retinoid signalling and inflammation in the skin of healthy volunteers (Study I)
- To study the effects of oral liarozole in lamellar ichthyosis and to gain further information about the mechanisms behind these effects (Study II)
- To investigate the effects of RA and CYP26 inhibitors on the expression of retinoid-regulated genes in organotypic epidermis and cultured keratinocytes to explore the usefulness of these in vitro models for evaluating pharmacological efficacy of novel CYP26 inhibitors (Study III and IV)
- To compare the effects of RA and CYP26-inhibitors on the metabolism of RA (Study III and IV)
- To study in more detail the function of enzymes involved in the catabolism or biosynthesis of RA with particular emphasis on CYP26 and CRABPII in cultures of normal keratinocytes (Study IV)
Material and methods

The following is a brief summary of the materials and methods used in this thesis. A more detailed description can be found in the individual studies.

Subjects (Study I and II)

All tissue specimens were collected at Uppsala University Hospital following the ethical principles from the Declaration of Helsinki. The local ethics committee at Uppsala University approved the studies and the volunteers signed a written informed consent at the beginning of the trials.

Study design

Study I

This randomized, double blind, left-right comparative, and placebo-controlled single centre study was performed on healthy volunteers, including both males (n=9) and females (n=7) (Caucasians), between the ages of 18 to 55 years. The participants had to be healthy and only women with a negative pregnancy test at screening and who were on oral contraception were included in the study.

Topical gel (200 mg) consisting of 0.07% or 0.35% of talarozole was applied to one designated gluteal area (40 cm²) and vehicle to the opposite gluteus, once daily for nine days. The applications were done by the investigators except for the weekend when the subject applied the test gels, after careful instruction. A timeline over the major events in this study is shown in Fig. 6.
Figure 6. Timeline for clinical study with topical talarozole.

Punch and superficial shave biopsies (consisting of epidermis and a minor proportion of papillary dermis (23)) were taken from both gluteal areas after infiltrating the skin with lidocain-adrenalin. At day 0, baseline samples were taken (one punch and one shave biopsy) and at day 9, biopsies from both placebo and talarozole treated skin were taken. The biopsies were kept at -70°C, until continuing with the histological, mRNA and protein expression analysis of retinoid and pro-inflammation biomarkers (Table 3).
Table 3. List of analysed biomarkers in the different studies.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Function</th>
<th>Study I</th>
<th>Study II</th>
<th>Study III</th>
<th>Study IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP26A1</td>
<td>RA catabolism</td>
<td>Xa</td>
<td>Xa</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CYP26B1</td>
<td>X</td>
<td>Xa</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CYP26C1</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2S1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>LRAT</td>
<td>Retinol storage</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDH16</td>
<td>Retinol and retinaldehyde metabolism</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>RalDH2</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRABPII</td>
<td>Binds to RA</td>
<td>X</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
</tr>
<tr>
<td>KRT2</td>
<td>RA-regulated keratin genes</td>
<td>Xa</td>
<td>Xa</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>KRT4</td>
<td>Xa</td>
<td>Xa</td>
<td>Xa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB-EGF</td>
<td>RA-regulated growth factor</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>Important in inflammation</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Examined both at the mRNA and protein level.

Study II

This study, which was part of a larger multicentre trial, included 12 volunteers both males (n=4) and females (n=8) (negative pregnancy test) no younger than 14 years of age, suffering from moderate to severe ichthyosis (judged from overall severity of scaling and/or erythema). Of these, 7 patients had TGM-1 mutations, 4 had ICHYN mutations and 1 had, to this date, unknown mutations causing the disease. Three groups of patients were randomly designated, one group received two tablets containing 75 mg of liarozole (total of 150 mg) once daily, the second group took one tablet containing 75 mg of liarozole and one containing a placebo tablet and the third, smaller group received two placebo tablets daily.

The schedule for the study is outlined in Figure 7 and the patient’s characteristics can be seen in Table 4. At baseline two punch biopsies (for different fixations, Lana’s fix and acetone) and one shave biopsy were obtained. This procedure was repeated after four weeks of treatment, i.e. at the first re-visit during therapy when the full clinical effect of the 3-month study was still pending. The biopsies were ran-
domized and stored at -70°C, followed by analysis of the histology, mRNA and protein expression of retinoid and pro-inflammation biomarkers (see Table 3).

Figure 7. Timeline for the liarozole clinical study.
Table 4. Patient’s characteristics, doses of liarozole and ichthyosis scores in the clinical trial.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Affected gene</th>
<th>Liarozole dose (mg)</th>
<th>Ichthyosis Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Baseline</td>
<td>1 month</td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>TGM1</td>
<td>Placebo</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>TGM1</td>
<td>150</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>TGM1</td>
<td>75</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>TGM1</td>
<td>150</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>TGM1</td>
<td>75</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>TGM1</td>
<td>150</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>TGM1</td>
<td>75</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>Ichthyin</td>
<td>150</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>Ichthyin</td>
<td>75</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>Ichthyin</td>
<td>75</td>
<td>28</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>Ichthyin</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>unknown</td>
<td>75</td>
<td>22</td>
</tr>
</tbody>
</table>

a) The baseline sample in this case was obtained first 1 mo. after stopping therapy, i.e. when the ichthyosis score had returned to pre-therapy value.
b) No biopsies were obtained from this patient.
c) This patient was excluded from the clinical trial but a baseline biopsy was taken and used in the comparison with healthy controls.
NA = not applicable

Study III

This study was performed using an organotypic epidermis containing normal human keratinocytes seeded on an insert (125) to assess the effects of RA and CYP26-inhibitors (RAMBAs) on retinoid-regulated genes and vitamin A metabolism in an in vivo-like skin model. One of the reasons why we chose to work with this model was the serum-free culture condition, which could be easily altered. This is an important factor when studying the metabolism and function of vitamin A. A disadvantage is the lack of a normal dermal-epidermal interaction, which possibly leads to a disturbed epidermal differentiation, compared to another model, where the keratinocytes are cultured on de-epidermised dermis (85).

First, the histology of the organotypic epidermis and the retinoid responsiveness of this model (1 μM RA for 48 h) was examined by looking at the expression of KRT4, which is known to be induced by RA in the skin. This was followed by a time study (8, 24, 48 or 72 h)
where the organotypic model was incubated with 1 μM RA or DMSO (as vehicle). Next, the effects of the two CYP26-inhibitors liarozole and talarozole on retinoid-regulated genes (see Table 3) were studied. In this case attention had to be paid to the RA levels in the culture medium because earlier experiments showed that liarozole and talarozole alone did not alter the expression of retinoid-regulated genes probably due to the low endogenous RA levels. A pre-incubation with 1 nM RA for 48 h was deemed necessary for the CYP26-inhibitors (1 μM) to increase RA and to alter the expression of the retinoid biomarkers. The uptake of [³H]RA and [³H]ROH by epidermis was examined beginning with a pre-incubation with unlabelled ROH (100nM) after 9 days of culture to build up the levels of vitamin A and imitate the natural vitamin A metabolism. After another 4 days, 5 μM of liarozole or talarozole were added to the culture medium and incubated for 24 h before adding 1 μCi of [³H]RA or [³H]ROH. Finally, the organotypic epidermis was harvested and analysed by HPLC.

In addition, some of the samples treated with ROH and CYP26 inhibitors in the metabolism experiments were also used for immunofluorescence analysis of KRT4 and CRABPII, to determine whether accumulation of RA in the organotypic epidermis affected the protein levels of these RA-regulated biomarkers.

Study IV

Normal human keratinocytes cultured submerged in a serum-free medium were used in this study. The retinol and calcium concentrations of the final medium were <1 nM and 0.06 mM, respectively. The first part paid attention to how the aspect of keratinocyte differentiation (achieved by adding 1.5mM CaCl₂) affects the response of genes involved in the metabolism of vitamin A (see Table 3). To compare proliferating and differentiating keratinocytes the cells were additionally incubated with RA. The second part of the study focused on the effects of CYP26 inhibitors (10μM ketoconazole, liarozole or talarozole) and CYP26A1 and B1 knock down (using siRNA, final conc. =60 nM) on [³H]RA metabolism, studied by incubation of keratinocytes with [³H]RA for 24 h prior to HPLC analysis. At the same time the mRNA expression of CYP26A1 and B1 were studied by incubating the cells with specific siRNA probes for 48 h. To examine the effects of CYP26B1-knock down and CYP26 inhibitors have on RA activity in the cell, the CRABPII expression (44) was assessed. The keratinocytes were incubated with RA (1μM), CYP26B1 siRNA or CYP26-inhibitors (1, 5 or 10μM) for 24 h. For immunofluorescence analysis the cells were seeded on cover slips. Lastly the influence of CYP26 inhibitors (1, 5 and 10μM) on the metabolism of vitamin A was exam-
ined by observing the expression of genes coding for enzymes involved in vitamin A metabolism.
Analysis of samples

In all four studies the effects of retinoids and CYP26 inhibition were analysed at the mRNA and protein level. In addition the epidermal thickness and signs of skin inflammation were evaluated in Study I and II. Here is a brief summary on the methods used.

Epidermal thickness and signs of proliferation (I, II)

In both studies the viable epidermis was measured and its thickness calculated from Haematoxylin stained sections from the biopsies. Also, in Study II the stratum corneum thickness was calculated. In both Study I and II, a dermatologist looked for any signs of adverse events such as erythema on each visit.

In Study I the expression of Ki67 in the basal layer was examined in sections where the number of Ki67 positive cells per length of basal membrane was calculated.

mRNA expression (I-IV)

Total RNA was extracted from shave biopsies (I and II), organotypic epidermis (III) or monolayer cultures (IV). cDNA was synthesised and the mRNA expression of the genes of interest (see Table 3) was analysed by using quantitative real-time PCR. For the two in vivo studies (I and II) the housekeeping genes cyclophilin and β-actin were analysed in parallel. A normalisation factor for each sample was calculated from the expression of the two housekeeping genes, using the geNorm software (126). All values for each gene were normalized using this factor. For the two in vitro studies (III and IV) cyclophilin was used as housekeeping gene.
Protein expression (Study I-IV)

Immunofluorescence

In both in vivo studies (I and II) the expression of KRT 2 and 4, and of the enzymes CYP26A1 and B1 were examined. In Study II, one additional punch biopsy from each patient was placed in Lana’s fix and sucrose for examination of CRABPII (44). The expressions were studied by immunofluorescence (IF) and the intensity evaluated by means of scoring. In Study III two punch biopsies were taken from the centre of each organotypic epidermis. One of the biopsies was fixed in 4% formaldehyde followed by methanol for 10 min at room temperature to be used in the protein expression analysis of KRT4. The other was placed in Lana’s fix for 2 h followed by incubation in 10% sucrose for 24 h and later sectioned and stained for CRABPII. In all four studies the samples were stored at -70°C. The monolayer cultures in Study IV were fixed with 2% paraformaldehyde for 10 min, before staining for CRABPII (44).

Immunoblotting

In Study I the protein expression of CYP26A1 and B1 was also examined by Western blot. Because no separate samples were taken for this analysis, the tissue extracts were obtained from sections of frozen punch biopsies in which dermis was microscopically removed and the remaining epidermis was lysed.

The cells in Study IV were homogenised and lysed using a common cell-lysing buffer, RIPA. In the two studies the lysed samples were centrifuged and the supernatant was stored at -70°C. The total protein extracts were then fractioned in SDS-PAGE followed by blotting and visualisation of CYP26A1, B1 or CRABPII protein by the use of specific antibodies.
Results and discussion

Study I
Effects of talarozole on retinoid biomarkers in healthy epidermis

In this study we examined whether two different doses (0.07% or 0.35%) of topically applied talarozole could alter retinoid-regulated genes, thus serving as a proof-of-principle for the drug. Talarozole increased the mRNA expressions of CRABPII and KRT4, whereas KRT2 expression was reduced. The changes were similar to those seen in skin treated topically with RA (83, 85). The magnitude of the changes was concentration-dependent; the highest concentration (0.35%) of talarozole being most effective. KRT4 protein expression was also affected by talarozole application, showing the appearance of a distinct immunostaining in stratum granulosum. KRT2, on the other hand, was only marginally down regulated by talarozole (Fig. 8).

Only two subjects showed mild signs of skin irritation after 9 days of exposure to the drug and analysis of epidermal thickness (and Ki67 staining), showed no major induction of epidermal hyperplasia.

Figure 8. Gene and protein expression of KRT2 and 4, in normal untreated human skin and after topical application of vehicle or 0.35% talarozole.
Effects of talarozole on enzymes involved in the metabolism of vitamin A

The CYP26 family and CYP2S1 are enzymes known to control the levels of RA in the cells and are also induced by RA. CYP26 enzymes are not only both primary targets for RAMBAs, but also positive biomarkers of increased RA activity (for review see (36)). Our results for CYP26 A1 and B1 mRNA expression are in total agreement with these concepts, the effects being dose-dependent and most pronounced for CYP26A1. However, the results of immunofluorescence staining and Western blot analysis showed no increased expression of the two proteins after exposure to RAMBAs, but neither did RA-exposed control skin. In theory the results could be explained by a rapid turnover of protein or by the fact that a large amount of mRNA is not translated into protein (Fig. 9). CYP2S1 had a lower expression and CYP26C1 was only marginally present in epidermis.

LRAT and RalDH2 are two enzymes that have a rate limiting function in retinol esterification and retinal oxidation respectively (127). They play an important role in the regulation of vitamin A metabolism and are also putative retinoid biomarkers. For example, it is known that exposure of normal skin to topical RA leads to increased retinol esterification (28), which may serve as an autoregulatory loop diverting retinol from oxidation to RA. In our analysis we detected a slight decrease of RalDH2 mRNA expression when applying the higher dose, while no alteration was detected in LRAT expression.
Although retinoid-like effects were observed at the mRNA and protein levels, only two subjects experienced signs of skin irritation, which is a common side-effect of RA and other retinoids (13). Even more surprisingly, IL-1α, a biomarker of early inflammatory processes (128) and known to be up regulated after RA application (129), was down regulated at the mRNA level. The expression of TNFα, another pro-inflammatory cytokine, was not significantly affected. Although the protein expression of IL-1α did not detect any reduction of this cytokine, it would be of interest in future clinical studies of talarozole treatment to be able to confirm or reject these findings.

Study II

In comparison with the talarozole study (I), the liarozole study on lamellar ichthyosis patients showed a less uniform response pattern of the retinoid biomarkers. One reason could be that out of the nine biopsied patients, only two had received the higher dose of liarozole (150 mg/day).

Comparison between normal and lamellar ichthyotic skin at baseline

One of the retinoid biomarkers that has been described before in LI epidermis and psoriasis lesions is CRABPII. The gene and protein expressions were both higher in the diseased epidermis compared to healthy skin, and expression was seen in all suprabasal layers instead of normally only in stratum granulosum (44, 54). These observations could be observed in our study as well (Fig. 10). Therefore, increased CRABPII expression may be secondary to epidermal hyperplasia in LI and not a reliable indicator of RA stimulation during liarozole therapy.
No systemic toxicity was noted in any of the patients that participated in this study and a comparison between baseline and treatment samples showed no significant difference in epidermal thickness. However, a tendency to decrease str. corneum thickness was observed after liarozole treatment consistent with an anti-keratinising effect of the drug.

The clinical results after 4 weeks of liarozole therapy were particularly good in the patients with ICHYN or other non-TGM1 mutations. Ichthyin is believed to act as a lipid transporter to the lamellar bodies (LB) essential for the formation of a skin water barrier (130). Interestingly, the absence of Ichthyin in ichthyosis patients (131) and retinoic acid receptor $\gamma$ in knockout mice (132), both lead to similar structural abnormalities in LBs. This implies a connection between retinoid signalling and the transport of lipids by Ichthyin, possibly explaining why patients with ICHYN mutations responded more promptly to increased endogenous RA levels during liarozole therapy than did patients with TGM1 mutations.
Effects of liarozole treatment on the expression of retinoid biomarkers

Five subjects that demonstrated a strong induction of KRT4 expression during liarozole treatment also had predominant changes in the mRNA levels of other genes, i.e. KRT2 and CYP26A1 indicating a retinoid-like response (Fig. 11).

Figure 11. KRT4, 2 and CYP26A1 gene expression in LI patients treated orally with liarozole. Five patients had a response in all three genes (black).

KRT4 expression

In contrast to the findings of Lucker et al (112), our KRT4 mRNA data showed only a mild induction, which could be explained by differences in the treatment duration and applied drug concentration in the two studies. To be sure that the effects of treatment were primary and not secondary to improvement of ichthyosis, we waited the shortest possible period of treatment before taking the biopsies. We also used lower liarozole concentrations (75 mg vs. 150 mg) since it has been reported that liarozole given at high doses elicits similar side-effects as seen with retinoid treatment (109, 112).

KRT2 expression

The gene expression of KRT2 is known to be down-regulated with RA treatment (85). This was also the case after liarozole treatment although no dose-related (75 mg and 150 mg) response could be observed and the overall change in keratin gene expression was small compared to the changes seen with topical RA application in normal human skin (85). Lucker et al (112) described the protein expression of keratins in ichthyosis patients treated with oral liarozole although KRT2 was not included in their study. In our study the KRT2 protein expression seems to be lower than in normal skin already at baseline, which could explain why no further reduction occurred after oral
liarozole treatment. Instead a small increase occurred, which suggests that the effects of liarozole tend to normalise the protein expression of this gene in epidermis (Fig. 12).

**CYP26A1**

One of the main targets of liarozole is believed to be CYP26A1. The mRNA of this enzyme tended to dose-dependently increase during liarozole therapy. Several LI patients displayed strong protein staining of CYP26A1, which was distributed all over epidermis before therapy in comparison to the specific basal layer staining in normal human skin (74, 133). The differences in CYP26A1 and CRABPII expressions (see above) suggest that the RA signalling is disturbed in LI patients.

After therapy the changes in CYP26A1 (and KRT2) expression indicate that ichthyotic epidermis strives to normalise the protein levels (Fig. 12).

![Protein expression of KRT2 and CYP26A1 in normal human skin (NS) and in lamellar ichthyotic skin, before and after liarozole treatment.](image)

**Figure 12.** Protein expression of KRT2 and CYP26A1 in normal human skin (NS) and in lamellar ichthyotic skin, before and after liarozole treatment.

On the other hand the mRNA expression of CYP26A1 does not correlate with its protein expression after liarozole treatment. This might be due to methodological problems, such as obtaining skin samples from a heterogeneous group of patients. However, the poor correlation between mRNA and protein levels is not exclusive to LI but was also observed in normal skin treated with talarozole or RA. Therefore, we cannot exclude the possibility that the influence of
CYP26 inhibitors, and their metabolites, or RA fundamentally altered the translation of CYP26A1 mRNA. Nevertheless, the fact that CYP26A1 reacted most strongly to the higher dose of CYP26 inhibitors suggests that this gene in particular has capacity to respond to excessive RA accumulation in epidermis and thereby “protect” the cell from cytotoxic levels of RA.

**LRAT and RalDH2**

In the present study we found no significant changes in the expression of two enzymes, LRAT and RalDH2 in LI skin, which implies that the RA levels during liarozole therapy remained below the threshold value needed to activate any autoregulatory mechanisms in the epidermal retinol metabolism.

**Pro-inflammatory cytokines**

The pro-inflammatory cytokine, TNF-α, showed a significant down regulation, whereas another cytokine, IL-1α, was more variably affected. Although a decreased IL-1α expression occurred in most patients on therapy, those with a poor clinical response or a high dose level of liarozole (150 mg per day) occasionally showed increased expression of this cytokine. At the moment it is not known if this effect is related to a mild retinoid-like dermatitis, which is sometimes observed as a side-effect in liarozole-treated patients (111, 112).

**Study III**

**Characteristics of organotypic epidermis**

A validation of the organotypic model showed typical features of epidermis on histology and a specific KRT4 staining in stratum granulosum after addition of RA to the culture medium, also observed in vivo (85). Other retinoid-regulated genes such as KRT2, CRABPII and HB-EGF were also affected after exposure to RA, in the same manner as reported after topical treatment in vivo (44, 85, 134).

**Time dependent RA effects on the mRNA expression of retinoid regulated genes and enzymes involved in vitamin A metabolism**

A time study showed that of the four retinoid-regulated genes, CRABPII had the weakest response to RA addition. In the same study genes encoding for enzymes involved in RA biosynthesis and catabolism, i.e. LRAT, RDH16, RalDH2, CYP26A1 and CYP26B1 were also
analysed. The two CYP26 enzymes had a rapid response to RA, which is in line with CYP26 being involved in the degradation of RA (135). The increased mRNA expression of CYP26A1, which has two RAREs in the promoter region (87), could not be confirmed at the protein level for unknown reasons.

Increased LRAT gene expression was a late event, suggesting a feedback mechanism where keratinocytes react to increased cellular levels of RA by increased retinol esterification and thus deviating retinol from oxidation to RA (28). A feedback regulation is further supported by the down regulation of RDH16 and RalDH2 (Fig. 13).

Figure 13. Time study - the gene expression of CYP26 enzymes (left) and the enzymes involved in the vitamin A metabolism (right).

The effects of CYP26 inhibitors on organotypic epidermis

Due to the low levels of RA in the culture medium a pre-incubation with 1nM RA had to be included to build up the amount of retinoid in the tissue before adding the inhibitors. Unexpectedly, the expression of CRABPII and the keratin biomarkers was maximally stimulated already at this low level of RA. This is probably because both the nuclear retinoid receptors have dissociation constants (Kd) in the nanomolar range (136) and the CRABPII gene has a RARE (82). However it could also be because in a virtually retinoid-deficient organotypic epidermis, the response to even minor increases in the tissue retinoid concentration is strong. Only the CYP26A1 expression was synergistically induced by a combination of RA and talarozole, which can be interpreted as a feed-forward control (137) of this gene mediated by its RARE sites (87). Paradoxically, RA or CYP26 inhibitors did not increase the CYP26A1 and B1 protein expressions in organotypic epidermis, an observation that confirmed the findings from Study I.
Effects of CYP26 inhibitors on the vitamin A metabolism

These effects were assessed by adding ROH and inhibitors to the culture medium followed by addition of [³H]RA or [³H]ROH, which showed that talarozole more effectively accumulated [³H]RA in the tissue than liarozole. Similar results could be seen with [³H]ROH (Fig. 14). In the same series of experiments the ROH- and talarozole-treated samples exhibited the strongest KRT4 and CRABPII protein staining.

![Figure 14](image)

**Figure 14.** Effects of RAMBAs on the cellular uptake of [³H]RA (left) and [³H]ROH (right).

Study IV

This last study explored the importance of the individual CYP26 members and how they are regulated in human keratinocytes. This was achieved by examining the mRNA expression of three CYP26 family members and other enzymes, which play a role in the vitamin A homeostasis (127) during keratinocyte differentiation. Furthermore their regulation by RA and CYP26 inhibitors was studied.

Normal mRNA expression of CYPs involved in RA catabolism

Our results show that CYP26A1 mRNA levels are low in normal keratinocytes, which supports previous reports on this subject (74, 138). We also found CYP26B1 to be expressed at a higher magnitude
than both CYP26A1 and CYP26C1. In fact CYP26A1 expression was barely detectable and no expression of CYP26C1 at all was found. We also examined the CYP2S1 expression, which showed much higher mRNA level than CYP26B1 (Fig. 15). This is not the same relationship we detected in vivo, where CYP26B1 was more expressed than CYP2S1.

![Gene expression of CYP26A1, B1 and CYP2S1](image)

**Figure 15.** Gene expression of CYP26A1, B1 and CYP2S1 in cultured human keratinocytes at resting state.

Smith et al (73) argued that CYP2S1 is mostly responsible for RA being catabolised into 4-OH-RA and 5,6-epoxy-RA and not at all for the oxidation into 4-oxo-RA. White et al (62) showed that CYP26 produces a broad range of hydroxylated forms of RA including 4-OH-RA and 4-oxo-RA although the work was performed in COS-1 cells transfected with human CYP26. This suggests that perhaps the main source of catabolism in vivo fluctuates with different epidermal conditions leading to various spectra of metabolites. During in vitro culture, on the other hand, a preference for 4-OH-RA, 5,6-epoxy-RA driven catabolism, mainly accomplished by higher levels of CYP2S1, may exist rather than 4-oxo-RA catabolism. These different metabolites have been shown to be more or less biologically active (58, 59); for example 4-OH-RA and 5,6-epoxy-RA are more active than 4-oxo-RA (58).
Effects of differentiation and RA on the expression of enzymes involved in vitamin A metabolism

In differentiated keratinocytes the mRNA levels of RDH16 increased, corroborating a previous report by Jurukovski et al (31). The expression of genes encoding other enzymes involved in vitamin A metabolism e.g. LRAT and RalDH2, also increased. This was however not the case for the CYP26B1, which was reduced, whereas CYP26A1 and CYP2S1 were unaffected by differentiation. On the other hand, CYP26A1 and B1 responded rapidly to RA addition, both showing the highest response in differentiated keratinocytes.

Interestingly our results in proliferating keratinocytes show a tendency of RA to early induce RDH16 levels. No corresponding induction was seen in differentiating cells. RalDH2 is marginally reduced in both cell types after RA exposure, implying that RA also controls its own biosynthesis. The increased LRAT mRNA levels were similar to previous described results (28) (Fig. 16). Thus, in supra-basal keratinocytes the whole machinery for RA synthesis seems to operate (high expression of RDH16 and RalDH2) together with degradation mechanisms in case the RA level is too high (RA-inducible CYP26 enzymes).

![Figure 16](image_url)

**Figure 16.** Time study of RA effects on the gene expression of CYPs (above) and enzymes involved in vitamin A metabolism (below), in proliferating (left) and differentiating (right) keratinocytes.
Effects of CYP26 knock-down on CYP26 and CRABPII mRNA expression

Targeting siRNA against CYP26A1 and B1 demonstrated that cellular accumulation of RA was slightly increased when inhibiting the B1 subtype, but not the A1 subtype. The reasons why CYP26A1 siRNA had no effect could be that the mRNA levels of CYP26A1 are low at resting state, and thus no further inhibition can be expected. It is also possible that the CYP26A1 protein, mainly confined to keratinocytes in the basal layer (both in vitro and in vivo) (74, 133), has a long turn-over time, leading to no changes in the amount of RA being catabolised.

The importance of CYP26B1 in controlling the cellular RA level was examined by studying the expression of CRABPII, a well-characterised biomarker of retinoid activity (49), in CYP26B1-silenced keratinocytes. The results show increased CRABPII mRNA and protein levels. Similar effects were also detected in cells exposed to talarozole. Interestingly, the cellular localisation of CRABPII protein was altered and a specific dotted staining pattern was observed after either CYP26B1-siRNA or talarozole treatment, but not in RA-exposed cells (Fig.17).

![Figure 17. CRABPII expression in normal human keratinocytes (far left) and after incubation with RA, CYP26B1-siRNA and talarozole (far right). Scale bar: 20 μm.](image)

As mentioned above the CYP26 family will be part of catabolising RA to a wide range of metabolites including 4-oxo-RA (73). This proposes that inhibiting the action of CYP26B1 in keratinocytes by either siRNA technique or talarozole would increase the amount of both RA and probably also other metabolites like 4-OH-RA and 5,6-epoxy-RA, but greatly reduce 4-oxo-RA. Reynolds et al (58) showed in mouse skin that CRABPII is mostly induced by RA followed, in order of potency, by 4-OH-RA, 5,6-epoxy-RA and 4-oxo-RA. Combining these data with our results concerning CRABPII expression (Study IV) may
suggest that the altered CRABPII expression in talarozole and siRNA exposed cells, not seen with RA, is a response to elevated levels of other more active metabolites than 4-oxo-RA. To clarify this matter, experiments should be performed where CRAPBII expression in keratinocytes is studied by culturing the cells with RA, talarozole, CYP26siRNA or RA together with different metabolites. Another interesting experiment to perform would be to examine the effects of CYP26B1-silencing on the uptake and metabolism of [3H]RA, determining the amount of RA, 4-OH-RA and 4-oxo-RA at different times.

Effects of CYP26 inhibitors on RA metabolism and vitamin A metabolising enzymes

Three different CYP26 inhibitors, ketoconazole, liarozole and talarozole, were examined by assessing [3H]RA metabolism and the changes in mRNA of enzymes involved in vitamin A metabolism. Once again talarozole was found to be the most potent RAMBA.

As seen in the studies mentioned above (I and III) CYP26A1 was highly induced by talarozole. This effect was not seen in the expression for CYP26B1, which was reduced in differentiated cells. This observation and the fact that both RDH16 and RalDH2 were induced by talarozole together suggest that the effects of talarozole are similar, but not identical to those seen with RA.
General Discussion and Future perspectives

Both talarozole and liarozole have already shown to be good candidates in the treatment of different keratinisation disorders, such as ichthyosis and psoriasis (101) and our results both confirm these findings in lamellar ichthyosis (liarozole) and show a good tolerance at topical treatment of normal skin (talarozole). Also by using an indirect approach, i.e. measurement of retinoid biomarkers, we can confirm the drugs’ ability to accumulate RA in human epidermis. Unfortunately direct analysis of the very low levels of RA in skin tissue is not possible with current technologies.

RA controls its own biosynthesis from retinol via an autoregulatory enzymatic system in keratinocytes (28), which balances the endogenous RA levels and counter acts excessive RA effects. In vivo (Study I) the higher dose of talarozole caused a minor down regulation in the gene expression of one of the enzymes involved in RA synthesis, but the retinol esterification was not altered. However, in vitro (Study III and IV) we found induction of retinol storage capacity and of other enzymes involved in RA synthesis in keratinocytes exposed to talarozole, which was not seen after RA exposure. The effects could be concluded as being talarozole derived instead of being a purely RA-derived response. Therefore future studies of long-term treatment should include analysis of a broader spectrum of enzymes involved in RA biosynthesis.

Adding to this understanding, results from our two in vivo studies (I and II), showed that both CYP26 inhibitors reduced the expression of certain pro-inflammatory mediators (IL-1α and TNFα) in both normal and ichthyotic epidermis. These findings are interesting because patients undergoing retinoid treatment often report skin irritation as side-effect, which could be circumvented by RAMBA treatment. Therefore larger studies of RAMBAs in patients with inflammatory skin conditions, i.e. psoriasis, where their effects are compared with those of retinoids, need to be performed to verify our findings.
One of the questions considered important to address in this project was that of CYP26 and CYP2S1 expression in epidermis. Our results show that CYP26A1, B1 and CYP2S1 are expressed both in vivo and in vitro in epidermis and that CYP26B1 plays an important role in RA catabolism in keratinocytes under physiological conditions. For many of these RA-degrading enzymes the expression was altered during keratinocyte differentiation (Study IV). This suggests that they have different roles during keratinisation aimed at keeping RA levels at steady state.

From our results we also conclude that the differences between the effects of RA and CYP26 inhibitors are not restricted to pro-inflammatory and RA metabolism auto-regulatory mechanisms, but also involve expression of retinoid-regulated biomarkers, e.g. CYP26 and CYP2S1. This difference was most dramatically seen in the protein expression of CRABPII, where talarozole completely altered its localisation and pattern compared to RA treated cells (Study IV). Unexpectedly, the same pattern was seen in CYP26B1 knock-down cells. This suggests that i) talarozole is specific to CYP26B1, ii) the outcome of talarozole and CYP26B1 knock-down on RA catabolism is similar, iii) talarozole and CYP26 knock-down affect the regulation of CRABPPII protein in a similar manner.

All these findings point to the fact that talarozole and liarozole have potential as novel drugs. Therefore it is of interest to understand more about their mechanism of action and effects to be able to understand their full potential.

It is also worth mentioning that for many patients suffering from ichthyosis it is important to find a treatment that allows them to live more normal and functional lives. It was therefore exciting to see the positive clinical response to the liarozole study (II), where many of the patients seemed content and happy with the results and wanted to continue with the treatment.
Conclusion

In this project both talarozole and liarozole have been shown to have the ability to increase the RA levels in keratinocytes and elicit effects on retinoid-regulated biomarkers, e.g. CRABPII and KRT4 that are similar but not identical to those obtained with RA. For instance, the intracellular distribution of increased CRABPII protein expression differs between RA-treated and talarozole-treated keratinocytes. The RAMBAs also alter the expression of CYP26 enzymes (their main target) and other vitamin A metabolising enzymes in a way that is both similar to RA, e.g. induction of CYP26 mRNA, and different, e.g. conditions under which CYP26 induction occurs compared to RA. Our overall results demonstrate that talarozole and liarozole are promising drugs that might in the near future complement retinoid therapy in different skin disorders. However, they should also be considered as substances that have their own effects unrelated to RA, which may yield unpredicted clinical and biological effects.
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Vitamin A och linknade molekyler (retinoider) har stor inverkan på cellers delning (proliferation) och utmognad (differentiering). I kroppens största organ, huden påverkar naturligt förekommande retinoider bl.a. differentieringsprocessen av de vanligaste hudcellerna i epidermis (keratinocyter). Med anledning av detta introducerades för ca 35 år sedan syntetiska retinoider i behandlingen av hudsjukdomar som kännetecknas av störd differentiering, tex medfödd iktyos. Denna sjukdom beror på specifika mutationer i keratinocyterna, som förorsakar kraftig fjällbildning. Nackdelen med retinoidbehandling är dess biverkningar. Kvinnor med iktyos som behandlas med retinoider måste undvika att bli gravida och bör fortsätta ta preventivmedel två år efter det att behandlingen avslutats.

Ett sätt att minska dessa problem är att istället för retinoider tillföra läkemedel som blockerar nedbrytningen av kroppens “eget” vitamin A och på så sätt få retinoidnivåer som är högre som förhoppningsvis ger mindre biverkningar. Detta kan uppnås genom att blockera det enzym (CYP26) i cellerna som bryter ner (katabolism) det hormonliknande vitamin A metabolit i cellen som heter retinsyra (RA). I djurförsök och kliniska studier på bl.a. psoriasis och iktyospatienter har man visat att denna form av blockering ger retinoidliknande effekter utan biverkningar. Denna doktorsavhandling har haft som mål att studera verkningsmekanismen av sådana substanser (ketoconazole, liarozole, talarozole) som blockerar CYP26 funktionen i epidermis, samt att undersöka funktionen av CYP26 i epidermis. Studierna på människa (delarbete I och II) var granskade och godkända av etisk kommitté.

Avhandlingen är indelad i fyra delarbeten (I-IV):

I I detta arbete studeras effekten av lokalbehandling med talarozole på 16 friska frivilliga försökpersoner. Två olika koncentrationer av talarozole jämfördes med kontroll. Ett hudområde behandlades med kräm med aktiv substans och ett annat med enbart krämbas. Hudbiopsier togs på bågge områdena och analyserades med hjälp av mikroskop, protein- och genuttrycksmetoder av olika retinoid biomarkörer. Efter nio dagars behandling kunde man iakta
signifikanta och i vissa fall dos-beroende förändringar i genuttrycket av keratiner (cytoskelettproteiner), CRABPII (protein som binder RA), CYP26A1 (medlem i CYP26 familjen) och andra retinoid markörer. Effekterna var inte förenade med den hudirritation man ser i samband med retinoidbehandling.

II


III

I denna in vitro studie undersöks effekterna av liarozole och talarozole. Effekten av substanserna studerades med avssende på uttrycket av flera retinoid reglerade gener och vitamin A metaboliserande enzymer i organotypiskt epidermis (bestående av keratinocyter i olika mognadsstadijer) i kombination med RA. Resultaten visar att talarozole är mer effektiv än liarozole-behandlande patienter. Studien visar också att CYP26A1 är den gen som är mest känslig för förändringar i vitamin A nivåerna.

IV

I denna studie analyserades uttrycket av retinoidmetaboliserande enzymer i prolifererande eller differentierande keratinocyter. CYP26A1 och CYP26B1 (en annan medlem av CYP26 familjen) bildningen förhindrades med hjälp av siRNA (korta RNA strängar), för att undersöka deras betydelse i RA katabolismen och RA signaleringen i cellen. Därutöver undersöks inverkan av ketoconazole, liarozole och talarozole på genuttrycket av retinoid-metaboliserande
Enzymer. Studien visar att CYP26 familjen och andra CYP-
enzymer noggrant kontrollerar RA katabolismen i såväl
prolifererande som differentierande keratinocyter. CYP26B1 är det enzym som verkar ha störst roll i keratinocyterna under mer fysiologiska omständigheter. CYP26A1 däremot verkar ha större roll vid höga doser av RA. Liksom i arbete III var talarozole den substans som mest effektivt påverkade RA metabolismen. Den påvisade en hög specificitet mot CYP26 och kraftigt inducerade CYP26A1. En annan slutsats är att substanserna har olika effekter i prolifererande och differentierande keratinocyter och att dessa effekter skiljer sig från retinoid (RA) effekterna.

Sammanfattningsvis har de fyra studierna visat att liarozole och talarozole kan vara goda ersättare till retinoider i behandlingen av hudsjukdomar. Vidare att deras effekter varierar i styrka och enbart delvis är identiska med retinoidernas. Dock måste fler studier genomföras där man fokuserar mer på RA katabolismen, för att tillfullo förstå dessa substansers verkningsmekanism och effekter i huden.
La vitamina A y las moléculas similares a esta (retinoides) tienen una gran influencia en la división celular (proliferación) y desarrollo (diferenciación). En el órgano más grande del cuerpo, la piel, van los retinoides a tener, entre otras cosas, influencia en el proceso de diferenciación de las células más comunes de la epidermis (keratinocitos). Es por ello que se utilizan retinoides sintéticos en el tratamiento de enfermedades de la piel que se caracterizan por la alteración en la diferenciación celular, por ejemplo la ictiosis. Esta enfermedad depende de mutaciones específicas en los keratinocitos. Dando, entre otras cosas, una gran escamocidad. La parte negativa del tratamiento con retinoides son sus secuelas. Mujeres que sufren de ictiosis y son tratadas con retinoides deben evitar quedar embarazadas y usar medios preventivos hasta lo menos dos años después de haber terminado su tratamiento con retinoides.

Una forma de disminuir esos problemas es que a cambio de administrar retinoides al cuerpo se bloquee la degradación de la “propia” vitamina A y de esta manera se obtengan niveles retinoidales más altos que causen menos daños. Esto se puede lograr a través de bloquear la enzima (CYP26) en la célula que cataboliza el ácido retinoico (AR). Con la ayuda de ensayos con animales y estudios clínicos, entre otros, pacientes con soriasis e ictiosis se ha podido comprobar que ésta forma de bloqueo da los resultados esperados sin efectos colaterales.

Esta tesis ha tenido como objetivo estudiar los efectos y mecanismos de esas substancias (ketoconazole, liarozole, talarozole) que bloquean la función de CYP26 en la epidermis, al mismo tiempo que se investigan las funciones de CYP26 en la epidermis. Los estudios hechos en personas (parte I y II) están aprobados por el comité ético.
La tesis está dividida en cuatro partes (I – IV)

I. En este trabajo se estudió la influencia de talarozole como tratamiento local en una piel normal. Dos concentraciones diferentes de talarozole se compararon con el control. Allí, a una parte de la piel se le administró una crema con la substancia y a la otra con sólo una crema base. Las biopsias se tomaron en ambas zonas y se hicieron análisis histológicos, proteínicos y de expresión genética de biomarcadores retinoidales. Luego de nueve días de tratamiento se pudo observar significativos resultados y en algunos casos, dependientes de la dosis en la expresión genética de los keratinos (proteínas de la estructura celular), CRABPII (proteína que acopla AR), CYP26A1 (miembro en la familia CYP26) y otros marcadores retinoidales. El efecto no estaba asociado con la irritación de la piel que se puede ver en relación con el tratamiento retinoidal.

II. En este trabajo se estudió la implicancia de liarozole en la epidermis de pacientes con ictiosis. Dos dosis diferentes de liarozole se compararon con placebo en el tratamiento de ictiosis laminar, la cual es una forma severa de ictiosis que a menudo es tratada con retinoides sintéticos. El trabajo fue parte de un estudio de multicentro-clínico donde los efectos clínicos de liarozole era el objetivo. Once pacientes (20-65 años) se escogieron al azar y se agruparon en tres diferentes grupos, allí se les administraron tabletas que equivalían dosis alta, baja y placebo. Las biopsias se obtuvieron después de cuatro semanas de tratamiento. En principio los mismos biomarcadores que se analizaron en el trabajo anterior se analizaron también en este estudio. Los resultados mostraron moderados efectos de parecido retinoidal en aquellos que obtuvieron liarozole, y también los efectos variaban entre individuos. Esto puede ser causa de que las biopsias tenían diferentes grados de piel callosa. Además, las muestras de referencias de los pacientes fueron en algunos casos, anormal en comparación con la epidermis normal.

III. En este item se ha estudiado los efectos de liarozole y talarozole en la expresión de varios genes regulados por los retinoides y enzimas metabolizadoras de vitamina A en un órgano tipo de epidermis (compuesta de queratinocitos en diversas etapas de madurez ) en combinación con AR. Los resultados muestran que talarozole es más eficaz que liarozole para aumentar la vitamina A y los niveles de AR en un órgano
El estudio muestra también que CYP26A1 es el gen más sensible a cambios de niveles de la vitamina A.

IV. En éste item se ha estudiado como las diferentes enzimas del metabolismo retinoico se expresaron en los cultivos de keratinocitos que se hayaban en fase de proliferación o diferenciación. CYP26A1 y CYP26B1 (otro miembro de la familia CYP26) fueron disminuidos con la ayuda del ARN de silenciamiento (fragmentos cortos de ARN), para investigar su importancia en el catabolismo de AR y la señalización del AR en la célula. Además fue examinada la influencia de ketaconazole, liarozole y talarozole en la expresión genética de enzimas del metabolismo enzimático retinoidal. El estudio muestra que la familia CYP26 y otras enzimas CYP parecen controlar el catabolismo de AR debido a que ambos tienen funciones en la proliferación y diferenciación de los keratinocitos. CYP26B1 es el que muestra tener un rol más activo en los keratinocitos en circunstancias más fisiológicas. CYP26A1 por otra parte parece tener un rol más de emergencia. Al igual que en item III talarazole fue la substancia más efectiva. También muestra una especificación contrá CYP26 y gran inducción de CYP26A1. Además quedó claro que las substancias tenian diferentes funciones en la proliferación y diferenciación de los keratinocitos los cuales difieren del efecto retinoidal (AR).

En resumen, los cuatro estudios mostraron que liarozole y talarozole podrían ser buenos reemplazantes para los retinoides en el tratamiento de enfermedades a la piel. Adicionalmente que los efectos de estas substancias varían en efectividad y son parcialmente iguales a los retinoides. Sin embargo, más estudios deben llevarse a cabo donde se enfoque el catabolismo del AR, con el fin de comprender el mecanismo de efecto de estas substancias y los efectos en la piel.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)