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Pharmacology of adapalene

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Summary Adapalene, a synthetic retinoid, is a new drug proposed for the treatment of acne patients. Studies on the *in vitro* and *in vivo* pharmacology of adapalene have shown that it is very active on cell and tissue proliferation and differentiation. Furthermore, adapalene has anti-inflammatory potential as determined by its anti-AP1 activity. Adapalene interacts selectively with the nuclear receptors RARβ and RARγ, and its activity on proliferation and differentiation can be blocked by a RARβ-γ antagonist. Because RARβ is not expressed in human keratinocytes, the effect of adapalene on the major cell type of the epidermis is certainly mediated by its interaction with RARγ. The unique pharmacological properties of adapalene may explain why, when compared to tretinoin, it has an improved therapeutic ratio due to its better tolerance.

Retinoic acid (RA), a metabolite of vitamin A, is a potent modulator of cellular proliferation and differentiation.[1,2] It is also considered a 'mediator' of morphogenesis and development.[3] These pleiotropic effects are known to be mediated by the interaction of RA with specific nuclear receptors which belong to the steroid/thyroid/vitamin D receptor superfamily. The RA receptors[4–6] (RARα, RARβ and RARγ) are ligand inducible trans-acting factors which heterodimerize with the 9*cis*-RA receptors[7,8] (RXRα, RXRβ, RXRγ) and interact with specific DNA sequences or retinoic acid response element (RARE) contained in the promoter region of target genes.[9,10] RXRs interact with each other to form homodimers able to bind to gene promoters containing a specific retinoid X response element (RXRE).[11]

Several natural and synthetic retinoids are used in the management of dermatological diseases.[12] For example, RA and one of its isomers, 13-*cis*RA, are used topically and orally, respectively for acne treatment; however, use of RA gives rise to substantial skin irritation showing the need for retinoids with an improved therapeutic/side-effect ratio. Our medicinal chemistry and pharmacology program led to the discovery of a new synthetic retinoid, adapalene, active in the treatment of acne and better tolerated than RA. The interaction of adapalene with RARs and RXRs, as well as its effect on cell proliferation and differentiation and its anti-inflammatory properties are described below.

Interaction of adapalene with RARs and RXRα:

The binding dissociation constants (Kd) of adapalene for RARα, RARβ, RARγ and RXRα were determined using human recombinant receptors produced by transfection of COS-7 cells with expression plasmids encoding the different receptors. Nuclear extracts prepared from transfected cells were used in competition binding experiments performed with the stable reference retinoid [H^3]-CD367.[13] The Kd values of adapalene and RA for the different RARs and RXRα are shown in Table 1. In contrast to RA, adapalene displayed selectivity for RARβ and RARγ and no affinity for RXRα.

The transactivation potential of adapalene was studied in HeLa cells co-transfected using the calcium–phosphate precipitation procedure with expression vectors coding for human RARα, RARβ, RARγ and RXRα. In the case of RARs, the cells were co-transfected with a TRE-tk-CAT reporter plasmid, and in

Table 1 Binding affinities for human RARs and RXRα of adapalene and RA

	Binding affinities (Kd* in nM)			
Compound	RARα	RARβ	RARγ	RXRα
RA	15	4	5	730
Adapalene	1100	34	130	N.B.†

*Kd values were derived from competition binding experiments using [3H]-CD367 as the reference retinoid.

the case of RXRα, a RXRE-tk-CAT vector was used. These plasmids consist of a thyroid hormone response element (TRE) or an RXRE cloned upstream of the minimal promoter of the HSV1 thymidine kinase (tk) and the reporter gene of the chloramphenicol-acetyl-transferase (CAT). After transfection, the cells were treated for 16 h with the retinoids. CAT expression was measured using an ELISA.

The transactivation potential of adapalene and of retinoic acid are shown in Table 2. Adapalene was less active via RARα than RA and did not transactivate through RXRα.

Activity of adapalene on cell and tissue differentiation

The effects of adapalene on cell differentiation *in vitro* were determined in F9 cells or normal human keratinocytes (NHK) in culture and in a reconstructed skin model.

F9 murine embryonal carcinoma cells are induced to differentiate into endoderm cells after treatment with retinoids.[14] This phenotypic change is concomitant with the secretion into the culture medium of the tissue type plasminogen activator which was used as a marker to quantify the biological effects of retinoids.[15] In the F9 differentiation test, adapalene was more active than RA showing that it is a good modulator of cell differentiation (Table 3). The treatment of NHK with retinoids inhibits the expression of several differentiation markers, i.e. keratin pair 1/10, involucrin, loricrin, filaggrin and plasma membrane associated transglutaminase (TG1). The latter is a characteristic enzyme of terminally differentiating keratinocytes which catalyses the formation of the cornified envelope constituting the membrane of corneocytes.[16] Corneocytes represent the final stage of keratinocyte differentiation and are found in the stratum corneum, the uppermost layer of the skin. A specific monoclonal antibody directed against TG1 was used to develop an ELISA in order to determine the level of TG1 expressed by keratinocytes.[17] Keratinocytes were grown up to sub confluency in low calcium semi defined medium and they were then shifted to high calcium medium. At the same time, retinoids were added to the culture medium. After four days, the ELISA assay was performed. As indicated in Table 4, adapalene displayed a very high activity in this differentiation model, while RA was less active.

In the reconstructed skin model, NHK were seeded on lattices made of type I collagen contracted by fibroblasts. The dermal equivalents were first kept submerged in the culture medium for a week to allow the epidermal cells to form a confluent monolayer and then were raised at the air–liquid interface on stainless steel grids over a second week, a time found to be sufficient to allow stratification and differentiation of the culture.[18] During this period, retinoids were added to the culture medium. The expression of keratinocyte differentiation markers was determined at the protein level by immunofluorescence staining of frozen sections. Adapalene inhibited the expression of several differentiation markers including keratin 10, filaggrin, loricrin, involucrin and transglutaminase (Fig. 1). The expression level of their mRNA was also studied by RT-PCR analysis. As an example, Fig. 2 shows that loricrin mRNA expression is down-regulated by adapalene.

The activity of adapalene on the differentiation of F9 cells and NHK was completely inhibited by the addition of the selective RARβ/γ antagonist CD2665. Furthermore, the antagonist CD2665, blocked the inhibitory effect of adapalene on epidermal differentiation in the reconstructed skin model (Fig. 2). Since RARβ is not expressed in human keratinocytes, this observation indicates that the action of adapalene on keratinocyte differentiation may be mediated exclusively by its interaction with RARγ.

Table 2 Transactivation potential of adapalene and RA

Compound	Transactivation potential (AC_{50} in nM)			
	RARα	RARβ	RARγ	RXRα
RA	2	4	2	1000
Adapalene	22	2	9	N.A.[a]

[a]N.A. not active

Table 3 Effect of adapalene and RA on cell differentiation *in vitro*

Compound	F9 cells Plasminogen activator production AC_{50} (nM)	Normal human keratinocytes Transglutaminase 1 expression IC_{50} (nM)
RA	200	24
Adapalene	40	2·5

Table 4 Effect of adapalene and RA in the rhino mouse assay

Compound	Number of epidermal comedones per cm of stratum corneum length	Epidermal thickness (μm)
Control (0%)	69	22
All-trans retinoic acid (0·1%)	32	64
Adapalene (0·1%)	20	58

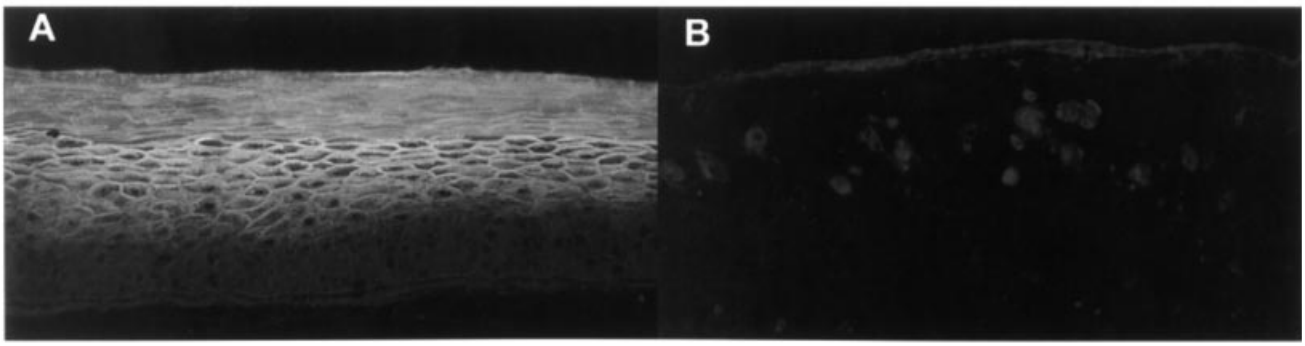

Figure 1. Effect of adapalene on the expression of transglutaminase in reconstructed skin. Immmunofluorescence staining of control (A) and adapalene (B) treated reconstructed skin sections.

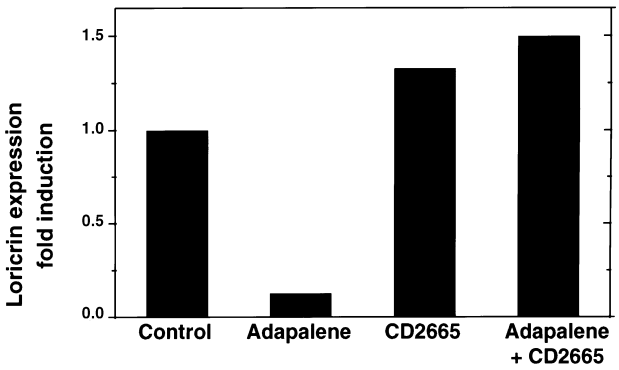

Figure 2. The inhibitory effect of adapalene on loricrin mRNA expression by reconstructed skin is blocked by the RARβ/γ antagonist CD2665 as shown by RT-PCR analysis.

The effects of adapalene on the differentiation process *in vivo* were studied using the rhino mouse strain, a mutant displaying some of the characteristic features of the skin of acne patients. At the age of 4 weeks, the rhino mouse loses hairs and the upper part of the original follicular unit gives rise to utricules filled with corneocytes and sebum. After 7–8 weeks, these sebaceous follicles, progressively distended by the production and accumulation of excessive horny material, are histologically reminescent of retentional human acneic lesions such as microcomedones. When applied topically for 3 weeks, retinoids reduce the number of epidermal comedones and induce an increase in epidermal thickness[19] (Fig. 3). Both parameters can be measured by image analysis of skin sections. Adapalene was more active than RA in the reduction of utricle number, but a little less active than RA in inducing epidermal hyperplasia (Table 4). In more detailed ultrastructural studies[20] it was observed that adapalene induces increased desquamation and decreased cohesiveness of corneocytes in the epidermis and epithelial wall of the pseudocomedones.

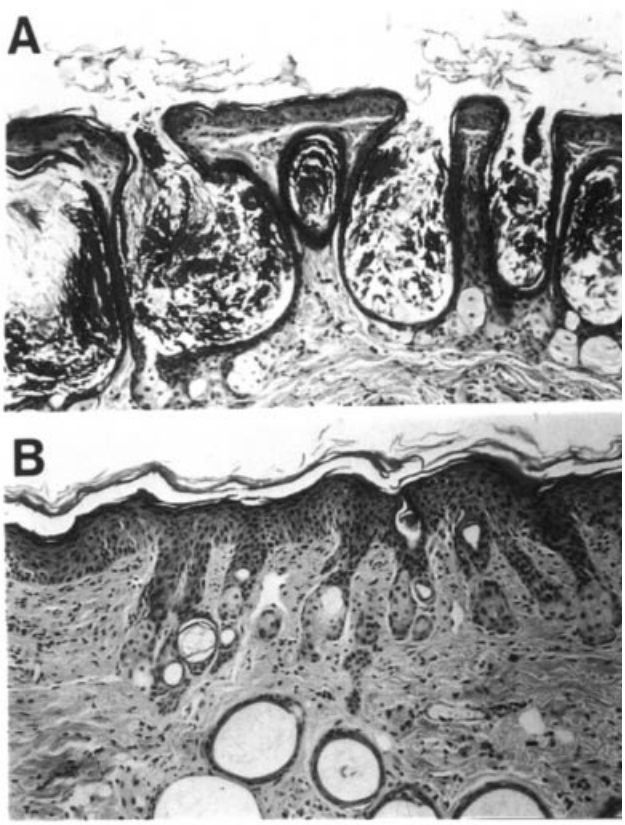

Figure 3. Comedolytic activity of adapalene on the comedonal lesions of the rhino mouse model. Histological sections of control (A) and adapalene (B) treated skin.

Anti-inflammatory activity of adapalene

The anti-inflammatory activity of adapalene was studied in comparison with known anti-inflammatory reference compounds, namely indomethacin (IN) and betamethasone valerate (BMV), and with RA, 13-*cis*RA, and etretinate.[21] *In vitro*, in both soya bean and human PMN enzyme systems, adapalene induced an inhibition of the oxidative metabolism of arachidonic

acid by 5-and 15-lipoxygenase pathways that was superior to the inhibitory activity produced by the reference compounds and the other studied retinoids. Adapalene produced a significant inhibition of f-met-leu-phe induced chemiluminescence of rabbit PMN, comparable to RA, and stronger than IN and BMV. In addition, it inhibited peripheral human blood PMN chemotaxis.

In several classical *in vivo* assays (see Table 5), adapalene proved to have moderate to strong anti-inflammatory activity, comparable to those of reference anti-inflammatory agents and generally superior to the other retinoids studied.

As indicated previously, retinoids exert their activities by interacting with RARs or RXRs and by activating genes which contain RARE or RXRE in their promoters. They can also regulate gene expression by inhibiting the activity of other transcription factors such as AP1.[22] AP1 is composed of jun/jun homodimers or fos/jun heterodimers and is inducible by growth factors, phorbol esters or ultraviolet (UV) radiation. AP1 sequences are found in the promoter region of many genes including matrix metalloproteinases (collagenases, stromelysin), growth factors (TGFβ, VEGF) and inflammatory mediators (IL1). The AP-1 transcription complex controls the expression of a subset of genes that are expressed early in response to extracellular mitogenic stimuli or to stress. AP1 is thus thought to play an important role in inflammation and immune response. Retinoids with anti-AP1 activity could block part of the inflammatory response.

The anti-AP1 activity of adapalene was tested in HeLa cells transfected with a plasmid-containing part of the collagenase promoter cloned upstream of the CAT gene.[23] AP1 activity was induced by treating the cells with TPA. Compared to RA, adapalene is a potent inhibitor of AP1 activity (Fig. 4A). Recently, it was shown that adapalene inhibits TPA-induced vascular endothelial growth factor (VEGF) and MMP1 mRNA expression by human keratinocytes in culture.

To confirm *in vivo* the anti-AP1 activity of adapalene, mouse ears were treated topically with TPA and the resulting oedema was measured 6 h later. The simultaneous treatment of mice with TPA and adapalene or RA led to the inhibition of the oedema response as shown in Fig. 4 (B). Adapalene displayed the same anti-AP1 activity as RA. Our recent unpublished data show that, in this model, the inhibition of the TPA-induced oedema by retinoids is well correlated with the decrease of the expression of VEGF mRNA.

Altogether, these results suggest that the anti-inflammatory activity of adapalene might be partly due to its anti-AP1 activity.

Table 5 *In vivo* animal models used for the evaluation of anti-inflammatory activity of adapalene

Models
UV irradiation induced erythema in the guinea pig
Croton oil induced ear oedema in the rat
Carrageenan induced foot paw oedema in the rat
Granuloma formation in the rat
Leucocyte migration and prostaglandin E2 synthesis in the rat after local application of polyester sponges impregnated with carrageenan
Passive cutaneous anaphylaxis in the rat

Anti-proliferative activity of adapalene

HeLa cells were chosen to develop a test system for the evaluation of the anti proliferative potential of synthetic retinoids.[24] In this assay, the cells were seeded at low density and were treated for 4 days with the test retinoid. Cell proliferation was then determined using a marker of mitochondrial activity (XTT assay). The inhibitory effect of the retinoids on cell proliferation was calculated from dose–response curves and expressed as IC_{50} values. Adapalene and RA showed a very high activity in the inhibition of Hela cell proliferation with IC_{50} values of 16 and 6 nM, respectively. The activity of adapalene on Hela cell proliferation was clearly linked to its interaction with the RARs since it was completely abolished by the addition of the selective RARβ/γ antagonist CD 2665. Recently, it was demonstrated that adapalene has a strong inhibitory activity on rat sebocyte proliferation *in vitro* (Prof. R.L. Rosenfield, personal communication).

In vivo, the anti proliferative potential of adapalene was assessed using the epidermal ornithine decarboxylase (ODC) assay.[25] ODC is a polyamine biosynthetic enzyme that plays a major role in growth and malignant transformation by catalysing decarboxylation of ornithine to putrescine, the first step and probably the

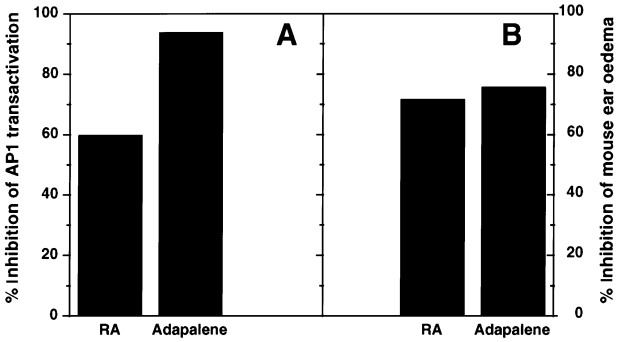

Figure 4. Inhibition by adapalene and RA of AP1 activity *in vitro* (A) and *in vivo* (B).

rate-limiting step in the pathway of polyamine biosynthesis. ODC activity was induced in the epidermis of hairless rats by cellulose tape stripping. Retinoids were applied topically immediately after tape stripping and the ODC activity of epidermal extracts was measured after 6 h. Adapalene and RA were able to significantly inhibit the tape-stripping induced ODC activity.

Conclusions

Two major factors have been proposed to account for the aetiology of acne, an altered keratinocyte differentiation in the infundibulum of the pilosebaceous unit and an increased sebum secretion. The abnormal differentiation of infundibular keratinocytes would lead to an increased cohesiveness between corneocytes, resulting in their retention and accumulation in the infundibulum and eventually in the obturation of the pilosebaceous duct. This would be followed by bacterial colonization and eventually could result in the disruption of the comedones and in an inflammatory response. It is now clearly established that adapalene is a very effective drug for acne treatment.[26] The data described above suggest that this effect could be related to the activity of adapalene on keratinocyte differentiation, sebocyte proliferation and inflammation.

In the treatment of acne patients, and compared to tretinoin, adapalene displays an improved therapeutic ratio mainly due to its better tolerance. This is certainly due to the unique pharmacological properties of this compound, which are characterized by a selectivity for the nuclear RARβ/γ, and a potent activity on cell differentiation.

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