

In Vitro Assessment of the Tolerance Profile, the Metabolism and the Potential Depigmenting Activity of New Retinoid Derivatives

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Abstract: Background: In spite of the numerous depigmenting agents available, there is an inverse relationship between safety and efficiency. Natural retinoids exert depigmenting activities *in vivo*, but have a narrow therapeutic window. There is thus a need for a new class of depigmenting agents that would be both safe and useful.

Objective: The aim of the study was to analyse the potential interest of new retinoid derivatives as topical depigmenting agents.

Methods: The tolerance profile of the new retinoids was analysed in murine BDVII keratinocytes and murine B16 melanocytes using the MTT assay. The uptake and the metabolism of the retinoids were analysed in keratinocytes and melanocytes by HPLC/UV. The cellular content of melanin was determined in melanocytes using a photometric assay.

Results: Methyl- and *t*-butyl-4-oxoretinoate, arbutin retinoate and retinoic acid were shown to have the best depigmenting potential according to the ratio between the IC₅₀ for MTT assay and that for melanin content. Retinaldehyde, 4-hydroxyretinaldehyde and methyl-4-hydroxyretinoate have a good depigmenting potential too, although the therapeutic window is narrower due to a higher toxicity.

Conclusion: 4-Oxoesters of retinoic acid, arbutin retinoate and 4-hydroxyretinaldehyde derivatives offer a new family of depigmenting agents acting *via* a different pathway than tyrosinase inhibitors.

Keywords: Keratinocytes, melanocytes, metabolism, pharmacology, pigmentation, retinoids.

INTRODUCTION

The pigmentation of the skin, due to melanin deposits, plays an important role in protecting the skin from the harmful effects of solar UV radiations. However, hyperpigmented lesions of the skin pose a problem to many people beyond a simple question of aesthetics [1-3]. In spite of a high demand, it's still difficult to find a safe and efficient treatment to lighten the skin. Although most of available depigmenting agents are based on their ability to inhibit tyrosinase *in vitro*, they did not prove to be effective when applied to human skin. Topical formulations shown to have a significant efficacy in some definite situations are not safe, in particular for a long period of time required to reach satisfying results [4, 5]. In this context it would be interesting to find a new class of safe topical depigmenting agents, acting *via* a different mechanism of action than tyrosinase inhibition.

Besides their known biological actions on morphogenesis, cell growth and differentiation, retinoids may have a depigmenting potential, as some of them have been shown to inhibit the pigmentation of human and animal skin, either alone or in combination with other compounds [6-8]. Retinoids are metabolised by hydroxylation and carbonylation

in position 4 of the cyclohexenyl cycle and by conjugation to glucuronate [9-11]. This oxidation does not abolish their biological activity, as demonstrated in a previous study in mice, where 4-oxoretinaldehyde, and to a lesser extent and 4-oxoretinol, were shown to have a lower but significant biological activity than their unmetabolised precursors [12]. On the other hand it seems that esterification of retinoic acid is not an endogenous process. Some retinoate esters have been synthesised in order to increase the stability or the chemopreventive property of retinoic acid [13], but these derivatives did not lead to a new family of therapeutic retinoids.

In this study we analysed in cell cultures the tolerance profile and the effects on cellular melanin of a new family of retinoids derived from 4-oxygenated forms of retinaldehyde and retinoic acid esterified to arbutin or fatty acids (Fig. 1).

MATERIALS AND METHODS

Chemicals

All retinoids, except all *trans*-retinoic acid (RA), all *trans*-retinaldehyde (RAL), and all-*trans* retinol (ROL), were provided by Pierre Fabre Dermo-Cosmétique (Toulouse, France). The other chemicals, if not specified differently, were purchased from Sigma (Buchs, Switzerland).

Monolayer Cell Cultures

Murine B16 melanocytes and BDVII keratinocytes, as well as human A431 cells, were cultured as previously

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described [14, 15]. Briefly, both BDVII and A431 keratinocytes were grown on DMEM/FCS 10%, and B16 melanocytes were grown in the same medium supplemented with 1 nm cholera toxin (Gentaur, Brussels, Belgium). Both media contained 100 U/ml penicillin G and 100 µg/ml streptomycin (Invitrogen, Basel, Switzerland). Cells were plated at 20'000 cells per well in 24-well plates and grown until confluence (3–4 days). The tested compounds were dissolved in ethanol and added to the cultures at the final indicated concentrations in 1% ethanol. This procedure was repeated daily for three consecutive days, then on day 5 cell viability was assessed using the MTT assay and melanin content determined spectrophotometrically as described below.

Melanin Determination

Cell cultures and reconstructed epidermis were rinsed with phosphate buffered saline (PBS), treated with trypsin 0.05%-EDTA for 10 minutes at 37°C or with trypsin 0.83% for one hour at 37°C, respectively, then melanin was extracted and its content determined spectrophotometrically at 405 nm and normalised by MTT assay as previously described [16].

Retinoid Analysis

Retinoids were applied at 10 µM for 4, 24 or 96 hours in BDVII keratinocytes or B16 melanocytes, then the cells were lysed and harvested by sonication in 50 mM acetate buffer pH 4, and intracellular retinoids were analysed by high performance liquid chromatography (HPLC) and UV detection as described (Table 1) [17]. Results shown in Figs.

(2-5) are expressed as (pmol retinoid/mg protein) and represent the means of duplicate experiments performed in the same series of culture. The whole experiments were repeated twice in two separate series of cultures with similar pharmacological profiles, although with different absolute values due to inter-experiment variability.

DOPA Oxidase Activity

DOPA oxidase activity (tyrosinase activity) was determined as previously described [14]. Briefly cultured B16 melanocytes were treated each day for 4 days by various concentrations of tested retinoids, then cells were harvested and DOPA oxidase activity was determined spectrophotometrically by the MBTH (3-methyl-2-benzothiazolinone hydrazone) assay [18].

Photometric Analyses

Retinoid derivatives were dissolved in ethanol to prepare 10 µM solutions, whereas arbutin was dissolved at 100 µM in water; 200 µl of these solutions were transferred into 96-well UV-STAR microplates (Greiner Bio One, Reinach, Switzerland), then absorption spectra were performed to determine the highest absorbance wavelength using an EnVision multiplate reader (Perkin Elmer, Schwerzenbach, Switzerland). For each solution, the absorbance was read at the respective maximum wavelength, and the molar extinction coefficient (ϵ) was calculated using the Beer-Lambert formula: $\epsilon = A/\ell \cdot c$, where A is the absorbance, ℓ the height of the solution in the microplate (0.56 cm) and c the molar concentration.

Table 1. Names and Spectral Parameters of the Tested Compounds

Abbreviated Names	Full Names of Compounds	λ_{max} (nm)	ϵ [(mol/l) ⁻¹ ·cm ⁻¹]
Arbutin	4-hydroxyphenyl- β -D-Glucopyranoside	280	1'750
ARA	arbutin retinoate	363	40'000
DIV374	<i>t</i> -butyl 4-oxoretinoate	353	59'000
DIV404	<i>t</i> -butyl 4-hydroxyretinoate	348	54'000
DIV405	4-oxoretinoic acid	353	60'000
DIV406	4-hydroxyretinoic acid	339	55'000
DIV407	<i>t</i> -butyl-4-acetoxyretinoate	346	59'000
DIV408	methyl 4-oxoretinoate	355	59'000
DIV419	methyl 4-hydroxyretinoate	354	54'000
DIV420	4-oxoretinyl acetate	336	55'000
DIV633	arbutin 4-retinate	343	50'000
DIV635	4-oxoretinaldehyde	377	65'000
DIV736	4-(4-methoxyphenyl)retinal	378	59'000
DIV857	4-hydroxyretinaldehyde	375	70'000
DIV1178	4-retinal and δ -tocopheryl azelate	375	3'600
DIV1181	4-retinal linoleate	368	12'000
RA	all- <i>trans</i> -retinoic acid	345	54'000
RAL	all- <i>trans</i> -retinaldehyde	383	60'000
ROL	all- <i>trans</i> -retinol	325	48'000

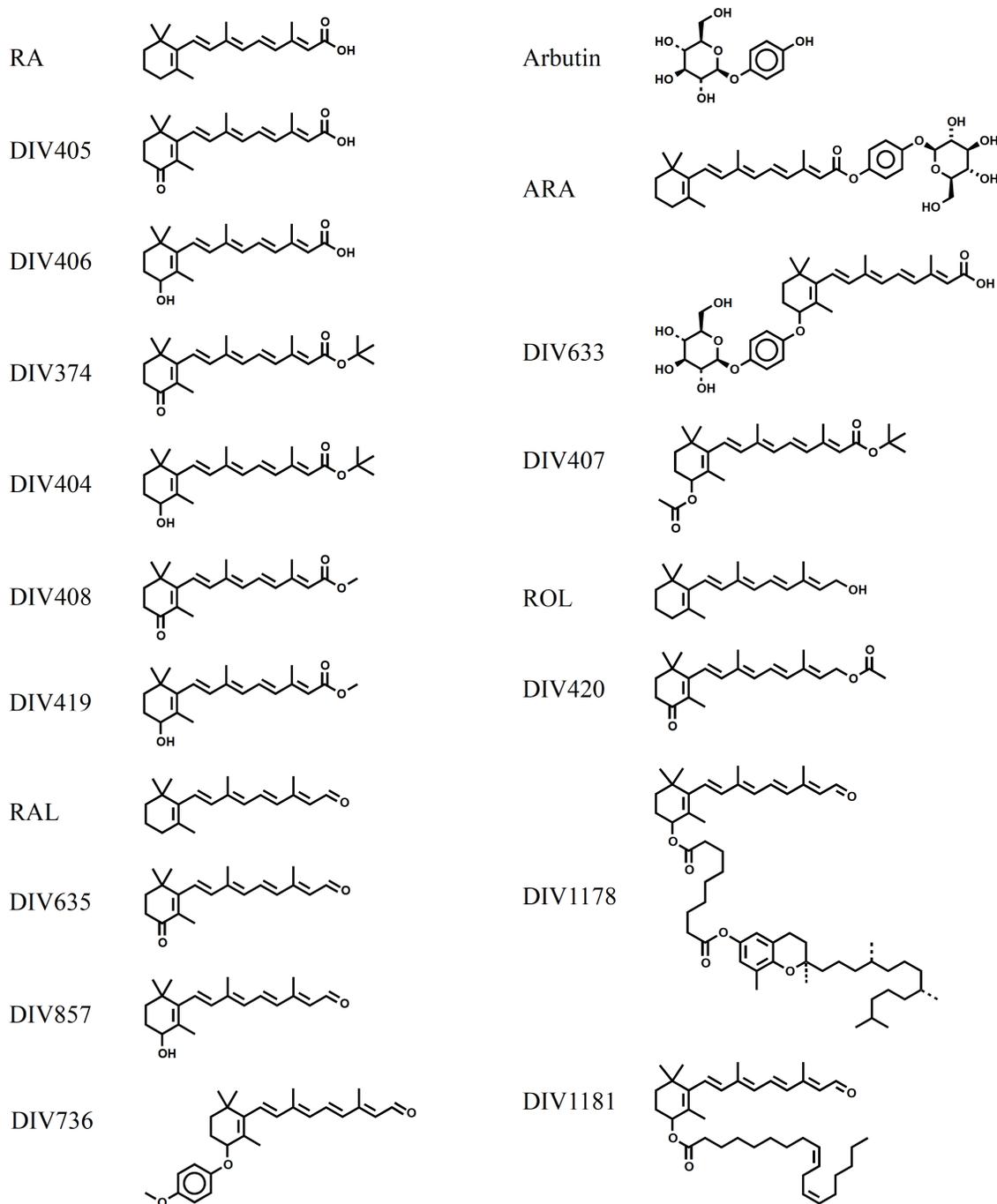


Fig. (1). Molecular structures of tested substances.

RESULTS

Uptake and Metabolism of Retinoid Derivatives by Keratinocytes and Melanocytes

RAL, ROL, as well as DIV374, DIV404 and DIV407 gave rise to a high cellular concentration of retinoids in both keratinocytes and melanocytes, i.e. a total of approximately 10'000 (pmol/mg prot) (Figs. 2-4). As expected, RA was not reduced to its precursors ROL and RAL, the bioconversion of RAL to RA being irreversible (Figs. 2A, 3A). RAL was reduced to ROL and ROL esters in both cells, and was oxidised to significant amounts of RA in keratinocytes only

(Figs. 2B, 3B). ROL was esterified to fatty acids in both cells, and was partly oxidised to RAL in melanocytes only (Figs. 2C, 3C). RA and its methyl esters of 4-oxo (DIV408) and 4-hydroxy (DIV419) derivatives were less taken up by the cells, with a total of retinoids of $\approx 1'000$ (pmol/mg prot) (Figs. 2A, G, H, 3A, G, H). The esters of 4-oxo RA were marginally reduced to their 4-hydroxy counterparts, whereas the 4-hydroxy derivatives were mostly oxidised to the 4-oxo ones (Figs. 2E, G, H, 3E, G, H). 4-OxoRAL, unlike RAL, was much less taken up by the cells, in particular by melanocytes, and was mostly reduced to 4-oxoROL in keratinocytes, the sum reaching 300 (pmol/mg prot) in keratinocytes and 30 (pmol/mg prot) in melanocytes (Figs.

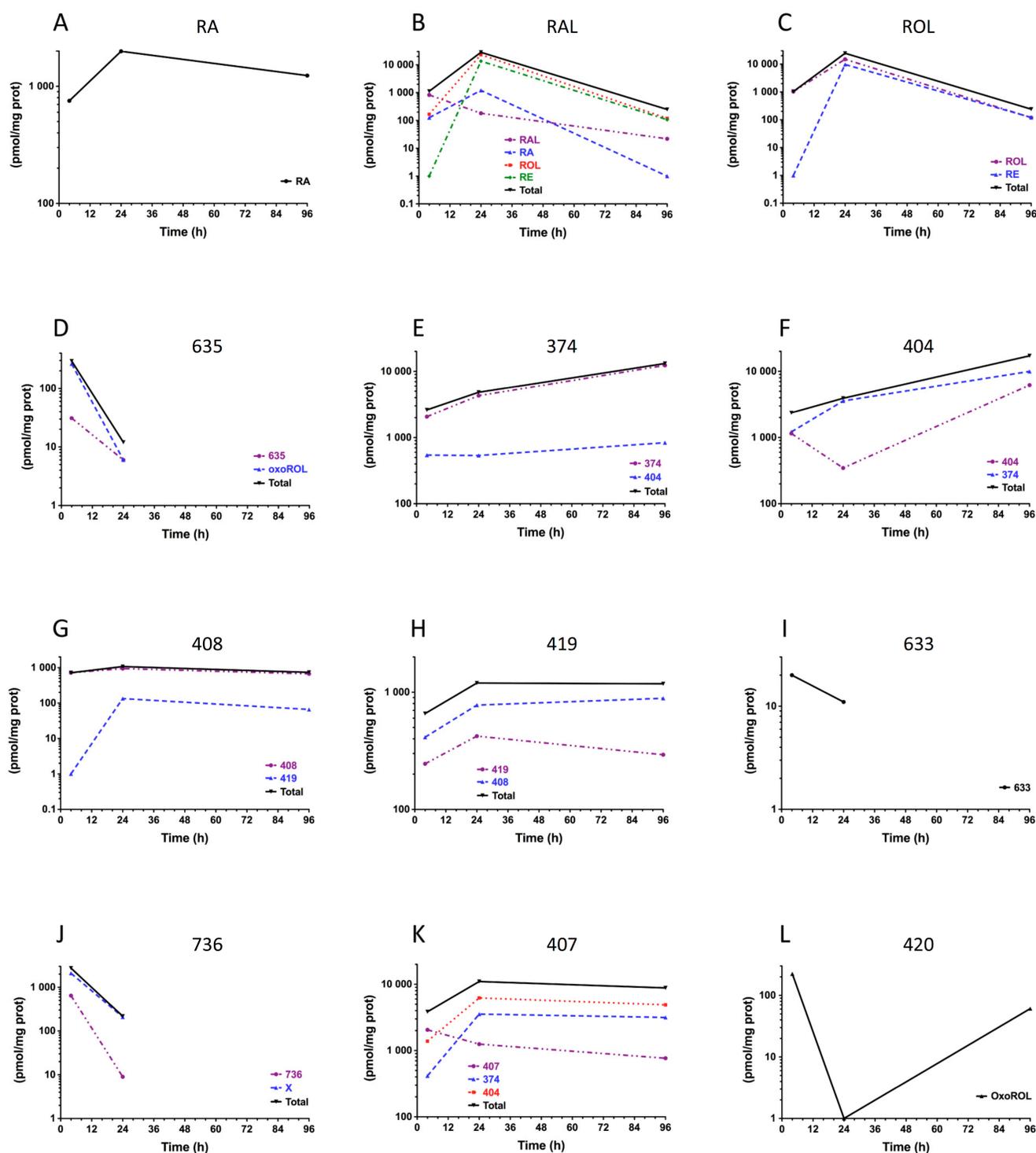


Fig. (2). Uptake and metabolism in BDVII keratinocytes. Retinoids were applied at 10 μ M for 4, 24 or 96 hours on BDVII keratinocytes, then intracellular retinoids were analysed by HPLC. Applied retinoids, indicated on the top of each graph, were RA (A), RAL (B), ROL (C), 635 (D), 374 (E), 404 (F), 408 (G), 419 (H), 633 (I), 736 (J), 407 (K) and 420 (L). Results, expressed as (pmol retinoid/mg protein), represent the means of duplicate experiments. The whole experiment was repeated twice with a similar pharmacological profile. RE: retinyl esters.

2D, 3D). A hydroquinone derivative of 4-hydroxyretinal (DIV736) was taken up and mostly metabolised to an unidentified metabolite by both cell types (Figs. **2J, 3J**). DIV1178 and DIV1181 did not produce detectable retinoids within keratinocytes or melanocytes at 10 μ M; they were then applied at 100 μ M, and at this high concentration

DIV1181 was taken up by both cell types (162 and 329 (pmol/mg prot) at 24h for B16 and BDVII, respectively) and converted to DIV857 and unidentified metabolites, whereas DIV1178 was still not taken up by the cells in these conditions (not shown).

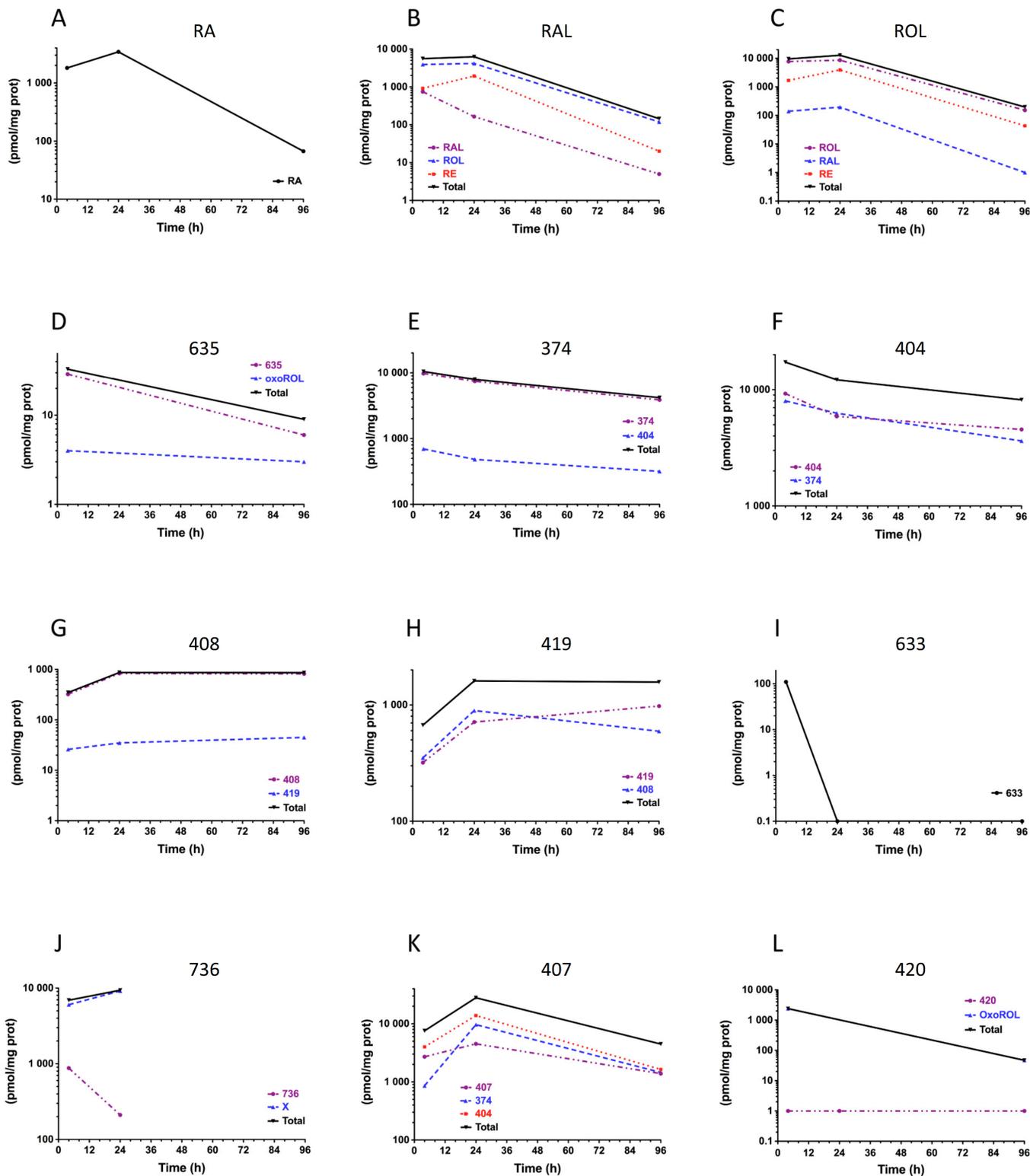


Fig. (3). Uptake and metabolism in B16 melanocytes. Retinoids were applied at 10 μ M for 4, 24 or 96 hours on B16 melanocytes, then intracellular retinoids were analysed by HPLC. Applied retinoids, indicated on the top of each graph, were RA (A), RAL (B), ROL (C), 635 (D), 374 (E), 404 (F), 408 (G), 419 (H), 633 (I), 736 (J), 407 (K) and 420 (L). Results, expressed as (pmol retinoid/mg protein), represent the means of duplicate experiments. The whole experiment was repeated twice with a similar pharmacological profile. RE: retinyl esters.

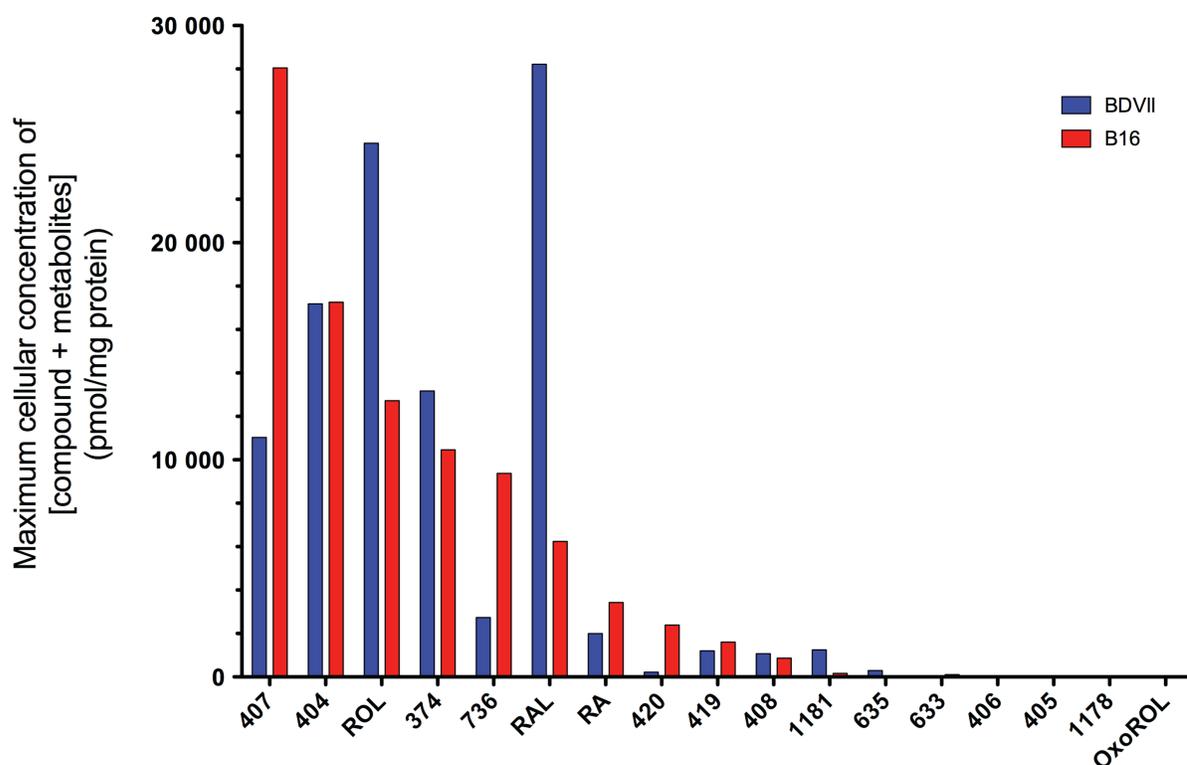


Fig. (4). Maximum total retinoid concentration. The histogram shows the maximum cellular retinoid concentration following incubation with the tested retinoids. Depending on the tested compounds, the maximum concentration is reached at 4h, 24h, or 96h. Results, expressed as (pmol retinoid/mg protein), represent the means of duplicate experiments, and were calculated from data shown in Figs. (2, 3).

Table 2. Cell Viability and Depigmenting Parameters

Compound	BDV II Keratinocytes	B16 Melanocytes			Pigmentation Index
	IC ₅₀ [toxicity] (μM)	IC ₅₀ [toxicity] (μM)	IC ₅₀ melanin (μM)	IC ₅₀ [depigmentation] (μM)	
RA	15	5	0.06	2	83
Arbutin	> 100	> 100	3	40	> 33
ARA	20	10	0.15	0.2	67
DIV 00374	50	30	1	2	30
DIV 00404	50	10	2	20	5
DIV 00408	10	50	0.4	0.5	125
DIV 00419	20	30	5	7	6
RAL	3	2	0.3	0.4	7
DIV 00857	10	4	0.6	1	7
DIV 00736	20	20	15	40	1

The pharmacological parameters shown here (IC₅₀ and pigmentation indexes) were calculated from concentration-response curves of two separate experiments performed in the same series of cultures. For cell viability the measured parameter was the value for MTT assay (i.e. MTT formazan, or MTTF) and for cellular melanin content the measured parameter was expressed as (μg melanin/μmol MTTF) [16]. From the corresponding concentration-responses curves the IC₅₀s for the toxicity and the depigmentation can be determined. The whole experiments were repeated twice in two separate series of cultures with similar pharmacological profiles, although with different absolute values due to inter-experiment variability. The IC₅₀s and pigmentation indexes however are very comparable from one experiment to another. The pigmentation index is defined as IC₅₀ [toxicity] divided by IC₅₀ (melanin) (see Results for details).

Tolerance Profiles of Retinoid Derivatives in Keratinocytes and Melanocytes

The MTT assay allows the determination of an IC₅₀ for the toxicity (IC₅₀ [toxicity]). RAL and its 4-hydroxyderivative were the most toxic to both cell cultures

with IC₅₀ in the low μM range, then came RA and its esters with IC₅₀s between 5 and 50 μM (Table 2). In keratinocytes the *t*-butyl esters of RA derivatives were less toxic than the methyl esters (Table 2). Arbutin was well tolerated in both cells with IC₅₀s > 100 μM (Table 2).

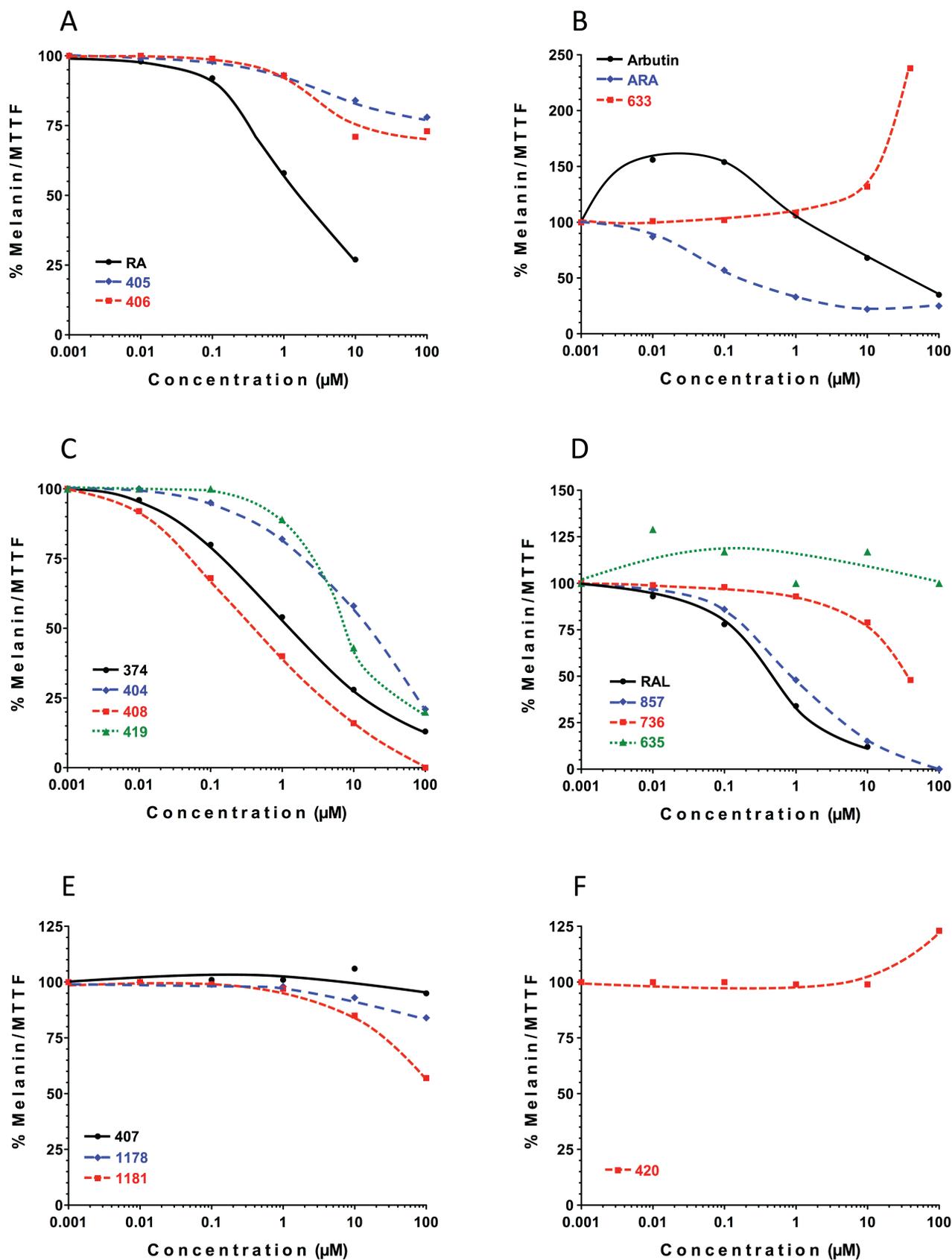


Fig. (5). Effects of retinoids on melanocyte melanin. Retinoids were applied for three consecutive days at various concentrations on B16 melanocytes, then cellular melanin content was determined and MTT assay performed two days following the last treatment. Results, calculated as (mg melanin/mol reduced MTT), are expressed as percentage of the value of vehicle (1% ethanol), and represent the means of duplicate experiment. The whole experiment was repeated twice with a similar pharmacological profile.

Depigmenting Potential of Retinoid Derivatives

To assess the depigmenting potential of the tested compounds, we defined a pigmentation index. This parameter gives an index of the specific action on cellular melanin content. Ideally, a compound that decreases specifically the melanin content in a concentration-dependent manner without affecting the viability of the cells would have a low IC₅₀ for melanin and a much higher IC₅₀ for the toxicity (MTT assay) [16]. We thus define the pigmentation index (PI): $PI \equiv IC_{50} [\text{toxicity}] / IC_{50} [\text{melanin}]$. According to the PI, esters of 4-oxoretinoate, arbutin retinoate and retinoic acid are the most interesting compounds, then come RAL and its 4-hydroxyderivatives, which have a strong effect on melanin, but with a narrower therapeutic window due to a lower IC₅₀ [toxicity] (Table 2, Fig. 5). The DOPA oxidase activity of the retinoid derivatives does not parallel the depigmenting activity, except for RAL, indicating that the mechanism by which these compounds decrease the cellular melanin is not mediated by an inhibition of tyrosinase (Table 3).

Table 3. DOPA Oxidase Activity vs Inhibition of Melanin

Compound	Concentration (µM)	% of Vehicle	
		Melanin/ MTT	DOPA Oxidase
RAL	1	34	22
DIV857	10	15	116
DIV374	10	28	104
DIV404	10	58	117
DIV405	10	84	102
DIV406	10	71	89
DIV407	10	106	158
DIV408	10	16	161
DIV419	10	43	117
DIV420	10	99	95
DIV1178	100	84	110
DIV1181	100	57	77

DOPA oxidase activity was measured as (µmol oxidised DOPA/min). For each compound the value of DOPA oxidase is indicated as the % of activity as compared to solvent alone (ethanol 1%). The indicated values represent the mean of two experiments performed in the same series of cultures. The whole experiment was repeated twice with similar % values.

DISCUSSION

Topical RA and its 13-*cis* isomer (isotretinoin) are used as anti-acne therapeutic agents, whereas RAL is used in cosmetic formulations to slow down skin ageing or to lighten skin pigmentation [19-21]. On the other hand, most, if not all, depigmenting agents target tyrosinase, the enzyme that catalyzes the rate-limiting step of melanogenesis [22]. However, there is a clear discrepancy between the anti-tyrosinase activity *in vitro* and the ability to lighten the skin *in vivo* [23].

Although retinoids are not known to be tyrosinase inhibitors, RA and RAL have been shown to potentiate the

depigmenting action of other agents such as hydroquinone [6], 4-hydroxyanisole [7] and glycolic acid [8], probably *via* a distinct mechanism of action. In our study, the conditions leading to a significant decrease of melanin content did not correlate to a decrease of DOPA oxidase activity, except for RAL (Table 3).

The problem with the use of systemic retinoids is their teratogenicity [24, 25]. However topical retinoids were proved to be safe, since no significant increase of circulating retinoids has been observed following topical retinoids [26-29]. Topical RA induces a skin irritation that may lead to cessation of treatment. Topical RAL is quite less irritant and may be used for longer periods of time [30-33]. In this context it is interesting to have at one's disposal a new family of branched retinoids with a potential depigmenting effect, which could be added, alone or in combination, in skin lightening formulations.

The tolerance profile, the uptake and the metabolism of the new retinoid derivatives were analysed in cultured keratinocytes and melanocytes. Most of them were rapidly taken up by the cells. The hydroxyl group at position 4 of the cyclohexenyl cycle was reversibly oxidised into a carbonyl. In the case of 4-oxoretinol, sizable amounts of the compound were found in keratinocytes at 4h and 96h, were it was almost undetectable at 24h. This could be due to the production of a metabolite not observed in the HPLC chromatogram; alternatively, it is possible that the cellular concentrations of a given compound are quite variable as a function of time. Our data indicate that methyl and *t*-butyl esters of 4-oxoretinoic acid have a good tolerance profile in both cultured keratinocytes and melanocytes and have a specific inhibitory action on cellular melanin in cultured melanocytes, as indicated by a high pigmentation index. This parameter gives a better information on the specific action on melanin, because the MTT assay is a better index of cell viability than the protein content. Indeed, dead cells still have proteins, which may be assayed using the Bradford method, whereas a corresponding MTT assay would give a value close to 0. Interestingly, 4-hydroxyretinoate esters were not hydrolysed into retinoic acid within the cells, since RA was always under the detection limit of 10 (pmol/mg prot), indicating that these 4-oxoRA esters have an inhibitory action on melanin *per se*. 4-Oxo- and 4-hydroxy-retinoic acids were not taken up by both keratinocytes and melanocytes, which precluded the analysis of their tolerance profile and depigmenting potential in our *in vitro* models; in these conditions, if they don't penetrate into cultured melanocytes, they appear as bad candidates for an application as topical agents. This is the same for DIV1178, a 4-hydroxyRAL derivative esterified on the position 4 with a δ -tocopheryl azelate. It is interesting to note that another 4-hydroxyRAL ester, DIV1181, was taken up by the cells and hydrolysed to 4-hydroxyRAL (DIV857), indicating a certain selectivity of the endogenous esterase that metabolised DIV1181.

As a conclusion, these retinoid derivatives have a development potential, and could be combined to tyrosinase inhibitors, but randomised controlled clinical trials are now expected to confirm the depigmenting activity of these new retinoids in topical formulations.

CONFLICT OF INTEREST

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