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Some 3-(4-Aminophenyl)pyrrolidine-2,5-diones as All-*trans*-retinoic Acid Metabolising Enzyme Inhibitors (RAMBAs)

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A series of (±)-3-(4-aminophenyl) pyrrolidin-2,5-diones substituted in the 1-, 3- or 1,3- position with an aryl or long chain alkyl function are weak inhibitors of the metabolism of all-trans retinoic acid (RA) by rat liver microsomes (68–75% inhibition) compared with ketoconazole (85%). Further studies with the 1-cyclohexyl analogue (1) (IC_{50} = 98.8 μM, ketoconazole, 22.15 μM) showed that it was not stereoselective in its inhibition. (±)-(1) was not an inhibitor of pig brain microsomal enzyme (ketoconazole, IC_{50} = 20.9 μM), had little effect on human liver microsomal enzyme (19.3%, ketoconazole, 81.6%) or human placental microsomal enzyme (9.8%, ketoconazole 73.9%) but was a weak inhibitor of human and rat skin homogenates (52.6% and IC_{50} = 211.6 μM respectively; ketoconazole, 38.8% and 85.95 μM).

In RA-induced cell cultures of human male genital fibroblasts and HaCat cells, (±)-(1) was a weak inhibitor (c. 53% at 200 μM) whereas ketoconazole showed high potency (c. 65% at 0.625 μM and 0.25 μM respectively). The nature of the induced target enzyme is discussed.

Keywords: Retinoic acid, RA; RA-metabolising enzyme inhibitors; Ketoconazole; 3-(4-Aminophenyl)pyrrolidin-2,5-diones; RA-metabolism blocking agents (RAMBAs)

INTRODUCTION

All-trans-retinoic acid (RA) is a naturally occurring retinoid responsible for growth and differentiation of epithelial tissues in mammals.[1] RA exerts its activity by binding to transcription-regulatory factors known as RAR (retinoic acid receptor) and RXR (retinoid X receptor), each having three subtypes, α, β and γ.[2] All are members of the steroid/thyroid hormone superfamily. All-trans retinoic acid has some specificity for the RAR type. These receptors are located in the nucleus of the cell and, upon RA binding, the activated receptor transcriptionally regulates its target genes via binding to its response element (RARE, retinoic acid response element, or RXRE, retinoid X response element).[3–5]

Retinoic acid has been used in a number of clinical situations, particularly in oncology and dermatology. RA has been shown to be spectacularly successful in acute promyelocytic leukaemia, which is associated with a specific translocation between chromosomes 15–17 that is responsible for an RAR-α receptor rearrangement.[6–8] Typically, there is complete remission within 1–3 months but this is followed by relapse from between 4–12 months. It seems that continued dosing with retinoic acid causes a fall in plasma levels, which is strongly associated with the relapse. It appears, therefore, that there is up-regulation of metabolism, which accounts for the decreased clinical activity.

Following this relative success, retinoic acid has been tried in a number of cancer types, with only moderate success, although it may improve the efficacy of other anticancer modalities such as radiation, cisplatin and interferon.[9,10]

Retinoids have a long established record in dermatology, particularly in the treatment of cutaneous malignancies, cystic acne, psoriasis, and other disorders of hyperkeratinization. Retinoic acid has also been used in the treatment of

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photodamaged skin: topically applied all-trans retinoic acid can repair and probably prevent photoageing of the skin by modulation of collagen synthesis and breakdown in the dermis.[11–13] This effect does not appear to be related to skin irritation.[14] There is emerging evidence that intrinsic ageing of the skin is also amenable to reversal by topical retinoids.[15]

Retinoic acid is metabolised via cytochrome P450 enzymes to various products, the most important being 4-oxo and 4-hydroxy retinoic acid. Retinoic acid can induce its own metabolism via a specific P450, CYP26,[2,16–18] and can also upregulate a number of other P450 enzymes.[19,20]

As mentioned above, retinoic acid metabolism is induced in acute promyelocytic leukaemia, and in other cancers, and in numerous cell lines, including tumour and skin cell lines. This rapid induction of metabolising enzymes causes a considerable problem for therapy using retinoic acid, and probably explains the relative lack of clinical success. Since cells very rapidly upgrade their metabolism, it would seem logical that an inhibitor of retinoic acid metabolism would increase the useful life of retinoic acid, and indeed show retinomimetic effects. This has been shown to be the case with several studies involving liarozole (Liazal).[21–26] Liarozole has been shown to have retinomimetic effects (e.g. antiproliferative) in cell culture, when administered with retinoic acid.[27] Liarozole alone has been shown to increase intratumour levels of retinoic acid *in vivo*. It has also been shown to be as effective as acitretin in clinical trials in psoriasis, decreasing inflammation and subsequently reducing epidermal proliferation and promoting differentiation.[25,26] It has been used with success in trials of icthyosis, and hormone resistant prostate cancer.[21–24,28]

Hence, it can be seen that retinoic acid metabolism inhibitors can be useful chemotherapeutic agents, either by increasing endogenous RA levels, or increasing the efficacy of exogenous RA.

Several CYP isozymes from different rat tissues have been shown *in vitro* to be capable of metabolising RA via 4-hydroxylation[29] but RA metabolism by rat liver microsomes is mainly by the 1A1/2,2A6 and 3A4 forms. However, RA administration induces another metabolising enzyme, CYP26[16,30] which recognises only RA as its substrate, and the expression and/or activity of this isozyme can be induced by RA both *in vitro* and *in vivo*.[31] The triazole R115866 (*N*-[4-[1-ethyl-1-(1*H*-1,2,4-triazol-1-yl)butyl]phenyl]-2-benzothiazolamine) has been described as a novel inhibitor of CYP26 which *in vivo* in a single dose study in rats increases endogenous tissue RA levels and mimics RA in several other of its biological actions.[31]

Here, we report studies on the screening of some 3-(4-aminophenyl)pyrrolidin-2,5-diones as inhibitors of mammalian liver and skin as well as human fibroblast and HaCat cellular RA-metabolising enzymes as potential lead agents for the development of agents for cancer or skin diseases.

MATERIALS

All reagents and solvents were general purpose grade. Optical rotations were measured with a Bellingham and Stanley ADP 220 polarimeter as 1% solutions in ethanol at ambient temperature and with sodium D light. The 3-(4-aminophenyl)pyrrolidin-2,5-diones (**1**–**5**) were synthesised as previously reported by us.[32] (+)- and (−)-Dibenzoyltartaric acid were obtained from Sigma.

A Milton Roy LC system was used consisting of a Model 3000 Constametric pump, a Rheodyne injection unit and a model 3100 variable wavelength spectromonitor. A model CL-4100 computing integrator was used to process the data. The HPLC column used was an amylose coated silica gel column (Chiralpak AD; 0.46 cm I.D. × 5 cm, Diacel Chemical Ltd.) using a precolumn (0.46 cm I.D. × 5 cm) both packed with identical material [amylose tris (3,5-dimethyl phenyl carbamate)]. Injection on the column was achieved using a Hamilton syringe (50 μL) into a Rheodyne 20 μL loop.

[^{3}H]-RA (1.92 TBq/mmol) and RA was purchased from NEN (Hounslow, UK) and Sigma Chemical Co. (Poole, UK) respectively. Dulbecco's Modified Eagle's medium, ketoconazole and butylated hydroxyanisole were also from Sigma. All solvents were HPLC grade and were obtained from Fisher (Leicestershire, UK).

METHODS

Chemistry

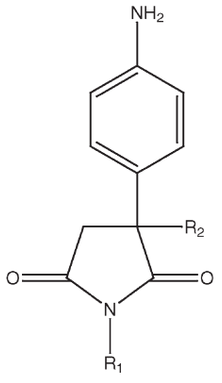
Resolution of 1-cyclohexyl-3-(4-aminophenyl)pyrrolidin-2,5-dione (1)

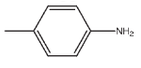
1-Cyclohexyl-3-(4-aminophenyl)pyrrolidin-2,5-dione (**1**, Table I) was stirred with a 1 molar equivalent of (+)-dibenzoyltartaric acid salt in the minimum amount of methanol needed for complete dissolution and the precipitated salt isolated. Seven recrystallisations with excess methanol gave the (+)-dibenzoyltartaric acid salt of the (−) enantiomer.

The free base was recovered from the salt by the method described below. $[\alpha]_{23}^{D}$(1% w/v, CH_3CH_2OH) = −33°.

The mother liquor from the (+)-dibenzoyltartaric acid salt was dissolved in dichloromethane (100 ml) and mixed with saturated sodium bicarbonate

TABLE I Inhibition of male rat liver microsomal RA-metabolising enzymes by some 3-(4-aminophenyl)pyrrolidine-2,5-diones



Compound*	R_1	R_2	% inhibition†
1		H	75
2	C_6H_{13}	H	79
3	H	C_5H_{11}	71
4	C_7H_{15}	H	80
5	C_6H_{13}		68
Ketoconazole			85

*100 μM. †RA = 3 μM. Results are the means of triplicate tubes.

solution (100 mL). The organic extracts were washed with water (2 × 50 mL), dried ($MgSO_4$) and concentrated to release the free base. The recovered mixture was added to an equimolar amount of (−)-dibenzoyl tartaric acid dissolved in methanol and the precipitated salt isolated. Seven recrystallisations from methanol gave the (−)-dibenzoyltartaric acid salt of the (+)-enantiomer. The enantiomer was recovered from the salt using the method below. $[\alpha]_{23}^{D}$(1% w/v, CH_3CH_2OH) = +33°.

HPLC Analysis

The salt (2 mg) was dissolved in dichloromethane (30 mL) and shaken with sodium bicarbonate solution. The organic layer was washed with water (2 × 10 mL), dried ($MgSO_4$) and concentrated. The residue was then diluted with propan-2-ol/hexane (1:1 v/v, 2 mL) and injected into the HPLC.

Chromatographic conditions were as follows: mobile phase: propan-2-ol/hexane (1:1 v/v); flow rate: 1 mL/min; detector UV at 243 nm; temperature: ambient; injection volume: 20 μl; pressure: 330 p.s.i.

The retention times for the (−) and (+) enantiomers were 8.18 and 12.34 min, respectively. The percentage purity was obtained by the relative integrals for the two peaks, and for the (+)- and (−)-base was 99.9%.

Biochemistry

General Assay for Metabolism of RA

Tubes in triplicate, with a total volume of 400 μL containing (11,12-^{3}H) retinoic acid (10 nM, 10 μL of 400 nM stock), unlabelled retinoic acid in methanol (10 μL of 120 μM stock to give 3 μM), inhibitor (8 μL of 5 mM ethanol stock to give 100 μM concentration in final assay volume), phosphate buffer 50 mM (pH = 7.4, 312 μL), NADPH solution (50 μL of 16 mg/mL) were prepared, and the tubes vortexed and preheated in a water bath for 4 min.

The enzyme reaction was initiated by addition of the respective tissue, and the mixture incubated at 37°C for 30 min. The enzyme action was arrested by addition of 1% formic acid (100 μL) and the tubes were placed in ice for 5 min. Then ethyl acetate containing 0.02% butylated hydroxy anisole (3 mL) was added and the tubes vortexed for 10 s. The tubes were then left for another 5 min at room temperature and the organic layer (2 mL) was removed from each tube, transferred to another set of tubes and the ethyl acetate extracts were evaporated using a Christ centrifuge connected to a vacuum pump and a multitrap at −80°C.

After 60 min the tubes were removed and the residue was reconstituted in methanol (100 μL) and 50 μL was injected into a HPLC machine equipped with a 10 μm C18μBondapak column (Waters), connected to a β-RAM online scintillation detector, connected to a Compaq PC running Laura data acquisition and analysis software (LabLogic Ltd.). A Milton-Roy pump was used, at a flow rate of 1.90 mL/min. The mobile phase was acetonitrile/1% ammonium acetate in water/formic acid (75:25:0.1 v/v/v). The scintillation fluid was Optiflow Safe 1 (Fisher).

Metabolites[29] were measured in terms of percentage activity relative to the total radioactivity (*i.e.* metabolite peak plus retinoic acid peak). Using a control with ethanol instead of inhibitor, these results were expressed as "percentage inhibition relative to control" = 100 − [(% metabolites with inhibitor/% metabolites control) × 100]. Ketoconazole was used as a standard inhibitor. Due to the photosensitivity of retinoic acid all the above assays were carried out in a dark room under yellow light.

Preparation of Rat Liver Microsomes

Phenobarbital induced: Male Wistar rats, 2 months old (200–250 g) were given phenobarbital (1 g/L) in their drinking water for 4 days. They were then killed by stunning (concussion) and the liver was removed.

Non-induced: Male Wistar rats, 2 months old (300–350 g) were killed by stunning and the livers were removed. Livers were frozen in liquid N_2 and stored at −80°C until the microsomal fraction was prepared.

Livers from either source were thawed rapidly and mixed with ice cold 50 mM phosphate buffer pH 7.45 containing 0.25 M sucrose. The mixture was finely chopped in a blender and homogenised on ice using an Elvejhm-Potter homogeniser. The homogenate was centrifuged for 20 min at 10,000 g, 4°C. Pellets were discarded and the supernatant spun for 60 min at 105,000 g, 4°C. The supernatant was discarded and the final pellet was resuspended in phosphate buffer pH 7.45 (50 mM). The suspension was homogenised on ice using an Elvejhm-Potter homogeniser, distributed into 1.5 mL capped vials, frozen in liquid N_2 and stored at −80°C until use. The general assay method was followed using a protein concentration of 0.25 mg mL^{-1}, as determined using a bicinchoninic acid protein assay kit (Sigma).

Preparation of Pig Brain Microsomes

Pig brain microsomes were obtained by the general method given for non-induced rat liver microsomes and the general assay method was followed.

Preparation of Human Liver Microsomes

The human liver was a generous gift from Dr Curtis (Biodynamics, Cardiff) and was processed as for the rat liver for the preparation of microsomes. A commercial sample of human liver microsomes was also obtained from Totam Biological in order to compare the results in terms of basic RA metabolism. The general assay method was followed using protein concentrations of 0.05–1.5 mg mL^{-1}.

The profile of metabolites was identical for both preparations. However, it differed from the previous profiles as only one metabolite was detected at an intermediate retention time between 4-oxo- and 4-hydroxy RA.

Preparation of Human Placental Microsomes

Human placental microsomes were prepared as described for the rat liver microsomes and the general assay method was followed using a protein concentration of 1.5 mg mL^{-1}.

Preparation of Human Skin Homogenate

A frozen skin sample (breast reduction) was cleared from fat and the epidermis roughly separated using a scalpel. 1 cm^2 pieces were cut and processed using a Powergen blender. Phosphate buffer, 50 mM, pH 7.4 was added and the preparation was then homogenised with a Teflon pestle and filtered through gauze. The general assay method was followed but using RA at 6.5 nM and 0.4 mg mL^{-1} protein concentration with a 90 min incubation.

Preparation of Rat Skin Homogenate

A rat skin homogenate was prepared from shaved skin from the back of Wistar male rats aged 2 months. The skin was removed, cut into 1 cm^2 pieces, phosphate buffer (50 mM pH 7.4) was added and the mixture was processed using a Powergen blender mounted with a large saw. Samples were further homogenised with a Teflon pestle and filtered through gauze. The general assay method was followed except that a protein concentration of 10 mg mL^{-1} with 120 min incubation was used.

Preparation of Fibroblast Cell Line

Human male genital epidermal fibroblasts were generously donated by Dr B. Evans, Heath Hospital, Cardiff. The assay method differed from the general method in that the metabolising enzymes were induced by prior incubation of the fibroblasts with RA.

Cold retinoic acid (RA) was added to Dulbecco's Modified Eagle's medium (DMEM) to a final concentration of 2.4 μM. 500 μL of this RA-medium was added to each well of the 12 well culture plate containing fibroblasts. The plates were wrapped in aluminium foil and incubated at 37°C for 24 h. The RA medium was then removed and replaced with medium (with no added RA) for a further 24 h.

The medium in each well was then removed and replaced with 500 μL of DMEM with ^{3}H-RA to a final concentration of 20 nM. 10 μL of test inhibitors (in acetonitrile) at different concentrations were added. Controls on each plate consisted of acetonitrile 10 μL. The plates were foil wrapped and incubated at 37°C for 6 h.

1% formic acid (500 μL) was then added to each well, and the medium was removed into separate tubes. Distilled water (500 μL)was added to each well and the cells scraped off with a rubber tipped glass rod and the contents added to the appropriate tube. This procedure was repeated with a further 500 μL of water but without scraping. Ethyl acetate containing 0.05% butylated hydroxyanisole (2 mL) was added to each tube. After vortexing, the tubes were spun down at 4000 rpm for 5 min. The organic phase was removed to other tubes, evaporated off and the general assay procedure was then followed.

Preparation of HaCat Cell Line

HaCat cells (transformed keratinocytes) were obtained from Dr M. Gumbleton, from within this

TABLE II Inhibition of normal and phenobarbital-induced male rat liver microsomal RA-metabolising enzymes by stereoisomers of 1-cyclohexyl-3-(4-aminophenyl) pyrrolidine-2,5-dione (**1**)

Compound	Normal liver % inhibition*,†	Normal liver IC_{50} (μM)†	Induced liver % inhibition*,†
(±)-(**1**)	67.0 (±15.8)	98.8 (±34.7)	71.2
(+)-(**1**)	74.4 (±17.1)	61.9 (±20.5)	88.1
(−)-(**1**)	71.4 (±18.1)	60.8 (±15.3)	89.8
Ketoconazole	94.1	22.15 (±1.6)	81.3 (±15.6)

*Compound 100 μM. †RA = 3 μM. Mean of single or duplicate determinations with triplicate tubes unless ±s.d given, in which case n = 3–5.

department. The methodology used was identical to that used in the fibroblast cell line.

RESULTS AND DISCUSSION

The current activity in the design and development of RA-metabolising enzyme inhibitors (RAMBAs) is concerned with increasing tissue cellular levels of endogenous RA, an epidermal anti-proliferating and differentiating agent, to control tumour and skin growth and so provide RA-mimetic agents for sex hormone—independent cancers and skin ailments/diseases. A moderate sustained increase in intracellular levels of endogenous RA rather than high systemic and extracellular levels attained with RA administration could lead to localised RA-effects without systemic toxicity. The proof of concept has been confirmed for the imidazole liarozole which has been used with some success in dermatological conditions and as an anti-prostatic cancer agent, however owing to its lack of selectivity towards other cytochrome P450s on the steroidogenesis pathway, the more selective triazole R115866 was recently developed.[31]

RA metabolism occurs in many tissues in the rat and we have found, on an organ weight basis, that activity in liver ⩾ skin = kidney > brain > intestinal mucosa > lung.[33] In rat liver microsomes metabolism occurs with a number of xenobiotic-metabolising enzymes, the order[29] of activity being 2A6 > 1A1/A2 > 3A4. Additionally, RA induces its own metabolism in living tissues and cell cultures through CYP26, a specific RA-metabolising enzyme.[30,34] It is not clear as to the relative importance to RA metabolism of non-induced metabolising P450s and induced CYP26 either in different living tissues (*i.e. in vivo*) or in isolated stored tissue systems (*i.e. in vitro*). Hence in different tissues the target enzymes, non-induced metabolising P450s and CYP26 may have different distributions and hence contributions to make to RA metabolism and furthermore have different structure-activity relationship requirements for their inhibitors. Consequently, a specific inhibitor of either target could be inadequate in attaining an optimal build up of RA in all living tissues.

We have examined some 3-(4-aminophenyl)pyrrolidin-2,5-diones (**1**–**5**), in comparison with ketoconazole, an established inhibitor, as inhibitors of rat liver microsomal enzyme and, with one of the inhibitors (**1**) extended this study to pig brain and human liver, human placental enzyme and human and rat skin homogenates. Also studied was the interaction of (**1**) and ketoconazole with RA-induced human male genital fibroblasts and HaCat cells.

Screening of the series of known 3-(4-aminophenyl)pyrrolidin-2,5-diones against rat liver microsomal RA-metabolising enzymes showed that substitution of the basic skeleton at N-1 or C-3 with hydrophobic long chain alkyl/aryl groups led to potent inhibitors (68–80% inhibition) compared with the established inhibitor ketoconazole (88%) (Table I). The unsubstituted compound was inactive.

As a representative of the series, the *N*-cyclohexyl analogue (**1**) was further studied in other tissue systems. Chiral inhibitors of steroidogenic cytochrome P450 enzymes normally exhibit enantioselectivity[35] and (**1**) was resolved into its enantiomers to study such an effect. Resolution of the racemate into its (+)- and (−)-forms was achieved using (−)-dibenzoyltartaric acid and (+)-dibenzoyltartaric acid respectively with repeated recrystallisation of the dibenzoyltartrates followed by final basification with sodium bicarbonate to recover the base. HPLC analysis showed 99.9% enantiomer purity for both forms.

TABLE III Inhibition studies on RA-metabolising enzymes in pig brain and human liver microsomes with stereoisomers of (**1**)

Compound	Pig brain % inhibition*,†	Pig brain IC_{50} (μM)†	Human liver % inhibition*,†
(±)-(**1**)	0.0	ND	19.3
(+)-(**1**)	0.0	ND	19.3
(−)-(**1**)	0.0	ND	8.3
Ketoconazole	91.4 (±1.9)	20.9 (±5.2)	81.6 (±10.3)

*Compound 100 μM. †RA = 3 μM. Mean of single or duplicate determinations with triplicate tubes unless ±s.d given, in which case n = 3 or 6. ND = not determined.

TABLE IV Inhibition studies on RA-metabolising enzymes in human placental microsomes with stereoisomers of (**1**)

Compound*	Placental microsomes % inhibition†
(±)-(**1**)	9.8
(+)-(**1**)	8.7
(−)-(**1**)	7.6
Ketoconazole	73.9

*100 μM. †RA = 3 μM. Mean of single determination with triplicate tubes.

TABLE V Inhibition studies on RA-metabolising enzymes in human and rat skin homogenates with (±)-(**1**)

Compound	Human skin homogenate % inhibition*,†	Rat skin homogenate IC_{50} (μM)‡
(±)-(**1**)	52.6 (±13.6)	211.6 (±17.1)
Ketoconazole	38.8 (±9.6)	85.95 (±4.7)

*Compound 100 μM. †RA = 6.5 nM, n = 3. ‡RA = 3 μM, n = 3.

With normal rat liver microsomal RA-metabolising enzyme, the (±)-, (+)- and (−)-forms of (**1**) had similar inhibitory potency (IC_{50} = 98.8, 61.9 and 60.8 μM respectively) and were about 3-fold less active than ketoconazole (22.15 μM). With phenobarbital-induced liver RA-metabolising enzyme(s) the 3 forms of (**1**) and ketoconazole showed similar activity (Table II). With either tissues the (+)- and (−)-forms of (**1**) had similar activity demonstrating a lack of enantioselectivity towards the xenobiotic-metabolising P450 enzyme(s).

Pig brain microsomal RA-metabolising enzymes were not inhibited by (±)-(**1**) but ketoconazole showed a high (91.4%) inhibitory effect (Table III), and a similar IC_{50} (20.9 μM) as that for rat liver enzyme (22.15 μM).

With human liver microsomal enzyme(s) a weak inhibitory effect (8.3–19.3%) was seen for (**1**) with, again, high activity by ketoconazole (81.6%) (Table III). A similar picture emerged with human placental microsomal enzyme (**1**, 7.6–9.8%; ketoconazole, 73.9%) (Table IV).

Human and rat skin homogenate RA-metabolising enzymes were also examined. (±)-(**1**) was a moderate inhibitor (52.6% inhibition) and was more potent than the weakly inhibitory ketoconazole (38.8%) towards human skin enzyme, but 2.5-fold less potent (IC_{50} 211.6 and 85.95 μM respectively) with rat skin homogenate (Table V). Ketoconazole generally was a much weaker inhibitor of the skin homogenate enzymes than the previously noted liver and brain enzymes from various mammalian sources.

Cultured human male genital epidermal fibroblasts and HaCat cells (transformed keratinocytes) were studied where the cells were initially incubated with RA to induce, presumably, the CYP26 enzyme, since this was identified as the RA-inducible enzyme in MCF-7 and HaCat cells.[36,37] (±)-(**1**) was a weak inhibitor of both RA-induced human fibroblasts and the HaCat cells (c. 53% at 200 μM) whereas ketoconazole was a very potent inhibitor in both cellular systems (c. 65% at 0. 625 μM and 2.5 μM respectively) and at least an order more potent than towards the pig brain, rat, and human liver enzyme (Table VI).

The triazole R115866 is a potent inhibitor (IC_{50} = 0.004 μM) of CYP26 expressed in yeast cells and significantly raises the endogenous RA content in rats after a single dose (p.o. 2.5 mg kg^{-1}) in plasma, skin, fat, kidney and spleen, but has little effect on liver except 6 h after dosing.[31] This suggests that R115866 may have only a partial RA-protective effect in liver by having a weaker effect on the xenobiotic-metabolising P450s and a more selective action on CYP26. This view follows from the fact that we have found that screening of potential RAMBAs using rat liver microsomes may give negative results whereas subsequent examination of such compounds in the RA-induced cellular systems mentioned here has shown high activity (Kirby and Smith, unpublished observations).

That ketoconazole, a known inhibitor of CYP3A4, is a fairly potent inhibitor ($IC_{50} \approx 20$ μM) of rat liver and pig brain microsomes but at least an order more potent as an inhibitor of RA-induced fibroblast and HaCat cells may suggest less selectivity than R115866 for the CYP26 target.

However, it is not clear that the increased RA obtained in the various rat tissues examined after single dose oral administration of R115866 is necessarily a direct effect of CYP26 induction and inhibition, since, (a) although CYP26 mRNA is expressed in the rat liver after dosing, the situation in other tissues was not reported;[31] (b) recent work with cultured human epidermal keratinocytes has suggested that only low levels of CYP26A1 mRNA

TABLE VI Inhibition studies on induced RA-metabolising enzymes in human male genital fibroblasts and HaCat cells with (±)-(**1**)

	Human fibroblasts % inhibition*		HaCat cells % inhibition*	
Compound	0.625 μM	200 μM	2.5 μM	200 μM
(±)-(**1**)	ND	53.1 ± 11.2	ND	52.8 ± 12.4
Ketoconazole	65.6 ± 15.3	ND	63.6 ± 11.5	ND

*RA = 3 μM. Mean of 3 or 4 determinations in triplicate tubes. ND = not determined.

are expressed and the enzyme is not RA-inducible so that it is unlikely that it contributes to RA-metabolism in these cells.[37]

A possible explanation of these seemingly contradictory views is touched on by Stoppie *et al.*[31] for fat tissue. Protection of RA-metabolism by systemic CYP26 inhibition by R115866 leads to enhanced RA plasma levels and its distribution to all tissues including those where there is little or no RA metabolism, such as adipose tissue, and, in the light of the work of Popa *et al.*[37] perhaps this includes skin.

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