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Inhibition of Retinoic Acid Metabolising Enzymes by 2-(4-aminophenylmethyl)-6-hydroxy-3,4-dihydronaphthalen-1(2H)-one and Related Compounds

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In a search for inhibitors of all-trans retinoic acid (RA)-metabolising enzymes as potential agents for the treatment of skin conditions and cancer we have examined 2-(4-aminophenylmethyl)-6-hydroxy-3,4-dihydronaphthalen-1(2H)-one (5). Compound (5) is a moderate inhibitor of RA-metabolising enzymes in mammalian cadaverous tissue microsomes and homogenates as well as RA-induced enzymes in cultured human genital fibroblasts and HaCat cells. Overall (5) was more potent than or equipotent with ketoconazole, a standard inhibitor, in the cadaverous systems but less active towards the RA-induced cell culture systems. Examination of the data suggests that RA-induction generates metabolising enzymes not present in the cadaverous systems, which are more susceptible to inhibition by ketoconazole than (5).

Keywords: Retinoic acid; RA, RA-metabolising enzyme inhibitors; Ketoconazole; RAMBAs, RA-metabolism blocking agents

INTRODUCTION

All-trans-retinoic acid (RA) is a naturally occurring retinoid responsible for growth and differentiation of mammalian epithelial tissues[1] and exerts activity by binding to transcription-regulatory factors in the cell nucleus known as RAR (retinoic acid receptor) and RXR (retinoid X receptor), each having subtypes α, β and δ.[2] Upon RA binding the activated receptor transcriptionally regulates its target genes by 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, especially oncology and dermatology. In oncology, RA has shown spectacular success in the treatment of acute promyelocytic leukaemia[6–8] although the complete remission seen within 1–3 months is followed by relapse within 4–12 months. The relapse appears to be due to increased RA-metabolism as a result of RA-induction so leading to decreased clinical efficacy. Moderate success has been achieved in other cancer types although it may improve the efficacy of other treatments such as radiation, cisplatin and interferon therapies.[9,10] Retinoids have been used for some time in the treatment of cystic acne, psoriasis, cutaneous malignancies and other disorders due to hyperkeratinisation as well as in the treatment of photo-damaged skin.[11–13]

Vitamin A (retinol) is oxidised through retinal by dehydrogenases in the cytoplasm of target cells in low yield to all trans-retinoic acid (RA). RA is at least 100 fold more active than retinol and is considered to account for its biological action. RA has a short half life (*c*. 1 h) and therefore the potency of RA is reduced when it is administered systemically, due to metabolism by human liver and intestine cytochrome P450s to the inactive 4-hydroxy-RA and thence by dehydrogenases to the partially active 4-keto-RA and inactive polar metabolites.[14]

The specific P450s (P450-RA) responsible for 4-hydroxylation of RA in the human liver have not been characterised, but several reconstituted P450s CY1A2/2B6/2C8/2D6/2E1/3A4, can catalyse the reaction.

A drug which can prolong and itensify the action of endogenous RA on the epidermal cell by

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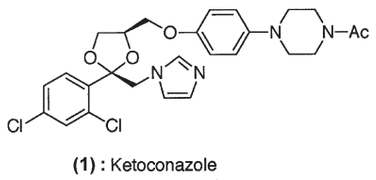
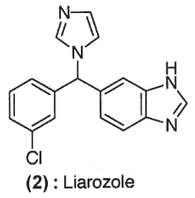
inhibiting P450-RA metabolising enzymes would have potential as a clinical agent in the treatment of certain skin conditions and as an anti-cancer agent.

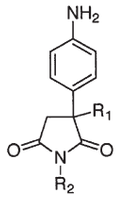
The imidazoles, ketoconazole (**1**) and liarozole (**2**), were reported as inhibitors of RA-metabolisng enzymes whilst being studied as inhibitors of 17α-hydroxylase: 17,20-lyase (P450 17α) as agents for the treatment of androgen-dependent prostatic cancer by lowering testosterone levels.[14] Ketoconazole is not a suitable oral agent as an RA-mimetic since it lacks specificity towards P450 17 and inhibits several other cytochrome P450 enzymes on the steroidogenic pathway of androgen synthesis and, furthermore, has a poor pharmacokinetic profile and produces gastro-intestinal effects at the frequent dosing required. Liarozole (Liazal®) inhibits testicular (but not adrenal) P450 17 and is a potent inhibitor of aromatase ($P450_{AROM}$) thus negating its potential as an oral RA-mimetic for sex hormone-independent cancers despite its effectiveness in clinical trails in psoriasis, decreasing inflammation and subsequently reducing epidermal proliferation and promoting differentiation[15,16] as well as in trials of icthyosis, and hormone resistant prostate cancer.[17–21]



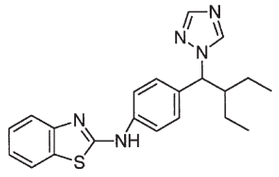
(1) : Ketoconazole



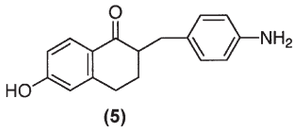
(2) : Liarozole



(4)



(3) ; R115866



(5)

Several CYP isozymes from different rat cadaverous tissues have been shown *in vitro* to be capable of metabolising RA via 4-hydroxylation[22] with RA metabolism by rat liver microsomes being mainly by the 1A1/2,2A6 and 3A4 forms. However, in living tissues, RA administration induces another RA-metabolising enyzme, CYP26[23–25] 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*.[26] The triazole R115866 (**3**) has been described as a novel inhibitor of CYP26 which *in vivo* in rats after a single oral dose increases endogenous tissue RA levels and mimics RA in several other of its biological actions.[26]

However, it is not clear that oral administration of R115866 producing skin effects is necessarily a direct effect of RA-induction of CYP26 and its subsequent inhibition in skin since recent work with cultured epidermal keratinocytes suggests that CYP26 is not RA-induced in this tissue;[27] perhaps plasma RA distribution from internal tissues accounts for the beneficial skin effects observed with R115866.[26]

We have recently described some substituted 3-(4-aminophenyl) pyrrolidine-2,5-diones (**4**) as inhibitors of rat liver microsomal RA-metabolism[28] and the *N*-cyclohexyl analogue (**4**) in other cadaverous systems (pig brain, human placenta and human liver microsomes; rat and human skin homogenates) as well as RA-induced cell cultures (human male genital fibroblasts and HaCat cells).[28] In general (**4**) was much less active than ketoconazole in the cadaverous *in vitro* systems and very weakly active in the cell culture systems where ketoconazole was a potent inhibitor. Here we describe a further phenylamine, compound (**5**) based on the tetralin structure, as an RA-metabolising enzyme inhibitor in these test systems.

MATERIALS AND METHODS

Chemistry

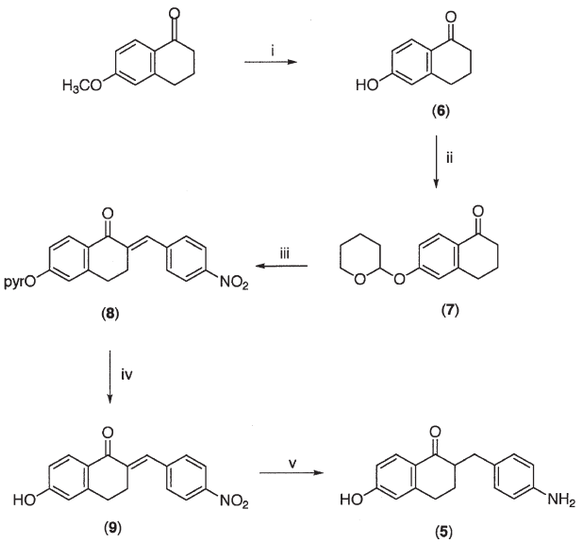
All reagents and solvents were general purpose grade. Melting points were determined on a Gallenkamp digital apparatus and are uncorrected. IR spectra were obtained as solids via a diffuse reflectance accessory using KBr matrix, or between NaCl plates using a Perkin Elmer 1600 series FTIR. ^{1}H-NMR spectra were recorded on a Bruker DPX 300 (300 MHz) spectrometer as dilute solutions in deuteriochloroform with tetramethylsilane as internal standard. 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.

Synthesis of 2-(4-aminophenyl)-6-hydroxy-3,4-dihydronapthalen-1(2H)-one (5)

The synthesis of (**5**) is shown in Scheme 1.

6-Hydroxy-3,4-dihydronaphthalen-1(2H)-one (**6**)

A solution of 6-methoxy-3,4-dihydronaphthalen-1 (2H)-one (17.6 g, 100 mmol) in 47% aqueous HBr was heated under reflux for 5 h and cooled in an ice bath. The precipitate was collected, washed with water and finally recrystallised from water to yield



SCHEME 1 (i) 48% HBr, reflux (ii) 3,4-dihydropyran, *p*-toluenesulfonic acid (iii) 4-nitrobenzaldehyde, KOH, EtOH (iv) 2M HCL, reflux (v) 10% Pd/charcoal, H_2.

the *phenol* as yellow crystals (9.5 g, 57%), m.p. 156–157°C. ν_{max} 3311 (O–H), 1661 (C═O), 1578 (C = C, Ar) cm^{-1}, δ_H (300 MHz; DMSO-d_6), 10.4 (1H, s, O–H), 7.74 (1H, d, $J = 8.55$ Hz, 8-*H*), 6.72 (1H, dd, $J = 8.58$ Hz and $J = 2.04$ Hz, 7-*H*), 6.65 (1H, br s, 5-H), 2.8 (2H, t, $J = 5.8$ Hz, CH_2–CH_2–*C*H_2–C = O), 2.5 (2H, t, $J = 6.75$, *C*H_2–CH_2–CH_2–C = O), 2.0 (2H, qi, $J = 6.21$ Hz, CH_2–*C*H_2–CH_2–C = O).

6-(Tetrahydropyranyl-2′-oxy)-3,4-dihydro-naphthalen-1(2H)-one (**7**)

A mixture of (**6**) (5 g, 30 mmol), 2,3-dihydropyran (6.0 g, 71 mmol), acetic anhydride (1 cm^3) and *p*-toluensulfonic acid (50 mg) in ether was stirred at room temperature for 3 h. The resulting brown clear solution was washed with 2% KOH (2 × 20 cm^3) and water (20 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The crystalline residue was recrystallised from ethanol to give the *ketone* as pale yellow crystals (6.6 g, 86.8%), m.p. 89.8–91.7°C. (Found: C, 72.97; H, 7.38. $C_{15}H_{18}O_3$ requires C, 73.14; H, 7.3%); ν_{max} 2960–2880 (C–H), 1670 (C═O), 1600 (C = C, Ar) cm^{-1}, δ_H (300 MHz; $CDCl_3$), 8.06 (1H, d, $J = 8.7$ Hz, 8-*H*), 7.1 (1H, dd, $J = 8.7$ Hz and $J = 2.4$, 7-*H*), 6.93 (1H, d, $J = 2.3$ Hz, 5-*H*), 5.57 (1H, t, $J = 3$ Hz, –O–C*H*–O), 3.9 (1H, m, 6′-H_AH_B), 3.69 (1H, m, 6′-H_AH_B), 2.95 (2H, t, $J = 6.1$ Hz, CH_2–CH_2–*C*H_2–C = O), 2.6 (2H, t, $J = 6.1$ Hz, *C*H_2–CH_2–CH_2–C = O), 2.16 (2H, quintet, $J = 6.4$ Hz, CH_2–*C*H_2–CH_2–C = O), 2.05 (1H, m, 3′-H_AH_B), 1.9 (2H, quintet, $J = 3.6$ Hz, 5′-*C*H_2), 1.8–1.6 (3H, m, 4′-*C*H_2, 3′-H_AH_B).

2-(4-Nitrophenylmethylene)-6(tetrahydropyranyl-2′-oxy)-3,4-dihydronaphthalen-1-one (**8**)

4-Nitrobenzaldehyde (3 g, 20 mmol) was added in portions over 15 min to a stirred solution of 6-(tetrahydropyranyl-2-oxy)-3,4-dihydro-napthalen-1 (2H)-one (4.8 g, 19.5 mmol) in 0.7% ethanolic KOH (100 cm^3) under nitrogen and the mixture was stirred at room temperature for 1 h. The resulting precipitate was collected, washed with cold water (3 × 10 cm^3) and crystallised from a solution of ethyl acetate–chloroform (1:1) to give the *ketone* as yellow crystals (2.2 g, 29.7%), m.p. 188.6–189.6°C. (Found: C, 68.01; H, 5.81; N, 3.37. $C_{22}H_{21}NO_5 \cdot 1/2\,H_2O$. Requires C, 68.01; H, 5.71; N, 3.60%); ν_{max} 2925 (C–H), 1662 (C═O), 1616 (C = C), 1594 (C = C, Ar), 1513, and 1343 (N = O) cm^{-1}; δ_H (300 MHz; $CDCl_3$) 8.35 (2H, d, $J = 8.7$ Hz, 3′-, 5′-*H*), 8.15 (1H, d, $J = 8.7$ Hz, 8-*H*), 7.85 (1H, m, CO–C = C*H*-Ph), 7.6 (2H, d, $J = 8.5$ Hz, 2′-,6′*H*), 7.1 (1H, dd, $J = 2.4$ Hz and $J = 8.7$ Hz, 7-*H*), 6.95 (1H, d, $J = 2.7$ Hz, 5*H*). 5.6 (1H, t, $J = 3$ Hz, O–C*H*–O) 3.9 (1H, m, 6″-CH_AH_B) 3.7 (1H, m, 6″-CH_AH_B), 3.15 (2H, m, *C*H_2–CH_2–(C = C)), 3.0 (2H, m, CH_2–*C*H_2–(C = C)), 2.05 (1H, m, 3″-CH_AH_B), 1.9 (2H, m, 5″-CH_2), 1.6–1.8 (3H, m, 4″-*C*H_2, 3″-CH_AH_B).

2-(4-Nitrophenylmethylene)-6-hydroxy-3,4-dihydronaphthalen-1-one (**9**)

Hydrochloric acid (5%, 10 cm^3) was added dropwise to a stirred solution of (**8**) (2.5 g, 6.7 mmol) in ethyl acetate (20 cm^3) and 2-butanone (10 cm^3). The mixture was stirred at room temperature for 1 h and at 60°C for 1 h. Cold water (20 cm^3) was then added and the resulting mixture concentrated. The resulting precipitate was collected, washed with water (2 × 10 cm^3) and dried to give the *phenolic ketone* (1.4 g, 73%) as a yellow crystalline powder, m.p. 253.2–257°C. (Found: C, 68.85; H, 4.57; N, 4.67. $C_{17}H_{13}NO_4$ requires C, 69.13; H, 4.44; N, 4.75%); ν_{max} 3341 (OH), 2939.5 and 2842 (C–H), 1669.5 (C═O), 1613 (C = C), 1593, 1571 (C = C, Ar), 1512 and 1342.8 (N = O) cm^{-1}, δ_H (300 MHz; DMSO-d_6) 10.2–10.8 (1H, br s, O–*H*), 8.3 (2H, d, $J = 8.74$ Hz, 3′-5,-*H*), 7.9 (1H, d, $J = 8.6$ Hz, 8-*H*), 7.75 (2H, d, $J = 8.74$ Hz, 2′-6′-*H*), 7.7 (1H, s, C = O–C = C*H*–), 6.8 (1H, dd, $J = 8.6$ and $J = 2.3$ Hz, 7-*H*), 6.7 (1H, d, $J = 2.19$ Hz, 5-*H*), 3.05 (2H, t, $J = 6$ Hz, *C*H_2–CH_2–(C = C)), 2.85 (2H, t, $J = 6$ Hz, CH_2–*C*H_2–(C = C)).

2-(4-Aminophenylmethyl)-6-hydroxy-3,4-dihydronaphthalen-1 (2H)-one (**5**)

A mixture of (**9**) (3.2 g, 10.84 mmol) and 10% palladium charcoal (320 mg) in methanol (100 cm^3) was shaken in an atmosphere of hydrogen at room temperature until 912 cm^3 of gas was absorbed (theory 915 cm^3). The catalyst was removed by filtration and the filtrate evaporated to dryness. The residue was crystallised from ether to give the *amino ketone* (2.2 g, 75.6%) as a white crystalline powder, m.p. 194.5–196.2°C.

(Found: C, 76.68; H, 6.49; N, 5.05%. $(M + H)^+$ 268.1338. $C_{17}H_{17}NO_2$ requires C, 76.37; H, 6.41, 5.24%. $(M + H)^+$ 268.1338). ν_{max} 3364 and 3298 (NH_2), 1667 (C═O), 1594 (C = C, Ar) cm^{-1}, δ_H

(300 MHz; DMSO-d_6) 10.1–10.6 (1H, s, O*H*), 7.8 (1H, $J = 8.6$ Hz, 8-*H*), 6.88 (2H, d, $J = 8.2$ Hz, 3′-5′*H* or 2′, 6′-*H*), 6.73 (1H, dd, $J = 2.2$ Hz and $J = 8.6$ Hz, 7-*H*), 6.6 (1H, d, $J = 2.01$ Hz, 5-*H*), 6.5 (2H, d, $J = 8.3$ Hz, 2′-, 6′-*H* or 3′-,5′-*H*), 4.9 (2H, s, NH_2), 3.05 (1H, dd, $J = 13.5$ Hz and $J = 3.8$ Hz, (C═O)–CH_x–CH_aH_b–Ph), 2.8 (2H, m, CH_2–CH_AH_B–CH_x–(C═O)) 2.6 (1H, m, CH_x–CH_aH_b), 2.4 (1H, dd, $J_{xb} = 9.7$ Hz and $J_{ab} = 13.5$ Hz (C═O)–CH_x–CH_aH_b–Ph), 1.9 (1H, m, CH_2–C$H_A$$CH_B$–$CH_x$–(C═O)), 1.6 (1H, m, CH_2–CH_AH_B–CH_x–C = O).

Resolution of (5) by HPLC

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. × 25 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.

Compound (**5**) was dissolved in the mobile phase and injected into the HPLC. Chromatographic conditions were as follows: mobile phase: isohexane/2-propanol (6:4 v/v); flow rate: 1 ml min^{-1} detector UV at 230 nm; temperature: 20°C; injection volume; 20 μl; pressure: 330 p.s.i.

The retention times for the (+)- and (−)-isomers were 5.87 and 7.30 min respectively. The percentage purity was obtained from the relative integrals for the two peaks and was 99.3 and 98.5%, respectively.

(+)-(**5**): cream coloured solid, m.p. 193–4°C, $[\alpha]_D^{17°}$ (1% EtOH) = +50°. ν (KBr) cm^{-1}, 3367 and 3296 (NH_2), 2920 (OH), 1650 (C═O). δ_H (DMSO-d_6) 10.29 (1H, brs, OH), 7.77 (1H, d, $J = 8.6$ Hz, Ar), 6.86 (2H, d, $J = 8.1$ Hz, Ar), 6.71 (1H, dd, $J = 2.1$, 8.6 Hz, Ar), 6.60 (1H, d, $J = 1.7$ Hz, Ar), 6.48 (2H, d, $J = 8.1$ Hz, Ar), 4.84 (2H, brs, NH_2), 3.06 (1H, dd, $J = 3.9$, 13.6 Hz, (C═O)CH_xC$H_a$$H_b$–Ar), 2.78 (2H, m, C$H_2$$CH_aH_bCH_x$ (C═O)), 2.55 (1H, m, C$H_x$$CH_aH_b$), 2.41 (1H, dd, $J = 9.5$, 13.5 Hz, (C═O) $CH_xCH_aH_b$–Ar), 1.92 (1H, m, CH_2C$H_a$$H_b$$CH_x$ (C═O)), 1.57 (1H,m, $CH_2CH_aH_bCH_X$ (C═O)). Found: C, 69.59; H, 6.90; N, 4.81. $C_{17}H_{17}NO_2$·1.5H_2O requires: C, 69.37; H, 6.85; N, 4.76%.

(−)-(**5**): peach coloured solid, m.p. 189–190°C, $[\alpha]_D^{18}$ (1% EtOH) = −28°. ν (KBr) cm^{-1}, 3368 and 3290 (NH_2), 2925 (OH), 1651 (C═O). δ_H (DMSO-d_6) 10.30 (1H, brs, OH) 7.78 (1H,d, $J = 8.6$ Hz, Ar), 6.86 (2H,d, $J = 8.1$ Hz, Ar), 6.71 (1H, dd, $J = 2.1$, 8.6 Hz, Ar), 6.60 (1H, s, Ar), 6.48 (2H, d, $J = 8.2$ Hz, Ar), 4.84 (2H, brs, NH_2), 3.06 (1H, dd, $J = 3.8$, 13.6 Hz, (C═O)CH_x C$H_a$$H_b$–Ar), 2.75 (2H, m, C$H_2$$CH_aH_bCH_x$(C═O)), 2.54 (1H, m, C$H_x$$CH_aH_b$), 2.41 (1H, dd, $J = 9.5$, 13.5 Hz, (C═O)$CH_XCH_aH_b$–Ar), 1.92 (1H, m, CH_2 C$H_a$$H_b$$CH_x$(C═O)), 1.58 (1H, m, $CH_2CH_aH_bCH_x$ (C═O)). Found: C, 75.34; H, 6.63; N, 4.97. $C_{17}H_{17}$ NO_2·O·2H_2O requires: C, 75.37; H, 6.47; N, 5.17%).

Biochemistry

[^{3}H]-RA (1.92TBq/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).

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^{-1}) were prepared, and the tubes vortexed and preheated in a water bath for 4 min.

The enzymic 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 100 μl of 1% formic acid and the tubes were placed in ice for 5 min. Then ethyl acetate (3 ml) containing 0.02% butylated hydroxy anisole 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 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 system equipped with a 10 μm C18 μBondapak 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^{-1}. The mobile phase was acetonitrile/1% ammonium acetate in water/formic acid (75:25:0.1). The scintillation fluid was Optiflow Safe 1 (Fisher).

Metabolites were measured in terms of percentage activity relative to the total radioactivity (i.e. metabolite peak plus retinoic acid peak).[22] 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.

Microsomal Preparations

Rat liver, human placental and pig brain microsomes were prepared as described previously[28] and the general assay method was followed using a protein concentration of 0.25 mg ml^{-1}, 1.5 mg ml^{-1} and 0.25 mg ml^{-1} respectively. Human liver microsomes were prepared from human liver supplied as a generous gift by Dr Curtis (Biodynamics, Cardiff) and 0.05–1.5 mg ml^{-1} was used in the assay.

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 then the preparation was 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.

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 then 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.

Fibroblast Cell Line

Human male genital epidermal fibroblasts were generously donated by Dr B. Evans, University Hospital of Wales, 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° 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.

500 μl of 1% formic acid was then added to each well, and the medium was removed into separate tubes. 500 μl of distilled water 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. 2 ml of ethyl acetate containing 0.05% butylated hydroxyanisole 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.

HaCat Cell Line

HaCat cells (transformed keratinocytes) were obtained from Dr M. Gumbleton, from within this department. The methodology used was identical to that used in the fibroblast cell line.

RESULTS AND DISCUSSION

The (±)-tetralone (**5**) (IC_{50} = 12.75 μM) was about two-fold more potent than the standard inhibitor ketoconazole (22.15 μM) against rat liver microsomal RA-metabolising enzyme(s) and about eight-fold more potent than the previously described *N*-cyclohexyl-3-(4-aminophenyl)pyrrolidine-2,5-dione (**4**) (Table I).[28] The (+)- and (−) forms of (**5**) had similar activity as previously noted for the isomers of (**4**).[28] (±)-(**5**) was equipotent with ketoconazole against pig brain microsomal enzyme whereas (**4**) was inactive. Ketoconazole was slightly more active than (±)-(**5**) against human liver (81.6 and 66.4%, inhibition respectively) and human placental microsomal enzyme (73.9 and 67.4% respectively) with (**4**) showing little inhibition. Using human skin homogenates the tetralone (±)-(**5**) was more potent than ketoconazole (71.8% and 38.8%) and this situation was mirrored with rat skin homogenate (IC_{50} = 14.75 μM and 85.95 μM respectively) (Table II). With RA-induced cultured cellular systems ketoconazole showed high potency (66% inhibition, 0.625 μM) being more than an order more potent than (±) (**5**) (IC_{50} = 18.5 μM) with human epidermal fibroblasts and just less than an order more potent (64% inhibition, 2.5 μM) than (±)-(**5**) (IC_{50} = 15.5 μM) with HaCat cells (Table III).

Overall, (±)-(**5**) was more potent or equipotent with ketoconazole in cadaverous *in vitro* microsomal or homogenised systems of RA-metabolism enzyme(s) but was about ten-fold, respectively, less

TABLE I Inhibition of RA-metabolism by various microsomal tissue sources by the tetralone (**5**) pyrrolidine-2, 5-dione (**4**) and ketoconazole

Compound	Rat liver		Pig brain		Human liver	Human placenta
	% Inhibition*	IC_{50} (μM)+	% Inhibition*,+	IC_{50} (μM)+	% Inhibition*	% Inhibition*
(±)-(**5**)	94.3	12.8	92.0	19.8	66.4	67.4
(+)-(**5**)	92.0	17.6	77.3	59.9	61.7	68.5
(−)-(**5**)	92.4	10.2	94.5	25.1	53.6	67.4
(±)-(**4**)	67.0	98.8	0.0	ND	19.3	9.8
Ketoconzole	94.1	22.15	91.4	20.9	81.6	73.9

*100 μM. +3 μM RA. The values are means of several determinations except for human placental microsomes (single determination in duplicate tubes).

TABLE II Inhibition of RA-metabolism by human and rat skin homogenates by tetralone (**5**), pyrrolidinedione (**4**) and ketoconazole

Compound	Human skin % inhibition*	Rat skin IC_{50} (μM)+
(±)-(**5**)	71.8 (±2.3)	14.8 (±1.2)
(±-(**4**)	52.6 (±13.6)	211.6 (±17.1)
Ketoconazole	38.8 (±9.6)	86.0 (±4.7)

*100 μM compound, 6.5 nM RA. +3 μM RA. Means ± S.D. ($n = 3$).

active in RA-induced cultural fibroblast and HaCat cell systems. Throughout the RA-metabolising enzyme systems used, (±)-(**5**) was much more potent than the previously reported pyrrolidine-2,5-dione (**4**).

The constituent RA-metabolising enzymes present in these systems is unclear. *In vitro* studies have shown that recombinant P450s CYP1A2, 2B6, 2C8, 2D6, 2E1 and 3AA as well as rat liver microsomal 1A1/2, 2A6, 3A4[22] can metabolise RA. A specific RA-metabolising enzyme, CYP26, is induced by RA in many tissues in the body. However, the actual mix of existing RA-metabolising enzymes present in microsomal fractions and tissue homogenates (i.e. cadaverous tissue) and the RA-induced enzymes (existing or new) in living cell culture systems and their relative contribution to RA-metabolism in different tissues (skin, liver) is not known. CYP can be RA-induced in rat liver,[26] MCF 7 cells[29] and HaCat cells[27] but the identification of other induced enzymes has not been described.

Since we know from unpublished screening studies that certain compounds may be highly active towards the RA-induced enzyme(s) in HaCat and human fibroblast cultures but inactive or show little activity towards rat liver microsomal enzymes, there would seem to be two groups of RA-metabolising enzyme as inhibition targets. Those enzymes existing in cadaverous systems may provide a base line metabolism of RA whilst the RA-inducible enzymes would provide a ready means of decreasing opportunist increases in RA levels.

The IC_{50} values obtained in this work for a specific compound are average values for a group of inhibited enzymes. Between different cadaverous tissues the background activity due to existing enzymes would vary as their relative composition changed but RA-induction of a new very active enzyme could be expected to shift the IC_{50} value for an inhibitor. Comparison of the ratio of IC_{50} values for two inhibitors with a different target enzyme spectrum in different tissues would emphasise this change.

Unfortunately, here, we were unable to obtain sufficiently reproducible IC_{50} values for ketoconazole with the RA-induced cellular systems to quantitatively show such shifts and thus the appearance of a new enzyme target. However the increase in potency of ketoconazole over the tetralone (**5**) by about an order in the cellular systems does suggest the emergence of a new enzyme target upon RA-induction for which ketoconazole is a superior inhibitor to (**5**). CYP26 could be a new component in the cellular systems since it is RA-inducible in cultured HaCat cells and human fibroblasts.[27]

TABLE III Inhibition of RA metabolism by RA-induced human genital fibroblasts and HaCat cells by tetralone (**5**), pyrrolidinedione (**4**) and ketoconazole

Compound	Fibroblasts % Inhibition			HaCat cells % Inhibition		
	0.625 μM	200 μM	IC_{50}*	2.5μM	200 μM	IC_{50}*
(±)-(**5**)	ND	ND	18.46 ± 5.39	ND	ND	15.48 ± 8.4
(±)-(**4**)	ND	53.1 ± 11.2	ND	ND	52.8 ± 12.4	ND
Ketoconazole	65.6 ± 15.3	ND	ND	63.6 ± 11.5	ND	ND

*20 nM RA. Means ± S.D. ($n = 3$–5). ND = not determined.

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