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SHORT COMMUNICATIONS

Liarozole fumarate inhibits the metabolism of 4-keto-all-*trans*-retinoic acid

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Abstract—The metabolism of 4-keto-all-*trans*-retinoic-acid (4-keto-RA), a biologically active oxygenated metabolite of all-*trans*-retinoic (RA), has been examined. *In vitro*, incubation of [^{14}C]4-keto-RA with hamster liver microsomes in the presence of NADPH produced two major radioactive metabolites which were more polar than the parent compound. Following isolation, appropriate derivatization and analysis by GC-MS, these compounds were tentatively identified as 2-hydroxy- and 3-hydroxy-4-ketoretinoic acid. Formation of both hydroxy-keto derivatives was suppressed by the imidazole-containing P450 inhibitor liarozole fumarate (IC_{50}, 1.3 μM). *In vivo*, an i.v. injection of 4-keto-RA (20 μg) into rats was followed by rapid disappearance of the retinoid from plasma with a half-life of 7 min. Pretreatment with liarozole fumarate (40 mg/kg, −60 min) reduced the elimination rate of 4-keto-RA: it prolonged the plasma half-life of the retinoid to 12 min, without affecting its distribution volume. These results indicate the important role of the P450 enzyme system in the metabolism of 4-keto-RA both *in vitro* and *in vivo*. The inhibitory effect of liarozole fumarate on this metabolic process may contribute to the reported retinoid-mimetic activity of this drug.

Key words: retinoic acid; metabolism; liarozole

RA* is one of the principal endogenous compounds that control growth and differentiation of epithelial tissues [1]. Probably because of its potent physiological activity, RA is subject to various metabolic transformations [2–4]. A primary route by which RA is metabolized consists of a cytochrome P450-dependent hydroxylation of RA to form 4-hydroxy-RA, whereupon this compound is oxidized to 4-keto-RA [5–11]. However, conversion of RA to 4-keto-RA does not always lead to total deactivation. Indeed, 4-keto-RA was found to be only about half as potent as RA in promoting cell differentiation of F9 embryonal carcinoma cells [12], in producing dysmorphogenesis in cultured rat conceptuses [13], in binding to cellular RA binding proteins [14, 15] and in provoking hypervitaminosis A side effects in rats [16]. Also, 4-keto-RA stimulated gene transcription at nanomolar concentrations, making it only 3–10 times less potent than RA [11]. Apart from some earlier conclusions implying that 4-keto-RA is metabolized by the P450 enzyme system [17], we know little about how this oxygenated RA metabolite is degraded.

Liarozole hydrochloride, formerly designated R 75 251, is a novel imidazole-containing drug which inhibits the cytochrome P450-dependent metabolism of RA: *in vitro*, it suppressed the conversion of RA by hamster liver microsomes [18] or by MCF-7 human breast cancer cells [19], whereas *in vivo* using rats, the compound increased the plasma half-life exogenously administered RA and even enhanced endogenous plasma levels of this retinoid [18]. Moreover, liarozole hydrochloride was found capable of exerting *in vivo* retinoic acid-mimetic effects on the differentiation process of rat vaginal epithelia [18]. This study was initiated to examine the effects of liarozole fumarate on the *in vitro* and *in vivo* metabolism of 4-keto-RA.

Materials and Methods

Chemicals. [15-^{14}C]RA (13.7 mCi or 507 MBq/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). The ^{14}C-labeled 4-keto-RA was prepared by incubation of [15-^{14}C]RA with hamster liver microsomes as described previously [18] and purified by reverse-phase HPLC as described below. Liarozole fumarate or 5[(3-chlorophenyl)(1*H* - imidazol - 1 - yl) - methyl] - 1*H* - benzimidazole (*E*)-butenedioate [20] is a product from Janssen Research Foundation (Beerse, Belgium). Unlabeled 4-keto-RA (RO 11-4824) was a generous gift from Dr M. Klaus (Hoffmann–La Roche, Basel, Switzerland). Stock solutions of 4-keto-RA were prepared in 0.9% NaCl containing 0.3% NaOH and appropriately diluted with 0.9% NaCl. Other materials were obtained from the following sources: NAD^+, $NADP^+$, NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase from Boehringer-Mannheim (Brussels, Belgium); *N*-methyl- *N*-nitroso-*p*-toluene-sulfonic acid to prepare diazomethane [21] from Janssen Chimica (Olen, Belgium); BSTFA and MOX (a 2% solution of methoxyamine hydrochloride in pyridine) from Pierce (Rockford, IL, U.S.A.). All other chemicals and solvents were of analytical or HPLC grade as required. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Rockville Center, NY, U.S.A.) with BSA as standard. To avoid isomerization of retinoids, all experiments were carried out under subdued yellow light.

In vitro *metabolism of 4-keto-RA.* Washed liver microsomes were prepared from male Syrian gold hamsters (Janssen Pharmaceutica Animal Breeding Centre, Beerse, Belgium) as described [18]. The assay buffer (pH 7.4) was composed of 0.05 M Tris, 0.15 M KCl, 0.01 M $MgCl_2$ and 0.02% (w/v) BSA. The standard reaction mixture (total vol. of 400 μL) was composed of (in μL): assay buffer, 260; microsomal suspension, 40 (9.8 mg of protein/mL); liarozole or ketoconazole, 40 (12.5–100 μM) or solvent

* Abbreviations: RA, all-*trans*-retinoic acid; 4-keto-RA, 4-keto-all-*trans*-retinoic acid; AUC, area under the plasma concentration–time curve; *CL*, clearance; V_d, volume of distribution; EI, electron impact; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide.

(1% DMSO); NADPH, 40 (20 mM) or a NADPH generating system, 40, containing $NADP^+$ (20 mM), glucose-6-phosphate (160 mM) and glucose-6-phosphate dehydrogenase (50 U/mL). The reaction was initiated by addition of 20 μL of [15-^{14}C]4-keto-RA (1 μCi/mL) or unlabeled 4-keto-RA (200 μM). Tubes were incubated open to air at 37°. After 60 min, the reaction was stopped by acidification with 0.1 mL of 1% formic acid and the samples were extracted twice with 2 mL of ethylacetate containing 0.05% butylated hydroxytoluene (Janssen Chimica). The combined organic layers were then lyophilized and residues were dissolved in 200 μL of methanol/water/acetic acid (68:32:0.05) containing 10 mM ammonium acetate, centrifuged and 150 μL of the supernatant were subjected to reverse-phase HPLC (see below).

In vivo *metabolism of 4-keto-RA*. Male Wistar rats (Janssen Pharmaceutica Animal Breeding Centre), weighing 200–220 g, were treated orally with vehicle (PEG 200) or test compound in a volume of 0.5 mL/100 g body wt. Sixty minutes later, the animals were anesthetized with diethylether and injected intrajugularly with 20 μg of 4-keto-RA. At designated times after this administration, rats were decapitated and trunk blood was collected on heparin (500 U/mL). After centrifugation (1000 *g*, 15 min), plasma fractions were recovered and extracted immediately. Therefore, plasma samples (1 mL) were diluted with 1 mL of water and mixed with 3 mL of acetonitrile to precipitate proteins. After centrifugations (1000 *g*, 10 min), supernatants were recovered and diluted with 120 mL of 20 mM acetic acid. The mixtures were then passed through 100 mg Bond Elut C_{18} minicolumns (Analytichem International, Harbor City, CA, U.S.A.) After washing the minicolumns with 20 mL of water, the absorbed material was eluted with 3.5 mL of methanol and the eluates were evaporated *in vacuo*. The residues were dissolved in 200 μL of methanol/water/acetic acid (68:32:0.05) containing 25 mM ammonium acetate, centrifuged and 150 μL of the supernatant were subjected to reverse-phase HPLC (see below). Recovery of 4-keto-RA for the total extraction procedure was higher than 90%.

HPLC analysis. HPLC analyses were carried out on a Varian 5500 Liquid Chromatograph equipped with a UV-200 detector set at 350 nm, a Perkin–Elmer ISS-100 Autosampler and a Vista 402 integration system. The *in vitro* samples were analysed on a Zorbax 5C8 column (4.6 mm i.d. × 250 mm; Chrompack) with methanol/water/acetic acid (68:32:0.05) containing 10 mM ammonium acetate as solvent at a flow rate of 1 mL/min. After 20 min, the solvent was changed to 100% methanol to elute 4-keto-RA. Radioactivity in the eluate was monitored on-line by β-counting (Berthold LB 504 radioactivity monitor) using Pico-Aqua as scintillation solvent. Metabolites of interest were isolated by recovering appropriate column fractions with an on-line fraction collector (LKB 2211 Helirac). The *in vivo* samples were analysed on a 10 μM C_{18} μBondapak (3.9 mm i.d. × 300 mm; Millipore) using methanol/water/acetic acid (68:32:0.05) containing 25 mM ammonium acetate as mobile phase at a flow rate of 1.3 mL/min. The effluent was monitored by UV absorbance at 350 nm and 4-keto-RA was quantified by peak-area integration and external standardization. The lower limit of detection was 8 ng/mL with an absolute recovery of more than 90%.

Derivitization procedures. Methyl esters were prepared by treating samples for 5 min at room temperature with an excess of ethereal diazomethane. Methoxime derivatives were prepared by reaction with MOX (50 μL) for 1 hr at 60°. Trimethylsilyl derivatives were formed by reaction with BSTFA (50 μL) in pyridine for 15 min at 60°.

GC-MS. GC-MS analyses were performed on a Hewlett Packard 5890 gas chromatograph equipped with an ULTRA 2 fused silica capillary column (0.32 mm i.d. × 12 m, film thickness 0.5 μm, Hewlett Packard) with helium as the carrier gas at a flow rate of 1 mL/min. The column was coupled directly to the ion source of an AutoSpec Q mass spectrometer which was provided with a VAX 4000-60 data system. The GC oven temperature was programmed from 50 to 310° at 12°/min. Mass spectra were recorded in the EI mode (ionization voltage 73 eV; source temperature 180°).

Data analysis. Plasma concentrations of RV vs time data were fitted to a monoexponential equation $C = C_0e^{-\gamma t}$ where C is the plasma concentration, t is time, C_0 the zero time concentration and γ the elimination rate constant. The parameters C_0 and γ were obtained by log-linear regression analysis. The plasma $T_{1/2}$, the CL and the apparent V_d were calculated according to standard equations [22]. The AUC was computed using the trapezoidal rule. If appropriate, data are expressed as means ± SE. IC_{50} values with 95% confidence limits were calculated by regression analysis.

Results and Discussion

Hamster liver microsomes (390 μg of protein) were incubated for 60 min at 37° with [15-^{14}C]4-keto-RA (0.02 μCi; 1.3 nmol) in the presence of NADPH and the reaction mixture was analysed by reverse-phase HPLC. The microsomal reaction produced two major polar products, designated metabolite I (retention time 9.9 min) and metabolite II (12.3 min). Combined, these metabolites accounted for 28 ± 3% (N = 12) of the total radioactivity recovered from the reaction mixture. The formation of both metabolites was strictly NADPH-dependent: omission of this nucleotide or addition of NADH, NAD^+ or $NADP^+$ did not support the microsomal reaction. Short preincubation (3 min) of hamster liver microsomes with liarozole fumarate resulted in a concentration-dependent inhibition of 4-keto-RA metabolism with an IC_{50} value of 1.3 (0.9–2.1) μM (N = 3). These *in vitro* data extend earlier observations by Roberts *et al.* [17] who identified the P450 system as a prime participant during the *in vitro* conversion of 4-keto-RA into more polar metabolites. Using hamster liver preparations, they showed that the 4-keto-RA converting activity was located in the microsomal fraction, dependent on the presence of oxygen and NADPH, and inhibited by carbon monoxide, all properties which are diagnostic of the involvement of P450 enzymes. We now found that hamster liver microsomes, fortified with NADPH, convert 4-keto-RA into two major, more hydrophilic compounds. Their formation was suppressed by micromolar concentrations of liarozole fumarate. This drug was earlier shown [18] to inhibit the P450-dependent conversion of RA to 4-hydroxy-RA (the immediate precursor of 4-keto-RA) and, as such, it interferes with the RA metabolic process on at least two levels.

To identify the structure of the metabolites I and II, we incubated hamster liver microsomes for 60 min with unlabeled 4-keto-RA in the presence of a NADPH regenerating system. The metabolites were isolated by reverse-phase HPLC and methylated with diazomethane. The EI mass spectra on the methyl esters of metabolites I and II (not shown) were virtually identical and both displayed molecular ions at m/z 344, i.e. 16 mass units higher than the molecular mass of the methyl ester of 4-keto-RA, suggestive of a monohydroxylation reaction. Other major fragmentation ions occurred at m/z 191 [$^+$CH=CH—C(CH$_3$)= CH—CH=CH—C(CH$_3$)=CH—COOCH$_3$], 159 [191 − 32, loss of CH$_3$OH], 125 [$^+$CH=CH—C(CH$_3$)=CH—COOCH$_3$], 105 [$^+$CH$_2$—CH=CH—CH=CH—C$_3$H$_3$] and 91 [$^+$CH$_2$—CH=CH—CH=CH—C≡CH], compatible with an unchanged side polyene chain of 4-keto-RA. A survey of published mass spectra of retinoids revealed that our spectra were virtually identical to those of the methyl esters of 2- and 3-hydroxy-4-keto-13-*cis*-retinoic acid [23], allowing us to characterize tentatively metabolites I and II as 2-hydroxy- and

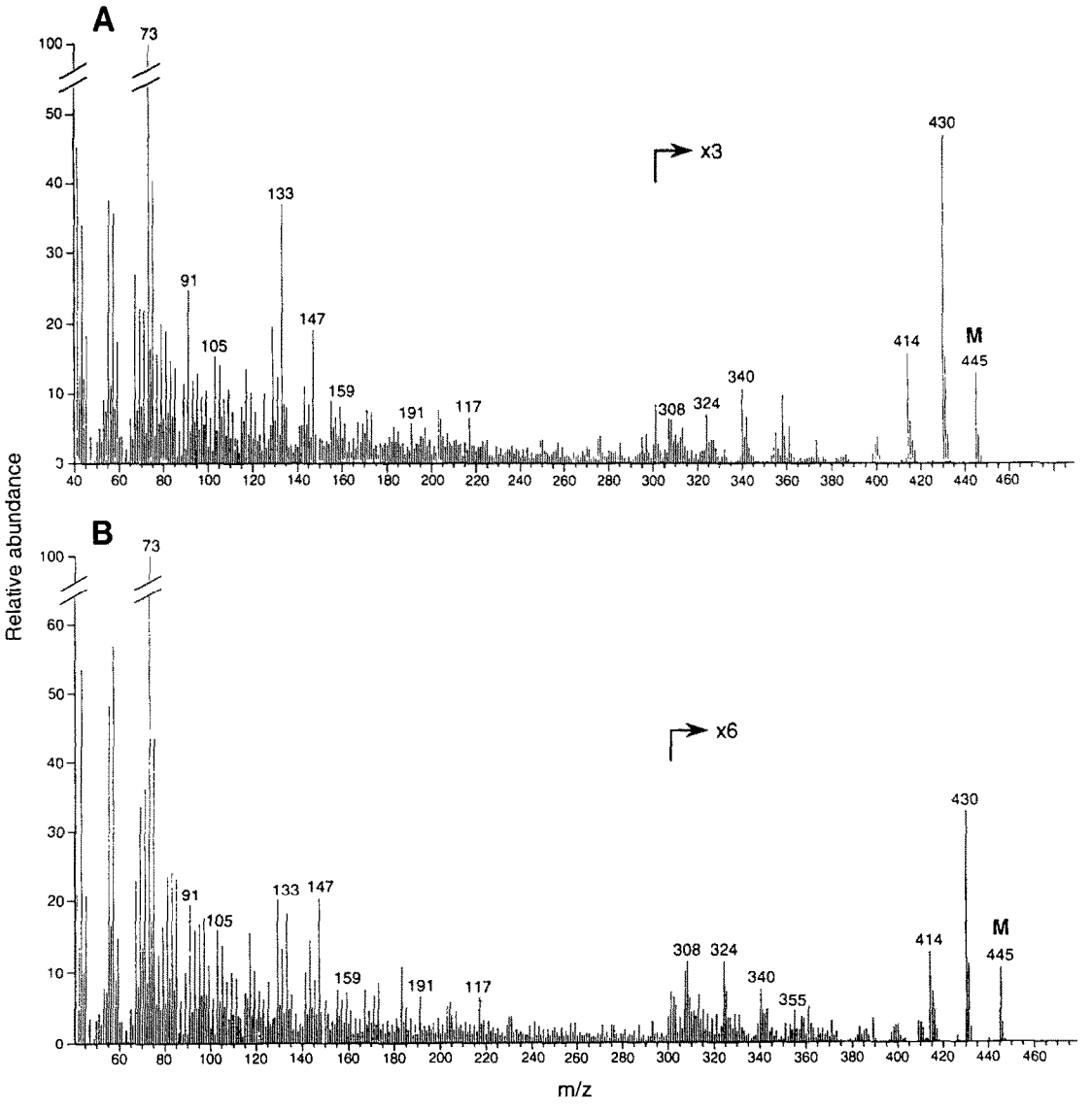

Fig. 1. EI mass spectrum of the methyl ester methoxime trimethysilyl derivative of metabolite I (2-hydroxy-4-ketoretinoic acid) (A) and of metabolite II (3-hydroxy-4-ketoretinoic acid) (B).

3-hydroxy-4-ketoretinoic acid, respectively. Additional findings corroborated this characterization. First, both metabolites absorbed UV light with a λ_{max} at 350–352 nm, suggesting the presence of a conjugated pentaene configuration linked to a keto function. Second, as expected for hydroxy-keto derivates, both methylated metabolites reacted with methoxyamine and BSTFA. The EI mass spectra of the methyl ester methoxime trimethylsilyl derivative of metabolites I and II are shown in Fig. 1A and B. Both spectra were very similar showing ions at m/z 445 [M], 430 [M-15, loss of $—CH_3$], 414 [M-31, loss of $—OCH_3$], 355 [M-90, loss of $(CH_3)_3SiOH$], 340 [355 − 15], 324 [355 − 31], 308 [340 − 32, loss of CH_3OH], 232 [$CH_3—CH=CH—CH=CH—C(CH_3)=CH—CH=CH—C(CH_3)=CH—COOCH_3$], 217 [232 − 15], 191, 159, 147 [191 − 44, loss of H_2O and C_2H_2], 133 [$^+CH_2—CH=CH—CH=CH—C(=CH_2)—CH=C=O$)], 125, 105, 91 and 73 [base peak, $Si(CH_3)_3$] in accordance with the proposed structures. Third, metabolite II resisted silylation by BSTFA unless its ketone function was first converted to a methoxime suggesting the presence of intramolecular hydrogen bond formation between the hydroxyl and ketone groups.

To examine the *in vivo* metabolism of 4-keto-RA, rats were treated with an oral dose of vehicle (PEG 200) or liarozole fumarate (40 mg/kg). One hour later, the animals were given an intravenous injection of 20 μg of 4-keto-RA and killed 5–40 min afterwards. Figure 2 shows that in vehicle-pretreated animals, 4-keto-RA injection was followed by its rapid, apparently first-order elimination from plasma. Its plasma $T_{1/2}$ of only 7 min (i.e. about four times shorter than that of similarly administered RA [10]) may explain the difficulty (if not the inability) of detecting 4-keto-RA in plasma samples from humans and experimental animals, even after administration of retinol or RA [8, 24–26]. As the V_d of the more hydrophilic 4-keto-RA (366 mL/kg) was about four times smaller than that of RA (1352 mL/kg), both retinoids had comparable AUC and *CL* values. Pretreatment with liarozole had a pronounced effect on the plasma elimination of 4-keto-

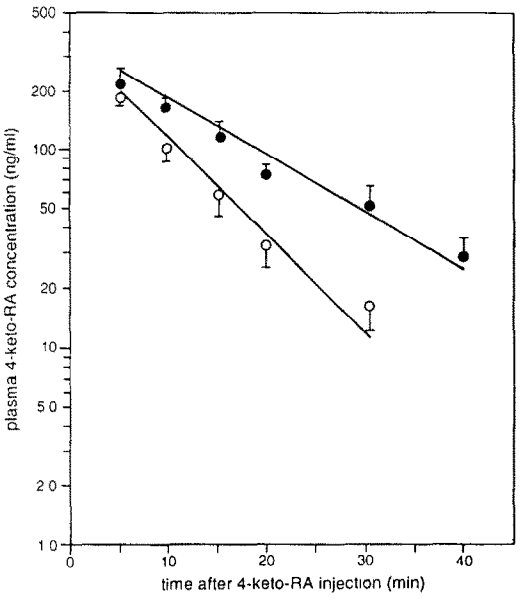

Fig. 2. Semilogarithmic plots of 4-keto-RA plasma concentrations (mean ± SE, N = 6 animals per treatment group and time point) as a function of time after i.v. injection of 4-keto-RA (0.1 mg/kg). Rats were pretreated (−60 min) with PEG 200 (vehicle) (○) or liarozole fumarate (40 mg/kg) (●).

RA: the drug nearly doubled the plasma $T_{1/2}$ to 12 min, while having no effect on they V_d (Table 1). The latter finding excluded the possibility that the effect of liarozole was due to interference with the binding of 4-keto-RA to plasma proteins. Taken together, this study identifies liarozole as a versatile P450 inhibitor of RA metabolism: together with inhibiting the 4-hydroxylation of RA, the drug also suppresses the monohydroxylation reactions of 4-keto-RA. Obviously, given this dual action, administration of liarozole may result in an enhancement of the "retinoid tone", i.e. the balance between the production and removal of biologically active endogenous retinoids. If correct, liarozole should be able to exert a retinoid-mimetic activity. Indeed, both liarozole and RA were recently reported [18] to produce similar morphologic and biochemical effects on the differentiation process of rat vaginal epithelium.

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Table 1. Pharmacokinetic parameters of 4-keto-RA (20 μg i.v.) in vehicle- and liarozole fumarate-pretreated rats

Parameter (dimension)	Pretreatment with PEG 200	Pretreatment with liarozole fumarate
AUC (ng min/mL)	2757	4761
$T_{1/2}$ (min)	7	12
CL (mL/min/kg)	36	21
V_d (mL/kg)	366	364

Animals were given an oral dose of PEG 200 (vehicle) or liarozole fumarate (40 mg/kg) 60 min before i.v. injection of 20 μg of 4-keto-RA.

Janssen Research Foundation
B-2340 Beerse
Belgium

J. Van Wauwe*
M.-C. Coene
W. Cools
J. Goossens
W. Lauwers
L. Le Jeune
C. Van Hove
G. Van Nyen

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* Corresponding author: J. Van Wauwe, Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium. Tel. 014/60.24.64; FAX 014/60.28.41.

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