

The antiproliferative activity of all-*trans*-retinoic acid catabolites and isomers is differentially modulated by liarozole-fumarate in MCF-7 human breast cancer cells

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Summary The clinical use of all-*trans*-retinoic acid (ATRA) in the treatment of cancer is significantly hampered by the prompt emergence of resistance, believed to be caused by increased ATRA catabolism. Inhibitors of ATRA catabolism may therefore prove valuable for cancer therapy. Liarozole-fumarate is an anti-tumour drug that inhibits the cytochrome P450-dependent catabolism of ATRA. ATRA, but also its naturally occurring catabolites, 4-oxo-ATRA and 5,6-epoxy-ATRA, as well as its stereoisomers, 9-*cis*-RA and 13-*cis*-RA, show significant antiproliferative activity in MCF-7 human breast cancer cells. To further elucidate its mechanism of action, we investigated whether liarozole-fumarate was able to enhance the antiproliferative activity of ATRA catabolites and isomers. Liarozole-fumarate alone up to a concentration of 10⁻⁶ M had no effect on MCF-7 cell proliferation. However, in combination with ATRA or the ATRA catabolites, liarozole-fumarate (10⁻⁶ M) significantly enhanced their antiproliferative activity. On the contrary, liarozole-fumarate (10⁻⁶ M) was not able to potentiate the antiproliferative activity of the ATRA stereoisomers, most probably because of the absence of cytochrome P450-dependent catabolism. Together, these findings show that liarozole-fumarate acts as a versatile inhibitor of retinoid catabolism in that it not only blocks the breakdown of ATRA, but also inhibits the catabolic pathway of 4-oxo-ATRA and 5,6-epoxy-ATRA, thereby enhancing their antiproliferative activity.

Keywords: catabolite; isomer; liarozole-fumarate; MCF-7; metabolism; retinoic acid

The therapeutic potential of all-*trans*-retinoic acid (ATRA) in cancer is obvious from a vast number of publications (for review see Hong and Itri, 1994; Moon et al, 1994). However, a major drawback to the clinical application of ATRA is the prompt emergence of resistance, most probably because of the induction of oxidative catabolism through cytochrome P450-dependent enzymes (Muindi et al, 1992; Smith et al, 1992; Warrell et al, 1993; Warrell, 1993; Kizaki et al, 1996).

Liarozole-fumarate, an imidazole-containing anti-tumour drug, was recently identified as an inhibitor of cytochrome P450-dependent retinoic acid (RA) catabolism (Van Wauwe et al, 1990; Wouters et al, 1992; Van Wauwe et al, 1994; Krekels et al, 1996). In vivo, liarozole-fumarate showed retinoid-mimetic effects (Van Wauwe et al, 1991; Mahler et al, 1993; Smets et al, 1995). In animals, the drug reduced the growth of both androgen-dependent and androgen-independent Dunning R3327 rat prostate adenocarcinoma cells (Van Ginckel et al, 1990; Stearns et al, 1993; Smets et al, 1995), and of androgen-independent PC-3ML-B2 human prostate carcinoma xenografts (Dijkman et al, 1994). In the Dunning AT-6sq, an androgen-independent rat prostate carcinoma, liarozole-fumarate reduced tumour weight, and concomitantly increased ATRA levels both in plasma and in tumours, resulting in a shift of their differentiation status (Smets et al, 1995). In patients, the drug is currently being tested in phase III clinical trials for the treatment of relapsed prostate cancer (Mahler et al, 1993; Wouters, 1994).

In vitro, liarozole-fumarate has no intrinsic retinoid-like activity, but enhances the beneficial effects of ATRA, most probably by inhibiting catabolism. The drug has been shown to potentiate the antiproliferative activity of ATRA in MCF-7 human breast cancer cells (Wouters et al, 1992; Van heusden et al, 1996) and to enhance the differentiation-inducing capacity of ATRA in F9 mouse teratocarcinoma cells (De Coster et al, 1992) and MCF-7 cells (Van heusden et al, 1996). The cancer chemopreventive activity of ATRA and β -carotene was potentiated in 10T1/2 mouse embryonal fibroblasts (Acevedo and Bertram, 1995) and retinoid-induced apoptosis was enhanced in DU145 human prostate cancer cells (Hall, 1996).

ATRA is well known to inhibit proliferation and to induce differentiation of malignant cells (Lotan, 1980). ATRA is rapidly metabolized in a catabolic pathway that includes the 4-hydroxylation of the β -ionone ring to yield 4-hydroxy-ATRA (Frolik et al, 1979; Roberts et al, 1980). This step is catalysed by a cytochrome P450-dependent ATRA 4-hydroxylase (Roberts et al, 1980; White et al, 1996). 4-Hydroxy-ATRA is then converted to more polar metabolites via 4-oxo-ATRA (Roberts et al, 1980), involving at least one, presently unknown, cytochrome P450-dependent enzyme (Roberts et al, 1980; Van Wauwe et al, 1994). Other catabolic pathways of ATRA have been described including epoxidation to yield 5,6-epoxy-ATRA (McCormick et al, 1978; Barua et al, 1991). Also, ATRA can isomerize in cell culture to 9-*cis*-RA and 13-*cis*-RA, an obviously non-enzymatic process (Urbach and Rando, 1994). ATRA and its naturally occurring catabolites and isomers possess significant antiproliferative activity in MCF-7 cells (Van heusden et al, 1998). To further explore its mechanism of action, we studied whether liarozole-fumarate was able to enhance the antiproliferative activity of these naturally occurring ATRA catabolites and isomers.

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MATERIALS AND METHODS

Drugs and chemicals

The fumarate salt of liarozole, {±5-[(3-chlorophenyl)(1*H*-imidazole-1-yl)methyl]-1*H*-benzimidazole (E)-2-butenedioate (2:3)} (R085246), was synthesized at the Janssen Research Foundation (Beerse, Belgium). ATRA was obtained from Serva (Heidelberg, Germany) and 13-*cis*-RA was purchased from Eastman Kodak (Rochester, NY, USA). 9-*cis*-RA, 4-oxo-ATRA and 5,6-epoxy-ATRA were generous gifts from Dr M Klaus (Hoffmann-La Roche, Basle, Switzerland). Stock solutions of liarozole-fumarate (10 mM) and retinoids (4 mM) were prepared in ethanol and appropriately diluted in culture medium. The final ethanol concentration did not exceed 0.5% (v/v). The retinoid stock solutions were checked for purity using high-performance liquid chromatography (HPLC) analysis. Experimental studies with retinoids were always performed in a dark room with yellow illumination.

Preparation of dextran-coated charcoal (DCC)-treated fetal bovine serum (FBS)

DCC-treated FBS was prepared as described by Horwitz and McGuire (1978). Briefly, FBS (Life Technologies, Paisley, UK) was heat-inactivated by a 30 min incubation at 56°C. Activated charcoal [0.25% (w/v); Sigma Chemical, St Louis, MO, USA] was coated overnight at 4°C with dextran [0.025% (w/v); Pharmacia, Uppsala, Sweden] in 0.01 M Tris-HCl (pH 8.0). Then, 100 ml of this suspension was pelleted by centrifugation and 50 ml of heat-inactivated FBS was incubated for 45 min at 45°C with the resulting DCC pellet. This procedure was repeated and finally the activated charcoal was removed from the FBS by centrifugation. DCC-treated FBS was sterilized by passage through a 0.22 µm Millipore filter (low protein binding) and stored at -20°C until use. The efficiency of this procedure was assessed by the addition of a trace amount of [6, 7 - ³H(N)]oestradiol (DuPont NEN, Boston, MA, USA), [11, 12 - ³H(N)]retinol (DuPont NEN) and [11, 12 - ³H(N)]ATRA (DuPont NEN). The DCC-treatment efficiently removed oestradiol and ATRA, and retinol to a lesser extent (Van heusden et al, 1998).

Cell culture

Stock cultures of MCF-7 human breast cancer cells, purchased from the American Type Culture Collection (Rockville, MD, USA), were cultured in Dulbecco's modified Eagle medium (DMEM) with 4.5 g l⁻¹ glucose supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 µg ml⁻¹ gentamicin (all reagents from Life Technologies). The MCF-7 subclone used in this study has been characterized previously (Van heusden et al, 1996). Cells were grown in a humidified incubator (5% carbon dioxide, 95% air) at 37°C and were *Mycoplasma* free as tested by the *Mycoplasma* TC kit (Gen-Probe Incorporated, CA, USA).

For the proliferation studies, MCF-7 cells were cultured for 6 days in phenol red-free DMEM containing 5% (v/v) DCC-treated FBS, 4.5 g l⁻¹ glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg ml⁻¹ gentamicin, 30 nM sodium selenite and 10 µg ml⁻¹ transferrin (all reagents from Life Technologies). Cells were seeded onto Chamber Slides (Nunc, Naperville, IL, USA) at a concentration of 15 000 cells per chamber. Chamber Slides had been coated with 50 µg ml⁻¹ poly-L-lysine one day before use.

Cells were allowed to attach for 24 h, and thereafter the medium was supplemented with growth factors, i.e. 10 µg ml⁻¹ final concentration insulin (Life Technologies) and 5 ng ml⁻¹ final concentration basic fibroblast growth factor (Life Technologies), retinoids (concentration ranging from 10⁻¹¹ M to 10⁻⁶ M) and liarozole-fumarate (concentration ranging from 10⁻⁷ M to 10⁻⁵ M). Cells were grown under these conditions for 7 days with medium changes 3 and 6 days after seeding.

Bromodeoxyuridine (BrdU) detection

Cell proliferation was measured using BrdU incorporation, which is considered an accurate method because it is a direct assay of DNA synthesis (Dolbeare, 1995). After 7 days of culture with retinoids as described above, MCF-7 cells were labelled with 100 µM BrdU for 2.5 h and fixed. Incorporated BrdU was visualized by immunofluorescence staining using the tyramide signal amplification (TSA)-direct kit (Green) (DuPont NEN Life Science Products, Boston, MA, USA) as described in detail previously (Van heusden et al, 1997).

The BrdU labelling index was defined as the proportion of BrdU-positive cells, representing cells in S-phase, and was estimated by counting cells under a fluorescence microscope (Axiophot, Zeiss, Germany) with a dual filter set for simultaneous visualization of fluorescein and propidium iodide signals. About 800 cells were counted twice for each test compound per experiment. Average results are presented as means ± s.d. of three to five experiments.

Microcolumn assay for ATRA catabolism

ATRA catabolism was quantitatively determined using the microcolumn assay as described previously (Krekels et al, 1997). Briefly, cells cultured in medium containing 5% DCC-treated FBS, were pretreated for 24 h with 10⁻⁶ M ATRA to induce the ATRA catabolic pathway (Wouters et al, 1992; Krekels et al, 1997). Cells were then washed with culture medium, harvested and resuspended at 4 × 10⁶ cells ml⁻¹. This cell suspension (450 µl) was incubated for 90 min in the presence of 10⁻⁷ M [11, 12 - ³H(N)]ATRA or 10⁻⁷ M [³H]9-*cis*-RA (Amersham Life Science, Buckinghamshire, UK), and thereafter 2 ml of 100% acetonitrile was added. After centrifugation for 10 min at 780 G, the resulting deproteinized supernatant was acidified with 2.5 ml of 40 mM acetic acid and applied to a 3 ml of C18 Bond Elut RC column (Varian, Harbor City, CA, USA; pretreated with 4 ml of distilled water) under a vacuum of 127 mmHg using VAC ELUT SPS-24 and the effluent was collected. The column was eluted with 1 ml of 40% acetonitrile and the effluent was collected in the same vial. The collected effluent, containing the polar metabolites, was counted for radioactivity in a Packard Tri-carb 4530 liquid scintillation analyser. Optiphase 'Hi Safe II' (Wallac, Milton Keynes, UK) was used as a scintillator.

HPLC analysis

The MCF-7 cell suspension was prepared as described above and 450 µl was incubated with 10⁻⁷ M [11, 12 - ³H(N)]ATRA or 10⁻⁷ M [³H]9-*cis*-RA for 4 h. After centrifugation for 10 min at 780 g, the supernatant was analysed for the presence of ATRA metabolites and isomers.

Analysis of ATRA metabolites

Reverse-phase HPLC analysis was carried out on a Varian HPLC system consisting of a HPLC pump 9010, an autosampler 9095 and a diode array detector (Polyview, 9065). The Star 4.0 data software (Varian) was used to analyse the chromatograms. Radioactivity in the eluate was monitored on-line by β -counting (Packard Radiomatic radioactivity monitor) using Ultima-flo M (Packard, Meriden, CT, USA) as the scintillation solvent. The samples (150 μ l) were analysed on a Zorbax 5C8 column (4.6 mm i.d. \times 250 mm, 5 μ m; Chrompack). The mobile phase was methanol–2% acetic acid–acetonitrile (1.5:93:5.5) containing 40 mM ammonium acetate (solvent A). Solvent B consisted of methanol–2% acetic acid–acetonitrile (15:30:55) containing 40 mM ammonium acetate and solvent C was 100% methanol. A linear gradient at a flow rate of 1 ml min⁻¹ was performed in 25 min from 24% A–76% B to 15% A–85% B. The solvent was then changed to 50% B – 50% C in 15 min. To elute unchanged ATRA the solvent was then changed to 100% C after 40 min.

Analysis of ATRA isomers

For the separation of the isomers of ATRA the same HPLC equipment was used. Samples (150 μ l) were analysed on a Novapak column (3.9 mm i.d. \times 300 mm). Solvent D was methanol–2% acetic acid–acetonitrile (15:30:55) containing 40 mM ammonium acetate. Solvent E consisted of methanol–2% acetic acid–acetonitrile (20:20:60) containing 40 mM ammonium acetate and solvent F was 100% methanol. The mobile phase was 50% D – 50% E for 30 min at a flow rate of 1 ml min⁻¹. Then a linear gradient was performed to 100% F.

Statistical analysis

Data were analysed using the two-tailed Mann–Whitney *U*-test using the Stat View II software (Abacus Concepts, Berkeley, CA, USA). Significance was defined at the level of **P* < .01 and ***P* < .001.

RESULTS

Liarozole-fumarate potentiates the antiproliferative activity of ATRA and its catabolites, but not of its stereoisomers

MCF-7 cells were cultured in steroid- and retinoid-free medium supplemented with growth factors. Under these culture conditions, MCF-7 cells showed a BrdU labelling index of 25.2 \pm 1.4% (*n* = 5). Liarozole-fumarate alone had no effect on MCF-7 cell proliferation up to a concentration of 10⁻⁶ M (Figure 1A). At 10⁻⁵ M, liarozole-fumarate decreased the BrdU labelling index to 21.5 \pm 2.8% (*n* = 4). In all following experiments liarozole-fumarate was therefore used at a concentration of 10⁻⁶ M.

Liarozole-fumarate (10⁻⁶ M) significantly enhanced the antiproliferative activity of ATRA (Figure 1B) and its naturally occurring catabolites 4-oxo-ATRA (Figure 1C) and 5,6-epoxy-ATRA (Figure 1D) as reflected by a further decrease in the BrdU labelling index. This potentiation was more pronounced at lower retinoid concentrations. Note that liarozole-fumarate was not able to enhance the antiproliferative activity at retinoid concentrations of 10⁻⁶ M (Figure B – D). In the case of 5,6-epoxy-ATRA, liarozole-fumarate was not able to enhance the antiproliferative activity at concentrations of

10⁻⁷ M and 10⁻⁶ M (Figure 1D). Liarozole-fumarate (10⁻⁶ M) did not enhance the antiproliferative activity of the stereoisomers 9-*cis*-RA (Figure 1E) and 13-*cis*-RA (Figure 1F).

5,6-Epoxy-ATRA competes with ATRA for ATRA catabolism

MCF-7 cells, cultured in medium containing 5% DCC-treated FBS, were pretreated for 24 h with 10⁻⁶ M ATRA to induce ATRA catabolism (Wouters et al, 1992; Krekels et al, 1997). ATRA catabolism was measured using 10⁻⁷ M [³H]ATRA as substrate. As shown in Figure 2, non-radioactive ATRA competed with [³H]ATRA with an IC₅₀ value of 129 \pm 40 nM (*n* = 5). 5,6-Epoxy-ATRA was a better competitor than ATRA and decreased the amount of polar [³H]ATRA metabolites with an IC₅₀ value of 50 \pm 28 nM (*n* = 5).

Liarozole-fumarate inhibits the catabolism of ATRA

Liarozole-fumarate concentration dependently increased the level of unmetabolized [³H]ATRA and concomitantly inhibited the formation of polar [³H]ATRA metabolites (Figure 3, Table 1). The unidentified apolar peak was concentration dependently increased by liarozole-fumarate. The isomerization process of ATRA to 9-*cis*-RA and 13-*cis*-RA was not affected by liarozole-fumarate (Table 1). A peak co-eluting with 4-oxo-ATRA was formed to only a limited extent (data not shown). No peak co-eluting with authentic 5,6-epoxy-ATRA could be detected with or without treatment with liarozole-fumarate at concentrations from 10⁻⁶ M to 10⁻⁵ M.

9-*cis*-RA is not metabolized in MCF-7 cells

[³H]9-*cis*-RA was not converted to polar metabolites in MCF-7 cells as measured by HPLC analysis. Similar results were obtained by the microcolumn assay (data not shown). The metabolism of 13-*cis*-RA, 4-oxo-ATRA and 5,6-epoxy-ATRA could not be studied because these retinoids are not available as tritiated compounds and unlabelled retinoids could not be used for these purposes (data not shown; Gubler and Sherman, 1990).

DISCUSSION

The present study further elucidates the mechanism of action of liarozole-fumarate by showing its enhancing effect on the antiproliferative activity of naturally occurring ATRA catabolites. These effects could only be properly studied in a steroid- and retinoid-free culture medium supplemented with growth factors. We have previously demonstrated that not only ATRA itself, but also its naturally occurring catabolites, 4-oxo-ATRA and 5,6-epoxy-ATRA, as well as its stereoisomers, 9-*cis*-RA and 13-*cis*-RA, significantly inhibit the proliferation of MCF-7 human breast cancer cells (Van heusden et al, 1998). Liarozole-fumarate, at a concentration of 10⁻⁶ M, enhanced the antiproliferative activity of ATRA and its catabolites, but not that of the stereoisomers of ATRA. As such liarozole-fumarate acts as a versatile inhibitor of the cytochrome P450-dependent ATRA catabolic pathway (Figure 4).

Liarozole-fumarate (10⁻⁶ M) significantly enhanced the antiproliferative activity of ATRA in MCF-7 cells. This potentiating effect was more than 100-fold at low retinoid concentrations (10⁻¹⁰ M to

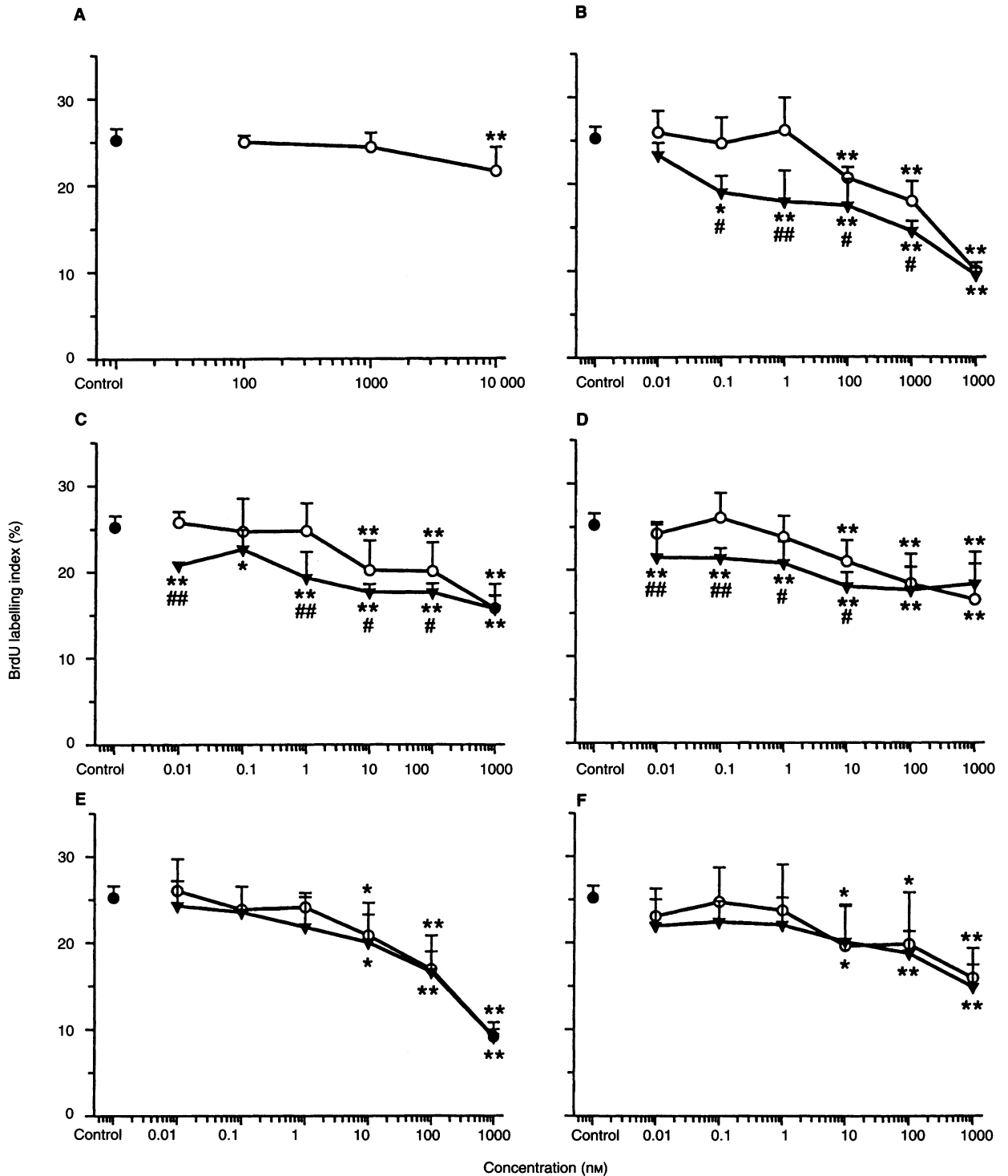


Figure 1 Concentration response curves showing the effect of liarozole-fumarate alone (A) or in combination with ATRA (B), 4-oxo-ATRA (C), 5,6-epoxy-ATRA (D), 9-cis-RA (E) and 13-cis-RA (F) in MCF-7 cells. Cells were cultured for 7 days in the presence of test compounds. Cell proliferation was measured by BrdU incorporation, as described in Materials and methods. Results are presented as means \pm s.d. of three (B, E, F), four (A) or five (C, D) experiments. * $P < .01$ and ** $P < .001$ vs control cells (Mann-Whitney U -test). # $P < .05$ and ## $P < .01$ vs retinoid-treated cells (Mann-Whitney U -test). (A) ○, Liarozole-fumarate. (B) ○, ATRA; ▼, ATRA + 1 μ M liarozole-fumarate. (C) ○, 4-oxo-ATRA; ▼, 4-oxo-ATRA + 1 μ M liarozole-fumarate. (D) ○, 5,6-epoxy-ATRA; ▼, 5,6-epoxy-ATRA + 1 μ M liarozole-fumarate. (E) ○, 9-cis-RA; ▼, 9-cis-RA + 1 μ M liarozole-fumarate. (F) ○, 13-cis-RA; ▼, 13-cis-RA + 1 μ M liarozole-fumarate

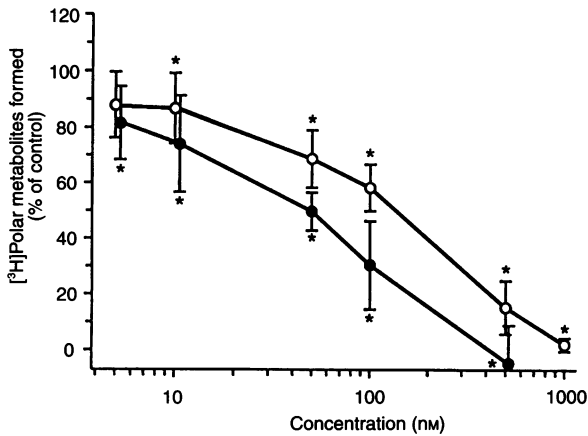


Figure 2 Competition between radioactive [^3H]ATRA and non-radioactive ATRA ($\text{IC}_{50} = 129 \pm 40 \text{ nM}$) and 5,6-epoxy-ATRA ($\text{IC}_{50} = 50 \pm 28 \text{ nM}$). MCF-7 cells were preincubated for 24 h with 10^{-6} M ATRA to induce ATRA catabolism. Concentration response curves for ATRA catabolism were determined using 10^{-7} M [^3H]ATRA as substrate. Results are presented as means \pm s.d. of five experiments. * $P < .01$ vs control. * $P < .05$ vs ATRA treatment (Mann-Whitney U -test), \circ , ATRA; \bullet , 5,6-epoxy-ATRA

10^{-9} M) in contrast to the tenfold enhancement, as previously demonstrated under growth conditions with culture medium containing untreated FBS (Wouters et al, 1992; Van heusden et al, 1996). The effect of liarozole-fumarate on ATRA metabolism was analysed using HPLC. Only the supernatant was studied. We have previously shown that in the cell extract the same overall metabolite profile can be found (Wouters et al, 1992). Liarozole-fumarate increased the level of RA and concomitantly inhibited the formation of more polar metabolites in a concentration-dependent manner. Together, these findings suggest that liarozole-fumarate enhances the antiproliferative activity of ATRA by inhibiting its catabolism, in agreement with our previous findings (Wouters et al, 1992).

Liarozole-fumarate (10^{-6} M) enhanced the antiproliferative activity of the naturally occurring catabolite 4-oxo-ATRA, confirming that its catabolism is inhibited by liarozole-fumarate. Indeed, Van Wauwe et al (1994) have shown that liarozole-fumarate inhibited the formation of more polar metabolites from 4-oxo-ATRA in hamster liver microsomes. In addition, liarozole-fumarate enhanced the plasma half-life of 4-oxo-ATRA in rats (Van Wauwe et al, 1994). Taken together, these findings suggest that catabolism of 4-oxo-ATRA involves at least one cytochrome P450-dependent enzyme (Roberts et al, 1980; Van Wauwe et al, 1994) that is inhibited by liarozole-fumarate.

The antiproliferative activity of low concentrations of 5,6-epoxy-ATRA was also potentiated by liarozole-fumarate (10^{-6} M). One possible explanation for this synergism is that liarozole-fumarate inhibits the cytochrome P450-dependent catabolism of 5,6-epoxy-ATRA. Although such enzyme(s) are unknown, it is not unlikely that 5,6-epoxy-ATRA is metabolized by the same enzyme(s) as ATRA as 5,6-epoxy-ATRA was a better competitor than ATRA itself in our assay for measuring ATRA catabolism. Although liarozole-fumarate was present at a tenfold higher concentration, i.e. 10^{-6} M , the drug was not able to enhance the antiproliferative activity of 5,6-epoxy-ATRA when tested at a concentration of 10^{-7} M . These data support the hypothesis that both liarozole-fumarate and 5,6-epoxy-ATRA compete for the same enzyme(s) and that the higher affinity of 5,6-epoxy-ATRA for these enzyme(s) does not allow enhancement.

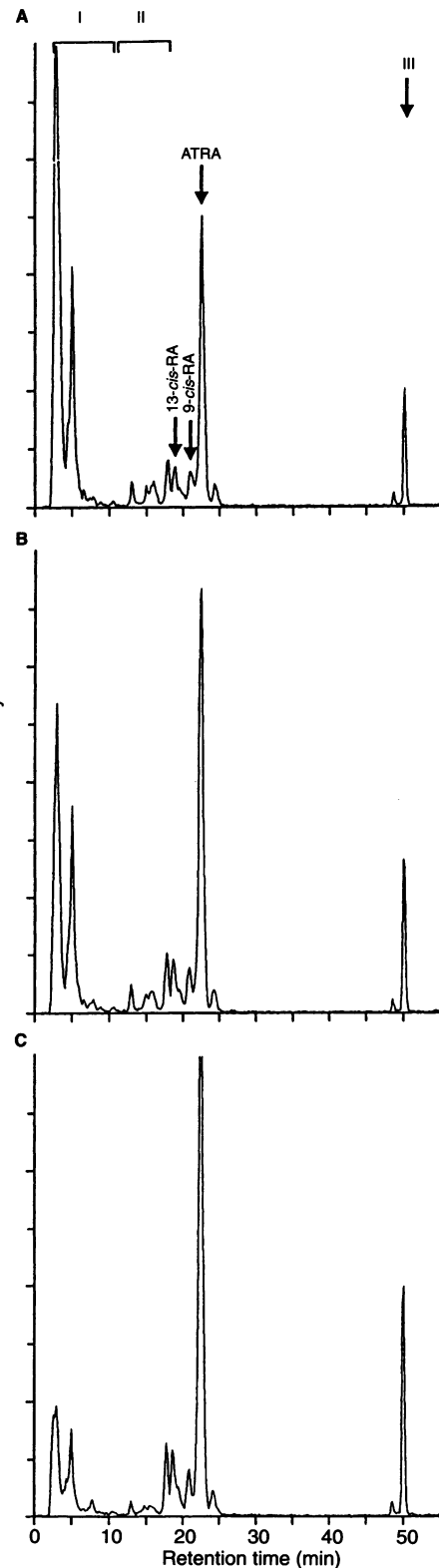


Figure 3 Chromatograms illustrating the metabolism of [^3H]ATRA in the absence (A) or presence of liarozole-fumarate at a concentration of 10^{-6} M (B) or 10^{-5} M (C). Cells were incubated for 24 h with 10^{-6} M ATRA to induce ATRA catabolism, washed twice and collected. Cells were then incubated for 4 h with 10^{-7} M [^3H]ATRA and the supernatant was analysed using reverse phase high-performance liquid chromatography (HPLC). I, very polar metabolites; II, intermediate polar metabolites; III, apolar metabolite

Table 1 Quantitative analysis of [³H]ATRA metabolism in MCF-7 cells

HPLC peaks	Radioactivity (%)		
	Control	+ 10 ⁻⁶ M liarozole	+ 10 ⁻⁵ M liarozole
RA (% ATRA, 9- <i>cis</i> -RA and 13- <i>cis</i> -RA)	43 (78, 13, 10)	52 (78, 13, 10)	73 (82, 10, 9)
Metabolites			
Very polar	36	28	10
Intermediately polar	15	12	5
Apolar	6	8	12

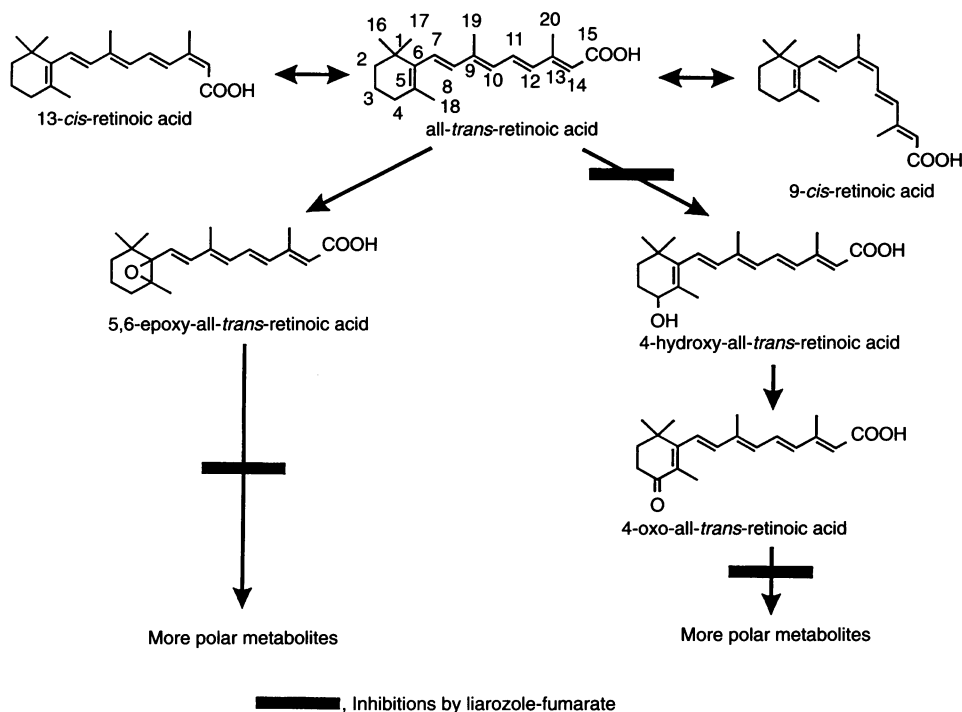


Figure 4 Proposed mechanism of action of liarozole-fumarate. The drug acts as a versatile inhibitor at different steps of the ATRA catabolic pathway. Liarozole-fumarate not only inhibits the catabolism of ATRA, but also of its catabolites 4-oxo-ATRA and 5,6-epoxy-ATRA, thereby enhancing their antiproliferative activity

The ability of ATRA to isomerize in cell culture has been described before (Urbach and Rando, 1994). Liarozole-fumarate (10⁻⁶ M) had no effect on isomerization of ATRA. The antiproliferative activity of 9-*cis*-RA and 13-*cis*-RA could not be enhanced by liarozole-fumarate (10⁻⁶ M). In contrast, *in vivo* liarozole-fumarate has been shown to enhance plasma levels of 9-*cis*-RA (Achkar et al, 1994) and 13-*cis*-RA (Westarp et al, 1994). As RA catabolism may be cell specifically regulated, the most likely explanation for this discrepancy is the absence of cytochrome P450-dependent enzymes that catabolize 9-*cis*-RA and 13-*cis*-RA in MCF-7 cells. In favour of this hypothesis is our finding that 9-*cis*-RA was not converted to polar metabolites in these cultures. Although, we have previously shown that 9-*cis*-RA and 13-*cis*-RA can isomerize to ATRA in MCF-7 cells (Krekels et al, 1997), the fact that their antiproliferative activity was not potentiated by liarozole-fumarate, indicates that their effect on MCF-7 cell proliferation was not due to conversion to ATRA.

We have shown that both 9-*cis*-RA and 13-*cis*-RA compete with ATRA in our assay for measuring ATRA catabolism in MCF-7 cells (Krekels et al, 1997). In this study, we show that liarozole-fumarate was not able to enhance the antiproliferative activity of these stereoisomers. Taken together, these findings suggest that although both stereoisomers are able to bind to ATRA-catabolizing enzyme(s), they are not used as substrates by these enzyme(s) because then an enhancing effect by liarozole-fumarate would be expected. These latter data are in agreement with the observations of Duell et al (1996) in mouse skin.

In conclusion, we can state that liarozole-fumarate is not only able to inhibit the catabolism of ATRA but also that of 4-oxo-ATRA and 5,6-epoxy-ATRA, thereby enhancing their antiproliferative activity. Liarozole-fumarate does not enhance the antiproliferative activity of the stereoisomers 9-*cis*-RA and 13-*cis*-RA in MCF-7 human breast cancer cells, most probably because of the absence of their oxidative catabolism in these cells.

Together, these findings extend and confirm the hypothesis that liarozole-fumarate acts as a versatile inhibitor in the ATRA catabolic pathway (Figure 4), and may show potential in circumvention of RA resistance in cancer.

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