Effects of Liarozole, a New Antitumoral Compound, on Retinoic Acid-induced Inhibition of Cell Growth and on Retinoic Acid Metabolism in MCF-7 Human Breast Cancer Cells

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ABSTRACT

Liarozole is a new imidazole derivative with antitumoral properties. Effects of the compound alone and in combination with all-trans-retinoic acid on proliferation of MCF-7 human breast cancer cells were examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay.

Following 9 days of drug exposure, MCF-7 cell growth was concentration dependently inhibited by all-trans-retinoic acid (drug concentration resulting in 50% growth inhibition, 2 × 10⁻⁸ M), while liarozole at 10⁻⁵ M inhibited cell growth by only 35%.

When MCF-7 cells were incubated with a combination of all-trans-retinoic acid and liarozole, the antiproliferative effect of all-trans-retinoic acid was clearly enhanced. This enhancement was dependent on the liarozole concentration and was more than 10-fold. A combination of 10⁻⁴ M all-trans-retinoic acid and 10⁻⁵ M liarozole resulted in a greater antiproliferative effect than that obtained with 10⁻³ M all-trans-retinoic acid alone.

When MCF-7 cells were incubated for 4 h with [³H]all-trans-retinoic acid, the radioactivity in the supernatant consisted of unaltered retinoid. However, when cells had been pretreated with 10⁻⁸ M all-trans-retinoic acid for 4 h, they were able to substantially metabolize [³H]all-trans-retinoic acid during a subsequent 4-h incubation. High-performance liquid chromatography analysis of the supernatants revealed that the reaction products consisted mainly of very polar metabolites. Liarozole inhibited the metabolism of all-trans-retinoic acid in MCF-7 cells with 10⁻⁵ M liarozole reducing the amount of polar metabolites by 87%.

It is concluded that the enhancement by liarozole of the antiproliferative effects of retinoic acid on MCF-7 human breast cancer cells is probably due to inhibition of retinoic acid metabolism. Further research into these effects in MCF-7 cells as well as in other cancer cell lines will provide more information concerning the exact mechanism of action of liarozole and the use of inhibitors of retinoid metabolism in cancer treatment.

INTRODUCTION

Liarozole (R 75 251) is a new imidazole derivative with antitumoral properties. The compound reduces tumor growth in the androgen-dependent Dunning G and H rat prostate adenocarcinoma models as well as in the androgen-independent Matlu, PIF-1, and H² sublines. The antitumoral effect of liarozole in the androgen-dependent Dunning G model cannot be reversed by supplementation with testosterone (1, 2).

In an open pilot study of 31 patients with actively progressing stage D prostate carcinoma after castration, liarozole induced subjective responses (marked improvement in pain and performance) in the majority of evaluable patients and partial remission (50% reduction in primary tumor or metastatic lymph node volume) in 2 of 2 patients with measurable disease. After 3 months of treatment, prostate-specific antigen levels decreased by at least 50% in more than half of the patients (1, 3).

The antitumoral effect of liarozole in androgen-independent rat prostate tumor models as well as in androgen refractory prostate carcinoma in men suggests the involvement of a non-androgen-related mechanism of action.

Retinoic acid is known to play a key role in proliferation and differentiation of epithelial tissues and has been shown to have anticarcinogenic and antitumoral properties (4-7). Retinoic acid is, however, rapidly metabolized, which results in its deactivation (8, 9). The first enzymatic reaction in the biodegradation of retinoic acid is its conversion to the less active 4-hydroxy metabolite. This reaction is catalyzed by the cytochrome P-450-dependent 4-hydroxylase (10-13) which has been shown to be present mainly in the liver. However, retinoic acid metabolism was also found in F9 teratocarcinoma cells (14) and LCC-PK cells (15), as well as in N-nitroso-N-methyleurea-induced rat mammary tumors (16).

Liarozole inhibits several cytochrome P-450-dependent enzymes (17, 18), and among these is 4-hydroxylase, which metabolizes retinoic acid. Oral administration of liarozole to experimental animals results in increased plasma retinoic acid levels (19), and this may contribute to the antitumoral effect of the compound.

The present study was undertaken to examine the effects of liarozole in relation to retinoic acid on cancer cells in tissue culture. For this purpose, the MCF-7 human breast cancer cell line was chosen since it is known that retinoids have profound effects on proliferation of this cell line (20, 21).

MATERIALS AND METHODS

Drugs and Chemicals. Liarozole [5-(3-chlorophenyl)[1H-imidazol-1-yl]methyl]-1H-benzimidazole (chemical structure depicted in Fig. 1), ketoconazole, miconazole, and vorozole (R 83 842) were synthesized by and obtained from the Janssen Research Foundation. Aminoglutethimide and metyrapone were obtained from Sigma Chemical Co. (St. Louis, MO). The compounds were dissolved at a concentration of 10⁻² M in ethanol or dimethyl sulfoxide. All-trans-retinoic acid (Serva, Heidelberg, Germany) was dissolved at an initial concentration of 4 × 10⁻³ M in ethanol. [11,12⁻³H(N)]All-trans-retinoic acid (1824 GBq/mmol, 49.3 Ci/mmol) was obtained from NEN (Dupont de Nemours, Brussels, Belgium). Retinoid stock solutions were regularly checked for purity using HPLC analysis. Retinoid manipulations were carried out in a dark room with yellow illumination. Further dilutions of all test compounds were made in culture medium. Final solvent concentrations during incubation were always ≤0.25% (v/v). These concentrations had no effect on cell growth.

Cell Cultivation. The MCF-7 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured as adherent monolayers at 37°C in 5% CO₂-95% air at 100% relative humidity in Falcon tissue culture flasks (Becton Dickinson, Aalst, Belgium). The culture medium was Dulbecco's modified Eagle's...
medium with 4.5 g/liter glucose and 3.7 g/liter sodium bicarbonate supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 \( \mu \)g/ml streptomycin sulfate (all reagents from Life Technologies, Gent, Belgium), and 10% fetal calf serum (Sera-Lab, International Medical Products, Brüssel, Belgium). Cells were subcultured once weekly at a split ratio of 1:10 using trypsin/EDTA solution and were regularly checked for Mycoplasma contamination. For the experiments described in this study, the MCF-7 cell line was used at passages between 158 and 174.

Growth Experiments. Stock cultures were trypsinized, and cells were counted using a hemocytometer and plated at 2000 cells/well in 96-well microtiter tissue culture plates (Falcon; Becton Dickinson, Aalst, Belgium) in 150 \( \mu \)l medium. For each experimental condition, 8 replicate wells were used. The cells were allowed to attach for 24 h. Then, the test compounds or the solvent were added to a final volume of 200 \( \mu \)l (day 0), and the cells were further incubated for the indicated times. Medium and test compounds were renewed on days 2, 5, and 7. Following the appropriate incubation period, the amount of metabolically active cells was measured using a tetrazolium-based (MTT) assay (22).

MTT Assay. MTT (Serva) was dissolved at 5 mg/ml in phosphate-buffered saline. At the end of the growth experiments, 25 \( \mu \)l of this solution was added to each well without removing the medium, and the cells were further incubated for 2 h at 37°C. The medium was then carefully aspirated, and the blue MTT-formazan product was solubilized by addition of 100 \( \mu \)l dimethyl sulfoxide. The microtiter plates were shaken for 10 min on a microplate shaker, and the absorbance at 540 nm was read using an automatic plate reader (Titertek Multiskan MCC/340; Flow, Asse-Relegem, Belgium). The MTT assay was validated for use with MCF-7 cells by comparing the MTT results obtained 3 h after plating different numbers of cells with those obtained by hemocytometer counting (data not shown). The method was linear up to the highest cell concentration tested (40,000 cells/well).

Retinoic Acid Metabolism. A 175-cm\(^2\) confluent MCF-7 culture, growing under normal conditions, was treated for 18 h with 10\(^{-8}\) M all-trans-retinoic acid. Cells were then washed twice with 20 ml culture medium and trypsinized. Cells were suspended at 6 \times 10\(^4\) cells/ml in Dulbecco's modified Eagle's medium without phenol red, containing 1 g/liter glucose and supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 \( \mu \)g/ml streptomycin sulfate, 2.5 \( \mu \)g/ml Fungizone, and 10% heat-inactivated fetal calf serum (all reagents from Life Technologies).

Aliquots (900 \( \mu \)l) of this cell suspension in glass test tubes were preincubated for 5 min at 37°C with 50 \( \mu \)l liarozole (final concentration 10\(^{-3}\) M) or its solvent. Incubation was then continued for 4 h with occasional shaking in the presence of 50 \( \mu \)l \([3H]\)all-trans-retinoic acid (final concentration 10\(^{-7}\) M). At the end of incubation, the cell suspension was centrifuged at 3000 rpm for 5 min. The cell pellet was extracted with 1 ml methanol. Aliquots of this extract and of the supernatant were counted for recovery; the rest was dried in vacuo (Savant Speed Vac Concentrator) and used for HPLC analysis.

HPLC Analysis. Reverse-phase HPLC analysis was carried out as described previously (23) with several modifications. A Varian 5560 liquid chromatograph equipped with a Perkin-Elmer ISS100 automatic injector, a UV-200 variable wavelength detector set at 340 nm, and a Vista 401 data system was used. Radioactivity in the eluate was monitored on-line by \( \beta \)-counting (Berthold LB504 radioactivity monitor) using Pico-Aqua (Canberra-Packard, Brüssel, Belgium) as the scintillation solvent. Samples were analyzed on a 10-\( \mu \)m C\(_{18}\) Bondapak column (3.9 mm inside diameter x 300 mm) (Waters Associates, Brussels, Belgium). Samples were eluted with methanol:water:formic acid (65:35:0.05) containing 10 mm ammonium acetate at a flow rate of 2 ml/min. After 20 min, the solvent was changed to 100% methanol in order to elute retinoic acid.

Statistical Analysis. Where appropriate, data were analyzed using the two-tailed Student's \( t \) test. Significance was defined at the level of \( P < 0.05 \).

RESULTS

The effects of all-trans-retinoic acid and liarozole on proliferation of MCF-7 cells are shown in Fig. 2. Continuous exposure of the cells to all-trans-retinoic acid for 9 days led to concentration-dependent inhibition of cell growth with >90% inhibition achieved at the highest retinoid concentration (10\(^{-5}\) M). The calculated drug concentration resulting in 50% growth inhibition was 2 \times 10\(^{-4}\) M. Liarozole up to 10\(^{-6}\) M had no effect on cell proliferation, and at 10\(^{-5}\) M a 35% inhibition was seen.

To study possible interrelations between liarozole and all-trans-retinoic acid, MCF-7 cells were continuously incubated with combinations of both substances during 9 days. Two low concentrations of all-trans-retinoic acid, which had no (10\(^{-9}\) M) or low (10\(^{-8}\) M) antiproliferative effect when used alone, were combined with increasing concentrations of liarozole (Fig. 3). This resulted in an enhancement of the antiproliferative effect that was dependent on the liarozole concentration. When 10\(^{-8}\) M all-trans-retinoic acid (producing no inhibition when used alone) was added, addition of 10\(^{-6}\) M liarozole significantly inhibited cell growth by 52% and, at 10\(^{-5}\) M liarozole, an 85% inhibition was obtained. With 10\(^{-5}\) M all-trans-retinoic acid (producing 28% inhibition when used alone), growth was significantly inhibited by 53, 83, and 93% with 10\(^{-7}\), 10\(^{-6}\), and 10\(^{-5}\) M liarozole, respectively.

The effects of all-trans-retinoic acid, liarozole, and combinations of both substances on MCF-7 cell growth were also compared by measuring cell proliferation using the MTT assay. Results are expressed as percentages of a control incubation with solvent (producing 28% inhibition when used alone), growth was significantly inhibited by 53, 83, and 93% with 10\(^{-7}\), 10\(^{-6}\), and 10\(^{-5}\) M liarozole, respectively.
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Several different inhibitors of cytochrome P-450 were tested for their ability to enhance the antiproliferative effects of 10^{-9} M all-trans-retinoic acid; the results are shown in Table 1. The drugs were used at 10^{-6} M. At this concentration when used alone, they had no antiproliferative effect. Liarozole, ketoconazole, and miconazole were able to enhance the effect of all-trans-retinoic acid, resulting in cell growth inhibition by 46, 26, and 19%, respectively. The effects of liarozole and ketoconazole were statistically different.

Since liarozole and ketoconazole are known to inhibit the 4-hydroxylation of retinoic acid, we next tried to assess retinoic acid metabolism in MCF-7 cells. When cells growing under normal conditions were incubated for 4 h with 10^{-7} M [{}^{3}H]all-trans-retinoic acid, the radioactivity recovered from the supernatant consisted of [{}^{3}H]retinoic acid (results not shown). Therefore, cells were pretreated overnight with 10^{-6} M all-trans retinoic acid, washed twice, collected, and subsequently incubated with [{}^{3}H]all-trans-retinoic acid. Fig. 5A shows a representative example of the radioactivity profile obtained by HPLC analysis of the supernatant of cells treated in this way. The quantitative analysis of this experiment is given in Table 2. Following a 4-h incubation, >85% of the radioactivity was consistently recovered in the supernatant. Only 47% of this...
consisted of retinoic acid (all-trans + 13-cis) (peak III), while 48% was recovered as very polar metabolites (peak I) and 3% as metabolites with intermediate polarity (peak II). One metabolite with less polarity than retinoic acid was also found, but this consisted of only 2% of total radioactivity (peak IV). In the cell extract, the same overall metabolite profile was found, but 29% of total radioactivity was recovered in the apolar peak (Table 2).

In the presence of $10^{-5}$ M liarozole, distribution of radioactivity between the cell supernatant and the cell pellet was unchanged. The radioactivity profile obtained by HPLC analysis, however, was completely different (Fig. 5B, Table 2). In the supernatant, only 6% of total radioactivity was recovered as very polar metabolites (peak I), and another 7% was found in the apolar peak (peak IV). The rest (87%) eluted in the retinoic acid peak (peak III). In the cell extract, the amount of polar metabolites was reduced to 3%. The rest of the radioactivity eluted as unchanged retinoic acid and in the apolar peak.

This metabolism experiment was repeated 4 times with essentially similar results. Liarozole ($10^{-5}$ M) reduced the amount of polar metabolites in the supernatant by 94 ± 6% (mean ± SD).

### DISCUSSION

The present study was undertaken to gain more insight into the as yet incompletely understood antitumoral mechanism of action of liarozole, a new compound showing antiproliferative effects in both steroid-dependent and steroid-independent animal tumor models.

Liarozole by itself had nearly no effect on the growth of MCF-7 cells but significantly potentiated the antiproliferative effects of all-trans-retinoic acid >10-fold. This potentiation was clearly a synergistic interaction since concentrations of all-trans-retinoic acid and liarozole, which by themselves were inactive, showed antiproliferative effects when combined. Moreover, this synergy is seen at physiologically relevant concentrations of both substances. Indeed, a concentration of 1–10 nM of all-trans-retinoic acid correlates with a normal human plasma level, and the active concentrations of liarozole ($10^{-7}$–$10^{-8}$ M) are within the range of the drug plasma levels obtained after oral intake of a standard liarozole dose (300 mg).

Very few other compounds have been reported to act synergistically with retinoids in MCF-7 cells. Valette and Botanch (24) showed synergistic potentiation of the antiproliferative effects of all-trans-retinoic acid by transforming growth factor-β. Although interactions between γ-interferon or α-interferon and retinoic acid were also described (25, 26), these effects were additive rather than synergistic and were obtained with retinoic acid concentrations 2–3 orders of magnitude higher than those used in the present study. The structure and pharmacological profile of liarozole do not suggest any similarity with these cytokines.

On the other hand, liarozole inhibits several cytochrome P-450-dependent enzymes. The two most prominent enzymes for which inhibition by liarozole has been shown are 4-hydroxylase (4-hydroxylation of retinoic acid) and aromatase (conversion of androgens to estrogens) (17, 19). Therefore, we compared the effects of liarozole to those of several other known P-450 blockers. Vorozole (R 83 842), a potent and selective aromatase inhibitor (27), had no effect on MCF-7 cell growth when used alone or combined with all-trans-retinoic acid. The same was true for aminoglutethimide, an inhibitor of aromatase, 11-hydroxylase, and side-chain cleavage (28), and metyrapone, a 11-hydroxylase inhibitor (29). Only ketoconazole and miconazole showed results similar to those obtained with liarozole, but the potentiation of the effect of all-trans-retinoic acid was less pronounced than with liarozole. Ketoconazole, which derives its antifungal properties from blocking 14α-demethylase in

### Table 2 Quantitative analysis of $[^{3}H]$all-trans-retinoic acid metabolism in MCF-7 cells

<table>
<thead>
<tr>
<th>HPLC peaks</th>
<th>Control + $10^{-7}$ M liarozole</th>
<th>Control + $10^{-5}$ M liarozole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>Radioactivity (% of total)</td>
<td>Cell extract</td>
</tr>
<tr>
<td>Retinoic acid (all-trans + 13-cis) (III)</td>
<td>47</td>
<td>24</td>
</tr>
<tr>
<td>Metabolites</td>
<td></td>
<td>87</td>
</tr>
<tr>
<td>Very polar (I)</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>Moderately polar (II)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Apolar (IV)</td>
<td>2</td>
<td>29</td>
</tr>
</tbody>
</table>

Roman numerals in parentheses refer to the HPLC elution positions indicated in Fig. 6.
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yeasts and fungi, also inhibits 17α-hydroxylase/17,20-lyase (conversion of progestins to androgens) and aromatase, as well as the 4-hydroxylation of retinoic acid (23, 30). Miconazole is an antifungal which is also a weak aromatase inhibitor (31). The results of this comparative experiment were in agreement with the activity of these compounds for inhibition of in vivo metabolism of all-trans-retinoic acid as described by Van Wauwe et al. (19) and with the data of Williams and Napoli (32) obtained for retinoid acid metabolism in F9 cells. This suggested that the effects of liarozole on MCF-7 cells could be related to inhibition of 4-hydroxylase, an enzyme activity not previously described in these cells.

We were unable to detect under basal conditions any retinoid acid metabolism in MCF-7 cells. However, pretreatment with all-trans-retinoic acid induced a very active conversion of all-trans-retinoic acid to more polar metabolites with most of the metabolized retinoic acid recovered as very polar metabolites. A similar induction by retinoic acid of its own metabolism was previously reported for some embryonal carcinoma cell lines (33). The overall HPLC profile was similar to that found in F9 embryonal carcinoma cells (14) and in rats treated in vivo with [14C]retinoic acid (23). Only a very small percentage of radioactivity eluted in the peaks between 12 and 15 min, which is the elution position of 4-hydroxy- and 4-keto-all-trans-retinoic acid in the HPLC system used (23). Positive identification of these peaks could not be made. The absence of substantial amounts of 4-hydroxy- and 4-keto metabolites (identified in hamster plasma and tissue as the main retinoid acid metabolites) may be due to the experimental conditions, their metabolic instability, or species differences. Further experiments with MCF-7 cells or their subcellular fractions are needed to clarify this point. The exact nature of the apolar peak also remains to be determined. Since this peak is present predominantly in the cell-bound radioactivity fraction, it may consist of esterified retinoic acid. Addition of liarozole did not change the distribution of radioactivity between supernatant and cell-bound fractions. Thus, liarozole does not seem to influence retinoid uptake by the cells. It did, however, dramatically change the metabolism of retinoic acid both in the supernatant and the cell-bound fraction. In both fractions, the formation of polar metabolites was almost completely blocked by 10−5 M liarozole, resulting in an enhanced level of unchanged retinoic acid and apolar material. Enhancement of the latter is probably not a direct effect of liarozole on the formation of this compound but rather just a consequence of a higher level of its substrate (retinoic acid).

Although it is not yet absolutely clear whether the formation of polar metabolites of retinoic acid in MCF-7 cells is due to the activity of a 4-hydroxylase enzyme, inhibition by the imidazole liarozole at least points toward involvement of some P-450 enzyme. This inhibition also explains the synergism of liarozole and all-trans-retinoic acid in MCF-7 cells. The potentiation by liarozole of the antiproliferative effect of all-trans-retinoic acid is not due to enhancement of the biological activity of all-trans-retinoic acid but rather to preventing it from being inactivated by metabolism.

The present study for the first time demonstrates the presence of retinoic acid metabolism in human epithelial cancer cells. The question remains whether this metabolism is a unique feature of MCF-7 cells or a more generally occurring phenomenon in epithelial cancer cells. If the latter is the case, this may have important implications for the use of retinoic acid metabolite inhibitors in cancer treatment. As was pointed out earlier, these compounds have potential use in cancer therapy by enhancing endogenous plasma retinoic acid levels through inhibition of liver retinoic acid metabolism (18). If tumor tissue also metabolizes retinoic acid, treatment with metabolism inhibitors may result in higher local (tumor) retinoid acid levels. In this way, the full antiproliferative activity of retinoic acid might be exploited while minimizing the side effects related to high systemic retinoid acid levels.

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REFERENCES

21. Fontana, J. A., Mezu, A. B., Cooper, B. N., and Miranda, D. Retinoid metabolism inhibitors in cancer treatment. As was pointed out earlier, these compounds have potential use in cancer therapy by enhancing endogenous plasma retinoic acid levels through inhibition of liver retinoic acid metabolism (18). If tumor tissue also metabolizes retinoic acid, treatment with metabolism inhibitors may result in higher local (tumor) retinoid acid levels. In this way, the full antiproliferative activity of retinoic acid might be exploited while minimizing the side effects related to high systemic retinoid acid levels.


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