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EXPERIMENTAL STUDIES WITH LIAROZOLE (R 75 251): AN ANTITUMORAL AGENT WHICH INHIBITS RETINOIC ACID BREAKDOWN

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Summary—Liarozole reduced tumor growth in the androgen-dependent Dunning-G and the androgen-independent Dunning MatLu rat prostate carcinoma models as well as in patients with metastatic prostate cancer who had relapsed after orchiectomy. *In vitro*, liarozole did not have cytostatic properties, as measured by cell proliferation in breast MCF-7 and prostate DU145 and LNCaP carcinoma cell lines. It did not alter the metabolism of labeled testosterone i.e. the 5α-reductase in cultured rat prostatic cells. In mouse F9 teratocarcinoma cells liarozole did not show any retinoid-like properties but enhanced the plasminogen activator production induced by retinoic acid. Furthermore, liarozole and retinoic acid similarly reduced the growth of the androgen-dependent Dunning-G tumor in nude mice and inhibited tumor promotion elicited by phorbol ester in mouse skin. These data have raised the hypothesis that the antitumoral properties of liarozole may be related to inhibition of retinoic acid degradation, catalyzed by a *P*-450-dependent enzyme that is blocked by the drug.

INTRODUCTION

Liarozole (R 75 251) is a new imidazole derivative which reduced the growth of androgen-dependent rat Dunning-G prostate adenocarcinoma similarly to castration but without alteration of serum testosterone levels and weight of androgen-dependent tissues such as prostate and seminal vesicles [1]. Furthermore, the compound still reduced tumor growth in castrated animals bearing a testosterone implant [1]. In a pilot study in orchiectomized stage D prostate cancer patients with hormone resistant tumors, liarozole (300 mg bid) induced both objective (serum PSA levels, tumor and lymph node reduction) and subjective (pain, performance status and urological complaints) improvements [2, 3]. In these patients, no significant reduction in the levels of adrenal androgens was detected but increased plasma retinoic acid levels as well as cutaneous reactions similar to those observed after high doses of vitamin A were observed [3]. Indeed, liarozole inhibits several cytochrome *P*-450-dependent enzymes, including the 4-hydroxylase, one of the main enzymes in the catabolic pathway of retinoic acid [4, 5].

Pharmacological data obtained so far with liarozole, as summarized in this paper, have raised the hypothesis that antitumoral effects may be related to the inhibition of the breakdown of retinoic acid by liarozole.

IN VITRO PHARMACOLOGY

Effect of liarozole on cell growth in tissue culture

The effects of liarozole on cell growth in tissue culture have been evaluated in DU145 and LNCaP human prostate and MCF-7 human breast carcinoma cell lines. DU145 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose and 3.7 g/l sodium bicarbonate supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate and 10% fetal calf serum. For LNCaP cells the medium consisted of RPMI 1640 supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate, non-essential amino acids and 10% heat-inactivated fetal calf serum. Furthermore, the antiproliferative effects of adriamycin, mitomycin C and etoposide were simultaneously evaluated in DU145 and MCF-7 cells. Cells were exposed to the drugs for 5 days with the medium containing drugs or vehicle being renewed on day 3. After 5 days, the amount of metabolically active cells was

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Table 1. IC_{50}-values (M) for inhibition of growth of MCF-7, DU145 and LNCaP cells after 5 days' exposure to different drugs

	MCF-7	DU145	LNCaP
Adriamycin	2.2×10^{-8}	2.3×10^{-9}	NP
Mitomycin C	4.3×10^{-8}	4.0×10^{-9}	NP
Etoposide	1.0×10^{-6}	1.9×10^{-7}	NP
Liarozole	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$

Each value represents the mean of three independent experiments
NP: not performed

measured using a tetrazolium based assay [6]. Table 1 shows that liarozole, unlike the classical cytostatics, marginally affected cell growth (inhibition of 10, 11 and 19% for respectively DU145, LNCaP and MCF-7 cells at a concentration of 10^{-5} M of liarozole).

Effect of liarozole on the metabolism of labeled testosterone in rat prostate

The effects of liarozole on the metabolism of testosterone in prostate tissue were investigated in short-term incubations of collagenase-dispersed rat prostatic cells incubated with 4.5×10^{-7} M of [^{14}C]testosterone, 4.5×10^{-4} M of NADPH with or without drug. After 4 h of incubation, 1.5 ml of culture medium was loaded on a silicagel column (Extrelut, Merck), extracted with ether, dried under nitrogen and dissolved in 600 μl of hexane–isopropanol (9:1, v/v). Separation of the steroids was carried out on a Varian 5560 high performance liquid chromatograph equiped with a Lichrosorb Diol column. Steroids were eluted with hexane–isopropanol in a stepwise linear gradient. Radioactivity was measured on-line by a Berthold LB-504 monitor.

Table 2 shows that liarozole up to 10^{-5} M does not modify the metabolism of labeled testosterone in primary culture of rat prostatic cells, i.e. mainly does not alter 5α-reductase activity.

Effect of liarozole on induction of plasminogen activator in F9 mouse teratocarcinoma cells

In the F9 cell line, originally initiated from a mouse testicular teratocarcinoma, retinoic acid induces differentiation which is reflected in morphological changes as well as the induction of several enzymes such as plasminogen activator [7]. The cells were cultured in gelatin-coated 6 well tissue culture plates at a density of 1000 cells/well in 2 ml of Dulbecco's modified Eagle's medium with 4.5 g/l glucose supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate and 15% fetal calf serum. After 24 h the medium was renewed, the test compounds were added in a volume of 200 μl and the cells were further incubated for 72 h. Then, the medium was discarded and replaced by 1 ml of serum-free medium and the cells were further incubated for 24 h. Plasminogen activator content of the supernatant was determined quantitatively with a spectrophotometric assay using human plasminogen activator as reference standard [8].

Up to a concentration of 10^{-5} M, liarozole did not induce enzyme activity ($\leqslant$100 mU/ml) whereas retinoic acid dose-dependently induced secretion of plasminogen activator between 10^{-9} and 10^{-5} M (up to 20,000 mU/ml; Fig. 1). However, a combination of 10^{-5} M of liarozole with 10^{-8} M of retinoic acid induced plasminogen activator 2.2-fold more than retinoic acid alone (Fig. 1). These results clearly show that liarozole has no intrinsic "retinoid-like" effect but enhances the retinoic acid induction of plasminogen activator secretion, probably by inhibiting retinoic acid metabolism as demonstrated in this cell line for other azole derivatives [9].

IN VIVO PHARMACOLOGY

Effects of liarozole on androgen-independent Dunning MatLu prostate adenocarcinoma

Since the antitumoral effects of liarozole in the androgen-dependent Dunning-G prostate adenocarcinoma were not related to androgen suppression [1], the compound was evaluated in the rapidly growing, androgen-independent MatLu subline of the Dunning R 3327 prostatic adenocarcinoma. In this experiment, 2×10^5

Table 2. Effects of liarozole on labeled testosterone metabolism in primary culture of dispersed normal rat prostate cells

		Liarozole (M)			
	Vehicle	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Testosterone	69 ± 5	69 ± 5	61 ± 8	67 ± 12	76 ± 2
Dihydrotestosterone	19 ± 4	21 ± 4	22 ± 5	24 ± 9	17 ± 1
Androstenedione	4.5 ± 0.7	4.2 ± 0.7	4.2 ± 0.9	2.9 ± 1.4	2.9 ± 0.6
Androstanedione	1.9 ± 0.8	1.5 ± 0.7	1.3 ± 0.8	1.5 ± 1.0	0.2 ± 0.1
Androsterone	3.6 ± 1.2	2.9 ± 1.0	3.1 ± 1.5	2.3 ± 0.4	2.6 ± 0.4

The results are expressed as per cent of total radioactivity (mean ± SEM, $n = 5$)

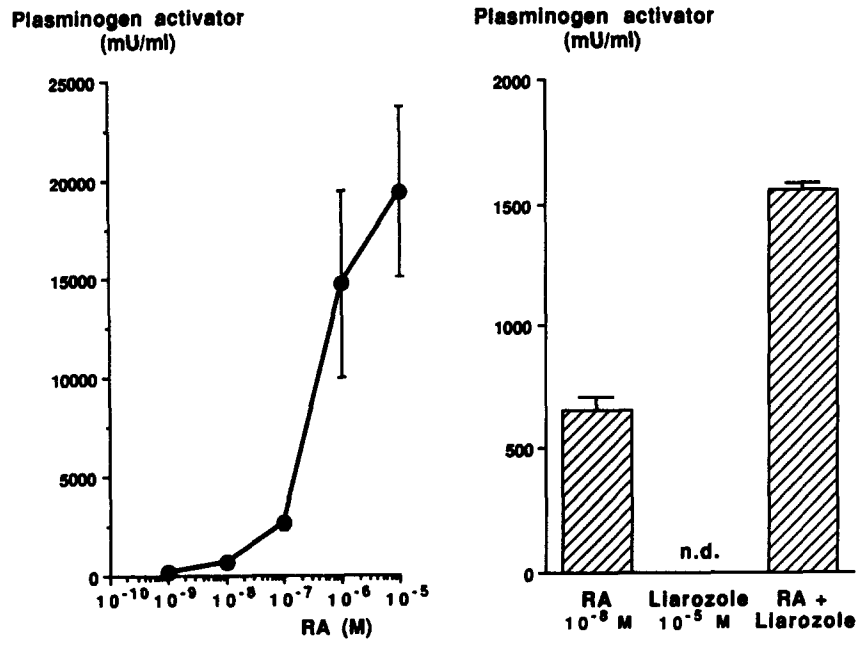

Fig. 1. Effects of retinoic acid (RA) and liarozole on plasminogen activator induction in F9 cells. Results are mean ± SD of 4 independent experiments, each performed in triplicate. n.d.: not detectable (≤ 100 mU/ml)

cells were injected s.c. in the subinguinal region. The next day treatment was initiated or orchiectomy performed in the appropriate groups of at least 8 animals each. Liarozole was administered admixed in food and tumor volume was measured biweekly [1]. On day 28, the experiment was stopped because of ulcerating tumors in the castrate group.

Liarozole at doses of 10 and 5 mg/100 g food lowered the median tumor volume of the subcutaneous tumors by 72 and 79%, respectively, whereas castration had no effect (median volume change: +14%; Fig. 2).

These results show that liarozole also reduces the growth of androgen-independent prostatic adenocarcinoma in rats.

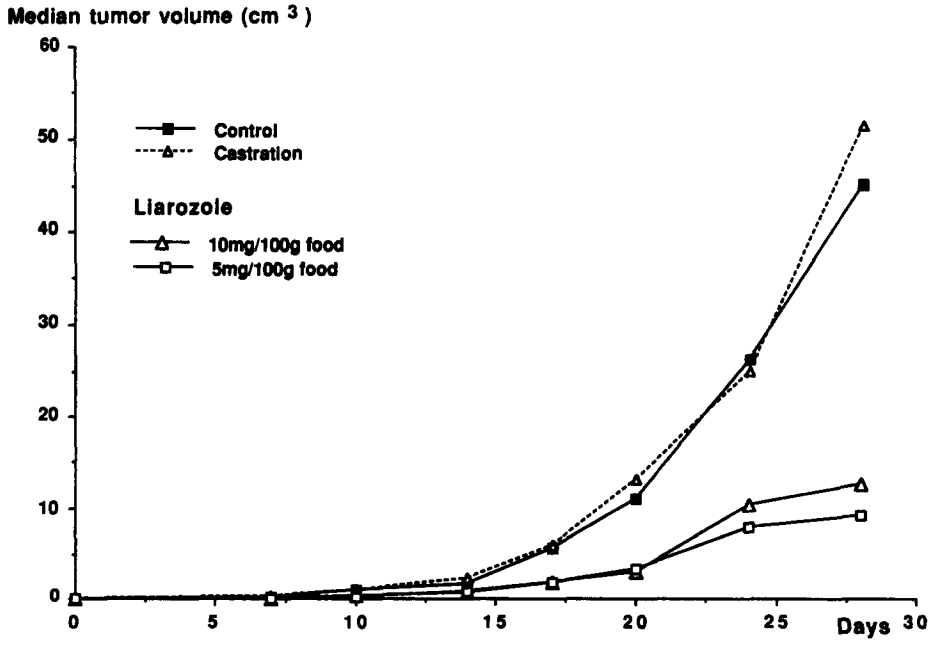

Fig. 2. Effects of dietary application of liarozole on the growth of R 3327 MatLu prostate adenocarcinoma in rats.

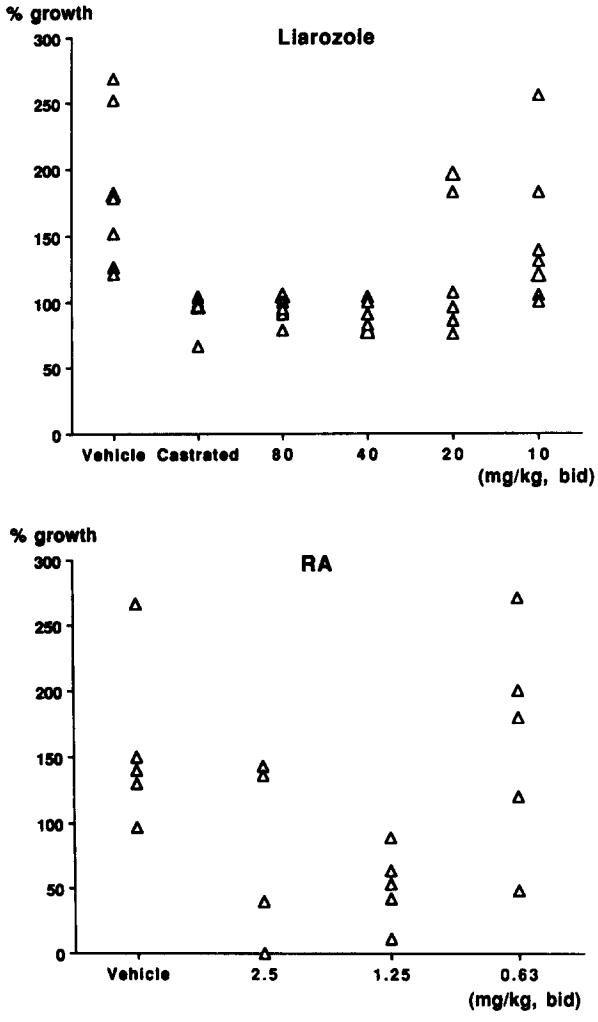

Fig. 3. Effects of a 21-day oral treatment with liarozole or retinoic acid (RA) on the growth of Dunning R3227-G rat prostate adenocarcinoma fragments implanted under the renal capsule of nude mice. Individual values are represented. Each group contains at least 5 animals

Effects of liarozole and retinoic acid on the growth of androgen-dependent Dunning-G prostatic adenocarcinoma in nude mice

To evaluate the effects of retinoic acid on Dunning-G adenocarcinoma, fragments of a s.c. grown tumor were inserted under the renal capsule of nude mice [1]. After 21 days of treatment, the growth of the grafts was expressed as percentage of the size of fragments at the day of insertion, which was considered to be 100%. Castration, retinoic acid (at 1.25 and 2.5 mg/kg p.o., bid) and liarozole (at 80 and 40 mg/kg p.o., bid) similarly reduced tumor growth (Fig. 3). These results confirm the effects of liarozole on the androgen-dependent Dunning-G prostate adenocarcinoma and demonstrate its sensitivity to retinoic acid.

Effects of liarozole and retinoic acid on tumor promotion elicited by 2-O-tetradecanoylphorbol-13-acetate (TPA) in mouse skin

Retinoids suppress the formation of papilloma in the initiation–promotion model by interfering with the promotion phase of mouse skin carcinogenesis [10–12]. Female CD-1 mice were initiated with a single topical dose of 50 μg of dimethylbenz(*a*)anthracene (DMBA) [13]. After 2 weeks, promotion with twice weekly applications of 5 μg TPA applied topically in 150 μl acetone was begun and continued for 17 weeks.

Treatment of mice with 5 μg retinoic acid topically 3 h prior to application of tumor promoter during 17 weeks of promotion, reduced both the incidence of tumors and tumor burdens (i.e. number of papillomas per mouse). Similarly, pretreatment of mice with 20, 40 or 80 mg/kg (p.o.) liarozole produced a reduction in tumor incidence and tumor burden (Table 3).

CONCLUSION

Data obtained to date show that liarozole altered tumor growth on androgen-dependent R 3327 Dunning-G prostate adenocarcinoma independently of androgen suppression [1], but without interfering with steroid receptors [5] or prostatic testosterone metabolism. In contrast to classical cytostatics, liarozole was also devoid

Table 3 Inhibition of TPA induced tumor promotion in mouse skin by liarozole and retinoic acid after 17 weeks of promotion

Initiation	Promotion	Treatment	*N*	Papillomas/ mouse	Incidence (%)
DMBA	Acetone	None	10	0	0
None	TPA	None	10	0	0
DMBA	TPA	None	14	6 6 ± 1 2	79
DMBA	TPA	Acetone	19	9 5 ± 1.5	89
DMBA	TPA	5 μg RA	19	2.9 ± 1 5	42
DMBA	TPA	Vehicle	20	8 0 ± 1 6	90
DMBA	TPA	Liarozole			
		20 mg/kg	15	5 4 ± 1 5	60
		40 mg/kg	19	2 4 ± 1 2	26
		80 mg/kg	18	1 3 ± 0 6	44

of any antimitotic activity and it does not show any "retinoid-like" properties. Furthermore, liarozole altered the growth of androgen-independent Dunning MatLu prostate adenocarcinoma. The finding that liarozole inhibited the 4-hydroxylase, a *P*-450-dependent enzyme involved in catabolism of retinoic acid [4], may shed more light on the mode of action of this drug. Indeed, liarozole inhibited growth of Dunning-G adenocarcinoma and suppressed phorbol ester induced tumor promotion model in mouse skin. These effects were qualitatively similar to the effects of retinoic acid. Furthermore, liarozole enhanced the effects of retinoic acid in F9 teratocarcinoma cells, an effect shared by other related azoles which inhibit the breakdown of retinoic acid [9]. Taken together, these data have raised the hypothesis that inhibition of retinoic acid degradation may play a role in the antitumoral effects of liarozole.

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