Liarozole and 13-cis-Retinoic Acid Anti-Prostatic Tumor Activity

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ABSTRACT

Liarozole fumarate (R85,246), a novel benzimidazole derivative, reduced s.c. and bone metastasis tumor growth by the androgen-independent PC-3ML-B2 human prostatic carcinoma clone in SCID mice. The drug inhibited cell invasion of Matrigel in Boyden chamber chemotactic assays and the secretion of type IV collagenase. In vivo, liarozole failed to inhibit cell proliferation and cell attachment to various substrates (Matrigel, laminin, type IV collagen, and fibronectin). In vivo, the drug also blocked type IV collagenase production in established s.c. tumors. Liarozole has been postulated by others (R. De Coster, W. Wouters, R. Van Ginckel, D. End, et al. J. Steroid Biochem. Mol. Biol., 43: 197-201, 1992) to inhibit retinoic acid catabolism. Our data indicate that liarozole treatment can increase the tumor retinoic acid levels in vivo. Studies of retinoic acid revealed that the drug independently reduced tumor growth in vivo and inhibited cell invasion of Matrigel and the secretion of collagenase IV. Surprisingly, liarozole and retinoic acid failed to exhibit measurable synergistic activity both in vitro and in vivo. Taken together these data suggest that liarozole might inhibit retinoic acid catabolism in vivo and consequently have significant therapeutic value as an anti-prostatic tumor agent.

INTRODUCTION

Liarozole fumarate (i.e., R85,246 or 5-[(3-chlorophenyl)(1H-imidazole-1-yl)methyl]-1H-benzimidazole fumarate) is an imidazole derivative and anti-cancer drug currently in clinical trials for the treatment of advanced prostate cancer. Animal studies revealed that liarozole decreased the growth of androgen-dependent rat Dunning G adenocarcinoma (1) and the androgen-independent rat Dunning MatLu prostate carcinoma (1). Similarly, in studies of orthotocmized stage D prostate cancer patients with hormone-resistant tumors, liarozole (300 mg twice a day) reduced the tumor size in primary tumors and in lymph node metastases (1-3). Increased levels of plasma retinoic acid plus cutaneous problems related to vitamin A were reported (3). The drug also reduced prostatic specific antigen levels but did not alter androgen levels in these patients (2,3). Overall, patients benefited from reduced pain, reduced urological complaints, and generally improved health.

Unfortunately, due to the limited number of studies, the mechanism of action of liarozole is incompletely understood. In vivo data have suggested that its antitumoral properties may arise from an inhibition of retinoic acid degradation. The drug, like other azole derivatives, inhibited several cytochrome P-450-dependent enzymes, including 4-hydroxylase, a key enzyme in retinoic acid metabolism (4, 5). However, in cultured mouse F9 teratocarcinoma cells, the drug failed to demonstrate retinoid-like properties (1). In fact, liarozole (10^-5 m) in combination with retinoic acid (10^-8 M) stimulated plasminogen activator secretion (1). Other experiments have revealed that liarozole did not exert cytostatic effects which inhibited proliferation of breast MCF-7 and human prostate DU 145 and LNCaP carcinoma cell lines in vitro (1). Clearly, the long-term effects of liarozole in vivo may be substantially different from that recorded in vitro with cultured cells.

We have examined the activity of liarozole and retinoic acid on human prostatic tumor cells in vitro and in vivo. Utilizing a bone-metastasizing PC-3 subclone (6), termed PC-3 ML-B2, we have investigated drug effects (a) on processes important for invasion in vitro (i.e., adherence, chemotactic response, collagenase IV secretion); and (b) on tumor growth and survival s.c. and in bone marrow metastases utilizing a SCID mouse model developed in our laboratory (6). Taken together, the data demonstrated that liarozole is an effective antitu morogenic agent for the treatment of prostate tumor growth and for prevention of tumor metastasis.

MATERIALS AND METHODS

For cell culture PC-3 ML-B2 cells, a highly invasive, bone-metastasizing clone, were derived from a PC-3 ML clone which metastasizes to the bone marrow in SCID mice (6). Basically we utilized two additional cycles of selection according to the original methods used to isolate the PC-3ML clones (6). The 3 X 1.7 PC-3 clone were selected using Boyden chambers as described previously (6). Cells were consistently kept below 10 passages and grown to about 80% confluence before using in experiments or harvesting the conditioned medium. The conditioned medium of PC-3 ML-B2 cells was prepared in serum-free medium, and protein levels were measured with a kit from Bio-Rad, Inc. (New Brunswick, NJ, and used at 10 mg/ml to induce invasion and/or protease secretion according to methods previously described (6, 7). The PC-3 ML-B2 clone has recently been serologically screened and found negative for 16 different viruses and for Mycoplasma infection (Quality Bio-Tech, Inc., Camden, NJ). Cell viability was greater than 95% as assessed by trypan blue exclusion assays.

For light microscopy, the Boyden chamber membranes were fixed and stained with Diff-Quick solutions (American Scientific, New Brunswick, NJ). Cells originally plated on the top of the membrane were removed with swipes of a cotton swab stick. Invasive cells on the bottom side of the membranes were photographed with a 25X lens using a Zeiss photomicroscope.

Liarozole was obtained from Janssen Research Foundation (Spring House, PA) and used according to the published protocols (1). All-trans-retinoic acid was from Sigma (St. Louis, MO). In brief, liarozole and retinoic acid were solubilized in distilled water and 100% DMSO, respectively. The retinoic acid stock solution was diluted in Dulbecco’s modified Eagle’s medium to 0.1% DMSO levels for experimental treatment of the cells. Control experiments were carried out with medium containing 0.1% DMSO.

For in vivo studies, the tumor cells were collected using trypsin-EDTA, washed with Dulbecco’s modified Eagle’s medium 3 times, diluted to 2 X 10^9/ml, and immediately injected s.c. (0.5 ml at 2 sites/mouse) or i.v. via the tail vein (0.2 ml/mouse). Mice were left for 5 or 10 days to establish small tumors (6) and then treated with drug at 40 mg/kg/day liarozole and 0.75 mg/kg/day retinoic acid for 21 days prior to sacrifice and excision of the tumor tissue. Aliquots of each drug were administered daily by gavage (~0.3-ml volume). Tumor volume was measured with calipers.

In some collagenase IV studies, 2-3-g tumors were allowed to grow to 10-20 mm and then treated with drug at 40 mg/kg/day liarozole and 0.75 mg/kg/day retinoic acid for 21 days prior to sacrifice and excision of the tumor tissue. Aliquots of each drug were administered daily by gavage (3-5 ml). Tumor volume was measured with calipers.

Received 3/1/93; accepted 4/27/93.

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assays. Routine HPLC assays were carried out on these extracts to measure the levels of retinoic acid in tumors. Purified retinoic acid was used as a standard to assess the amounts of retinoic acid in tumor extracts.

RESULTS AND DISCUSSION

Cell Adherence to Substrate

We examined the influence of liarozole and retinoic acids on PC-3 ML-B2 cell attachment to Matrigel (Fig. 1A), laminin (Fig. 1B), type IV collagen (Fig. 1C), and fibronectin (Fig. 1D) for increased periods of 0 to 8 h. Retinoic acid alone or in combination with liarozole significantly reduced the percent of cell attachment over 8 h (i.e., by 75%) in comparison to that observed in the absence of drug where 99.8% of the cells attached. In comparison, liarozole alone had little or no demonstrable effect on the degree of cell attachment. Similar results were obtained for all four substrates tested (Fig. 1) and for plastic (data not shown), albeit total cell adherence to Matrigel was relatively more efficient than for the individual constituents. For example, adherence to fibronectin (Fig. 1D) was uniformly lower than to laminin or type IV collagen. Correlative measurements of the percent of nonattached cells (Fig. 1) and attached cells (data not shown) were in close agreement. The substrate adherence assays clearly showed that liarozole did not significantly alter cell attachment.

In agreement with our work, others have shown that retinoic acid decreased the adherence of B16-F1 melanoma cells to extracellular matrix components, including laminin, entactin, collagen IV, and collagen I (9). Hendrix et al. (10) have also shown that retinoic acid-treated human melanoma cell lines exhibited a 10-40% decrease in adherence to extracellular matrix components, including laminin, entactin, collagen IV, and collagen I (9). Hendrix et al. (10) have also shown that retinoic acid increased cell surface laminin receptors and decreased a gp78 motility factor receptor. Since the cells in our studies were preexposed to the drug for 5 days to ensure optimal exposure and response, it is possible that cell surface integrins or receptor composition were also altered in the PC-3ML-B2 clones. However, because retinoic acid had a similar dosage-dependent effect for each substrate tested, it is unlikely that such changes in specific receptor number and affinity would account for the overall drug effect.

Boyden Chamber Chemotactic Invasion Assays

The PC-3 ML-B2 clone was, in part, selected for an ability to migrate across Matrigel barriers in response to chemotactic factors in the conditioned medium (6). We have measured the inhibitory activity of liarozole and retinoic acid on cellular invasion in response to the conditioned medium of the PC-3ML-B2 clones (Table 1). The data showed that both drugs partially blocked invasion in a dosage-dependent fashion. For example, liarozole, retinoic acid, and retinoic acid plus liarozole together reduced the extent of invasion by about 57, 58, and 55%, respectively, at 20 μM levels. By comparison, taxol, a potent inhibitor of invasion (11), totally blocked cell penetration of Matrigel at about 1.0 μM levels. Recovery from liarozole and retinoic acid was observed if the cells were washed and allowed to recover for 24 h in fresh medium. The extent of invasion by the recovered cells was comparable to untreated PC-3 ML-B2 cells (~8.5 ± 0.8% (SD) of the total cells plated).

The antimitogenic effects of ketoconazole have been shown to be mitigated by serum (12). One possibility is that there is binding of the
drug by serum factors. The action of liarozole on invasion (and collagenase IV secretion; see Table 2) was observed in the absence or presence of 10% fetal calf serum in the culture medium, indicating that the drug probably has inhibitory activity independent of growth factor receptor cascade mechanisms. In the presence of serum, a greater concentration of drug (i.e., 20 μM versus 10 μM) was required to obtain maximum inhibition in the same time frame, however (data not shown). Note that in the studies reported here the conditioned medium of PC-3 ML-B² cells was serum free and did not contain factors which bind or were competitive with liarozole or retinoic acid.

Qualitative light microscopic studies of fixed and Diff-Quick-stained membranes supported the radio-label cell invasion studies (data not shown). At low drug levels (i.e., 0.1 μM liarozole plus 0.1 μM retinoic acid) large numbers of the PC-3 ML-B² cells penetrated the Matrigel barrier and migrated to the bottom side of the membrane filters. In the presence of higher levels (1 to 20 μM) of liarozole plus retinoic acid, the number of cells which crossed the Matrigel barrier were increasingly reduced as reported in the quantitative studies in Table 1.

The mechanism of inhibition by liarozole and/or retinoic acid is unclear, especially given the multitude of events involved in cell migration through the reconstituted basement membrane material (i.e., cell detachment, motility, and protease release). Recently, retinoic acid has been shown to inhibit human melanoma tumor cells in their ability to penetrate Matrigel-coated membrane filters (i.e., in a dose and time dependent manner over 72 h) (10). Retinoic acid failed to alter melanoma cell attachment kinetics significantly but did stimulate increased laminin secretory expression. No correlation between these events and invasion was observed, however. Three possible targets were identified which might account for the anti-invasive activity of the drug, including drug blockage of expression of (a) a motility factor receptor (i.e., gp78); (b) collagenase IV; and (c) plasminogen activator (10, 11).

**Drug Effects on Type IV Collagenase**

**In Vitro.** ELISA measurements with polyclonal antibodies specific for the prozymogen Mr 72,000 type IV collagenase (7, 8) showed that liarozole (20 μM for 6 h) partially reduced the total amounts of collagenase IV secreted by (a) the parent PC-3 cells (i.e., by 14%); (b) a 3 X 1. PC-3 clone (i.e., by 27%); and (c) the PC-3 ML-B² clone (i.e., by 68%) in culture (Table 2). Retinoic acid (i.e., 10 μM for 6 h) also reduced the detectable levels of collagenase IV secretion in the PC-3 parent cells (i.e., by 53%), the 3 X 1 PC-3 clone (i.e., by 85%), and the PC-3 ML-B² clone (i.e., by 85%). Liarozole and retinoic acid together had no detectable synergistic influence on the collagenase IV levels secreted. Note that the inhibitory effects were more noticeable in the PC-3ML-B² clones presumably because they secrete greater amounts of collagenase than the parent PC-3 cells.

In agreement with our data, Hendrix et al. (10, 12) reported that retinoic acid inhibited the production of type IV collagenolytic and plasminogen activator by human melanoma tumor lines after treatment with 10 μM retinoic acid for 72 h. Further, they found that retinoic acid inhibited mRNA expression by the type IV collagenase gene (10, 12).

**In Vivo.** ELISA analysis of whole cell extracts of tumors grown in SCID mice revealed that liarozole and liarozole plus retinoic acid had a similar activity in vivo on s.c. tumors grown from the parent PC-3 cells, the 3 x 1. clone, and the PC-3 ML-B² clone (Table 2). Liarozole had a much less significant inhibitory effect than retinoic acid (Tables 2 and 3). For example, in PC-3 ML-B² tumors the collagenase IV levels reduced by 64% with liarozole treatment (40 mg/kg/day for 72 h) and by 91% with retinoic acid treatment (0.75 mg/kg for 72 h). Again, liarozole and retinoic acid together had no significant additive influence on collagenase IV production in the s.c. tumors. Interestingly, liarozole had a more dramatic effect on collagenase IV levels in bone metastasis (as opposed to s.c. tumors) and liarozole plus retinoic acid completely depleted the collagenase to undetectable levels (Table 3). Thus, these studies indicate that the drugs have synergistic effects on bone tumors.

We measured the retinoic acid levels by HPLC in the tumors treated with liarozole alone in Table 3. V. Average levels of 2.3 ± 0.2 and 1.8 ± 0.3 μM/mg protein were found for the s.c. and bone metastasis, respectively, in Table 3. Similarly, levels of 2.4 ± 0.3 μM/mg protein were measured in the s.c. tumors of liarozole-treated mice in Table 4. Since the retinoic acid levels detected in control tumors of untreated mice were < 0.001 μM/mg protein, the data suggest that liarozole...
might induce increased levels of retinoic acid in tumors in vivo as its primary mechanism of action.

**Drug Effects on Tumor Growth in SCID Mice**

Cell proliferation and survival studies have revealed that neither liarozole (0 to 10 μM) nor retinoic acid (0 to 10 μM) influenced PC-3 ML-B² cell proliferation or survival in vitro (13). In comparison, the growth kinetics of mammary tumor cells (13) and human melanoma lines (10) were inhibited by retinoic acid in vitro. We have tested the effects of both drugs alone and in combination on the s.c. growth of PC-3 ML-B² cells in SCID mice. Surprisingly, liarozole and retinoic acid independently reduced tumor burden substantially (i.e., to less than 3 mm³) in comparison to controls (≈3000 mm³). Likewise, the two drugs together reduced the total tumor burden further in a total of 20 mice tested (i.e., to <0.3 mm³) by at least an order of magnitude (Table 4). HPLC measurements of the retinoic acid levels in the residual tumors of 13 of the mice again revealed levels of 5 ± 1 μM/mg protein. Untreated tumors had levels of 0.001 μM/mg protein. Also, the tumors in the 2 mice which failed to respond to treatment (see Fig. 1 legend and Table 4) had retinoic acid levels of 0.001 μM/mg protein, indicating that (i.e., inexplicable) poor drug uptake in these tumors accounted for the total lack of response to treatment.

The studies were extended to assess drug activity on PC-3 ML-B² metastatic bone tumors in SCID mice. Control experiments demonstrated that the PC-3 ML-B² clones colonized the lumbar vertebrae marrow by 5 days and formed large rapidly growing tumors (i.e., in 8 of 10 mice) after about 21 days following i.v. injection of 2 × 10⁶ cells/ml via the tail vein. In drug studies, the tumors were allowed to establish for 5 and 10 days prior to drug treatment for 21 days. Liarozole treatment eradicated any grossly detectable tumors in the bone marrow of the lumbar vertebrae. However, small tumors (≈1 mm in diameter) were detected by light microscopic analysis of H&E stained tissues in the lumbar bone marrow in 2 of 49 and 2 of 10 drug-treated mice, respectively. In comparison, retinoic acid (0.75 mg/kg/day) reduced the incidence of lumbar tumors by about 40 and 20% in mice inoculated for 5 and 10 days, respectively. Liarozole and retinoic acid in combination reduced the tumor incidence by about 90% in both cases in a total of 49 mice tested (Table 4). In sum, retinoic acid was partially effective, but liarozole, alone or in combination with retinoic acid, was a very effective inhibitor of tumor growth and survival. HPLC measurements of the retinoic acid levels in the residual bone tumors of 2 mice exposed to liarozole and retinoic acid for 21 days (Table 4B) revealed levels of 1.1 and 8.0 μM/mg protein.

Liarozole has been shown to block phorbol ester-induced skin tumors in mice (1). DeCoster et al. (1) have suggested that liarozole may work to prevent retinoid catabolism in vivo, raising the available retinoic acid levels. Retinoids and agents that alter the metabolism of retinoids might therefore be of therapeutic value in mediating the course of advanced prostate cancer metastases which are resistant to endocrine ablative therapy (e.g., as was the cancer from which the PC-3 cells were derived).

Retinoids have been found to suppress the growth of carcinoen-induced tumors, including skin papillomas in mice (14, 15). Likewise, fenretinide, a derivative of retinoic acid, inhibited the process of carcinogenesis (16) and decreased tumor growth (17) in a rat carcinogenesis model of prostate cancer. Fenretinide (2.5 to 25 μM levels was cytotoxic to rat MLL cells, human PC-3 prostate cancer cells, and the CPAE endothelial cell line. All trans-retinoic acid and 13-cis-retinoic acid were less cytotoxic by at least an order of magnitude (17). Because fenretinide inhibited endothelial cell migration and angiogenesis (at 25 to 250 nM), the prevention of neoangiogenesis was believed to be a critical function of the drug during tumor growth.

Mechanistically, the activity of retinoic acid is poorly understood (18). For example, it binds to cellular retinoic acid-binding protein and the complex moves to the nuclear membrane but does not seem to penetrate the nucleus directly (19). Retinoid-inducible genetic activity has been observed in mouse F9 teratocarcinoma cells (20), but again the mechanism of action is unclear. Sporn et al. (21) have also demonstrated that retinoic acid mediates growth factor levels (i.e., including transforming growth factor) in a variety of cells.

Therapeutically, 13-cis-retinoic acid has been used both as a chemopreventative and chemotherapeutic agent. For example, doses between 50 and 100 mg/m² have been successful in the treatment of premalignant oral leukoplakia (22), head and neck cancers (23), squamous cell carcinoma (24), cutaneous T-cell malignancies (25), acute promyelocytic leukemia (26), and myelodysplastic syndrome (27).

The overall failure of hormonal therapy in improving patient survival has stimulated a continued search for new preventative strategies. Intervention in the early stages of prostate cancer metastases (i.e., to the lymph nodes) with nontoxic chemotherapeutic agents is highly desirable. Liarozole treatment to prevent prostate cancer growth and spreading at early, nonpalpable stages is therefore worthwhile. In this study, mice (≈35 g) given daily dosages of 40 mg/kg showed no toxic response over the entire 21-day treatment, and over longer periods of 3 months (data not shown). Only with twice daily treatments at 40 mg/kg did mice exhibit mucocutaneous dryness, hair loss, weakness, and occasionally death. If liarozole serves to increase tumor retinoid acid levels as a primary mechanism of action (as our data suggest in Table 4) the two drugs in combination (i.e., at nontoxic levels) might effectively serve to block tumor growth and spread in patients. The in vivo data suggest that liarozole can effectively raise endogenous retinoic acid levels in hormone refractory cancer and that this is the principle mechanism of action of the drug.

**ACKNOWLEDGMENTS**

We gratefully acknowledge financial support from the Janssen Research Foundation, Springhouse, PA.

**REFERENCES**


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