Chemopreventive effects of the aromatase inhibitor vorozole (R 83842) in the methylnitrosourea-induced mammary cancer model

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The chemopreventive activity of the highly specific non-steroidal aromatase inhibitor, vorozole, was examined in the methylnitrosourea (MNU)-induced rat model of mammary carcinogenesis. Various doses of vorozole (0.08–1.25 mg/kg body wt/day) were administered daily (by gavage) to female Sprague–Dawley rats starting at 43 days of age. Seven days later, the rats were given a single i.v. dose of MNU (50 mg/kg body wt). Rats were continually treated with vorozole until the end of the experiment (120 days post-MNU). Vorozole caused a dose dependent inhibition of mammary cancer multiplicity. The highest dose of vorozole (1.25 mg/kg body wt/day) decreased cancer multiplicity by ~90%, and simultaneously decreased cancer incidence from 100 to 44%. The next two highest doses of vorozole (0.63 and 0.31 mg/kg body wt/day) inhibited MNU-induced mammary cancer multiplicity by 70–80%. Even the two lowest doses of vorozole (0.16 and 0.08 mg/kg body wt/day) decreased cancer multiplicity ~50%. Serum testosterone levels were significantly elevated in rats given vorozole (0.16 and 0.08 mg/kg body wt/day) decreased cancer multiplicity ~50%. Serum testosterone levels were significantly elevated in rats given vorozole (0.16 and 0.08 mg/kg body wt/day). This result presumably reflects the limited half-life of testosterone and androstenedione in post-menopausal women, mediates the conversion of testosterone and androstenedione to estradiol and estrone, respectively. Tests for aromatase activity were performed on a variety of endpoints at either 4 or 24 h following the last dose of vorozole. Insulin-like growth factor (IGF)-1 levels were slightly, but significantly, increased by vorozole treatment. Vorozole induced striking increases in serum testosterone levels at 4 h at all the dose levels employed. Testosterone levels were significantly elevated over controls at 24 h in rats given the lower doses of vorozole (0.08–0.31 mg/kg body wt/day), but were significantly lower than in rats administered the higher doses of vorozole (0.63 or 1.25 mg/kg body wt/day). This result presumably reflects the limited half-life of vorozole in rats. In a second series of experiments, the effects of limited duration of dosing with vorozole (2.5 mg/kg body wt/day) or intermittent dosing with vorozole were determined. Treatment of rats with vorozole for limited time periods, from 3 days post-MNU administration until 30 or 60 days post-MNU treatment, resulted in significant delays in the time to appearance of palpable cancers. However, these limited treatments did not greatly affect the overall incidence or multiplicity of mammary cancers when compared with the MNU controls at the end of the study (150 days post-MNU). Finally, the effects of intermittent dosing with vorozole (2.5 mg/kg body wt/day) were examined. Rats were administered cycles of vorozole daily for a period of 3 weeks followed by treatment with the vorozole vehicle for the next 3 weeks (total of four cycles). Although this intermittent treatment did inhibit the appearance of new tumors during each of the periods that vorozole was administered, it did not cause regression of palpable cancers.

Introduction

The majority of locally invasive ductal breast cancers in women are estrogen receptor positive (1). Almost 100 years ago it was first observed that many pre-menopausal women with advanced breast cancer were responsive to hormonal therapies (e.g. ovariectomy) (2). The development of anti-estrogens has perhaps been the primary emphasis in the field of hormonal therapy. Thus, the anti-estrogen/estrogen tamoxifen is the most commonly employed adjuvant agent in the treatment of breast cancer (3,4).

Hormonal therapy can also be accomplished by inhibiting the production of estrogens, e.g. inhibition of the cytochrome P450-mediated enzyme aromatase (5). This cytochrome P450 enzyme, which is primarily functional peripherally (i.e. fat, liver, muscle) in post-menopausal women, mediates the conversion of testosterone and androstenedione to estradiol and estrone, respectively. One method for inhibiting aromatase activity is the use of highly specific competitive inhibitors. This approach, although difficult because of the relatively high affinity (low K_m) of aromatase for the endogenous substrates testosterone and androstenedione, has been employed clinically with aminoglutethimide (6,7). However, aminoglutethimide has numerous side effects including neural toxicity as well as inhibition of other cytochromes P-450 involved in adrenal steroid synthesis (8). More recently, a number of highly potent and more specific inhibitors have been developed, including vorozole (5,9,10). Vorozole, a triazole analog, is a highly potent and specific non-steroidal inhibitor of aromatase both in humans and in rats in vitro and in vivo. In contrast with aminoglutethimide, vorozole at effective doses does not inhibit other cytochrome P450 isozymes (10), including those cytochromes involved in steroid metabolism in the adrenal glands. Vorozole was effective in the therapy of primary dimethylbenzanthracene (DMBA*)-induced tumors in rats as well as blocking the formation of new secondary tumors (11). Most recently, vorozole has proven to be effective in a subset of women who failed on tamoxifen therapy (12).

We have previously demonstrated that relatively high doses of vorozole (2.5 or 5.0 mg/kg body wt/day) are extremely effective when administered as a chemopreventive agent in the methylnitrosourea (MNU)-induced mammary cancer model (13). In the present study, we examined the effects of vorozole on the development of MNU-initiated mammary cancers to answer the following questions: (i) are relatively low doses of vorozole effective in a chemopreventive setting?; (ii) what relationship exists between vorozole-induced alterations of hormone levels and the preventive efficacy of vorozole in this model?
model?; (iii) is limited exposure to relatively high doses of vorozole effective in a chemopreventive setting?; and (iv) is intermittent treatment with vorozole an effective chemopreventive regimen?

**Materials and methods**

**Chemicals and animals**

Vorozole was supplied by the Janssen Research Foundation (Spring House, PA). MNU was obtained from Ash Stevens (Detroit, MI). Female Sprague–Dawley rats were obtained from Harlan Sprague–Dawley (Indianapolis, IN; virus-free colony number 202). The rats were arrived at 34 days of age and were placed immediately on Teklad (4%) diet. Rats were housed five per cage in a room maintained at 22 ± 2°C and artificially-lighted for 12 h/day. Two animals from each shipment were randomly selected for complete health status evaluation. The rats exhibited no major lesions and were pathogen-free. Animals were allowed free access to the basal diet and drinking water throughout the duration of the experiment.

**Experiment I: Treatment with various doses of vorozole**

Rats were treated by gavage with the various levels of vorozole (0.08, 0.16, 0.31, 0.63 or 1.25 mg/kg body wt/day), 7 days per week. Control animals were treated with the vehicle (4% ethanol, 40% polyethylene glycol-400, 56% saline) daily for the duration of the experiment. Vorozole was initially prepared at a stock solution of 20 mg/ml in vehicle. Lower doses were obtained by dilution in excess vehicle. Vorozole solutions used during weeks 1 and 9 of the studies were analyzed for vorozole and found to contain the appropriate dose levels. Vorozole was analyzed by HPLC (LDC Constandem II pumps with Gradient Master, Waters 490E Programmable Multi-wavelength UV-Vis detector and Waters 745 Data Module Integrator) and was resolved employing a Spherisorb ODS-2 3 micron column (150×4.6 mm). For vorozole (λ 265 nm), the mobile phase was a linear gradient (A: 0.25% aqueous ammonium acetate, B: acetonitrile, C: tetrahydrofuran) from a ratio of 80:10:10 to a final ratio of 60:15:25 in 10 min using a high-pressure mixing method. Employing a flow rate of 0.75 ml/min, the retention time of vorozole was 14.1 min. The amount of vorozole was determined by external standardization.

Rats were administered the indicated doses of vorozole beginning at age 43 days. One week later (50 days of age), rats were injected i.v. with MNU as previously described (14). Rats were weighed weekly, palpated for mammary tumors twice per week, and checked daily for signs of toxicity. Estrus cycles of rats receiving only the chemopreventive agent alone (no MNU) or vehicle alone (no MNU) were completely necropsied. Chemopreventive agents were administered vorozole (2.5 mg/kg body wt/day) intermittently beginning 3 days after MNU. That is, rats were administered vorozole by gavage for a period of 3 weeks beginning at the age of 53 days, and then were administered the vorozole vehicle for the subsequent 3 weeks. This cycling process was continued for the duration of the experiment. For both the limited exposure and intermittent exposure, rats were weighed weekly, palpated for mammary cancers twice per week, and checked daily for signs of toxicity. The studies were terminated 150 days following MNU administration. Mammary tumors were removed and examined histopathologically employing previously published criteria (15).

**Results**

**Experiment I: Body weights (Table I)**

Vorozole caused a dose-dependent increase in body weight gain at the doses employed. Although the gains in final body weight were quite striking (20%) in the rats treated with the higher doses of vorozole alone (0.63 and 1.25 mg/kg body wt/day), there were more limited effects (7% increase) observed in rats treated with the lower doses of vorozole (0.08 and 0.16 mg/kg body wt/day).

**Estrus cycles**

The highest dose of vorozole (1.25 mg/kg body wt/day) inhibited the normal estrus cycle in all rats. However, a lower dose of vorozole (0.31 mg/kg body wt/day) altered the estrus cycle in less than half of the rats. At the doses employed in these studies, vorozole did not cause gross pathological manifestations (based on necropsy) but did cause any overt adverse pharmacological effects.

**Chemopreventive effects (Table I)**

Vorozole caused a dose-dependent decrease in cancer incidence. The three highest doses of vorozole (1.25, 0.63 and 0.31 mg/kg body wt/day) decreased cancer incidence from 100 to ~50%. In contrast, the lower doses of vorozole (0.16 and 0.08 mg/kg body wt/day) did not greatly decrease final cancer incidence. However, even these relatively low doses of vorozole significantly increased tumor latency (Figure 1). Vorozole caused a profound dose-dependent decrease in the multiplicity of rat mammary cancers. Mean cancer multiplicity was 4.5, 0.6, 0.7, 1.3, 2.4 and 2.0 for rats treated with 0.00, 1.25, 0.63, 0.31, 0.16 and 0.08 mg/kg body wt/day of vorozole, respectively. These results demonstrate that even relatively low doses of vorozole inhibit the growth of mammary cancers in a chemoprevention setting.

**Levels of various hormones and IGF-1**

The effects of vorozole on multiple biomarkers (estradiol, testosterone and IGF-1) were determined. Rats were killed at the end of the chemoprevention study at either 4 or 24 h (Figures 2–4) following the last administration of vorozole. The objective of having both time points was to determine whether extended (24 h) effects of exposure to the various doses of vorozole were observed or whether altered levels of various hormones reflected primarily the transient effects of the last dose of vorozole. Estradiol levels displayed a dose-dependent decrease of up to 80% at 24 h following the last dose of vorozole (Figure 2). However, because of relatively high variance within groups only the results with the two highest doses were statistically significant. At 4 h post-vorozole, all doses strikingly decreased estradiol levels. The effect on testosterone was a strong increase in serum levels at day of vorozole by gavage for either the subsequent 30 or 60 days, or were administered vorozole continuously until the end of the experiment.

**Experiment II: Intermittent exposure to vorozole**

Rats were administered MNU i.v. at 50 days of age. The animals were administered vorozole (2.5 mg/kg body wt/day) intermittently beginning 3 days after MNU. That is, rats were administered vorozole by gavage for a period of 3 weeks beginning at the age of 53 days, and then were administered the vorozole vehicle for the subsequent 3 weeks. This cycling process was continued for the duration of the experiment. For both the limited exposure and intermittent exposure, rats were weighed weekly, palpated for mammary cancers twice per week, and checked daily for signs of toxicity. The studies were terminated 150 days following MNU administration. Mammary tumors were removed and examined histopathologically employing previously published criteria (15).
tamoxifen in a prevention setting. Furthermore, although the still remain, e.g. increased endometrial cancer (19), hepatic cancer in rats (20) and certain estrogen agonist effects (21). Nevertheless, a significant number of the individuals responsive to alternative hormonally based therapies (11,23). In view of these considerations, the continued development of other types of hormonal therapies (e.g. pure anti-estrogens, antiprogestins and aromatase inhibitors) seem justified in both treatment and prevention settings.

As discussed in the Introduction, vorozole is a highly specific non-steroidal inhibitor of aromatase. Previous studies in both humans and animal models have demonstrated that vorozole can be an effective hormonal treatment in a therapeutic setting (11,12). Our previous studies had demonstrated that when administered continually at relatively high doses (5.0 or 2.5 mg/kg body wt/day) vorozole was virtually 100% effective in a large chemoprevention trial. Despite its relatively wide use and low overall toxicity certain potential problems still remain, e.g. increased endometrial cancer (19), hepatic cancer in rats (20) and certain estrogen agonist effects (21). Although this spectrum of problems is likely to be deemed acceptable for a woman who has breast cancer, these are still significant potential problems if one wishes to employ tamoxifen in a prevention setting. Furthermore, although the majority of women with ER+ tumors respond to tamoxifen therapy, most individuals still fail within 5 years on therapy (21,22). Nevertheless, a significant number of the individuals who relapse following tamoxifen therapy still appear to be responsive to alternative hormonally based therapies (11,23).

Table I. Efficacy of vorozole in the prevention of MNU-induced mammary cancers

<table>
<thead>
<tr>
<th>Group</th>
<th>Carcinogena</th>
<th>Treatmentb</th>
<th>Final body wt (g)</th>
<th>Adenocarcinomasc</th>
<th>Percent incidence</th>
<th>Average no./rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MNU</td>
<td>Vorozole, 1.25 mg/kg body wt per day</td>
<td>293±4</td>
<td>4⁴</td>
<td>0.56 ± 0.14⁴</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MNU</td>
<td>Vorozole, 0.63 mg/kg body wt per day</td>
<td>279±4</td>
<td>4⁴</td>
<td>0.68 ± 0.21⁴</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MNU</td>
<td>Vorozole, 0.31 mg/kg body wt per day</td>
<td>270±4</td>
<td>5²</td>
<td>1.28 ± 0.34²</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MNU</td>
<td>Vorozole, 0.16 mg/kg body wt per day</td>
<td>265²</td>
<td>80⁴</td>
<td>2.36 ± 0.40⁴</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MNU</td>
<td>Vorozole, 0.08 mg/kg body wt per day</td>
<td>262²</td>
<td>88</td>
<td>2.00 ± 0.27²</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>MNU</td>
<td>Vehicle</td>
<td>245</td>
<td>100</td>
<td>4.52 ± 0.58</td>
<td></td>
</tr>
</tbody>
</table>

aMNU (50 mg/kg body wt) was administered by i.v. injection to female Sprague–Dawley rats at 50 days of age (25 rats/group).
bBeginning at 43 days of age, vorozole was administered daily by gavage, 7 times a week.
cIncidence and no. of mammary cancers at end of study (120 days after MNU).

Fig. 1. Effects of continual exposure to various doses of vorozole on mammary cancer multiplicity. Rats were administered the indicated doses of vorozole by gavage, 7 times a week, beginning at 43 days of age.

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Fig. 2. Serum levels of estradiol at either 4 or 24 h following the last dose of either vehicle or vorozole. Veh, vehicle; 0.08, 0.16, 0.31, 0.63, 1.25, vorozole dose in mg/kg body wt/day administered by gavage; box plots, median, 25–75th and 10–90th percentiles of estradiol levels; ○, individual values below 10th or above 90th percentiles.

Fig. 3. Serum levels of testosterone at either 4 or 24 h following the last dose of either vehicle or vorozole. Veh, vehicle; 0.08, 0.16, 0.31, 0.63, 1.25, vorozole dose in mg/kg body wt/day administered by gavage; box plots, median, 25–75th and 10–90th percentiles of estradiol levels; ○, individual values below 10th or above 90th percentiles.

as a chemopreventive agent in the MNU-induced rat mammary cancer model (13).

In the present studies, vorozole was administered at a wide variety of doses (0.08–1.25 mg/kg body wt/day) beginning 7 days prior to MNU treatment and was continued for the duration of the study (120 days). The relatively wide dose-range was employed with the expectation that a ‘no effect’ dose would be observed. This expectation was based on prior findings that low doses of vorozole (<0.32 mg/kg body wt/day given twice per day) were relatively ineffective for therapy of palpable tumors induced by treatment of rats with dimethylbenzanthracene (DMBA) (11). The DMBA-induced tumors, similarly to the MNU-induced cancers, are hormonally responsive. We have recently examined the ability of vorozole to act as a therapeutic agent employing palpable MNU-induced mammary cancers (unpublished data) and found a high dose of 2.5 mg/kg body wt/day was highly effective (causing ≥70% decrease in tumor volume of all palpable tumors). In contrast, the lowest dose examined (0.16 mg/kg body wt/day) decreased tumor size ≥70% in only 30% of the tumors. As can readily
be seen in Table I, while the two higher doses of vorozole profoundly inhibited final cancer incidence and inhibited cancer multiplicity (>85%), even the two lowest doses of vorozole inhibited cancer multiplicity by 45–55%.

Two other observations can be made about the dose-dependent effects that were observed. First, vorozole caused a dose-dependent increase in final body weight (Table I). This increase was ~20% in the case of the highest dose of vorozole, but was only 7% in animals given the two lower doses of vorozole. Second, although the highest dose of vorozole (1.25 mg/kg body wt/day) inhibited normal estrus cycles, a dose of 0.31 mg/kg body wt/day did not affect cycling in the majority of rats. These two results are in agreement with the results of the serum hormone levels obtained in this study. Specifically, the higher doses of vorozole (0.63 and 1.25 mg/kg body wt/day) strikingly increased the levels of testosterone at both 4 and 24 h following the last vorozole treatment. In contrast, while the lower doses of vorozole (0.08–0.31 mg/kg body wt/day) strongly increased testosterone levels at the 4 h time point, there were more limited effects at the 24 h time point. The observation of sustained high levels of androgens at the higher doses, but more minimal effects at the lower doses, would appear to agree with the relatively great increases in body weight observed at the higher doses. These differences in hormonal levels at 4 versus 24 h presumably reflect the relatively short half-life of vorozole in a normal cycling rat (24). The other parameters that were measured in the serum were estradiol and IGF-1. The growth factor IGF-1 has been shown to enhance the growth of mammary tumor cells, and has been shown to be decreased by tamoxifen treatment (25,26). In contrast, vorozole caused a limited, but significant, increase in serum IGF-1 levels. Vorozole caused striking decreases in levels of serum estradiol at 4 h. There appeared to be some recovery of estradiol levels at 24 h in all rats.
We next attempted to determine whether limited exposure to vorozole might be highly effective following this intermittent dosing. However, the effects were more limited. While no new mammary cancers became palpable during periods of vorozole treatment, we nevertheless failed to observe complete regression of previously existing palpable cancers. This may imply that even though this dose of vorozole is partially effective therapeutically, the 3 week treatment is insufficient to accomplish complete regression of palpable tumors.

Cancers derived from the chemically induced rat mammary cancer models have many characteristics similar to that observed in the human disease, e.g. ductal origin, similar histopathology (28), hormonal responsiveness (29) and increased levels of cyclin D (30). Perhaps the most fully characterized genetic alteration in the MNU-induced rat mammary model are alterations in the Ha-ras oncogene (31), an alteration infrequently observed in human breast cancer. Fifty percent of the palpable cancers derived from rats treated with MNU alone displayed mutations in the 12th codon of the Ha-ras oncogene. MNU-induced cancers derived from rats treated with the vorozole vehicle or the highly-effective doses of vorozole, which decreased cancer multiplicity 50–80%, had similar levels of Ha-ras mutations in their cancers. These data demonstrate that this highly effective chemopreventive agent does not appear to select either for or against the outgrowth of tumors bearing Ha-ras oncogenes.

As shown in the present studies, very high chemopreventive efficacy is associated with increased body weights in hormonally intact rats receiving vorozole. However, prior work by De Coster et al. (11) demonstrated that these androgenic effects are observed only in the presence of functional ovaries. More importantly, studies have shown that increased levels of androgens are not observed in post-menopausal women treated with vorozole (32).

Vorozole has demonstrated efficacy in a subset of women who were previously treated and failed on tamoxifen therapy (12). The results obtained here strongly suggest that vorozole would be a prime candidate for further development both in a neo-adjuvant setting as well as in a chemopreventive setting. The potential use of aromatase inhibitors is particularly interesting since human breast epithelial cells, tumor cells, adipocytes and even stromal cells, may express aromatase (33,34). Based on recent studies that have examined various co-regulators of the steroid superfamily it would appear that combinations of agents that decrease the levels of estrogen (e.g. vorozole, anti-estrogens) combined with agents that use the same class of co-regulators (e.g. vitamin D, retinoids, glucocorticoids) may show synergistic chemopreventive efficacy (35).

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