The Antitumor Effects of the Quinoline-3-Carboxamide Linomide on Dunning R-3327 Rat Prostatic Cancers

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ABSTRACT

Linomide (N-phenylmethyl-1,2-dihydro-4-hydroxyl-1-methyl-2-oxo-quinoline-3-carboxamide) is a quinoline 3-carboxamide which previously has been demonstrated to produce immunomodulator and antitumor effects when given in vivo. To test the possible antitumor effects of linomide against prostatic cancers, rats bearing five distinct Dunning R-3327 rat prostatic cancer sublines were treated daily with i.p. injections of linomide. These studies demonstrated that linomide has a reproducible antitumor effect against all of the prostatic cancers tested regardless of their growth rate, degree of morphologic differentiation, metastatic ability, or androgen responsiveness. This antitumor effect is observed only in vivo, not in vitro, and involves a cytotoxic response of the prostatic cancer cells. This cytotoxic response results in the retardation of the growth rate (i.e., increased tumor volume doubling time) of primary prostatic cancers and in metastatic lesions. Linomide's growth retardation is reversible, and thus continuous daily treatment with linomide is required for maximal antitumor response. Pretreatment of rats with linomide before tumor inoculation has no effect in addition to that produced by initiating linomide treatment at the time of tumor inoculation. No enhancement of either natural killer cell number or natural killer cell cytotoxic activity is induced by linomide treatment in the tumor-bearing rats. In addition, depletion of natural killer cell activity via injections of asialo-GM1 antiserum does not prevent the antitumor effects of linomide in vivo. Likewise, the antitumor effects of linomide are also produced in prostatic cancer-bearing athymic nude rats. These results suggest that the requirement for host involvement in the antitumor effects of linomide against rat prostatic cancers may involve both immune and nonimmune host mechanism(s) (e.g., antiangiogenesis).

INTRODUCTION

Metastatic prostatic cancer is highly responsive to androgen ablation therapy; however, this therapy is rarely curative (1). This failure is due to the nearly universal progression of prostatic cancer to an androgen-independent state (2). The major reason for this progression is that metastatic prostatic cancer within an individual patient is usually heterogeneous, being composed of both androgen-dependent and -independent prostatic cancer cells (3-5). Thus, androgen ablation does not eliminate the androgen-independent prostatic cancer cells within the patient (6, 7). To increase the cure rate for metastatic prostatic cancer, an effective therapy for androgen-independent prostatic cancer cells is needed which can be combined with any of the large variety of available forms of effective androgen ablation to affect all of the heterogeneous tumor cell populations present within an individual patient (8).

Regimens which have combined chemotherapeutic agents targeted at the androgen independent cancer cell with androgen ablation are currently producing only modest increases in response rates and survival times in patients with metastatic prostatic cancer (9). This has led to a search for new approaches to controlling androgen-independent prostatic cancer cells. A successful treatment for androgen-independent cancer cells can be obtained by either lowering the rate of cell proliferation and/or by raising the rate of cell death to a point where this death rate exceeds the rate of cell proliferation. Since the rate of prostatic cancer cell proliferation is remarkably low in untreated patients (i.e., less than 5% of the cell proliferating/day) (10-13), it may be easier to achieve a therapeutic effect by raising the low rate of prostatic cancer cell death than by lowering the already low rate of prostatic cancer cell proliferation.

There are at least three approaches to increasing the death rate of androgen-independent prostatic cancer cells. The first approach is to activate the "programmed cell death" pathway within these cells, leading to their suicide (14). Such an approach is actively being pursued (15, 16). The second approach is to block the host development of the tumor blood supply. Such an antiangiogenetic approach could induce the hypoxic death of the cells. The third approach is to stimulate the host immune system to evoke/enhance an antitumor response. It is this latter approach using linomide, a recently discovered immunomodulator, which is the focus of the present study.

Linomide is a quinoline-3-carboxamide (Fig. 1). When given systemically in vivo to mice, linomide enhances NK cell activity (17), inhibits autoimmunity in MRL/1 mice (18), enhances the proliferation of mitogen-stimulated T-cells (19), and reduces metastases of murine B16 melanoma cells (20, 21). The in vivo antitumor effects of linomide in mice do not involve the enhancement of serum interferon levels (17) and are mediated both by enhanced NK activity (20, 22) and non-NK (i.e., macrophage) activity (20). When given systemically in vivo to rats, linomide enhances the delayed-type hypersensitivity reaction to bacterial antigens (23), enhances mitogen-stimulated proliferation of T-cells (24), inhibits the growth of dimethylbenzanthracene-induced mammary tumors (24), and reduces metastases of Lewis lung carcinoma cells (25). In the rat, the cell type(s) (i.e., NK, macrophage, and/or T-cell, etc.) responsible for the antitumor effects have not been resolved. In the present study, the ability of linomide to elicit an antitumor effect against a series of androgen-independent Dunning R-3327 rat prostatic cancers was tested in vivo and in vitro. In addition, various analyses were performed to clarify the mechanism of such antitumor effects.

MATERIALS AND METHODS

Tumors. The PAP, AT-1, AT-2, MAT-Lu, and MAT-LyLu tumors utilized in the present study were all members of the Dunning R-3327 system of serially transplantable rat prostatic cancer. The developmental history and characteristics of each of these sublines have been described previously (26, 27). Each of the sublines is serially transplantable in inbred Copenhagen (Cop) rats or F1 hybrids produced by breeding Cop with inbred Fischer rats. All of the sublines except the...
PAP are androgen-independent, anaplastic prostatic cancers. The PAP is an androgen-responsive, well-differentiated, nonmetastatic prostatic cancer (27). The AT-1 and AT-2 are nonmetastatic cancers, while the MAT-Lu and MAT-LyLu are highly metastatic cancers (27). The PAP tumor was a gift from Dr. Norman Alunan (Papanicolaou Institute, Miami, FL).

Animals. All animals used in these studies were maintained in accordance with the NIH guide for the care and use of laboratory animals, and the specific protocols used were approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee. Animals used in the AT-2, MAT-Lu, and MAT-LyLu studies were inbred Copenhagen male rats obtained from Harlan Sprague-Dawley (Indianapolis, IN) and random-bred athymic nude rats (i.e., N:NIH-nu/nu) obtained from the Veterinary Resources Branch Division of Research Services, NIH (Bethesda, MD). Animals used in the AT-1 and PAP experiments were C57 x Fischer F1 hybrids obtained from ALAB (Solna, Sweden).

Animals were housed in groups of 2-3 animals/individual microisolator cage under controlled temperature (21-22°C), humidity (50%), and lighting conditions (12 h light, 12 h dark, lights at 0700 h). Animals were fed a commercially available chow (i.e., 5053 Rat Chow; Purina Mills, Inc., Richmond, IN) for the AT-2, MAT-Lu, and MAT-LyLu experiments and R34 rodent food (EWOS, Sodertalje, Sweden) for the AT-1 and PAP tumor-bearing rats, and water was available ad libitum.

Method of Tumor Transplantation. Animals were anesthetized with Metofane. The animals were shaved, and their skin was sterilized with alcohol. Using sterile equipment, an incision was made in the flank, and the specific protocols used were approved by the Johns Hopkins Institutional Animal Care and Use Committee. Animals used in the AT-2, MAT-Lu, and MAT-LyLu experiments and R34 rodent food (EWOS, Sodertalje, Sweden) were inbred Copenhagen male rats obtained from Harlan Sprague-Dawley (Indianapolis, IN) and random-bred athymic nude rats (i.e., N:NIH-nu/nu) obtained from the Veterinary Resources Branch Division of Research Services, NIH (Bethesda, MD). Animals used in the AT-1 and PAP experiments were C57 x Fischer F1 hybrids obtained from ALAB (Solna, Sweden).

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Substance. Linomide (Fig. 1), supplied by Kabi Pharmacia Thera-peutics (Helsingborg, Sweden), was dissolved in sterile isotonic saline. Delivery of linomide was via either daily 1 ml bolus i.p. injection, daily 1 ml bolus feeding p.o. via a gastric tube, or daily continuous feeding p.o. via the drinking water.

Tumor Volume Determination and Doubling Time Calculations. Following the development of palpable tumors, individual tumor dimensions in centimeters were serially measured using calibrated microcallipers at various times following tumor inoculation. From these measurements, tumor volumes in cm3 were calculated using the formula \(V = \frac{4}{3} \pi r^3\), where \(r\) is the radius of the tumor. Additionally, the total number of tumor cells was determined by flow cytometry.

In Vivo Response of Dunning AT2 Tumors to Linomide. To determine initially if linomide could produce an antitumor effect against prostatic cancer cells, Cop rats were inoculated with the androgen-independent, anaplastic Dunning R-3327 AT2 rat prostatic cancers and immediately began on daily i.p. injections of linomide at various doses (Fig. 2). These results demonstrated a dose-response antitumor effect of linomide upon the growth rate of the AT2 cancers. Additional studies demonstrated that the maximal AT-2 tumor growth retardation was induced at a dose of 100 mg/kg/day. At this dose, the AT2 tumor growth rate was decreased by ~2-fold (\(P < 0.05\)) as compared to that on untreated control animals (i.e., tumor volume doubling time increased from 2.5 ± 0.3 days in control animals to 4.9 ± 0.4 days in animals given daily i.p. injections of 100 mg of linomide/kg). Linomide treatment did not produce a decrease in body weight or food intake and did not produce any obvious outward signs of toxicity even when given daily at the highest dose of 125 mg/kg for more than 60 days.

The antitumor response of the AT2 prostate cancer to the dose of 100 mg of linomide/kg/day was essentially identical regardless of the route of daily administration of the drug (i.e., i.p. injection versus oral gavage versus in the drinking water).
Table 1 Effect of starting daily i.p. linomide treatment at various times on the response of AT-2 rat prostatic cancer

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor volume at 4 weeks post-inoculation (cm³)</th>
<th>Tumor volume doubling time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>17.4 ± 2.1</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Daily i.p. linomide (100 mg/kg/day) initiated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 days before tumor inoculation</td>
<td>4.7 ± 1.1*</td>
<td>4.8 ± 0.5*</td>
</tr>
<tr>
<td>At time of tumor inoculation</td>
<td>4.5 ± 1.0*</td>
<td>4.9 ± 0.4*</td>
</tr>
<tr>
<td>10 days after tumor inoculation</td>
<td>8.0 ± 0.7*</td>
<td>4.6 ± 0.9*</td>
</tr>
</tbody>
</table>

* n = 5 animals/group; mean ± SE.
* P < 0.05 as compared to control group.

Fig. 2. Dose response of Dunning R-3327 AT-2 tumor to daily i.p. injections of linomide.

Fig. 3. Effect of linomide on Dunning R-3327 AT-1 tumor. Linomide was administered 5 days a week starting 10 days post-tumor inoculation and was continued during the entire 4-week observation period. There were 6 AT-1 tumor-bearing animals/group.

After 4 weeks of treatment, the tumors were about one-third the size of those in the untreated host regardless of the route of daily administration (i.e., tumors were 17.4 ± 2.1 cm³ in the control untreated group; 5.29 ± 1.1 cm³ in the i.p. injected group; 6.74 ± 1.3 cm³ in the oral gavage group; and 5.83 ± 0.91 cm³ in animals given linomide in the drinking water).

Thus, in all further experiments the standard linomide treatment was by i.p. injection of 100 mg of the drug/kg/day.

To achieve the maximal antitumor effect, linomide had to be given daily continuously. This was demonstrated by the following observations. If animals were inoculated with the AT2 tumor and immediately begun on daily i.p. injections of 100 mg of linomide/kg, the growth of tumor was inhibited (i.e., tumor volume doubling time increased from 2.5 ± 0.3 days for untreated controls to 5.9 ± 0.8 days in treated animals). By 10 days of treatment, the tumors were less (i.e., P < 0.05) than one-half (i.e., 0.56 ± 0.13 cm³; n = 10) the size of those in untreated animals (i.e., 1.25 ± 0.31 cm³; n = 5). If daily i.p. injections with linomide were stopped, within 1 week the tumors resumed a faster growth rate (i.e., 2.9 ± 0.5 day) essentially equal to that of tumors in untreated rats (i.e., 2.5 ± 0.3 days). This resumption of maximal growth resulted in a tumor size of 13.2 ± 1.9 cm³ (n = 5) 20 days after discontinuation of the daily linomide as compared to 5.01 ± 1.1 cm³ (n = 5) in the group maintained continuously on daily linomide and 19.2 ± 2.4 cm³ in the untreated control group (n = 5).

Using the standard linomide treatment, the effect of varying the time of initiation of daily linomide injections on AT-2 growth was tested (Table 1). Pretreatment of animals (i.e., starting daily i.p. linomide injections 10 days before tumor inoculation) did not prevent AT-2 tumors from developing (i.e., 100% tumor take) and reduced the tumor growth rate to the same extent as initiation of treatment at the time of tumor inoculation. Delaying initiation of treatment until 10 days after tumor inoculation (i.e., time at which tumors were ~1 cm³ in size) did not reduce the ability to decrease the growth rate (i.e., increase tumor volume doubling time) (Table 1). This growth inhibition was detectable within 1 week of linomide treatment in the delayed treatment group. Since delayed treatment did not induce tumor regression, only a slowing of the growth rate, the AT-2 tumors were larger in the delayed treatment group versus the pretreatment and immediate treatment groups even though the growth rate retardation was similar in the three treatment groups.

Generality of the in Vivo Antitumor Effect of Linomide on Prostatic Cancers. To determine the generality of the ability of the linomide to inhibit the growth of nonmetastatic rat prostatic cancers, an additional androgen-independent, nonmetastatic anaplastic R-3327 subline, the AT-1, was tested. Animals were inoculated with AT-1 tumor tissue and allowed to go untreated until the tumors were ~0.5 cm³ in volume (i.e., 10 days post-tumor inoculation). Animals (n = 6 rats/group) were then randomized into a control untreated group, and two groups of animals were treated 5 days a week with daily i.p. injections of either 10 or 100 mg/kg of linomide. These results (Fig. 3) demonstrate that the growth rate of the AT-1 tumor was decreased by more than 2-fold (P < 0.05) from a doubling time of 4.1 days observed in the control group to values of 9.4 and 10.6 days in the 10 and 100 mg/kg linomide-treated groups, respectively.

To determine if linomide could inhibit the growth of metastatic as well as nonmetastatic prostatic cancers, the highly metastatic Dunning MAT-Lu and MAT-LyLu sublines were used. The MAT-Lu subline metastasizes to the lung while the MAT-LyLu metastasizes to lymph nodes and lung (26). Copenhagen male rats were inoculated with 1 × 10⁶ cells of either the MAT-Lu or MAT-LyLu sublines in the flank. A group of animals bearing each tumor subline was left untreated as a control, a group was immediately started on daily i.p. injections
of 100 mg/kg/dose of linomide, and another group was allowed to go untreated for 10 days following tumor inoculation before being started on 100 mg/kg/dose of linomide. All animals were followed for 4 weeks. These studies (Table 2) demonstrated that both of the highly metastatic Dunning R-3327 rat prostatic cancers were growth inhibited (P < 0.05) by linomide treatment. Linomide treatment did not produce any regression of established tumor size, however; it only inhibited growth when initiated 10 days post-tumor inoculation. Such growth inhibition (i.e., increase in tumor volume doubling time) was essentially identical regardless of whether daily linomide treatment was initiated at the time of tumor inoculation or delayed 10 days. In the delayed treatment group, retardation of the growth rate was apparent within 1 week of treatment.

Ability of Linomide to Affect Prostatic Metastasis. To determine if linomide affects the metastatic process, male Cop rats were inoculated with the androgen-independent, highly metastatic MAT-Lu subline. Groups of MAT-Lu animals were either untreated or begun on daily i.p. injections of 100 mg linomide/kg/dose immediately at the time of inoculation or 10 days post-tumor inoculation when the primary tumor was ~1 cm³ in size. At 48 days post-MAT-Lu inoculation, or at the time of spontaneous death, rats were analyzed for lung metastases (Table 3). These results demonstrate that both the percentage of animals with lung metastases and the average number of lung metastases are reduced by daily linomide treatment. That delaying the initiation of linomide treatment by 10 days results in the same degree of metastasis reduction as that produced in the group initiated on linomide at the time of tumor inoculation suggests that the MAT-Lu cells are just beginning to metastasize when the primary tumor reaches ~1 cm³ in size on day 10 post-inoculation. This suggestion is consistent with the previous demonstration that the majority of MAT-Lu lung metastases develop when the primary MAT-Lu tumor is larger than 1 cm³ in size (31). Besides decreasing the number of lung metastases, the size of lung metastases were also decreased by both the delayed and immediate linomide treatment (Table 3). In the control group, the majority (i.e., 64%) of the lung metastases were >5 mm in diameter. In contrast, less than 25% of metastases in the linomide-treated group were >5 mm. These data demonstrate that linomide treatment delays the rate of initial establishment and slows the rate of growth of established MAT-Lu metastases.

In Vivo Response of the Slow-growing Androgen-responsive R-3327 PAP Tumors to Linomide. All of the previously tested Dunning sublines were anaplastic, fast growing (i.e., doubling time ≤ 4 days), and androgen-independent in their phenotype. To determine whether linomide treatment could also affect slower-growing, well-differentiated, androgen-responsive prostatic cancers, the Dunning PAP subline was used. This subline previously has been demonstrated to be androgen responsive and well differentiated histologically and to be much slower growing (i.e., doubling time 2–3 weeks in intact male rats) than the anaplastic Dunning sublines (27). Animals were inoculated with PAP tumor tissue and allowed to grow untreated until the tumors were ~0.5 cm³ (i.e., 130 days post-tumor inoculation). Animals (n = 6 animals/group) were then randomized into a control group of untreated rats and two groups of PAP tumor-bearing rats given i.p. injections 5 days a week for 4 weeks with either 20 or 40 mg/kg of linomide. These studies (Fig. 4) demonstrated that growth of the PAP sublines was nearly completely inhibited during the 4-week treatment period (i.e., day 130–150 post-tumor inoculation). Within 1 week after stopping linomide treatment, however, the growth of the PAP tumor resumed in both groups of animals previously treated with linomide. These results demonstrated that linomide treatment also inhibits the growth of slow-growing, well-differentiated rat prostatic cancers and that this inhibitory effect is reversible once linomide treatment is discontinued.

In Vitro Effects of Linomide on Dunning R-3327 Rat Prostatic Cancer Cells. To determine if linomide directly inhibits the growth of the rat prostatic cancer cells, in vitro cell culture experiments were performed. Initially 5 × 10⁴ viable AT-2, MAT-Lu, or MAT-LyLu cells were inoculated into flasks containing media lacking linomide. After 24 h to allow cell attachment, flasks were randomized into those remaining unexposed to linomide and those receiving media containing 300 ng of linomide/kg/dose. Daily i.p. linomide (100 mg/kg) initiated either untreated or begun on daily i.p. injections 5 days a week for 4 weeks with media containing 300 ng of linomide/kg/dose immediately at the time of inoculation or 10 days post-tumor inoculation, for the time < 4 days), and androgen-independent in their phenotype.
linomide/ml of media. This in vitro dose of linomide was chosen since pharmacokinetic studies demonstrated that 244 μg/ml is the maximal plasma concentration of linomide after in vivo dosing with 160 mg/kg of the drug. Thus the 300 μg of linomide/ml used in these in vitro studies exceeds the maximal achievable dose which produces maximal in vivo efficacy. Appropriately formulated medium was replaced every 2 days, at which time the total number of viable cells was determined for four separate flasks. These in vitro studies demonstrated that there was no inhibition in the rate of cell growth during 6 days of linomide exposure for any of the cell lines. Thus, at the end of 6 days of in vitro treatment, the total number of viable cells was equal in the control and linomide-exposed cultures of all three cell lines (Table 4). Thus, linomide does not have direct growth-inhibitory effects on the rat prostatic cancers without the involvement of the host animal.

Cytostatic versus Cytotoxic Effect of Linomide in Vivo. The retardation in the in vivo growth rate of the rat prostatic cancers could be due to a cytostatic and/or cytotoxic effect. To differentiate between these possibilities, a group of five Cop rats were inoculated with 2 × 10⁶ AT-2 cells, and 5 days later daily i.p. injections of 100 mg of linomide/kg/dose were begun. A second group of five control rats were inoculated with 5 × 10⁶ AT-2 cells and given no further treatment. After 10 days of linomide treatment (i.e., 15 days postinoculation), the growth rate of the AT-2 tumors was maximally reduced (i.e., doubling time increased to 4.8 days). Even though the growth rate of the AT-2 tumor in control untreated animals was 2-fold faster (i.e., doubling time of 2.5 days), the tumor volumes were ~3 cm³ in both the linomide-treated and untreated control groups at 15 days post-tumor inoculation. This was due to the fact that initially 4 times the number of AT-2 cells were inoculated into the linomide-treated animals as compared to the control rats. At 15 days post-tumor inoculation, the comparably sized AT-2 tumors were excised from both groups of animals, and the rate of tumor DNA synthesis determined as an index of the rate of cancer cell proliferation. These analyses demonstrated that there was no difference in the rate of cell proliferation in equally sized AT-2 tumors from linomide-treated and untreated animals, e.g., rates of DNA synthesis were equal; 265.5 ± 58.5 dpm of [³H]thymidine incorporated into AT-2 tumor DNA/h/μg DNA from the linomide group (n = 5) versus 204.3 ± 7.2 dpm of [³H]thymidine incorporated into AT-2 tumor DNA/h/μg DNA for the control group (n = 5). The observations that the net growth rate of the AT-2 tumors in the linomide-treated group is one-half that of the untreated control group even though the rates of tumor cell proliferation are equal in the two groups is consistent with linomide inducing a cytotoxic effect in vivo in the AT-2 prostatic cancer cells.

Mechanism of in Vivo Cytotoxic Effects of Linomide. Since linomide has no apparent direct cytotoxic effect when tested in vitro against rat prostatic cancer cells, this suggests that the cytotoxic effects of linomide observed in vivo are indirectly mediated by host cells. Previous in vivo studies in mice demonstrated an enhancement of both peripheral blood and spleen cell NK activity (17, 20, 22). To determine if similar increases in NK numbers and/or NK activities are induced in rats by linomide treatment, peripheral blood and spleen cells were analyzed from linomide-treated and untreated AT-2 tumor-bearing rats. Analysis of the peripheral blood demonstrated that linomide treatment did not enhance any of the white cell types quantitated (Table 5). The only statistically significant difference in peripheral blood was a rise in total WBC in the untreated and linomide-treated tumor-bearing rats 21 days post-tumor inoculation compared to age-matched non-tumor-bearing animals. This rise in total WBC was correlated with the size of the AT-2 tumor. For example, when the AT-2 tumor reached a size of ~40 cm³ in untreated hosts, the WBC number was elevated nearly 3-fold (P < 0.05) to a value of 34.2 ± 3.4 × 10⁴ cells/ml. When tumor-bearing animals were treated daily with linomide, this rise in WBC was significantly retarded (P < 0.05). The WBC was only 18.4 ± 0.2 × 10⁴ cell/ml when AT-2 tumors reached a similar size (~40 cm³) in the linomide-treated animals (i.e., 56 days post-inoculation). Determination of the percentage of spleen cells which are T-helper lymphocytes, T-cytotoxic lymphocytes, and NK cells in linomide-treated and untreated animals also demonstrated no difference in the portion of any of these cells. To test whether linomide induces an increase in functional NK activity, as opposed to NK cell number, spleen cells from AT-2 tumor-bearing animals treated for 21 days with daily i.p. linomide (100 mg/kg/dose) and from tumor-bearing animals not given linomide were tested using a YAC-1 cytotoxic NK assay. These assays did not demonstrate any induction in the functional NK activity of spleen cells of linomide-treated versus untreated tumor-bearing rats (percentage lysis of YAC-1 cells after 6 h incubation at a target effective ratio of 1:12, 1:25, and 1:100 were 5 ± 1.0, 11.0 ± 1.6, and 21.4 ± 2.7% for untreated and 3.9 ± 1.5, 13.4 ± 2.1, and 23.4 ± 2.0% for the linomide group, respectively).

Role of Natural Killer Cells in the Antitumor Effects of Linomide. Based upon the peripheral blood NK numbers and the spleen cell NK functional activity, NK cell enhancement does not occur in tumor-bearing rats following linomide treatment. To clarify if baseline NK activity, independent of enhancement, is required for the antitumor effects of linomide in vivo, the
antitumor effects of linomide were tested in vivo in AT-2 tumor-bearing animals with normal and greatly reduced NK activity. To reduce the NK activity, AT-2 tumor-bearing animals were injected i.v. with antiserum to asialo-GM1. Injections of 100 μl of antiserum have previously been demonstrated to effectively reduce the NK activity in rats by more than 50% for at least 1 week (32). AT-2 tumor-bearing rats were treated with (a) daily i.p. injections of 100 mg linomide/kg, (b) i.v. injection of 100 μl of anti-asialo-GM1 antiserum every 5 days, or (c) combination of the two treatments. The NK activity of the spleen cells and tumor volumes at 28 days post-tumor inoculation are presented in Table 6. These data demonstrate that linomide alone does not enhance NK activity and that a ~90% depression in the NK activity induced by asialo-GM1 antiserum does not inhibit the antitumor effects of linomide.

Antitumor Response to Linomide in Nude Rats. To determine if functional T-cells are required for the growth-retarding effects of linomide on prostatic tumors, athymic nude rats were inoculated with AT-2 tumors. Athymic nude rats lack a functional thymus and thus have a defective T-cell immune response; however, their NK activity is unimpaired (33). When such nude rats were begun on daily i.p. injections of 100 mg of linomide/kg/dose at the time of AT-2 tumor inoculation, the growth rate of the tumors decreased (P < 0.05) from a doubling time of 3.0 ± 0.5 days in the untreated control nude rats (n = 6) to a value of 5.2 ± 0.6 days in the linomide-treated nude rats (n = 6). Due to this growth inhibition, the AT-2 tumors in linomide-treated nude rats at 28 days post-inoculation were significantly smaller (P < 0.05), being only about one-half the size (4.5 ± 0.8 cm³) of those in untreated control nude rats (8.8 ± 1.4 cm³).

**Table 6: NK Activity of spleen cells and AT-2 tumor size after 28 days of various treatments**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>NK activity of spleen cells from AT-2 tumor-bearing rats at 28 days post-tumor inoculation (% of YAC-1 cells lysed at 1:100 target:effector cell ratio)</th>
<th>AT-2 tumor size (cm³) at 28 days postinoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>21.4 ± 2.7</td>
<td>38.5 ± 6.5</td>
</tr>
<tr>
<td>Daily linomide*</td>
<td>23.4 ± 2.0</td>
<td>15.7 ± 5.9</td>
</tr>
<tr>
<td>Anti-asialo-GM1 serum every 5 days</td>
<td>2.9 ± 1.0*</td>
<td>35.7 ± 5.7</td>
</tr>
<tr>
<td>Daily linomide plus anti-asialo-GM1 serum every 5 days</td>
<td>1.7 ± 0.5*</td>
<td>16.4 ± 3.1*</td>
</tr>
</tbody>
</table>

*Mean ± SE of 6 tumor-bearing rats/group.

**DISCUSSION**

The results presented demonstrate that the quinoline 3-carboxamide linomide has a reproducible antitumor effect against all of the rat prostatic cancers tested regardless of their growth rate, degree of morphological differentiation, metastatic ability, or androgen responsiveness. This antitumor response is observed only in vivo, not in vitro, and involves a cytotoxic response of the tumor cells. This cytotoxic response results in the growth retardation of both the primary prostatic cancer and its metastases. For maximal tumor growth retardation, daily treatment had to be given continuously (linomide growth retardation is reversible). Pretreatment of rats with linomide before tumor inoculation had no additional effect other than that produced by initiating linomide treatment at the time of tumor implantation.

The mechanism of such an antiprostatic cancer response in rats is not entirely clear, but it definitely requires the involvement of the host. One possible explanation of the host requirement for this in vivo antitumor activity of linomide is that the parent compound is converted in vivo to an active cytopathic metabolite and that production of this metabolite does not occur in the tumor cells themselves either in vitro or in vivo. This possibility is currently being tested. Another possibility is that the host immune system is required for the antitumor effects of linomide. This would be consistent with previous studies in mice suggesting that antitumor effects of linomide against murine B16 melanoma involve the enhancement of the NK activity and a lymphoproliferative response to mitogenic stimulation (17, 20, 22). In the present rat studies, linomide treatment was not found to enhance either peripheral or splenic NK numbers or splenic NK cytotoxic activity. In addition, in vivo depletion of the NK cytotoxic activity via asialo-GM1 antiserum did not reduce the antitumor effect of linomide against rat prostatic cancers. These latter results suggest that the antiprostatic cancer effects of linomide in rats involves cell types in addition to NK cells (e.g., B-lymphocytes, T-lymphocytes, or macrophages). The observation that a similar antiprostatic cancer response to linomide is elicited in athymic nude as well as normal rats further suggests that only minimal T-lymphocyte function is required for the in vivo response to linomide. The demonstration that continuous daily treatment with linomide is required to maintain maximal growth retardation of the prostatic cancers in rats (i.e., linomide's effects are reversible with little memory) also suggests that B-lymphocytes are not involved in the antitumor response to linomide. Combining these observations implicates macrophages as a possible intermediate in the antitumor effects of linomide. There is precedent for a linomide effect on macrophage function in rats. Earlier studies demonstrated that daily treatment of rats with linomide...
at a dose of ≥80 mg/kg/day maximally stimulated the delayed-type hypersensitivity response to bacterial antigens (23). These studies suggested that delayed-type hypersensitivity enhancement by linomide involves increasing the efficiency of the macrophage/T-cell interaction, possibly by affecting the production of lymphokines (e.g., interleukin 2). Along these lines, it is significant that linomide is able to enhance the delayed-type hypersensitivity response even in T-cell-depleted rats (23). In addition, it has been demonstrated that linomide increases the stimulatory capacity of macrophages in mitogen-stimulated mixed lymphocyte cultures (19). These studies also demonstrated that spleen cells from linomide-treated animals produced more interleukin 2 when incubated with concanavalin A than spleen cells from untreated animals and that this interleukin 2 enhancement is due to the effect of linomide on macrophages (19).

Studies are currently under way to clarify the role of macrophages in the in vivo antiproliferative cancer response to linomide. These studies will include the determination of the number of T-helper lymphocytes, T-cytotoxic lymphocytes, NK cells, and macrophages in rat prostatic cancers from linomide-treated and untreated hosts using appropriate immunochemical staining of histological sections of tumors and morphometric quantitation. Preliminary examination of standard hematoxylin and eosin histological sections have demonstrated that AT-2 tumors from linomide-treated animals have more focal necrosis than identically sized tumors from untreated hosts (e.g., comparing AT-2 tumors grown for 3 weeks in linomide-treated animals versus tumors grown for 2 weeks in untreated hosts). These results suggest that linomide treatment may have an antiangiogenic effect upon the neovascularization occurring in vivo in the rat prostatic cancers. The possibility that such an antiangiogenic effect is involved in the mechanism of action of linomide could explain why the antitumor effects of linomide are only produced in vivo and not in vitro and why linomide treatment has no effect against L1210 leukemia growing in mice. The antiangiogenic effects of linomide treatment in vivo are also currently being tested.

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The Antitumor Effects of the Quinoline-3-Carboxamide Linomide on Dunning R-3327 Rat Prostatic Cancers

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