Combination Therapy: Lonidamine, Hyperthermia, and Chemotherapy against the RIF-1 Tumor in Vivo

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ABSTRACT

Lonidamine enhances the cytotoxicity in vitro of several conventional antitumor drugs as well as that of hyperthermia (HT). We have investigated the possibility that such enhancement can also be demonstrated in vivo against the RIF-1 tumor system. Two assays were used to examine antitumor activity: tumor growth delay and clonogenicity of cells obtained from tumors treated with different agents. We used drug (and HT) doses that by themselves did not achieve significant cell killing. The drugs whose interaction with lonidamine was tested were: cis-diamminedichloroplatinum (CDDP), mitomycin C (MMC), bleomycin, 5-fluorouracil, and nitrosourea. Of these only CDDP and MMC yielded positive data. Both assays gave essentially the same results, showing that antitumor activity reflected direct cell killing. CDDP and MMC activity was also enhanced by HT. When we combined all three modalities, however, the results of the trimodality therapies were no better than that of individual bimodality treatments. These last results suggest that lonidamine and HT have similar mechanisms, most likely inhibition of repair of DNA damage. Our studies show that lonidamine may have a role in multidrug therapies that include either CDDP or MMC as a component of the treatments.

INTRODUCTION

Lonidamine, 1-(2,4-dichlorobenzoyl)-1-H-indazol-3-carboxylic acid, is an interesting antitumor drug. It is said to exert its cytotoxic effect by interfering selectively with energy metabolism of tumor cells (1, 2), and it apparently shows little or no toxicity against the human normal hemocytogenic system (3, 4). When used alone, however, it shows few activities against most tumors (5). For this reason, combined applications with other drugs and therapies such as hyperthermia or radiation have been tested (6-9). We have systematically studied the potential in vitro of lonidamine in combination with several other drugs against mouse (RIF-1) and human (HT1080) fibrosarcoma cells (10). We observed that lonidamine can appreciably enhance the cytotoxicity of CDDP2 and of some antitumor antibiotics, particularly MMC. For optimum effect, lonidamine followed these drugs.

Based on these initial in vitro studies, we have tested the ability of lonidamine to enhance drug efficacy in the RIF-1 tumor system in C3H mice. We used two assays: tumor growth delay and the ability of cells from tumors treated in vivo to form colonies in vitro. Because the efficacy of LND is enhanced by hyperthermia, we also explored the efficacy of trimodality regimens. Our results indicate that (a) CDDP and MMC are good drugs to combine with lonidamine; (b) lonidamine-induced enhancements are dependent both on sequencing and doses of the combined drugs; (c) the short-term (1 day) and long-term (5-10 days) exposures to lonidamine induced similar enhancement; and (d) trimodality of LND, HT, and CDDP (or MMC) does not further improve the therapeutic results, although either lonidamine or hyperthermia can significantly augment the antitumor activity of CDDP and MMC. We believe that these data could provide a rationale for the use of the combined administration of lonidamine and other drugs in the treatment of cancer.

MATERIALS AND METHODS

Drugs. Lonidamine, obtained from F. Angelini Research Institute of Rome, was dissolved in a glycine (0.5 m) and trizma base (0.2 m) solution. LND (5 mg) was added to 1 ml glycine-trizma base solution immediately before injection. The final dose of LND used was 50 mg/kg body weight and was injected i.p. into C3H mice in 0.2- to 0.4-ml volume. The glycine-trizma base solution was well tolerated and did not appear to affect the activity of other drugs.

CDDP, MMC, BLM, BCNU (Bristol-Myers, Evansville, IN), and 5-FUra (Solo Park Laboratory, Franklin, IL) were purchased from the Stanford Hospital pharmacy. All drugs, except BCNU, were dissolved and diluted in 0.9% sodium chloride solution. BCNU was dissolved in ethanol and diluted in 0.9% sodium chloride solution. All these drug solutions were freshly made and injected i.p. in 0.1- to 0.2-ml volumes.

Mice. Male C3H/Km mice were obtained from the Stanford Radiation Biology Mouse Facility. The age of the mice was 12-14 weeks and body weight was 30-35 g at the beginning of each experiment. These mice were normally bred and were maintained under specific pathogen-free conditions.

Tumor. The transplantable mouse RIF-1 tumor, derived from a radiation-induced fibrosarcoma, was used in this study. Maintenance and characteristics of this tumor have been described elsewhere (11). For each experiment, 2 x 10^5 cells in 0.05 ml Hanks' solution were implanted intradermally into the left flanks of mice approximately 10 days before treatment.

Treatment Procedure. When tumor volumes were approximately 100 mm³, tumor-bearing mice were randomly allocated into groups. At this time, treatment was initiated. In preliminary experiments to test dose-response effects of each drug, three combination schedules were used: LND+drug, LND→drug, and LND→LND+drug. LND+drug means that LND and drugs were injected simultaneously; for LND→drug, LND was injected 24 h before the other drugs; LND→LND+drug means that LND was given 24 h before LND+drug.

To test alternative regimens in detail, different schedules of LND, drugs, and HT combination were evaluated. The day on which a single dose of drug (and/or HT) was delivered was designated as Day 0 (Table 1). LND (50 mg/kg) was given in one dose daily for 1, 5, or 10 days. One-day LND administration means that LND was injected once into mice on Day 0 with (or without) other antitumor drugs. When called for, this was immediately followed by the HT treatment. Five-day LND means that the injection on Day 0, LND was given once daily from Day 1 to Day 4. Ten-day LND means that in addition to the above, LND was given daily for 5 days before Day 0. Hyperthermia was given locally at 43°C for 30 min. Mice were lightly anesthetized with pentobarbital (68 mg/kg) and then placed on specially designed water bath "boats" that permitted the tumors to be immersed in a circulating water bath (Neslab Instruments, Inc.) kept at 43.5°C.

In Vivo/in Vitro Clonogenic Assay. Mice were treated with drugs and local hyperthermia as described. Mice were then kept in their cages for 2 or 24 h to allow for the expression of drug cytotoxicity and possible repair of potentially lethal damage. Mice were then sacrificed and their tumors were excised, weighed, and minced to a fine brei with scissors.
under sterile conditions. Two tumors for each group were pooled, suspended, and stirred in Hanks' buffered salt solution containing appropriate dilutions into Petri dishes in duplicate for the colony-forming assay in vitro. Ten days later the dishes were stained and colonies of more than 50 cells were counted. We then calculated the number of clonogenic cells/tumor, i.e., the product of the plating efficiency and the total cell number/tumor. This was expressed relative to the untreated control. The plating efficiency of the untreated control tumor cells was 10–20%.

Tumor Growth Delay Assay. Six to 8 mice were used for each group. The volume of each tumor was measured with calipers every 2 days until it reached at least 4 times its original volume. Tumor growth delay of each group was expressed as the difference in the average number of days taken by treated tumors to reach 4 times their original volume compared to that of untreated control tumors. Mice dying from calculations of tumor growth delay.

Additionally, we attempted to quantify the toxicity of each treatment regimen by using body weight loss as an indicator of toxicity. Body weight loss was determined by subtraction of tumor weight from body weight. Tumor weight was estimated from 2-dimensional measurements according to the formula,

\[ Tumor \text{ wt (mg)} = \frac{A \times B^2}{2} \times K \]

where \( A \) is length of longitudinal axis (mm), \( B \) is width of vertical axis (mm), and \( K \) is a coefficient of conversion.

**RESULTS**

Effect of LND in Combination with CDDP and HT. The initial experiment was designed to determine the sequencing effect of LND administration when combined with CDDP. Tumor-bearing mice were treated with 0, 2.5, 5, and 10 mg/kg CDDP alone or plus a fixed dose of LND (50 mg/kg), and then, survival of tumor cells was evaluated in vitro. The single LND treatment (50 mg/kg) gave about 80 and 45% survival in tumors excised 2 h and 24 h after LND injection, respectively. The dose–response curves (Fig. 1) demonstrate the exponential decrease of survival with increasing CDDP doses. In CDDP+LND and LND—>CDDP+LND, CDDP cytotoxicity was potentiated by the concomitant administration of LND, as demonstrated by a 1- to 1.5-log decrease in survival. However, 24-h pretreatment of LND (LND—>CDDP) did not show any effects on CDDP killing. These results indicate that the enhancement of CDDP cytotoxicity by LND is sequence and CDDP dose dependent.

Table 2 shows results of the combination therapy of LND, CDDP, and HT, after tumor-bearing mice were treated with 50 mg/kg LND, 7.5 mg/kg CDDP, and 43°C 30-min HT on Day 0. In this experiment, several observations are of interest. First, the number of clonogenic cells of tumors from mice that received CDDP alone and the CDDP combined therapy significantly decreased 24 h after CDDP administration. The CDDP-LND combination (with or without HT) produced the greatest cell killing (~10^{-3}–10^{-4} in survival). When we compared the potentiation produced by the different schedules of LND administration, we found that 1-, 5-, and 10-day LND resulted in similar cell killing. In fact, preadministration of LND for 5 days (LSPLH.L5) showed less potentiation of CDDP cytotoxicity than did the simultaneous 1-day administration.

Similar results were obtained with tumor growth delay experiments (Table 2). Treatment with either LND or HT alone, or with both produced little effect on the tumor regrowth. A single dose of 7.5 mg/kg CDDP produced a tumor growth delay of 3.8 days. When CDDP was combined with LND, the tumor growth delay was about 8.4 days (P < 0.05, versus CDDP alone); when combined with HT, the tumor growth delay was 10.7 days (P < 0.01, versus CDDP alone). Treatment with CDDP+HT plus 1-, 5-, or 10-day LND resulted in 14.1, 11.8, and 14 days of tumor growth delay, respectively. There was no statistically significant difference in tumor growth delay between CDDP+HT with and without LND (P > 0.05). However, either LND or HT individually enhanced the CDDP effect.

Table 1 A schematic representation of the experimental protocols

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>-5</th>
<th>-4</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td>LND</td>
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<tr>
<td>1-day (LNDa)</td>
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<tr>
<td>5-day (LNDa)</td>
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<tr>
<td>10-day (LNDa)</td>
<td>^</td>
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<td>^</td>
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<td>Other drugs</td>
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<td>Hyperthermia*</td>
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</table>
| ^ The local hyperthermia treatment in water bath immediately followed the administration of drugs.

Fig. 2 shows the body weight loss of treated tumor-bearing mice. A slight loss of body weight was seen in the mice treated with LND, HT alone, and LND+HT. Combination therapy of LND with CDDP or CDDP+HT resulted in certain weight loss, especially for the 5-day LND treatment. In addition, mice died during the first week of posttreatment in combination therapy (Table 2). These results suggest that the LND treatment aggravated the side effect of CDDP, or reduced the tolerance of host mice to CDDP toxicity.

Effects of LND in Combination with MMC and HT. Preliminary experiments evaluated the dose-response effect of MMC. Mice bearing RIF-1 tumors were treated in vivo with either 0, 2.5, 5, or 10 mg/kg MMC alone or plus 50 mg/kg LND. As shown in Fig. 3, the results obtained from the combination therapy showed that LND enhanced the MMC cytotoxicity dependent upon sequencing. In MMC+LND and LND—>MMC+LND, LND expressed maximum enhancement of MMC cytotoxicity. At the high dose of MMC (7.5–10 mg/kg), it caused a 1-log decrease in cell survival compared to MMC alone. Twenty-four h preadministration of LND (LND—>MMC), however, did not show any enhancement.

To investigate the interaction of LND, MMC, and HT in combination, tumor-bearing mice were treated with either 50 mg/kg LND, 4 mg/kg MMC, or 43°C 30-min HT alone, or in combination. Both the in vivo/in vitro clonogenic assay and tumor growth delay experiments gave essentially the same results (Table 3). Lonidamine alone or plus 43°C 30-min HT showed only marginal cytotoxicity. Treatment of 4 mg/kg MMC alone led to 94% cell killing and 3.1 days of tumor growth delay. The combination therapy of MMC and HT led to the greatest amount of cell killing and 7–8 days of tumor growth delay (P < 0.01, versus MMC alone). The cytotoxicity produced by the MMC-HT combination was intermediate, i.e., more than that produced by MMC alone but less than that by MMC+LND. These results show that LND enhanced MMC antitumor activity against RIF-1 tumor more effectively than did HT. Again, as seen in the CDDP-LND experiments, the short-term (1-day) or long-term (5–10 days) administration of LND produced similar potentiation of MMC antitumor activ-
ity. Cell killing and TGD time obtained from mice that received trimodality therapy of MMC-LND-HT were similar to that of mice that received bimodality therapy of MMC-LND. Toxicity of treatment to host mice showed no significant difference among all the treated groups.

Effects of LND in Combination with BLM, BCNU, and 5-FUra. In our earlier in vitro study we showed that lonidamine potentiated BLM, BCNU, and 5-FUra cytotoxicity against mouse RIF-1 and human HT1080 cells (10). We, therefore, tested these drugs in in vivo RIF-1 tumor system.

Unfortunately, the results from BLM-LND combination indicate that the enhancement of BLM cytotoxicity by LND was minor, and that was seen only at the highest dose of BLM (40 mg/kg) in the schedule BLM+LND in tumors excised 2 h after drug injection.

The combination of BCNU and LND also did not show any statistically significant enhancement from survival after BCNU only (P > 0.05).

The dose-response curves of LND-5-FUra are shown in Fig. 4. The combination treatment produced a 0.5- to 1-log increase of survival at high doses of 5-FUra, as compared to 5-FUra alone. These results suggested an antagonistic interaction between 5-FUra and LND. The protection was independent of scheduling.

DISCUSSION

We used two assays, the tumor growth delay and the in vivo/in vitro clonogenic assay, to evaluate the effect of lonidamine on antitumor activity of drugs and hyperthermia. Lonidamine at 50 mg/kg for up to 5 days showed little or no activity against RIF-1 tumor; when combined with other drugs, the effects of lonidamine were both drug and schedule dependent. Among agents tested in our present study, cis-platinum and mitomycin C yielded positive results in combination with lonidamine. The two assays produced essentially the same results, showing that antitumor activity of CDDP and MMC reflected direct cell killing. The combination of lonidamine with BLM, BCNU, or 5-FUra did not show any significant enhancement over activity of the drugs alone. In fact, LND protected RIF-1 tumor cells against 5-FUra and perhaps against BLM cytotoxicities. These data suggest that one should carefully choose the agents which will be used to combine with lonidamine in multimodality therapy in clinical situations.

Cis-platinum and mitomycin C are two important cytotoxic drugs in the treatment of a variety of human cancers. Based on the independent observations that (a) CDDP and MMC are the two optimum drugs to combine with lonidamine; (b) hyperthermia enhances the cytotoxicity of CDDP and MMC (12-14); and (c) lonidamine is a hyperthermic sensitizer (6), we studied in detail the possibility of combination of CDDP or MMC with LND and HT. Our data clearly indicate that either lonidamine or hyperthermia was able to enhance the cytotoxicity of these drugs and prolonged the tumor regrowth time (P < 0.01, versus each drug alone). However, the trimodality therapies of CDDP (or MMC), LND, and HT were no better than that of individual bimodality treatments of CDDP (or MMC) plus LND or HT. We also found that the extent of lonidamine-induced enhancement was dependent both on sequencing of its administration and on doses of drugs, but was
COMBINATION THERAPY OF LND, HT, AND DRUGS

Fig. 2. Effect of treatment with 50 mg/kg LND (L), 7.5 mg/kg CDDP (P), or 43°C 30min HT (H) alone, or in combination on the body weight of tumor-bearing C3H mice. L1, one dose of LND; L5, 50 mg/kg LND daily for 5 days. Data from a representative experiment.

Fig. 3. Survival of RIF-1 tumor cells treated in vivo with schedules of MMC-LND combination. MMC alone (O); MMC+LND (O); LND→MMC (O); LND→MMC+LND (O). The survival of LND alone treatment was about 75–80% and 45% for 2-h and 24-h experiments, respectively. Bars, SEM.

Table 3 Effect of single and combined treatments of LND, MMC, and HT on tumor growth delay (TGD) and clonogenic cells in RIF-1 tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>TGD (days)</th>
<th>Clonogenic cells/tumor</th>
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</thead>
<tbody>
<tr>
<td>LND₄₅ alone</td>
<td>6</td>
<td>0.1 ± 0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>HT alone</td>
<td>6</td>
<td>1.1 ± 0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>MMC alone</td>
<td>6</td>
<td>3.1 ± 0.8</td>
<td>0.06</td>
</tr>
<tr>
<td>HT+LND₄₅</td>
<td>6</td>
<td>1.3 ± 0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>MMC+LND₄₅</td>
<td>8 (2)</td>
<td>7.1 ± 1.7</td>
<td>0.007</td>
</tr>
<tr>
<td>MMC+HT</td>
<td>8</td>
<td>4.8 ± 1.0</td>
<td>0.02</td>
</tr>
<tr>
<td>MMC+HT+LND₄₅</td>
<td>8</td>
<td>7.7 ± 3.2</td>
<td>0.003</td>
</tr>
<tr>
<td>MMC+HT+LND₄₅</td>
<td>8</td>
<td>8.9 ± 2.6</td>
<td>0.005</td>
</tr>
<tr>
<td>LND₄₅+MMC+HT+LND₄₅</td>
<td>8</td>
<td>5.3 ± 1.8</td>
<td>0.009</td>
</tr>
</tbody>
</table>

* LND (50 mg/kg) was given once daily on Day 0 to Day 4; MMC (4 mg/kg) and HT (4°C 30 min in water bath) were given once on Day 0.
* Untreated control tumors reached 4 times their original volume in 7.7 ± 1.7 days mean ± SE.
* The relative number of clonogenic cells per tumor was obtained with the in vivo/in vitro clonogenic assay on Day 5 and is expressed relative to the number of clonogenic cells in untreated control tumors on Day 1 (6.9 ± 0.2 × 10⁶).
* Numbers in parentheses, dead mice/treatment. These are excluded from calculation of TGD. All deaths occurred within 7 days after the initial treatment.
* LND was given once on Day 0.
* LND was given once daily for 10 days (from Day 0 to Day 4).

not dependent on the duration of LND administration. For example, the maximum enhancement was obtained when lonidamine was given during and immediately following CDDP or MMC delivery; and this enhancement increased with increasing the doses of CDDP or MMC. No significant difference was seen in the extent of enhancement produced by 1-, 5-, or 10-day administrations of lonidamine. These in vivo results are consistent with our previous observations (10) and other reports in in vitro systems (15). Although lonidamine, by itself, is relatively nontoxic, it increased the toxicity of CDDP to host mice, or perhaps reduced the tolerance of host mice to CDDP toxicity, especially in trimodality treatment. Altogether, our data suggest that short-term (1 dose) delivery of this agent may avoid aggravating the side effects and yield the best enhancement ratio in combination treatment.

The mechanisms by which LND potentiates the anticancer activity of CDDP and MMC are unclear. Our data reported here suggest that lonidamine has similar mechanisms as hyperthermia does, most likely inhibition of repair of DNA damage. Both CDDP and MMC have been reported to produce DNA

5913

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cross-links, which may be the molecular injury associated with cell killing (16, 17). In the presence of lonidamine or under the hyperthermic condition, the repair process for DNA damage produced by CDDP or MMC would be prolonged or inhibited, because the repair of DNA damage is an energy-requiring process, and LND is reported both to interfere with the energy metabolism (1–3) and to reduce the capacity of target tumor cells to synthesize ATP (18).

In conclusion, the potentiation by LND varies from drug to drug. It potentiates the response of RIF-1 tumors to CDDP and MMC in a sequence-specific manner. Considering side effects, LND should be given for a short period. Predelivery of LND is not advantageous. Trimodality therapy of LND, HT, and CDDP (or MMC) is no better than that of individual bimodality treatments. These data may serve as a rationale for designing clinical trials of LND in multidrug therapies that include either CDDP or MMC as a component of the treatments.

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