Cytotoxicity of Lonidamine Alone and in Combination with Other Drugs against Murine RIF-1 and Human HT1080 Cells in Vitro

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ABSTRACT

Lonidamine is an agent that is reported to inhibit recovery from potentially lethal damage. By itself, it has only mild anticancer activity. We have examined the ability of lonidamine to enhance the cytotoxicity of several drugs against a mouse and a human fibrosarcoma cell line in vitro. By itself, lonidamine showed only a limited cytotoxic effect with drug exposure up to 100 µg/ml and 24-h duration. Lower concentrations and shorter term exposures were not toxic to either of these tumor cell lines. When tested against the mouse line, the cytotoxicity of 5-fluorouracil, methotrexate, and etoposide was enhanced by lonidamine if the latter drug was given either before or after the exposure of the cells to the cytotoxic agents. For cisplatinum, bleomycin, mitomycin C, doxorubicin, and Actinomycin D, cytotoxicity was also enhanced, but only if lonidamine followed the other agents. In contrast, potentiation of 1,3-bis(2-chloroethyl)-1-nitrosourea toxicity was maximum when lonidamine preceded the nitrosourea. The human cells were more resistant to lonidamine and to the combination treatments than were the mouse cells. Nevertheless, substantial enhancement was seen particularly for cisplatin and mitomycin C. We examined in more detail the enhancement of cisplatin. Maximum interaction was obtained when lonidamine was given immediately following (or in conjunction with) the platinum agent. Our results suggest that lonidamine enhances the effects of several other agents in a time- and concentration-dependent manner and indicate a potential usefulness for lonidamine in multidrug therapy.

INTRODUCTION

Lonidamine is an interesting drug. It is said to be an antimitochondrial agent and has been found to possess antisupermetagonetic, embryotoxic, and modest anticancer properties (1–3). The mode of action of lonidamine as an anticancer agent is said to be via interference in energy metabolism (4, 5). Its range of side effects also appears to be different from that of most other drugs. When used in conjunction with radiation therapy or hyperthermia, it enhances the effectiveness of the X-irradiation or heat, perhaps by interfering with repair processes (6–8). Lonidamine enhances effectiveness of X-irradiation against both proliferating and nonproliferating cells (6, 8), another feature that makes it an attractive candidate for combined therapy.

The possible ability of lonidamine to enhance drug cytotoxicity has only been studied in a few experiments and with a small number of drugs (9, 10). From these studies, none of which were systematic, it appears that potentiation of lonidamine activity is dose and schedule dependent and varies from cell line to cell line. The lack of systematic data caused us to undertake a study to assess the combined activity of lonidamine and several other chemotherapeutic agents against a mouse and a human fibrosarcoma line. Because, in general, the plasma levels of lonidamine stay high for long periods of time, while those of most other drugs decay rapidly, we combined long term (24-h) exposures of cells to lonidamine with short term (1-h) exposures to other drugs.

MATERIALS AND METHODS

Cell Lines. All experiments were performed with RIF-1 and HT1080 cells. RIF-1 cells were originally derived from a radiation-induced fibrosarcoma (11) and have been widely used for the study of radiation, hyperthermia, and chemotherapy in vivo and in vitro. The cells were grown as a monolayer in RPMI 1640 medium supplemented with 15% fetal calf serum. Plating efficiencies were 60–70%.

HT1080 cells, a human fibrosarcoma line obtained from the American Type Culture Collection, were grown as a monolayer in α-medium supplemented with 10% calf serum and 0.1% RNA. Plating efficiencies were also in the 60–70% range.

The cultures were equilibrated at 37°C in a humified incubator with a mixture of 95% air and 5% CO2 and were routinely checked for Mycoplasma. For all experiments presented here, cells were used 3 days after plating of 2 x 10⁵ cells in a 60-mm Petri dish, when the cells were in asynchronous exponential growth.

Drugs. LND, obtained from F. Angelini Research Institute of Rome, was dissolved in DMSO immediately before the experiments (10 mg LND in 1 ml DMSO) and diluted to the desired concentrations with fresh medium. Control experiments with DMSO (maximum concentration, 1.5%) alone (without LND) were always done. The results show that DMSO as a solvent alone, at the doses and exposure times used in this study, was not cytotoxic against the RIF-1 and HT1080 cells, nor did it enhance the activity of other drugs.

CDDP, BLM, BCNU, MMC (Bristol-Myers, Evansville, IN), 5-FU (Solo Pak Lab, Franklin Park, IL), MTX (Lederle Parenterals Inc., Corolina, Puerto Rico), ACD (MDM, West Point, PA), Amph.B (Squibb & Sons Inc., Princeton, NJ), and DOX (Farmitalia Carlo Erba S.P.A., Milan, Italy) were purchased from the Stanford hospital pharmacy. VP-16 was obtained from the Division of Cancer Treatment, National Cancer Institute. All drugs, except BCNU, were dissolved in 0.9% sodium chloride and protected from light. BCNU was dissolved in ethanol and was also protected from light. Appropriate drug concentrations were made by dilution with fresh medium immediately before each experiment.

Survival Assays. Cell survival was assayed by Puck's cloning technique. Following the final drug exposure, monolayers cells were rinsed 3 times with phosphate-buffered saline, trypsinized, counted, and plated into 60-mm tissue culture dishes containing fresh medium, after appropriate dilutions had been made. The dishes were incubated at 37°C for 10 days and then stained with crystal violet. Only colonies containing more than 50 cells were counted. Whenever possible, dishes containing 50–200 colonies were used to calculate surviving fractions. All experiments were performed at least twice. Error bars represent the standard error of at least two replicate experiments.

RESULTS

LND Toxicity against RIF-1 and HT1080 Cells. To observe the toxicity of LND alone on RIF-1 and HT1080 cells, exponentially growing cells were exposed to graded concentrations of LND for 1 or 24 h at 37°C. Fig. 1 shows the concentrations...
and time-response effects of LND. One-h short term exposure to LND showed no killing of either RIF-1 or HT1080 cells over the concentrations examined. Twenty-four-hour exposures showed that the toxicity of the drug increased with increasing drug concentration. RIF-1 cells were more sensitive to LND than were HT1080 cells.

Potentiation by LND of Drug Cytotoxicity. Preliminary experiments were carried out to determine the effects of varying experimentally. We made no attempt to obtain complete survival concentrations and sequences of LND in combination with other drugs on cell survival. The concentration of each agent was normalized to the surviving fraction for each drug (Table 1) alone, in order to illustrate directly the effect of LND. The results of experiments on RIF-1 cells (Figs. 2A and 3, left), show that maximal potentiation of LND was seen when the highest concentration of LND (150 μg/ml) was used. Also, sequencing was important for MMC, BLM, DOX, CDDP, ACD, and BCNU. The cytotoxicities of MMC, BLM, DOX, CDDP, and ACD were significantly enhanced by LND in the sequence drug→LND. The reverse sequence did not enhance cytotoxicity; in fact, for ACD and possibly DOX, the earlier LND exposure may have actually protected the cells. The cytotoxicity of BCNU was only enhanced for the LND→BCNU protocol. 5-FU, MTX, and VP-16 were potentiated by high concentrations (100–150 μg/ml) of LND, regardless of what exposure sequence was used. Finally, the effect of Amph.B on RIF-1 cells was not enhanced by LND in either sequence.

We then tested LND effects on human HT1080 cells (Figs. 2B and 3, right). Qualitatively, the results were consistent with those found with RIF-1 cells. Quantitatively, however, the HT1080 cells were appreciably more resistant to all of the drugs used than were the RIF-1 cells. The equitoxic concentrations of LND and other drugs used on HT1080 cells needed to be 2–4-fold higher than those required for RIF-1 cells.

Sequencing Effect of LND and CDDP. Because LND and CDDP showed appreciable interaction, we studied the results of sequencing of LND and CDDP in more detail. Fig. 4 shows the interaction between LND and CDDP as a function of the interval between exposures to the two agents. The left part of each panel depicts data from experiments in which LND preceded CDDP. Here the time shown on the abscissa indicates the number of hours between the end of the 24-h LND exposure and the beginning of the 1-h CDDP pulse. The right part of the panels represents experiments where the order was reversed, and the time shown here is the interval between the end of the CDDP pulse and the beginning of the LND exposure. Note that the dose used was 1 μg/ml for RIF-1 cells (Fig. 4A) but 4 μg/ml for HT1080 cells (Fig. 4B), and yet survival values were quite similar. Survival after exposure to the individual drugs is shown on the left ordinate of each panel (see also Table 1). Several observations are of interest. First, it appears that the sequence LND→CDDP (left part of each panel) results in little or no interaction; survival was similar to that predicted based upon additive cell killing. This was true even when CDDP followed immediately after the LND exposure. Second, maximum cytotoxicity occurred when the cells were exposed to LND immediately after CDDP exposure; the interaction disappeared if 8 h (RIF-1) or 4 h (HT1080) or more separated the two drug exposures. Third, except for minor quantitative differences, the pattern of interaction was similar for the rodent and human cells.

Fig. 5 shows complete dose-response curves of RIF-1 and HT1080 cells to CDDP with or without LND (100 μg/ml). The sequences tested were CDDP→LND, LND→CDDP, and CDDP+LND. CDDP→LND and LND→CDDP have their defined meaning; the sequence CDDP+LND describes an experiment in which cells were exposed simultaneously to CDDP and LND for 1 h and the medium was then replaced with fresh medium containing only LND and was so maintained for 23 h. The LND→CDDP sequence did not enhance CDDP cytotoxicity, consistent with earlier results. The reverse sequence, however, showed considerable enhancement, with the sequence CDDP+LND leading to intermediate levels of cell killing.

**DISCUSSION**

We examined the interaction of LND with conventional anticancer agents for two reasons. First, the mechanism of action of LND is different from that of other anticancer agents. LND is said to interfere with energy metabolism, perhaps via damage to mitochondrial or other membranes (12, 13). Thus,
Fig. 2. Sequence and concentration effects of LND in combination with other drugs on RIF-1 (A) and HT1080 (B) cells. Cells were exposed to LND for 24 h and to the other drugs for 1 h. Exposure to the test drugs directly preceded (or followed) the LND treatment. Concentrations: LND, 50 (C), 100 ( ), and 150 (O) μg/ml; ACD, 2.5 μg/ml; DOX, 2.0 μg/ml; Amph.B, 10 μg/ml; BCNU, 50 μg/ml; BLM, 50 μg/ml; 5-FU, 1000 μg/ml; MMC, 1.0 μg/ml; MTX, 20 μg/ml; and VP-16, 20 μg/ml. Points, geometric mean of survivals determined in 2 or 3 independent experiments; SEs are small (Table 1) and not shown in order to simplify the figure.
Our results show that LND is a chemopotentiation agent as well as a (modestly) cytotoxic drug. By itself, LND possesses weak activity, and that only at high concentrations (>100 \( \mu \text{g/ml} \)) and with long term (24-h) exposure. When LND is combined with other drugs, the combined cytotoxicity is, in general, a function of dosage, time, and sequence of the drug exposures. For the majority of the combinations tested, only the high dose of LND (150 \( \mu \text{g/ml} \); 100 \( \mu \text{g/ml} \) for a few drugs) enhanced cytotoxicity. Only for a few agents (5-FU, MTX, and VP-16) was the enhancement of cytotoxicity independent of sequencing. For BCNU maximum cytotoxicity was seen when LND preceded this agent, while for the remaining drugs optimum sequencing was reversed. The drugs showing the greatest interactions in both human and murine cells were CDDP and MMC. For both these agents, the sequence drug—LND resulted in cytotoxicity that was appreciably more than additive.

When we investigated the timing of LND and CDDP in detail, we found that cell killing was maximum when CDDP administration was immediately followed by LND. When LND was given at later times, the interaction was progressively reduced, consistent with the notion that LND interfered with a repair mechanism. The enhancement by LND of the cytotoxicity of 5-FU, MTX, and VP-16 suggests, however, that other mechanisms need to be considered as well.

In summary, our results show that LND can appreciably enhance the activity of some drugs against the murine and human test systems. Clinically, the most promising appears to be the combination of LND with CDDP or MMC in the sequence CDDP (or MMC)—»LND. We are currently testing these ideas using RIF-1 tumors in vivo.

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