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Effect of lonidamine on the cytotoxicity of four alkylating agents in vitro*

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Summary. We examined the ability of lonidamine, which has been described as an inhibitor of cellular respiration and glycolysis, to enhance the cytotoxicity of alkylating agents to MCF-7 human breast-carcinoma cells. Lonidamine was increasingly cytotoxic to MCF-7 cells with increasing time of exposure. With a 12-h exposure, the IC_{50} for lonidamine was about 365 μM, and with a 24-h exposure it was about 170 μM. A drug concentration of 250 μM was chosen for use in the drug combination studies. Lonidamine appeared to have a dose-modifying effect on cisplatin (CDDP), producing increasingly supraadditive cell kill with increasing CDDP concentration. When simultaneously incubated with lonidamine for 1 h, 500 μM CDDP yielded a cell kill that was 2 log greater than additive cytotoxicity. Extending the exposure to lonidamine for 12 h after CDDP treatment led to a small, additional aliquot of cell kill of about 2.5-fold over the CDDP concentration range. Lonidamine also appeared to have a dose-modifying effect on melphalan cytotoxicity in the melphalan concentration range of 100–500 μM. Between concentrations of 10 and 100 μM melphalan, the drug combination survival after 1 h exposure fell within the envelope of additivity for the two agents. However, maintaining the presence of lonidamine for an additional 12 h increased the effect such that the combination was supraadditive over the entire concentration range of melphalan. Simultaneous exposure to 4-hydroperoxycyclophosphamide (4-HC) and lonidamine for 1 h resulted in greater than additive cell kill, and extending the lonidamine exposure period such that lonidamine was present during and 12 h after 4-HC treatment further increased this effect. Lonidamine had a moderate effect on the cytotoxicity of carmustine (BCNU) with a 1 h simultaneous exposure; however, this treatment combination reached greater than additive cytotoxicity only at the highest concentration of BCNU tested. Extending the lonidamine exposure time for an additional 12 h resulted in supraadditive cell kill over the BCNU concentration range. Therefore, when lonidamine was present during exposure to the alkylating agent and its presence was then extended for an additional 12 h, a synergistic cell kill was produced with all four alkylating agents tested.

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Introduction

Lonidamine, 1-[(2,4-dichlorophenyl)methyl]-1H-indazol-3-carboxylic acid, affects the energy metabolism of cells [5, 6, 9–12]. In both normal and neoplastic cells, oxygen consumption is strongly inhibited by this drug; furthermore, in tumor cells, aerobic and anaerobic glycolysis are additionally affected [5, 9–12]. Based on these data, mitochondria have been considered the primary intracellular targets of the drug [5, 9–12]. Recent studies by DeMartino et al. [6] indicate that the plasma and mitochondrial membrane of cells are the primary targets of lonidamine. It may be that the inhibition by lonidamine of energy metabolism of cells is a consequence of structural damage to the inner and outer mitochondrial membranes, which leads to inhibition of respiration and glycolysis and, finally, loss of cell viability [6].

Lonidamine could be an important component of a combined modality regimen if repair of damage due to cytotoxic treatment is an energy-dependent process. Working with Chinese hamster HA-1 cells in culture, Hahn et al. [13] showed that at concentrations achievable in vivo, lonidamine inhibited the repair of potentially lethal damage caused by X-rays, methyl methane sulfonate, bleomycin, and hyperthermia. Kim et al. [14–16] showed that lonidamine potentiated the effects of radiation as well as hyperthermia [15] in murine tumor models. This drug has also been shown to enhance the cytotoxicity of Adriamycin in culture [23].

We are searching for drugs that albeit relatively noncytotoxic themselves, may positively and selectively modulate the cytotoxicity of alkylating agents in tumor cells. The present studies were undertaken as an initial step to examine the potential of lonidamine to enhance the cytotoxicity of four alkylating agents in vitro in MCF-7 human breast-carcinoma cells.

Materials and methods

Drugs. Lonidamine was obtained as a gift from DeSanctis Consultants (Montreal, Canada), prepared in 0.9% phosphate-buffered saline (PBS), and stored at −20° C. *cis*-Diamminedichloroplatinum(II) (CDDP; cisplatin) was a gift from Drs. Donald H. Picker and Michael J. Abrams (Johnson Matthey, Inc., West Chester, Pa) and was prepared in PBS and stored at −20° C. 4-Hydroperoxycyclophosphamide (4-HC) was a gift from Dr. Michael Colvin (Johns Hopkins University School of Medicine, Balti-

more, Md) and was prepared in PBS just prior to use. Melphalan (L-phenylalanine mustard) was purchased as a pure powder from Sigma Chemical Co. (St. Louis, Mo) and was dissolved in HCl-acidified ethanol and diluted with PBS just prior to use. Carmustine (BCNU) was purchased from the Dana-Farber Cancer Institute pharmacy; the lyophilized powder was resuspended in HCl-acidified ethanol, then diluted with PBS just prior to use.

Cell line. MCF-7 human breast-carcinoma cells grow as monolayers in Dulbecco's Modified Eagle's (DME) medium supplemented with antibiotics, L-glutamine, and 10% fetal bovine serum (FBS). These cell lines have a plating efficiency of 25%–40% and a doubling time of 32–36 h in vitro. For cloning, cells were suspended by trypsinization, diluted in complete growth medium, and plated onto 60 × 15-mm tissue-culture dishes containing 5 ml complete growth medium. Colonies grow to a countable size (> 50 cells) in 2 weeks [21].

Survival studies. MCF-7 cells in exponential growth were exposed for 1, 12, or 24 h to concentrations of lonidamine ranging from 10 to 1,000 μ*M* in DME media with sera (pH 7.4). Combination studies of lonidamine and CDDP, 4-HC, melphalan, or BCNU were carried out for 1 h at drug concentrations ranging from 10 to 500 μ*M*; in addition, cells were exposed for 1 h to 250 μ*M* lonidamine alone or for 1 h to 250 μ*M* lonidamine during alkylating agent exposure as well as for an additional 12 h or 24 h thereafter in DME containing 10% FBS. The cells were then washed with PBS and plated for colony formation as described above. Each survival curve was determined in three independent experiments.

Data analysis. Using the method of Deen and Williams [4], isobolograms were generated for the special case in which the dose of one agent is held constant. This method produces envelopes of additive effect of different levels of the variable agent; it is conceptually identical to generating a series of isobolograms and replotting the results at a constant dose of one agent on a log effect by dose of the second-agent coordinate system. Dose-response curves for each agent alone were first generated. The envelopes of additivity shown in the figures were generated from a series of isoeffect curves derived from the complete dose-response curves for each agent alone. Overall, combinations that produce the desired effect and are within the envelope boundaries of mode I and mode II are considered additive. Those displaced to the left are supraadditive, and those displaced to the right are subadditive [2, 20]. This general approach can be extrapolated to the special case in which the level of an agent is held constant. Under these conditions, an isobologram can be derived that plots the expected effect (mode I and mode II) for any level of the variable agent plus the agent combinations [7]. Experimentally, this approach is far simpler and readily facilitates the determination of additive and nonadditive combinations.

To facilitate these analyses, a flexible, interactive computer program in BASIC was written for the Apple II+ microcomputer. The program first derives the best-fitting dose-response curves using dose or log dose, effect, log effect, probit percent effect, or logit percent effect relations. For cell-survival dose-response curves, correlations of ≥ 0.96 have been obtained. The program then calculates an isobologram at a constant level of the selected agent and plots the data [21].

Results

Lonidamine was increasingly cytotoxic to the MCF-7 cells with increasing time of exposure (Fig. 1). With a 1-h exposure up to a concentration of 1 m*M* lonidamine, < 50% of the cells were killed. The IC_{50} for lonidamine with a 12-h exposure was about 365 μ*M*, and with a 24-h exposure it was about 170 μ*M*. A lonidamine concentration of 250 μ*M* was used for all of the drug combination studies.

CDDP was increasingly cytotoxic to MCF-7 cells with increasing drug concentration (Fig. 2). About 1 log MCF-7 cells were killed by 100 μ*M* CDDP, and about 2.5 log MCF-7 cells were killed by 500 μ*M* CDDP. The envelope of additivity for CDDP and 250 μ*M* lonidamine with simultaneous exposure or simultaneous exposure plus 12 h lonidamine is also shown (*shaded area*). Simultaneous exposure to CDDP and lonidamine had greater than additive cell killing over the entire CDDP concentration range. Lonidamine appeared to have a dose-modifying effect on CDDP, producing increasing supraadditivity with increasing CDDP concentration, which reached a maximum of 2-log greater than additive cell kill at 500 μ*M* CDDP. Extending the exposure to lonidamine for 12 h after CDDP treatment led to a small, additional aliquot of cell kill of about 2.5-fold over the CDDP concentration range.

4-Hydroperoxycyclophosphamide (4-HC) was used as an activated analog of cyclophosphamide for these in vitro studies (Fig. 3) [21]. 4-HC killed 1 log MCF-7 cells at a 60-μ*M* concentration in 1 h and 3 log MCF-7 cells at a 500 μ*M* concentration in 1 h. The *shaded area* (Fig. 3) shows the envelope of additivity for the simultaneous exposure of MCF-7 cells to 4-HC and 250 μ*M* lonidamine for 1 h or 4-HC and 250 μ*M* lonidamine followed by an additional 12-h exposure to 250 μ*M* lonidamine. Simultaneous exposure to 4-HC and lonidamine resulted in greater than additive cell kill over the 4-HC concentration range examined. The additional level of cell kill increased

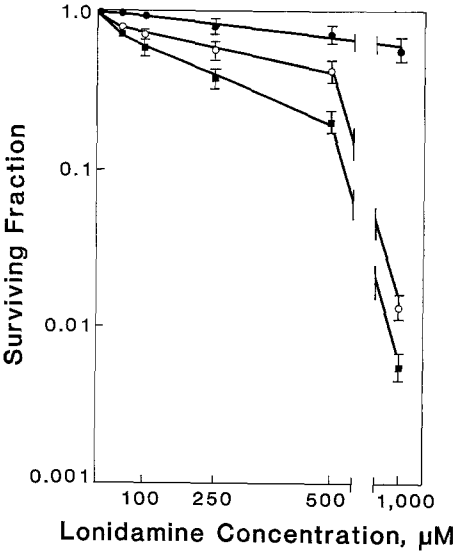

Fig. 1. Survival of human MCF-7 breast carcinoma cells exposed to various concentrations of lonidamine for 1 (●), 12 (○), or 24 h (■). *Points* represent the means of three independent experiments and *bars*, the SEM

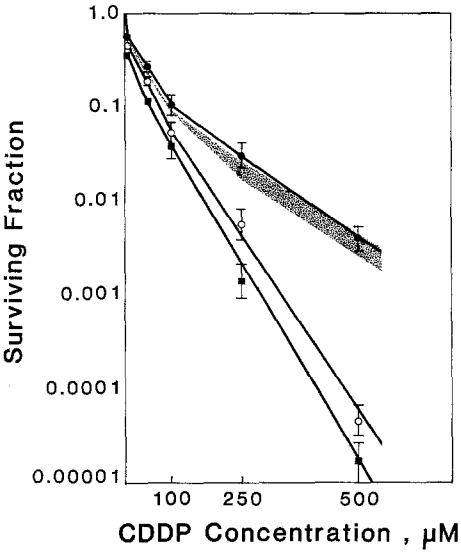

Fig. 2. Survival of human MCF-7 breast carcinoma cells exposed to various concentrations of CDDP alone for 1 h (●), with simultaneous exposure to 250 μ*M* lonidamine for 1 h (○), or with simultaneous exposure to 250 μ*M* lonidamine for 1 h followed by an additional 12-h exposure to lonidamine. The *shaded area* is the envelope of additivity for CDDP plus either 1 h or extended exposure to lonidamine. *Points* represent the means of three independent experiments and *bars*, the SEM

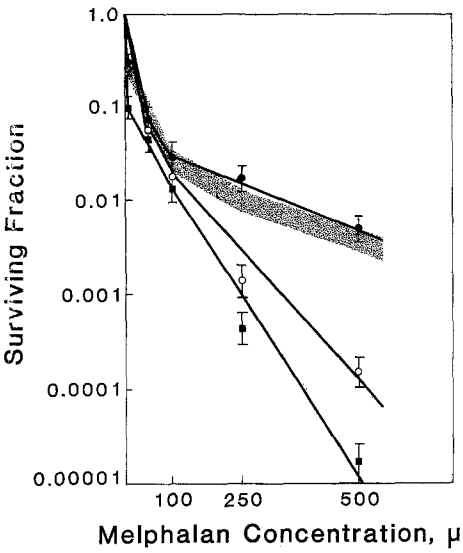

Fig. 4. Survival of human MCF-7 breast carcinoma cells exposed to various concentrations of melphalan alone for 1 h (●), with simultaneous exposure to 250 μ*M* lonidamine for 1 h (○), or with simultaneous exposure to 250 μ*M* lonidamine for 1 h followed by an additional 12-h exposure to lonidamine. The *shaded area* is the envelope of additivity for melphalan plus either 1 h or extended exposure to lonidamine. *Points* represent the means of three independent experiments and *bars*, the SEM

with increasing 4-HC concentration to a maximum of 1 log with 500 μ*M* 4-HC. Extending the lonidamine exposure period such that lonidamine was present during and 12 h after 4-HC treatment enhanced 4-HC cytotoxicity: at a concentration of 500 μ*M* 4-HC, >5 log MCF-7 cells were killed, compared with a 3-log cell kill obtained with 4-HC alone.

The survival of MCF-7 cells exposed to various concentrations of melphalan is shown in Fig. 4. The IC_{90} for melphalan with a 1-h exposure in these cells was about 45 μ*M*. There was an approximately 2.5-log cell kill with a 500-μ*M* concentration of melphalan. The *shaded area* (Fig. 4) indicates the envelope of additivity for a 1-h exposure to various concentrations of melphalan and simulta-

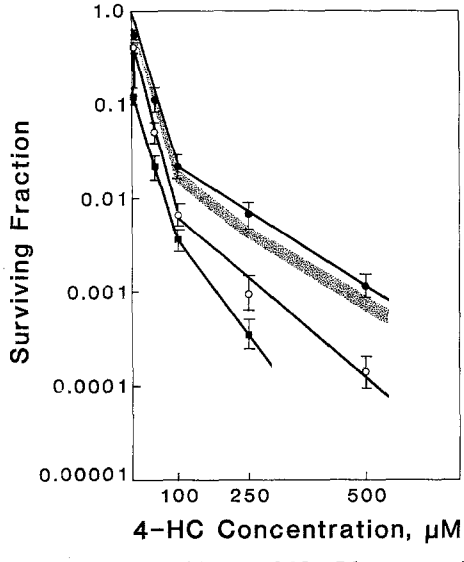

Fig. 3. Survival of human MCF-7 breast carcinoma cells exposed to various concentrations of 4-HC alone for 1 h (●), with simultaneous exposure to 250 μ*M* lonidamine for 1 h (○), or with simultaneous exposure to 250 μ*M* lonidamine for 1 h followed by an additional 12-h exposure to lonidamine. The *shaded area* is the envelope of additivity for 4-HC plus either 1 h or extended exposure to lonidamine. *Points* represent the means of three independent experiments and *bars*, the SEM

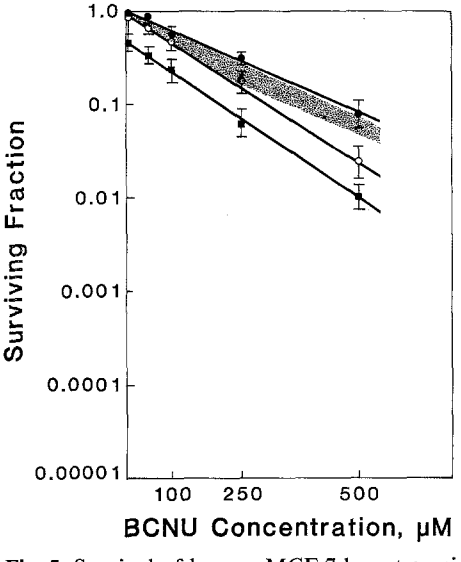

Fig. 5. Survival of human MCF-7 breast carcinoma cells exposed to various concentrations of BCNU alone for 1 h (●), with simultaneous exposure to 250 μ*M* lonidamine for 1 h (○), or with simultaneous exposure to 250 μ*M* lonidamine for 1 h followed by an additional 12-h exposure to lonidamine. The *shaded area* is the envelope of additivity for BCNU plus either 1 h or extended exposure to lonidamine. *Points* represent the means of three independent experiments and *bars*, the SEM

neous exposure to 250 μM lonidamine or for simultaneous and extended exposure to lonidamine for an additional 12 h. Lonidamine appeared to have a dose-modifying effect on melphalan cytotoxicity in the concentration range from 100 to 500 μM. Between 10 and 100 μM, the drug combination survival fell within the envelope of additivity. Extending the lonidamine exposure time for an additional 12 h beyond the 1-h exposure to melphalan and lonidamine increased the effect of lonidamine on the cytotoxicity of melphalan, such that the combination was supra-additive over the entire concentration range of melphalan. With a 1-h simultaneous exposure at a 500-μM concentration of melphalan, there was a 1.5-log greater than additive cell kill; with extended exposure to lonidamine, this increased to 2.5-log greater than additive cell kill.

Of the four alkylating agents examined, BCNU was the least cytotoxic to MCF-7 cells (Fig. 5). Lonidamine (250 μM) had a moderate effect on the cytotoxicity of BCNU with a 1-h simultaneous exposure; however, this treatment combination reached greater than additive cytotoxicity only at the highest concentration of BCNU tested. Extending the lonidamine exposure time for an additional 12 h beyond the simultaneous exposure resulted in an additional aliquot of cell kill over the BCNU concentration range. With the extended lonidamine exposure in combination with BCNU, greater than additive cell kill was obtained over the entire BCNU concentration range.

Discussion

Agents that are relatively nontoxic but can modulate the cytotoxicity of bifunctional alkylating drugs in tumors could be very useful in combination chemotherapeutic regimens for the treatment of clinical neoplastic diseases. Modulators of alkylating agent cytotoxicity include: (a) the topoisomerase II inhibitors such as etoposide, VM-26, and novobiocin; (b) the nitroimidazole radiosensitizers such as misonidazole and etanidazole; (c) agents that inhibit or prevent DNA repair, such as 3-amino benamide and pentoxyphylline; and (d) agents that interfere with cellular energy production, such as rhodamine-123 and lonidamine. Most of these drugs, even those that are highly cytotoxic in their own right, have been shown to be highly effective additions to other single treatments and combinations. Lonidamine has undergone phase II testing in clinical trials [1, 8, 18, 22] and has shown promising results in preliminary reports of clinical trials in combination with radiation therapy [17] and hyperthermia [3]. There has been limited work with lonidamine and combination chemotherapy in clinical treatment [19].

To varying degrees, lonidamine enhanced the cytotoxicity of all four of the alkylating agents tested here to MCF-7 human breast-carcinoma cells in culture. The greatest enhancements were seen with CDDP and melphalan, for both of which lonidamine was dose-modifying, i.e., the degree of enhancement increased markedly with increasing alkylating agent levels. The enhancement of 4-HC as well as BCNU was greater than additive with extended exposure to lonidamine. This may be indicative of the inhibition of the repair of DNA cross-links and/or strand breaks by these drugs. The extension of these studies to in vivo preclinical tumor models, with positive results, could lead to very promising enhancement of the antitumor effects of alkylating agents in the clinic.

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