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Efficacy of lonidamine combined with different DNA-damaging agents in the treatment of the MX-1 tumor xenograft

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Abstract Lonidamine is an antitumor agent with a peculiar mechanism of action, since it differentially impairs the energy metabolism of normal and neoplastic cells. We investigated the effects of lonidamine on the activity of DNA-damaging antitumor agents against the MX-1 human breast carcinoma xenograft. Athymic mice bearing measurable s.c. tumors were treated by a single injection of doxorubicin (i.v.), cyclophosphamide (i.v.), or cisplatin (i.p.) followed by repeated daily injections of lonidamine (i.p. or p.o.). A potentiation of the activity of all these DNA-damaging drugs was achieved when each was given in combination with lonidamine, but for doxorubicin and cyclophosphamide the increase in antitumor activity paralleled the increase in lethal toxicity. In contrast, a therapeutic advantage of the combination was achieved for cisplatin and lonidamine as compared with cisplatin alone. Indeed, 6 mg/kg of cisplatin plus lonidamine cured all tumors, whereas the maximum tolerated dose of cisplatin alone (12 mg/kg) cured only six of eight tumors. In addition, the study indicated that the duration of lonidamine administration after injection of the cytotoxic drug influenced the tumor response and that prolonged treatment resulted in greater efficacy. These results document the ability of lonidamine to modulate the pharmacological activity of DNA-damaging drugs, thus suggesting that lonidamine may be a clinically useful cisplatin modulator.

Key words Lonidamine · Cisplatin · Doxorubicin · Cyclophosphamide · Human tumor xenograft

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Introduction

The antitumor action of lonidamine (LND), a dichlorinated derivative of indazole-3-carboxylic acid, has been ascribed to its effects on energy metabolism [29]. The drug is known to inhibit oxygen consumption in malignant and normal cells but to inhibit aerobic glycolysis only in tumor cells [12, 14, 25]. This selective action of LND may be attributed to its inhibitory effect on mitochondria-bound hexokinase, which is present in tumor cells but not in normal differentiated cells [13, 17]. A study on human tumor xenografts showed that LND inhibited the tumor growth of advanced gliomas only when mitochondrial hexokinase activity accounted for more than 30% of the total activity [26]. Further studies have demonstrated that LND may affect cell membrane structure [20, 21], ion flux [11], electrical properties [28], as well as cell shape and morphology [22].

When used as a single agent, LND showed in preclinical studies a marginal activity against most tumors tested [5]. However, LND is a very promising drug for combination studies with DNA-damaging antitumor agents, since repair of damage is an energy-dependent process. Its capability to inhibit the recovery from potentially lethal damage induced by X-rays or drugs has been described elsewhere [16]. Many preclinical studies in different tumor cell lines have shown that LND usually increases the cytotoxic efficacy of different antitumor drugs [6, 27, 32, 34, 35]. In vitro and in vivo studies have shown the importance of timing in the combination of LND and other treatment modalities, and the results indicate that LND is generally more effective when given after the therapeutic agent [8, 32]. However, the optimal schedule and timing in combination treatment as well as the importance of LND treatment duration remain to be identified. In a recent review, Teicher [31] questioned the predictivity for clinical use (in which LND is given chronically

for a long time) of the few preclinical studies in vivo where LND was given concurrently or shortly after the cytotoxic therapy.

The aims of the present study were to investigate in a human tumor xenograft the interaction of LND with DNA-interacting antitumor drugs (cisplatin, cyclophosphamide, or doxorubicin) and the influence of the timing of LND treatment. The MX-1 mammary carcinoma was chosen for its differential sensitivity to the three drugs employed in the study (i.e., hypersensitivity to alkylating agents and resistance to doxorubicin). Moreover, the study examined in an in vivo system whether LND induced a true therapeutic advantage, i.e., a tumor response greater than the highest achievable by the drug alone at maximum tolerated doses (MTD), since such information is not revealed by in vitro studies. The results of our study showed a different modulation of therapeutic efficacy in relation to the duration of LND treatment and a synergistic interaction for all three drugs tested and demonstrated that only the combination of LND and cisplatin afforded a therapeutic advantage as compared with the drug alone.

Materials and methods

Animals

Female athymic Swiss nude mice aged 6–10 weeks were employed in this study. The animals, obtained from Charles River Italia (Calco, Italy), were maintained in laminar air-flow rooms. Sterilized cages, bedding and acidified water were used for mouse care. The air was conditioned at a temperature of 24–26° C and at 50% humidity. The experiments were approved by the Ethics Committee of our institute according to the UKCCCR guidelines [33].

Tumor line

The human breast MX-1 tumor line, from an infiltrating duct-cell carcinoma of an untreated patient, was established in nude mice in the National Cancer Institute (Bethesda, Md., USA). The original MX-1 tumor was estrogen receptor-negative and marginally positive for progesterone receptors [18]. For line maintenance and experimental purposes, tumor specimens were grafted s.c. into both flanks of athymic mice by a 13-gauge trocar. Growth of s.c. tumors was followed by biweekly caliper measurements of tumor length and width. The tumor volume (TV) was calculated in cubic millimeters using the formula $TV = width^2 \times length/2$ according to Geran et al. [15]. The mean doubling time (DT) of the tumor line was evaluated from the semilogarithmic best-fit curve of each control tumor plotted versus time from the day on which tumors became measurable to the day on which the curves began to level off. In the different experiments of this study, the mean DTs were 6–8 days. Histological examination of the tumor line was routinely performed by hematoxylin and eosin staining, and the original histology was well maintained. The pattern of human lactic dehydrogenase could be detected persistently in the tumor extracts.

Drugs

Cisplatin and doxorubicin (kindly supplied by Pharmacia, Milan, Italy) were dissolved in saline and in sterile distilled water, respectively. Cyclophosphamide (clinical preparation, Asta, Frankfurt, Germany) was dissolved in sterile distilled water and then diluted in saline. LND (kindly supplied by Angelini, Rome, Italy) was dissolved in 2.3% *N*-methyl-D-glucamine (NMG) water solution; 10 mg of LND was previously dissolved in 0.31 ml of the NMG solution and then diluted in sterile distilled water to the desired concentration. All drugs were given in a volume of 10 ml/kg body weight.

Chemotherapy studies

Chemotherapy studies were carried out in mice bearing s.c. tumors transplanted in both flanks, and each experimental group consisted of at least eight evaluable tumors. Treatment in the different experiments started when the tumors had reached a mean volume of 150–250 mm^3. Single injections of different doses of the cytotoxic drugs were given via different routes: cisplatin, i.p.; and doxorubicin and cyclophosphamide, i.v. LND was given i.p. or orally daily for different periods starting immediately after the DNA-acting drugs. In all experiments, control mice were treated i.p. or orally with NMG solution. Drug activity was evaluated as (1) the percentage of TV inhibition (TVI%) occuring at, 7–8 days after the last LND treatment, calculated by the formula $100\text{-}(T^*C \times 100)$, where T is the mean TV of treated tumors and C is that of control tumors; and (2) the log cell kill (LCK), calculated by assessing the difference in the mean time required for tumors of treated and control mice to reach 1000 mm^3 and dividing that value by the product of 3.32 and the median DT [9].

The body weight of mice was measured at the beginning of and during each experiment, and in general a weight loss of greater than 10% was not observed (see Table 4). Death occuring in treated mice before the death of the first control mouse was ascribed to toxic effects.

Statistical evaluation

Student's *t*-test (two-tailed) was used for statistical comparison of tumor volumes in mice treated with the drug combination versus a single DNA-acting drug.

Results

LND given alone according to the same schedule employed in the combination studies (i.e., daily 10–20 times) did not affect tumor growth when given i.p. or p.o. The MTD was tested in intraperitoneal treatment studies (Table 1).

When i.p. LND was combined with doxorubicin, a DNA-intercalating topoisomerase II-inhibitory drug (Table 2), it increased the drug's efficacy (5 mg/kg; from 11% to 44% TVI). However, a higher dose of doxorubicin (7 mg/kg) alone achieved a similar activity (45% versus 44% TVI), and this dose plus LND was equally active and more toxic, therefore discouraging the use of higher doxorubicin doses on this schedule.

Cyclophosphamide alone was very active against the MX-1 tumor xenograft (Table 3). The combination of the alkylating drug and i.p. LND achieved a marked increase in the antitumor efficacy of a suboptimal dose of cyclophosphamide (100 mg/kg). The combination of a higher dose of cyclophosphamide (200 mg/kg) with

Table 1 Efficacy of LND given daily for 10–20 days on MX-1 mammary tumor xenografts

LND		Tox/Tot[a]	TVI%[b]	LCK[c]
mg/kg	Route			
50	i.p.	2/20	29[d]	0.2[d]
50	p.o.	0/5	0	0
100	p.o.	0/5	8	0

[a]Number of deaths for toxicity/total number of mice
[b]Tumor volume inhibition percent in treated/control mice
[c]Log cell kill (see Materials and methods for calculation)
[d]Mean value of five experiments (range 13–40% for TVI, 0.2–0.25 for LCK)

Table 2 Efficacy of doxorubicin given i.v. once ± LND given i.p. for 10 days on MX-1 mammary tumor xenografts

Doxorubicin (mg/kg)	LND (mg/kg)	Tox/Tot[a]	TVI%[b]	LCK[c]
–	50	1/5	13	0.2
5	–	0/5	11	0
5	50	1/5	44	0.4
7	–	0/5	45	0.4
7	50	2/5	49	0.4

[a]Number of deaths for toxicity/total number of mice
[b]Tumor volume inhibition percent in treated/control mice
[c]Log cell kill (see Materials and methods for calculation)

Table 3 Efficacy of cyclophosphamide given i.v. once ± LND given i.p. for 16 days on MX-1 mammary tumor xenografts

Cyclophosphamide (mg/kg)	LND (mg/kg)	Tox/Tot[a]	TVI%[b]	LCK[c]
–	50	1/5	30	0.2
100	–	0/5	76	0.7
100	50	0/5	89*	1.0
200	–	0/5	97**	2.0
200	50	4/5		

*$P < 0.005$ vs cyclophosphamide at 100 mg/kg (Student's *t*-test); **$P < 0.001$ vs cyclophosphamide at 100 mg/kg + LND (Student's *t*-test)
[a]Number of deaths for toxicity/total number of mice
[b]Tumor volume inhibition percent in treated/control mice
[c]Log cell kill (see Materials and methods for calculation)

LND caused an unacceptable increase in lethal toxicity. Thus, on this schedule, no therapeutic advantage was evident for the combination, since the alkylating agent itself produced a tumor growth inhibition superior to that achieved by the MTD of the combination.

LND was combined with cisplatin, a DNA-damaging agent that, like cyclophosphamide, was very active against the MX-1 tumor xenograft (Table 4). At a very low cisplatin dose, 4 mg/kg, tumor growth was marginally inhibited (42% and 53% TVI in two separate experiments); at this dose level, LND markedly increased cisplatin's efficacy when delivered i.p. or p.o.

Table 4 Efficacy of cisplatin given i.p. once ± LND, given for 20 days on MX-1 mammary tumor xenografts

Cisplatin (mg/kg)	LND		Max%[a] b.w. loss (day)	Tox/Tot[b]	TVI%[c]	LCK[d]
	mg/kg	Route				
–	50	i.p.		0/10	33–40	0.2–0.2
–	50	p.o.		0/5	0	0
–	100	p.o.		0/5	8	0
4	–		5 (7)	1/10	42–53	0.4–0.5
4	50	i.p.	6 (7)	1/5	88*	1.1
4	50	p.o.	9 (11)	0/5	68**	0.8
4	100	p.o.	12 (11)	0/4	71	0.9
6	–		0	0/5	99	3.4
6	50	i.p.	6 (7)	0/5	100	> 7.3
12	–		14 (7)	0/5	99	3.6
13	–		30 (6)	4/9	–	–

*$P < 0.01$ vs cisplatin alone (Student's *t*-test); **$P < 0.05$ vs cisplatin alone (Student's *t*-test)
[a]Maximal percentage of mouse body weight loss. In parentheses is shown the day (after cisplatin treatment) in which it was observed
[b]Number of deaths for toxicity/total number of mice
[c]Tumor volume inhibition percent in treated/control mice
[d]Log cell kill (see Materials and methods for calculation)

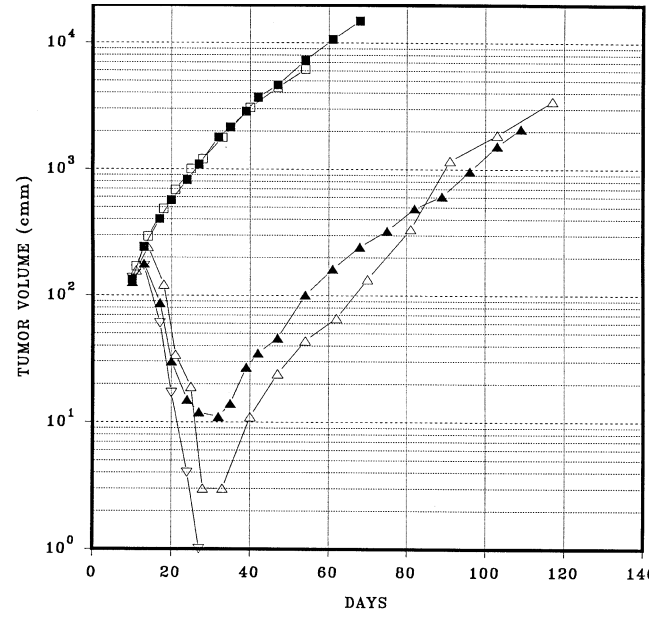

Fig. 1 Activity of cisplatin and LND on MX-1 human mammary carcinomas growing s.c. in female Swiss nu/nu mice. Mice were treated with i.p. cisplatin given at 6 (*black triangles*) or 12 (*white triangles*) mg/kg or with i.p. cisplatin given at 6 mg/kg + i.p. LND given at 50 mg/kg for 20 days (*inverted white triangles*). Data represent mean values for 8/9 tumors. (*white and black squares* solvent-treated controls)

When mice were treated with 6 mg/kg (half the MTD) of cisplatin, all tumors stopped growing and two of the five mice were cured (no evidence of disease at 180 days). However, when LND was delivered i.p. for 20 days after the single injection of cisplatin, all tumors stopped growing and, moreover, all mice (5/5) were cured (Fig. 1). Mouse weight loss was negligible

($\leq 6\%$) for this dose of cisplatin given either alone or in combination with LND. Even though no mouse died of toxicity and no sign of toxicity was observed at necropsy (180 days) in the cured mice, doses in the combination were not further escalated. A single treatment of i.p. cisplatin at 12 mg/kg (MTD, since four of nine mice died after injection of 13 mg/kg) also completely inhibited tumor growth in all animals for up to 30 days. At this time, one of eight tumors began to grow again, as did another at 100 days (Fig. 1). It can be concluded that in the case of cisplatin, LND determines not only an increased antitumor activity but, more importantly, a therapeutic advantage.

For investigation of the relevance of LND treatment duration, the low single dose (4 mg/kg) of cisplatin was combined with LND delivered p.o. for as long as 5, 12, or 19 days (Fig. 2). LND given alone at 100 mg/kg was inactive and devoid of toxicity for up to 19 treatments. Cisplatin alone was more active than in the previous experiments (TVI% = 83). When it was combined with LND, the efficacy of the combination was apparently related to the duration of LND treatment, since 5 days of LND administration did not increase cisplatin's efficacy and treatment for 12 days was less effective than that for 19 days. Probably due to the small number of tumors investigated, no difference reached statistical significance as compared with cisplatin therapy alone. This trend should be confirmed in a larger number of animals.

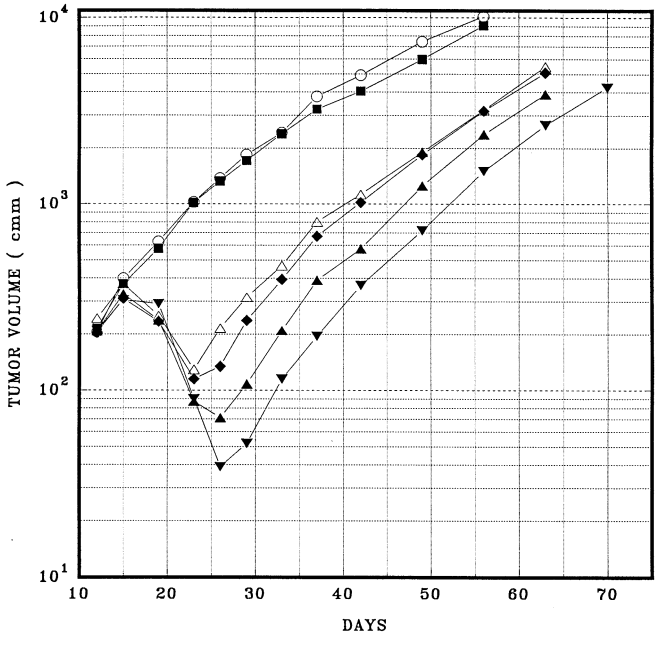

Fig. 2 Activity of cisplatin and LND on MX-1 human mammary carcinomas growing s.c. in female Swiss nu/nu mice. Mice were treated with i.p. cisplatin given at 4 mg/kg alone (*black diamonds*) or in combination with oral LND given at 100 mg/kg for 5 (*white triangles*) 12 (*black triangles*), or 19 days (*inverted black triangles*). Data represent mean values for 8/9 tumors. (*white circles* solvent-treated controls, *black squares* controls given oral LND at 100 mg/kg for 19 days)

Discussion

Although LND alone exerts a marginal activity in the treatment of experimental tumors [5], the drug has been reported to enhance the cytotoxic effects of several antitumor agents, including alkylating agents and DNA topoisomerase II inhibitors [6, 27, 32, 34, 35]. The drug's ability to potentiate the cytotoxicity of DNA-damaging agents could be related to its unique mechanism of action (interference with energy metabolism). The efficacy of LND in enhancing tumor cell killing by the cytotoxic agents is variable, depending on the individual DNA-damaging agent involved (i.e., on the nature of the induced DNA lesion) and, presumably, on the tumor cell type.

In the present study the efficacy of LND combined with different clinically useful agents was studied in the treatment of a human breast cancer xenograft in nude mice. The model was chosen since it is highly sensitive to alkylating agents but is refractory to doxorubicin. The results document that LND was most effective in enhancing the antitumor effects of alkylating agents (cyclophosphamide and cisplatin). However, a true therapeutic advantage of the combination over single treatment with the cytotoxic drug was documented only for cisplatin. When LND was combined with cisplatin, an increase in drug activity was observed at all the dose levels tested, whereas toxicity was not modified. Moreover, half the MTD of i.p. cisplatin (6 mg/kg, single treatment) cured 100% of treated mice when the alkylating agent was combined with LND and cured only 70% when it was given alone. Moreover, the MTD of cisplatin (12 mg/kg) did not cure all the mice, since 2/8 tumors had regrown by 100 days. The curative potential of the LND-cisplatin combination may be related to the presence in the MX-1 tumor of cell subpopulations less responsive to cisplatin, and the addition of LND might overcome this partial resistance. Such a hypothesis is in keeping with previous studies in which cisplatin-resistant cells were most sensitive in the presence of LND [30]. In addition, cisplatin-resistant tumor cell lines have been reported to have a greater mitochondrial mass or activity than do the parent cells [1].

In the case of the LND-cyclophosphamide combination, an improvement in the therapeutic outcome was observed only at suboptimal doses of the alkylating agent (100 mg/kg). However, at this dose level of the combination, tumor growth inhibition was less marked than that produced by a higher dose of cyclophosphamide alone (200 mg/kg). The combination of the latter dose with LND was associated with unacceptable toxicity. The different interaction of LND with cisplatin and cyclophosphamide could be interpreted in terms of the different toxicity profiles of the two drugs. In contrast to cisplatin, cyclophosphamide is known to be a myelotoxic drug. Such toxicity could be potentiated

by combination with LND, as has been reported by Teicher et al. [32]. Preliminary observations in our laboratory in mice treated with cisplatin and LND suggest no increase in cisplatin-induced bone marrow toxicity.

The potentiating activity of LND with cisplatin and alkylating agents could be related to a specific interference with DNA energy-dependent repair processes. Indeed, phosphorylation of nuclear proteins is known to be a part of the cell response to drug-induced DNA damage. Since this event may reflect the relative cellular sensitivity/resistance status [24], LND could sensitize cells to alkylating agents by interfering with protein phosphorylation through adenosine triphosphate (ATP) depletion. Such an effect could result in a fixation of DNA damage or a reduced ability of cells to repair drug-induced DNA cross-links, thus activating a cascade of damaging biochemical events that might result in cell death by apoptosis. This interpretation is consistent with the observation that the cisplatin-LND combination induced greater numbers of apoptotic cells than were observed in tumors treated with cisplatin alone (unpublished results). LND has also been described to enhance the cytotoxicity of doxorubicin in MCF-7 cells expressing a multidrug-resistant(MDR) phenotype [7] through hyperpolarization of the plasma membrane and increased intracellular drug level [3]. Modulation of the P-glycoprotein (P-gp) function through phosphorylation has also been proposed as a molecular basis of the synergistic interaction [3]. In the MX-1 system derived from an unpretreated patient, unknown for *mdr*-1 expressior, LND showed only a moderate ability to enhance the tumor response to doxorubicin. Taken together, these observations further support the concept that variable cellular mechanisms are operative in modulating the cytotoxicity of different DNA-damaging agents.

Finally, the results of this preclinical study may have relevant pharmacological implications. They indicate that i.p. administration of LND was more toxic and more effective than oral administration. However, they also show that oral administration is well tolerated, even over a long treatment period, and is highly effective in increasing the therapeutic efficacy of suboptimal cisplatin doses. The importance of the LND treatment duration after cisplatin injection, showing that a long period is better than a short period of administration, supports the clinical results of trials in which LND was given chronically for long periods [2, 4, 23]. Clinical trials have been recently activated in which LND has been combined with cisplatin, and the results seem promising [10, 19]. Only a single cisplatin injection was delivered in the present study; the effect of LND combined with cytotoxic drugs in multiple-dose chemotherapeutic regimens is being investigated.

In conclusion, in the MX-1 human tumor model, LND differently affects the cytotoxic properties of DNA-interacting agents. A synergistic interaction was documented for all drugs tested, but a substantial therapeutic advantage was achieved only with the LND-cisplatin combination. This observation may have clinical implications also, considering the different profile of toxicity of these agents. The most relevant side effect of lonidamine is myalgia [3].

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