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In vitro Effect of Lonidamine on the Cytotoxicity of Mitomycin C and BCNU in Human Colon Adenocarcinoma Cells

R. Villa, N. Zaffaroni, L. Orlandi, A. Bearzatto, A. Costa and R. Silvestrini

The ability of lonidamine (LND), an energolytic derivative of indazole carboxylic acid, to modulate the cytotoxic activity of mitomycin C (MMC) and 1,3,bis(2-chloroethyl)-1-nitrosourea (BCNU) was investigated in two human adenocarcinoma cell lines (LoVo and HT29) expressing different sensitivity profiles to the drugs. After a 1-h treatment with MMC or BCNU, cells were postincubated for 24 h with 150–225 μM LND. In the LoVo cells, a synergistic interaction between LND and MMC or BCNU was observed at both LND concentrations. In HT29 cells, only additive effects of the drugs given in sequence were seen. Flow cytometric analysis indicated that LND was generally able to stabilise the cell cycle perturbations induced by MMC and BCNU in the two cell lines. The ability of LND to potentiate anticancer drug activity, and the consideration that LND causes side-effects different from those of conventional antitumour drugs, make this compound an attractive candidate for multidrug combination therapy in colon cancer.

Key words: lonidamine, mitomycin C, BCNU, colon adenocarcinoma cells
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INTRODUCTION

COLON CANCER is one of the human tumour histotypes most refractory to systemic treatment [1] due to the inherent resistance of cells to conventional drugs [2, 3]. Thus there is great interest in new drugs with different molecular targets, other than DNA, for clinical control of this malignancy.

LND, [1-(2,4-dichlorophenyl)methyl]-1H-indazole-3-carboxylic acid, is a non-conventional anticancer drug which interferes with the energy metabolism of tumour cells. In fact, it reduces oxygen consumption in both normal and neoplastic cells [4] and increases aerobic glycolysis of normal cells and inhibits that of tumour cells [5]. Such a selective action has been ascribed

to the inhibition of mitochondria-bound exokinase, which is linked to the outer membrane of tumour mitochondria and is usually absent in normal differentiated cells [6]. Moreover, recent ultra-structural studies have revealed that not only mitochondria but also cell membranes and the cytoskeleton are targets for LND action [7, 8].

Since LND interacts with cellular energy metabolism, there probably is no cross-resistance with drugs acting on DNA or the DNA-replicating system. This observation, and the consideration that LND causes side-effects different from those observed for conventional antitumour drugs (myalgia is the dose-limiting toxicity), makes this compound an attractive candidate for multidrug combination therapy.

It has been demonstrated in experimental studies that LND enhances the cytotoxic activity of several drugs including alkylating agents [9, 10] and doxorubicin [11], as well as physical agents such as ionising radiation [12] and hyperthermia [13]. The present study was undertaken to assess the antiproliferative effect of LND alone or associated with MMC and BCNU, two drugs frequently used in the clinical treatment of colon cancer, on two human colon adenocarcinoma cell lines (LoVo and HT29) characterised by different chemosensitivity profiles.

MATERIALS AND METHODS

Cell lines

LoVo and HT29 human colon adenocarcinoma cell lines were used in the study. Their biological characteristics have been described previously [14, 15]. Cell lines were maintained in logarithmic phase at 37°C in a 5% CO_2 humidified atmosphere in air, using Ham's F12 medium supplemented with 15% fetal calf serum, 2 μM L-glutamine, a 1% vitamin solution and 0.1% gentamycin for LoVo cells, and RPMI 1640 medium supplemented with 10% fetal calf serum, 2 μM L-glutamine and 0.04% gentamycin for HT29 cells. The two cell lines showed a similar doubling time: 37 and 40 h for LoVo and HT29, respectively. For both the lines all experiments were performed within the tenth passage after thawing [14, 15].

Drugs

LND, obtained from F. Angelini Research Institute, Rome, was dissolved in a 2.3% glucamine solution immediately before each experiment and then diluted with fresh medium. Control samples were always run with glucamine alone. At the doses and exposure times used, glucamine alone was neither cytotoxic against either cell line nor did it influence the activity of MMC or BCNU. Moreover, glucamine did not induce any change in the cell cycle distribution compared to controls treated with a saline solution. MMC (Kyowa Hakko, Tokyo, Japan) and BCNU (Sintesa S.A., Bruxelles, Belgium) were initially dissolved in 0.9% sodium chloride and absolute ethanol, respectively, and then diluted with 0.9% sodium chloride to the desired concentration, immediately before each experiment. Control samples for BCNU were always run with ethanol. LND was used at concentrations of 150 and 225 μM in both cell lines. MMC concentrations ranged from 0.3 to 3 μM for HT29 and from 1.5 to 15 μM for LoVo. BCNU was used at concentrations of 50–200 μM in HT29 and 25–100 μM in Lovo cells.

Correspondence to R. Silvestrini.
The authors are at the Oncologia Sperimentale C, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy.
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Cell survival assay

LoVo and HT29 cells growing exponentially in 6-well plates were exposed to different concentrations of MMC and BCNU for 1 h. Medium was then removed and the cells were rinsed with phosphate buffer solution (PBS) and incubated in fresh medium, with or without LND, for an additional 24 h. At the end of the incubation period, cells were washed again and fresh medium was added. After 4 days of incubation at 37°C, plates were washed with PBS to remove detached cells and monolayers were trypsinised with trysin–EDTA solution. Single cell suspensions were obtained by repeated pipetting in Isoton solution (azide-free balanced electrolyte solution, Coulter Scientific, Luton, U.K.), and checked microscopically prior to counting in a particle counter (Coulter Counter model, Coulter Electronics Limited, Luton, U.K.). In addition, the percentages of viable cells were determined by the Trypan blue dye exclusion test. The viability always exceeded 95%. Each experimental point was run in triplicate. The results were expressed as the cell number of treated samples compared with control samples. *In vitro* activities of MMC and BCNU were expressed in terms of concentrations able to inhibit cell proliferation by 50% (IC_{50}).

Cell cycle distribution analysis

At the end of the different treatments or after various intervals of incubation in drug-free medium, cells were washed and harvested by trypsin, and samples of 1×10^6 cells were stained in a solution containing 50 μg/ml propidium iodide, 50 mg/ml RNase and 0.05% Nonidet P-40 for 30 min at room temperature. Samples were filtered through a 30 μm pore polyester filter and analysed by a FACScan (Becton Dickinson, Sunnyvale, California, U.S.A.). At least 3×10^4 cells were collected for each sample. The percentages of cells in the different cycle phases were evaluated from DNA plots according to a rectangular model (RFIT) provided by Becton Dickinson.

Statistical analysis

The type of interaction between LND and MMC or BCNU in paired experiments was assessed by the method of Drewinko *et al.* [16]. For this evaluation, the agents were assumed to provide independent effects. For a given dose of LND, we observed a surviving fraction of cells (SFa); likewise, for MMC or BCNU we observed SFb. For the combination of MMC or BCNU and LND, we observed SFab. If additivity holds, then SFab = SFa × SFb, so that our estimate of deviation from additivity is given by SFab−(SFa × SFb). Since the estimate of deviation from additivity divided by the square root of its variance was normally distributed, Z was used as a score to quantify the deviations and to evaluate their statistical significance.

Differences in the percentage of cells in the different cycle phases between control and treated samples were analysed by Student's *t*-test. All hypothesis tests were two-sided.

RESULTS

A 24-h treatment with 150 μM LND had only a negligible effect on cell growth of the two cell lines (Figure 1), whereas an appreciable inhibition of cell proliferation was achieved in both cell lines after exposure to 225 μM LND. The cytotoxic effect was slightly higher in HT29 cells although not significantly different from that observed in LoVo cells.

The sensitivity of LoVo and HT29 cell lines to MMC and BCNU is shown in Figure 2. A marked difference in response to a 1-h treatment with each drug was observed between the two

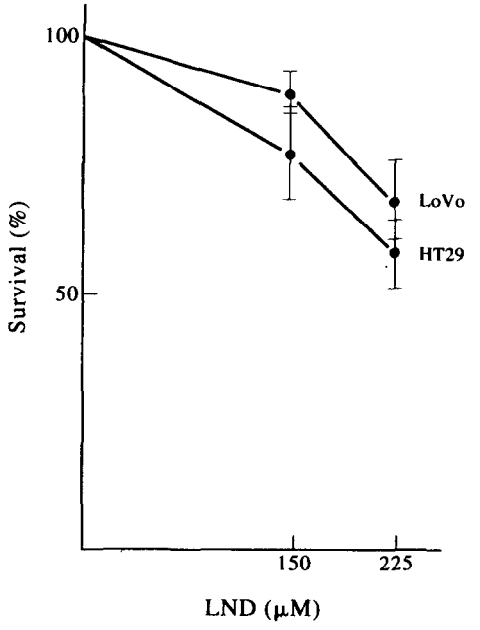

Figure 1. Dose–response survival curves of LoVo and HT29 cells exposed to LND for 24 h. Each value is the average ± S.E. of six independent experiments.

cell lines, indicating different inherent drug sensitivity profiles. In particular, LoVo cells were more resistant to MMC than HT29 cells, as indicated by a 6-fold IC_{50} value (9.9 versus 1.62 μM). Conversely, HT29 cells were less responsive to BCNU and showed an IC_{50} value twice that of LoVo cells (167 versus 79 μM).

Sequential treatment with MMC (1 h) and LND (24 h) induced a more marked cytotoxic effect than that obtained with MMC alone in both cell lines (Figure 3). In LoVo cells the effect provided by the drug combination was greater than that expected by simple additivity of the effects of the two agents. This synergistic interaction was observed at both LND concentrations (Table 1). Conversely, in HT29 cells, the combined effect of MMC and LND was only additive (Table 1). In particular, postincubation with LND reduced MMC IC_{50} values by 2.06–5.5 times in LoVo cells, and by 1.5–4.0 times in HT29 cells.

The cytotoxic effects induced by the BCNU–LND sequence are shown in Figure 4. In LoVo cells, a 24-h post-treatment with LND significantly enhanced BCNU activity at both concentrations used (Table 2). Conversely, in HT29 cells, the effects of the drug combination on cell survival were very close to those expected by additivity at both LND concentrations (Table 2). More precisely, post-incubation with LND lowered BCNU IC_{50} values by 1.8–5.6 times in LoVo cells and by 2.0–12.0 times in HT29 cells.

To explain the different patterns of drug interaction in the two cell lines, the perturbations induced on the distribution of cells throughout the different cycle phases were analysed by flow cytometry. In LoVo cells (Table 3), a 1-h exposure to 10 μM MMC caused a significant accumulation of cells in G_2 + M phases at 24 h and 48 h, which was partially resolved at 72 h. Treatment with LND did not induce any detectable perturbation in cell kinetics, whereas sequential treatment with the two agents maintained a significant G_2 + M cell block, which remained constant in degree at least up to 72 h.

In HT29 cells (Table 3), 1.5 μM MMC treatment caused an almost complete depletion of the G_0/G_1 compartment and an accumulation of cells in S/G_2 + M, which was progressively but not yet completely resolved at 72 h. Again, LND did not affect cell cycle distribution, whereas MMC followed by LND induced a block of cells in S/G_2 + M, qualitatively similar to that induced by MMC alone, but which was more pronounced at 48 h.

As regards BCNU, in LoVo cells the nitrosourea induced a temporary block of cells in the G_2 + M phases detectable only at 24 h (Table 4). Post-treatment with LND prolonged this block up to 48 h. In HT29 cells (Table 4), BCNU alone caused an accumulation of cells in S/G_2 + M compartments, appreciable only 24 h after treatment. In cells postincubated

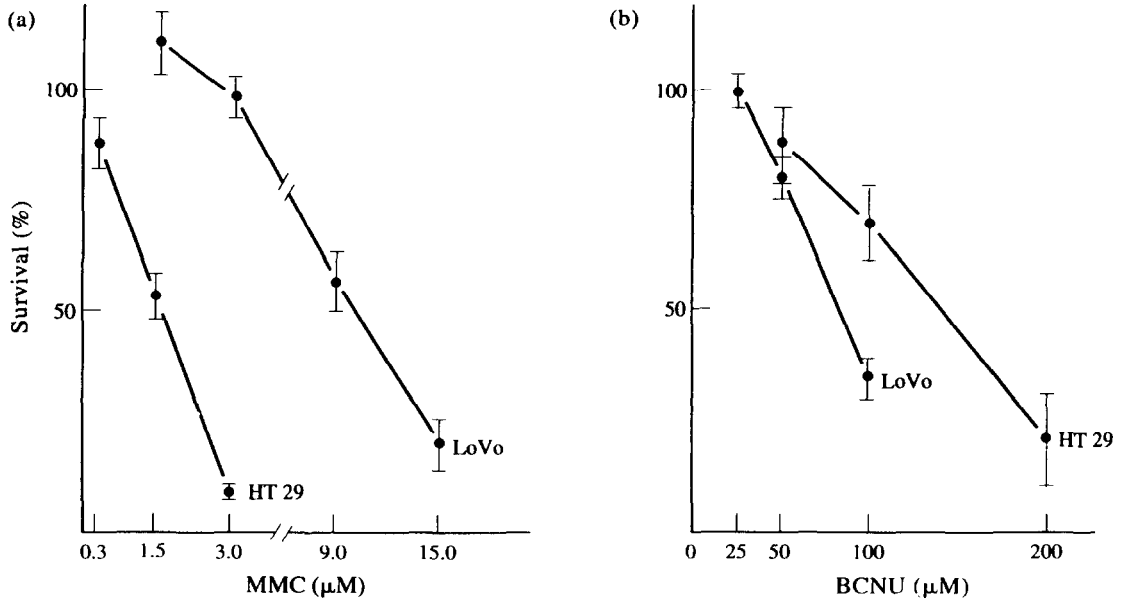

Figure 2. (a) Dose–response survival curves of LoVo and HT29 cells exposed to MMC for 1 h. Each value is the average ± S.E. of six independent experiments. (b) Dose–response survival curves of LoVo and HT29 cells exposed to BCNU for 1 h. Each value is the average ± S.E. of six independent experiments.

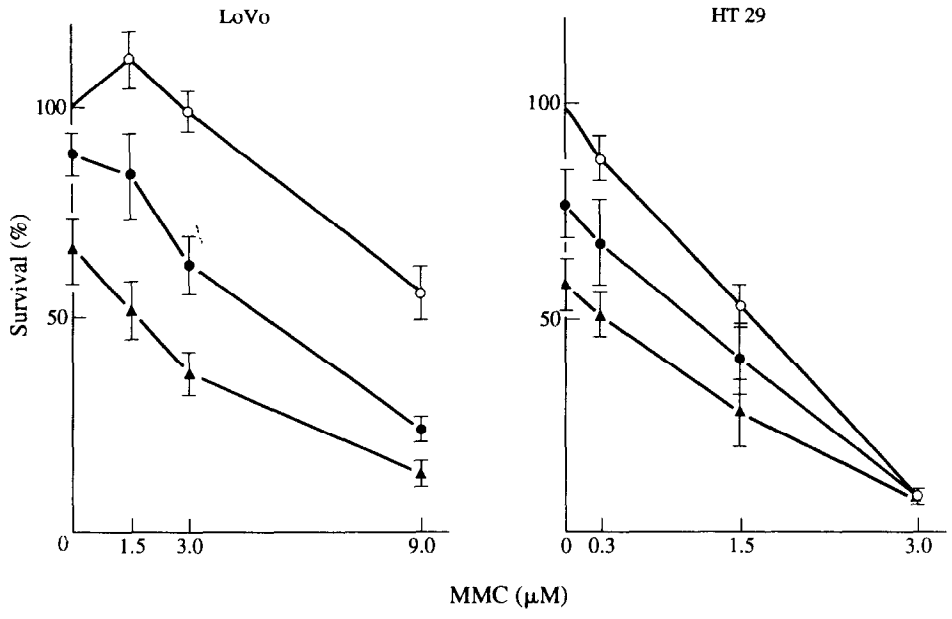

Figure 3. Dose–response survival curves of LoVo and HT29 cells exposed to MMC alone (○—○) for 1 h; MMC for 1 h followed by 150 μM (●—●) or 225 μM (▲—▲) LND for 24 h. The effect of LND alone on cell survival can be seen as the change in survival at 0 μM MMC. Each value is the average ± S.E. of six independent experiments.

with LND, a slight accumulation of the cells in S phase was still evident after 72 h.

A spontaneous increase, not statistically significant, in the percentage of LoVo and HT29 cells in G_0/G_1 phase was observed in control samples after 72 h in culture. This finding may reflect a slight cell kinetic instability of cell lines at the longest time of *in vitro* culture.

DISCUSSION

We evaluated the antiproliferative activity of LND as well as its interference with MMC and BCNU activity in two human colon adenocarcinoma cell lines characterised by different chemosensitivity profiles. In this study, the cytotoxicity of drugs was assessed by using a particle counter to record the variation induced in the cell number. This methodology has already proved its reliability in giving chemosensitivity information in several established cell lines through a direct comparison with other assays that also measure total cell kill. In particular, a good agreement has been observed between colorimetric assays, such as the MTT assay, and total cell counts in murine [17] and human [18] tumour cell lines.

In agreement with previous findings [19], our results indicated

Table 1. Cytotoxic effects of treatment with MMC versus MMC followed by LND

IC_{50} ratio*	MMC (μM)	% survival with LND (mean ± S.E.) SFa	% survival with MMC (mean ± S.E.) SFb	% expected survival in combination SFab = SFa × SFb	% observed survival in combination (mean ± S.E.) observed SFab	P†
LoVo						
2.06	1.5	89 ± 3.8‡	111 ± 6.4	99	84 ± 10.0	0.01
	3.0		99 ± 4.6	81	63 ± 6.4	
	9.0		56 ± 5.8	40	24 ± 2.8	
5.5	1.5	67 ± 7.0§	111 ± 6.4	74	52 ± 6.7	<0.01
	3.0		99 ± 4.6	61	37 ± 5.2	
	9.0		56 ± 5.8	30	14 ± 1.4	
HT29						
1.5	0.3	76 ± 8.0‡	87 ± 5.3	66	67 ± 9.6	0.849
	1.5		53 ± 5.0	40	41 ± 7.9	
	3.0		9 ± 1.3	7	9 ± 3.7	
4.0	0.3	57 ± 6.3§	87 ± 5.3	49	51 ± 5.3	0.833
	1.5		53 ± 5.0	30	29 ± 7.9	
	3.0		9 ± 1.3	5	8 ± 2.8	

* IC_{50} MMC/IC_{50} MMC followed by LND. † Calculated on the overall curve. ‡ 150 μM. § 225 μ M.

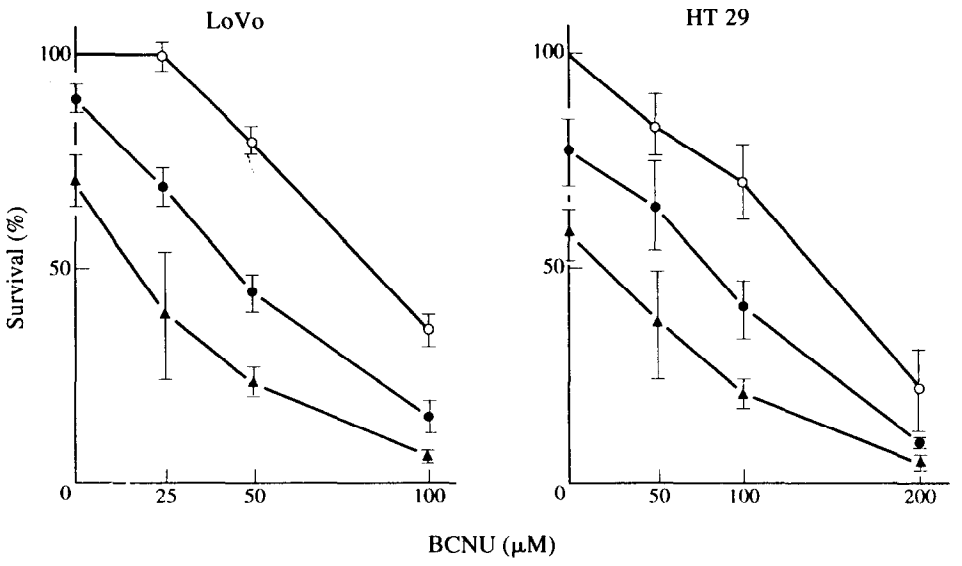

Figure 4. Dose–response survival curves of LoVo and HT29 cells exposed to BCNU alone (○—○) for 1 h; BCNU for 1 h followed by 150 μM (●—●) or 225 μM (▲—▲) LND for 24 h. The effect of LND alone on cell survival can be seen as the change in survival at 0 μM BCNU. Each value is the average ± S.E. of six independent experiments.

that LND exhibited a certain degree of activity only at high concentrations and after long-term treatment. The activity was slightly higher in HT29 cells, which also exhibited a higher mitochondria-bound hexokinase activity than LoVo cells (unpublished data, A. Floridi). Moreover, we found that LND modulated the cytotoxic activity of MMC and BCNU in the two cell lines to different extents.

A synergistic interaction between MMC and LND, when given in sequence, has been reported by Ning and Hahn [20] on murine and human fibrosarcoma cell lines. With respect to BCNU, Rosbe *et al.* [9] found a supra-additive interaction between the nitrosourea and LND in human breast cancer MCF7 cells simultaneously exposed to the two drugs for 1 h and then postincubated with LND for an additional 24 h. No studies aimed to ascertain the modulating effect of LND on MMC and BCNU have been performed on colon carcinoma cell lines, even though MMC and BCNU are drugs largely used in the clinical treatment of this malignancy.

The experimental scheme we adopted is consistent with the pharmacokinetic characteristics of the drugs. In fact, since the plasma levels of MMC and BCNU rapidly decay [21, 22], whereas that of LND remains high for long periods of time [23], we combined a short-term exposure to the anticancer drugs (1 h) with a long-term exposure to LND (24 h).

Table 2. Cytotoxic effects of treatment with BCNU versus BCNU followed by LND

IC_{50} ratio*	BCNU (μM)	% survival with LND (mean ± S.E.) SFa	% survival with BCNU (mean ± S.E.) SFb	% expected survival in combination SFab = SFa × SFb	% observed survival in combination (mean ± S.E.) observed SFab	*P*†
LoVo						
1.8	25	90 ± 3.2‡	100 ± 3.8	88	69 ± 3.2	<0.001
	50		80 ± 4.0	70	44 ± 4.9	
	100		34 ± 4.0	24	15 ± 3.6	
5.6	25	71 ± 5.7§	100 ± 3.8	65	39 ± 15	<0.001
	50		80 ± 4.0	50	23 ± 3.6	
	100		34 ± 4.0	16	6.2 ± 1.0	
HT29						
2.0	50	76 ± 7.9‡	88 ± 7.2	54	64 ± 9.9	0.218
	100		70 ± 8.8	40	40 ± 6.4	
	200		21 ± 9.5	12	8.5 ± 0.7	
12.0	50	57 ± 6.3§	88 ± 7.2	37	37 ± 12	0.05
	100		70 ± 8.8	30	21 ± 3.9	
	200		21 ± 9.5	7.5	4.5 ± 2.1	

* IC_{50} BCNU/IC_{50} BCNU followed by LND. † Calculated on the overall curve. ‡ 150 μM. § 225 μM.

Table 3. Cell cycle perturbations induced by exposure to MMC and LND either singly or in combination

Time (h)	Phase	Control	MMC*	LND†	MMC* → LND†
Lovo					
24	G_0/G_1	48.0 ± 4.2‡	24.0 ± 8.5	56.7 ± 8.0	40.0 ± 6.2
	S	36.5 ± 3.5	31.0 ± 5.6	29.3 ± 1.5	24.3 ± 5.0
	G_2 + M	16.3 ± 7.1	45.0 ± 3.0§	14.0 ± 8.9	35.7 ± 3.8§
48	G_0/G_1	50.0 ± 6.2	45.7 ± 5.5	40.3 ± 4.6	32.0 ± 11.8
	S	36.3 ± 4.9	16.3 ± 7.1	44.7 ± 3.0	20.3 ± 5.5
	G_2 + M	13.6 ± 1.5	38.0 ± 8.7§	15.0 ± 2.0	47.7 ± 6.6§
72	G_0/G_1	61.3 ± 12.6	55.3 ± 7.5	53.3 ± 7.4	44.0 ± 1.0
	S	25.7 ± 5.1	16.7 ± 8.0	28.7 ± 5.8	14.3 ± 4.7
	G_2 + M	13.0 ± 8.7	28.0 ± 10.6	18.0 ± 8.7	41.7 ± 5.7§
HT29					
24	G_0/G_1	47.0 ± 6.2	4.7 ± 5.5§	53.0 ± 14.1	14.0 ± 7.0§
	S	37.0 ± 6.6	49.3 ± 2.1	30.7 ± 11.0	47.3 ± 8.5
	G_2 + M	16.0 ± 4.0	46.0 ± 3.5§	19.0 ± 2.0	38.7 ± 3.5§
48	G_0/G_1	48.0 ± 5.6	38.7 ± 2.5	48.7 ± 6.4	23.7 ± 15.2
	S	38.0 ± 8.2	39.0 ± 2.6	38.0 ± 5.6	50.0 ± 15.5
	G_2 + M	14.0 ± 7.5	22.3 ± 3.5	13.3 ± 7.1	26.3 ± 3.8
72	G_0/G_1	66.0 ± 6.6	44.0 ± 12.1	54.7 ± 7.4	41.3 ± 1.1
	S	23.3 ± 7.5	37.0 ± 8.0	33.7 ± 5.0	37.7 ± 2.9
	G_2 + M	10.7 ± 1.2	19.0 ± 4.4	11.7 ± 3.5	21.1 ± 3.6

* 10 μM (LoVo) or 1.5 μM (HT29), 1 h. † 225 μM, 24 h. ‡ Mean + S.D. of three independent experiments. § $P < 0.05$, Student's *t*-test, compared to controls.

Table 4. Cell cycle perturbations induced by exposure to BCNU and LND either singly or in combination

Time (h)	Phase	Control	BCNU*	LND†	BCNU* → LND†
LoVo					
24	G_0/G_1	48.0 ± 4.2‡	43.0 ± 3.6	56.7 ± 8.0	40.7 ± 5.5
	S	36.5 ± 3.5	23.7 ± 2.1	29.3 ± 1.5	32.0 ± 1.0
	G_2 + M	16.3 ± 7.1	33.3 ± 5.7	14.0 ± 8.9	27.3 ± 5.1
48	G_0/G_1	50.0 ± 6.2	52.3 ± 11.5	40.3 ± 4.6	45.3 ± 4.9
	S	36.3 ± 4.9	29.3 ± 9.0	44.7 ± 3.0	25.0 ± 2.6
	G_2 + M	13.6 ± 1.5	17.7 ± 3.0	15.0 ± 2.0	29.7 ± 3.5§
72	G_0/G_1	61.3 ± 12.6	60.0 ± 11.3	53.3 ± 7.4	60.7 ± 4.9
	S	25.7 ± 5.1	24.0 ± 4.0	28.7 ± 5.8	23.0 ± 6.2
	G_2 + M	13.0 ± 8.7	16.0 ± 8.2	18.0 ± 8.7	16.3 ± 2.3
HT29					
24	G_0/G_1	47.0 ± 6.2	23.7 ± 2.3	53.0 ± 14.1	28.4 ± 8.4
	S	37.0 ± 6.6	49.0 ± 4.2	30.7 ± 11.0	27.3 ± 19.8
	G_2 + M	16.0 ± 4.0	28.7 ± 5.5§	19.0 ± 2.0	30.7 ± 6.8
48	G_0/G_1	48.0 ± 5.6	43.7 ± 3.2	48.7 ± 6.4	36.3 ± 3.8
	S	38.0 ± 8.2	36.6 ± 7.8	38.0 ± 5.6	42.7 ± 3.8
	G_2 + M	14.0 ± 7.5	20.3 ± 6.1	13.3 ± 7.1	21.0 ± 7.0
72	G_0/G_1	66.0 ± 6.6	61.3 ± 11.2	54.7 ± 7.4	47.3 ± 3.8
	S	23.3 ± 7.5	27.3 ± 11.5	33.7 ± 5.0	38.0 ± 6.0
	G_2 + M	10.7 ± 1.2	11.3 ± 2.1	11.7 ± 3.5	14.7 ± 3.8

* 79 μM (LoVo) or 167 μM (HT29), 1 h. † 225 μM, 24 h. ‡ Mean + S.D. of three independent experiments. § $P < 0.05$, Student's *t*-test, compared to controls.

In the LoVo cell line, when given after a 1-h exposure to MMC or BCNU, LND significantly enhanced the cytotoxicity of both antitumour agents. This potentiating effect is of particular importance since it was obtained at a clinically relevant 150 μM concentration, i.e. in the range of peak plasma levels [24]. In HT29 cells, combined treatment with MMC (or BCNU) and LND gave only additive effects. However, comparison of the IC_{50} values obtained with MMC (or BCNU) alone and those obtained with the sequence MMC– (or BCNU–) LND indicated the potential clinical usefulness of these combinations, since they may allow a dose reduction of MMC (or BCNU) and thus avoid consequent side-effects without reducing the therapeutic efficacy.

To explain the difference in the potentiation of anticancer drug activity by LND in the two cell lines (synergy versus additivity), we analysed by flow cytometry cell cycle perturbations induced by the drugs singly or in combination. We found that LND was generally able to stabilise the cell cycle perturbations induced by MMC and BCNU. Results were qualitatively similar in the two cell lines and suggested that the different degree of potentiating effect by LND on MMC and BCNU cytotoxicity cannot be ascribed to a different interference of LND on the cell cycle. Differences in cellular energy metabolism of the cell lines could be responsible for the differential susceptibility to LND as a modulator of anticancer drug activity. In fact, previous evidence [25, 26] has suggested that in cell lines with an enhanced rate of glycolysis, LND can selectively impair cellular energy-dependent mechanisms, such as recovery from lethal damage induced by drugs. Future experiments are planned to define whether differences in regard to energy-dependent mechanisms are also responsible for the differential modulation exerted by LND on the activity of conventional antitumour drugs.

Results from this and other studies [19, 27, 28] concerning the ability of LND to potentiate anticancer drug activity, together with clinical evidence of a lack of myelosuppression [29], strongly suggest the importance of clinically verifying the role of LND in multidrug combination therapies.

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