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Experimental report

Modulation of melphalan cytotoxic activity in human melanoma cell lines

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The aim of the present study was to potentiate the cytotoxic effects of melphalan through pharmacological and physical modulators. The combination of the cytotoxic agent with ethacrynic acid, a glutathione-S-transferase π (GSTπ) inhibitor, or topotecan, a topoisomerase I inhibitor, or mild hyperthermia was investigated. The selected cell lines exhibited variable levels of expression of GSTπ, DNA topoisomerase I and heat-shock proteins. Mild hyperthermia (42°C) alone potentiated melphalan cytotoxicity, especially in the two cell lines exhibiting low basal levels of HSP70 expression. The combination of the GST inhibitor with melphalan resulted in a potentiation of drug cytotoxicity only in JR8 cells, one of the two cell lines which expressed high levels of GSTπ mRNA and which were the less responsive to ethacrinic acid alone. A synergistic interaction between topotecan and melphalan was observed only in the cell lines expressing low levels of topoisomerase I even if all cell lines exhibited a comparable sensitivity to this agent. The results support an involvement of GST and DNA topoisomerase in cell defense and response to the alkylating agent. However, the variable potentiation of the cytotoxic effects of melphalan achieved in different cell systems suggests that factors other than the level of expression of the modulation target are responsible of such potentiation.

Key words: Cytotoxicity, drug resistance, melanoma, modulation.

Introduction

Bifunctional alkylating agents, such as melphalan (L-phenylalanine mustard or L-PAM), are effective antitumor drugs currently used in the treatment of several human neoplasms, including melanoma.[1,2] However, intrinsic or acquired cellular resistance to such antitumor agents limits considerably their clinical efficacy in tumor therapy.[3–5]

Several mechanisms have been suggested to be involved in alkylating agent resistance, including alterations in drug transport,[6] increased drug detoxification[7,8] and enhanced repair of DNA damage.[9,10] Thus far, there are only preliminary clinical approaches to overcome resistance to alkylating agents. Attempts to modulate the function of specific cellular targets involved in cellular defense or response to alkylating agent treatment[1] are expected to provide a rational basis for pharmacological intervention.

Regional chemotherapy with L-PAM under hyperthermic conditions has allowed an effective treatment of malignant melanoma.[4,11,2] Experimental studies have demonstrated that mild hyperthermia (HPT) is able to significantly increase L-PAM cytotoxicity in different cell lines.[12–14] The enhancement has been related to an increased accumulation of the drug and a reduced repair of DNA interstrand cross-links induced by L-PAM.[14,15]

In an attempt to identify other effective modulators of L-PAM cytotoxicity that can be included in chemotherapeutic regimens, in the present study we investigated the effects of ethacrynic acid (EA)[16,17] and topotecan (TPT)[18,19] as potential enhancers of L-PAM activity on four established melanoma cell lines under normothermic and hyperthermic conditions. EA is a well-known inhibitor of glutathione transferase (GST). Available evidence supports a role of the GSH-related enzyme in determining cell response to alkylating agents.[7,8] TPT, a topoisomerase I inhibitor, has been used since the enzyme has been implicated in the mechanism of DNA repair.[18,20] The effects of HPT, EA and TPT have been examined in relation to the level of expression of target enzymes.

Materials and methods

Cell lines

Two human melanoma cell lines, JR8 and M14, and two human melanoma cell clones, 2/21 and 2/60

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(selected by micromanipulation in soft agar from 665/2 cell line), were used in the study. Their biological characteristics were previously described.[21–23] The doubling time was around 30–34 h for all cell lines. Cell lines were grown in a monolayer at 37°C in a 5% CO_2 humidified atmosphere in air in RPMI 1640 medium (Bio Whittaker, Verviers, Belgium) containing 10% fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel), 2 mM L-glutamine and gentamycin (0.1 mg/ml) for JR8 and M14 cells. For 2/21 and 2/60 cells, RPMI 1640 containing 10% fetal calf serum (Gibco BRL, Life Technologies, Gaithersburg, MD), 10 mM HEPES buffer and 4 mM L-glutamine was used. For all the cell lines the experiments were performed within the 10th passage after thawing.

Drugs

L-PAM (Sigma, St Louis, MO) was dissolved at 50 mg/ml just before use in perchloric acid and ethanol solution (1 : 20) and diluted in 0.9% NaCl saline solution. Final concentrations of perchloric acid and ethanol did not exceed 0.01 and 0.19%, respectively, and they did not influence drug cytotoxicity. EA was provided as a pharmaceutical formulation (Reomax) from Bioindustria Farmaceutici (Novi Ligure, Italy); it was dissolved at 2.5 mg/ml in 5% glucose solution and then diluted in saline solution just before use. TPT (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) was dissolved in H_2O and diluted in saline solution.

Probes

Human *hsp60* fragment cDNA subcloned into pGem-7(zf) was kindly provided by Dr RS Gupta (Ontario, Canada) and *hsp70* fragment cDNA cloned into pH2.3 was a gift from Dr S Fox (Evanston, IL). A 0.750 kb GSTπ-1 cDNA fragment, subcloned into pHD (a 5 kb vector derived from pGEM 2), was kindly supplied by Dr JA Moscow (Bethesda, MD); a 0.7 kb human topoisomerase I cDNA fragment (topo 1), purified from the plasmid pGEM-4-DI, was kindly provided by Dr L Liu (Baltimore, MD); the pHDR5A plasmid containing the human *mdr1* (H-*mdr1*) insert was a gift from Dr MM Gottesman (Bethesda, MD). The human γ-actin cDNA[24] was used to normalize mRNA levels. Deoxycytidine-5′[α-^{32}P]triphosphate (3000 Ci/mmol) was purchased from Amersham International (Amersham, UK).

Schedule of treatment and cell survival determination

Cells (9×10^4/well) were seeded in 6-well plates (Costar, Cambridge, MA) and after 24 h samples were exposed for 1 h to L-PAM (from 1 to 5 µg/ml for JR8 and M14 cell lines, and from 1 to 100 µg/ml for 2/21 and 2/60 cell lines) under normothermic (37°C) or hyperthermic (42°C) conditions.

In the combination experiments, cells, 24 h after seeding, were preincubated for 3 h with EA; then L-PAM, at different concentrations, was added to the culture medium already containing EA; the incubation with L-PAM and EA was performed for 1 h at 37 or 42°C. Preliminary experiments performed on melanoma clones showed that addition of EA following L-PAM treatment did not enhance the cytotoxicity of alkylating agent. In the combined treatments with TPT, cells, 24 h after seeding, were exposed for 1 h to L-PAM under normothermic or hyperthermic conditions and, after washing, treated with TPT for 24 h. Preliminary experiments on melanoma clones showed that treatments with TPT followed by L-PAM or with TPT simultaneously to L-PAM did not induce any significant potentiation. For all the experiments, cell number was determined 96 h after seeding. Cells were trypsinized and then single cell suspensions were obtained by repeated pipetting in Isoton solution (azide-free balanced electrolyte solution; Kontron Instruments, Milan, Italy). Samples were counted in a particle counter (Coulter Counter; Coulter Electronics, Luton, UK). Each experimental point was run in triplicate. The results were expressed as the percentage of cell number in treated samples compared to untreated samples.

RNA extraction and Northern hybridization

Total RNA of human melanoma cells (JR8, M14, 2/21 and 2/60) was prepared by the LiCl–guanidine monothiocyanate method according to Cathala *et al.*[25] from cells harvested in the logarithmic phase of growth. Northern gel electrophoresis and blot hybridization were performed according to reported procedures.[26] Briefly, total RNAs were size fractioned on denaturing 1% agarose gel; then gels were equilibrated in 20 × SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7) before overnight blotting to Hybond nylon membrane (Amersham). The filter was UV cross-linked and based at 80°C, and then prehybridized for at least 4 h at 42°C in 50% formamid, 5 × SSC, 0.2% SDS, 5 × Denhart's

solution and 250 μg/ml DNA salmon sperm (SS DNA). DNA probes were [^{32}P]deoxycytidine labeled with a random primer kit (specific acitivity, 2.5×10^8 c.p.m./μg of DNA) (Amersham) and denatured, and SS DNA was added. Hybridization was carried out for 18–20 h in the same buffer used for prehybridization. The final membrane wash was at 65°C in 0.1 × SSC. Filters were exposed to autoradiography Hyperfilm (Amersham). Gene transcript levels were calculated with an ultrascan XL laster densitometer (LKB, Uppsala, Sweden) and normalized to human γ-actin expression.

Data analysis

In combination experiments, the type of interaction between L-PAM and EA or TPT was assessed by a modified method of Drewinko *et al.*[27] For this evaluation, the agents were assumed to provide independent effects. For a given dose of L-PAM at 37–42°C, we observed a survival fraction of cells (SF_a); likewise, for EA or TPT at 37 or 42°C, we observed a SF_b. For the combination of EA or TPT and L-PAM at 37 or 42°C we observed a SF_{ab}. By this way interaction between two agents at a time was examined. In such an analysis, $SF_a \times SF_b/SF_{ab} = 1$ indicates an additive effect, $SF_a \times SF_b/SF_{ab} > 1$ indicates a potentiation and $SF_a \times SF_b/SF_{ab} < 1$ indicates a subadditive effect. Synergistic indices ($SF_a \times SF_b/SF_{ab}$) > 1.5 were considered to indicate a significant potentiation.

Results

Initial experiments were performed on the four human melanoma cell lines (JR8, M14, 2/21 and 2/60) in the logarithmic phase of growth to determine the antiproliferative effects of HPT alone. One hour exposure to 42°C did not modify, in comparison with 37°C, the cell proliferation of any tested

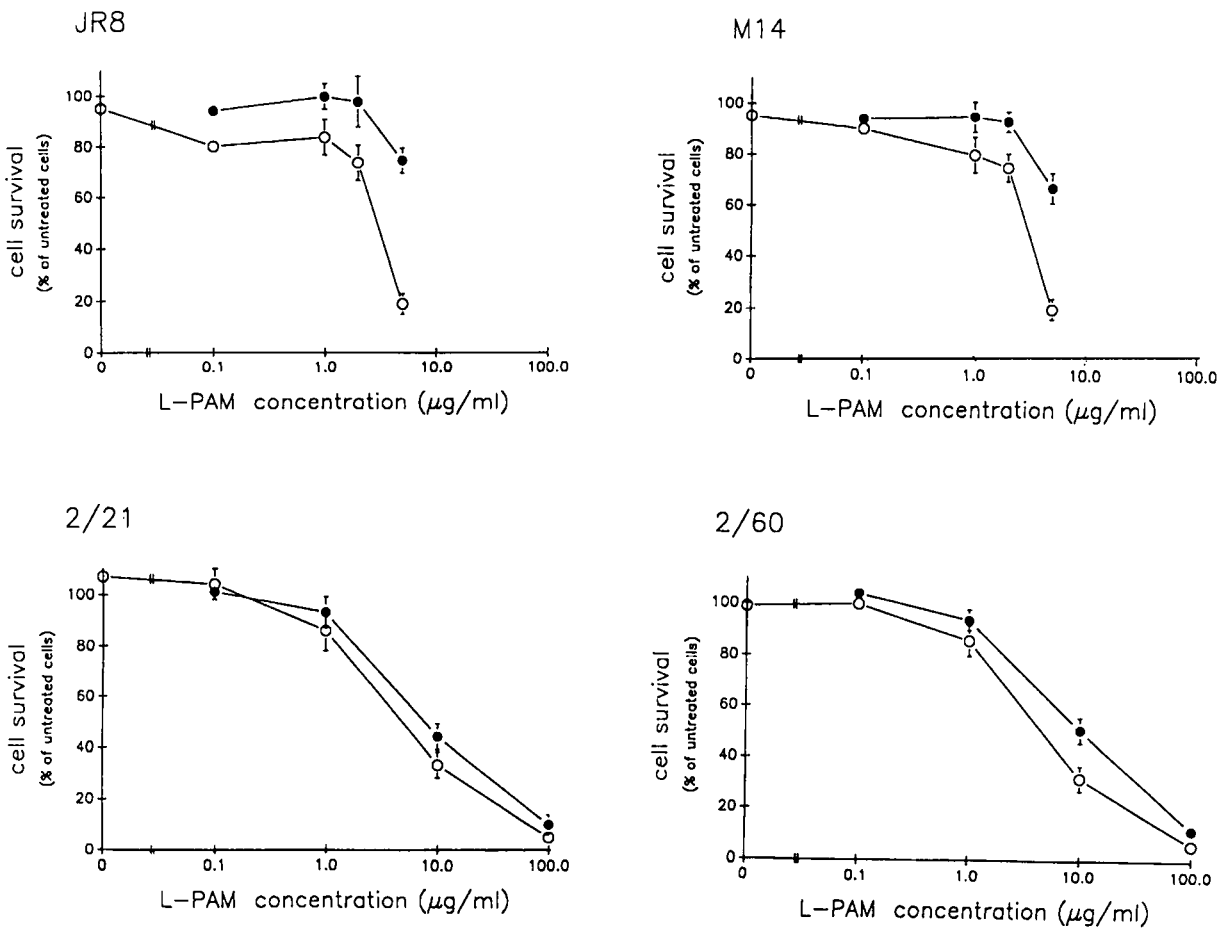

Figure 1. Effect of HPT (1 h at 42°C) on the sensitivity to 1 h exposure to L-PAM. , L-PAM at 37°C; , L-PAM at 42°C. Points represent the mean ± SD (bars) of at least three independent experiments.

Table 1. Sensitivity of melanoma cell lines to hyperthermia[a]

Temperature (°C)	Cell number × 10^3/cm^2			
	JR8	M14	2/21	2/60
37	95 ± 25	84 ± 38	72 ± 8	77 ± 16
42	90 ± 25	80 ± 32	77 ± 7	78 ± 9

Data represent the mean ± SD of at least three independent experiments.
[a] Cells were exposed to hyperthermic treatment for 1 h; cell number was evaluated 3 days later.

cell line (Table 1). When these cells were treated for 1 h with L-PAM under normothermic conditions (Figure 1), a relatively low cytotoxic effect was observed in the four cell lines, as expected. The IC_{50} values (required for 50% cell growth inhibition) were found in the range of 8–10 μg/ml (values below 1 μg/ml were observed in cell lines of other tumor types). However, a remarkable enhancement of L-PAM cytotoxicity was induced by HPT in JR8 and M14 cell lines with a reduction of the IC_{20} of L-PAM from 4.5 to 1.5 μg/ml, a concentration which reduced cell survival by about 30% under normothermic conditions. In 2/21 and 2/60 cells, the enhancement of L-PAM cytotoxicity induced by HPT was modest, but consistent in all experiments. IC_{50} values were reduced by 30–50% under hyperthermic conditions.

The effect of EA alone or in combination with L-PAM under normothermic and hyperthermic conditions is shown in Figure 2. A different sensitivity to a 4 h exposure to EA was observed among the tested cell lines. In JR8, M14 and 2/21 cell lines, 30 μg/ml EA produced a 60% inhibition of cell growth. In JR8 cells, combined treatment with L-PAM and EA at 37°C provided a cytotoxic effect greater than that expected by the product of effect of individual agents (synergistic index 1.93 at L-PAM 5 μg/ml). Moreover, in this cell line a further

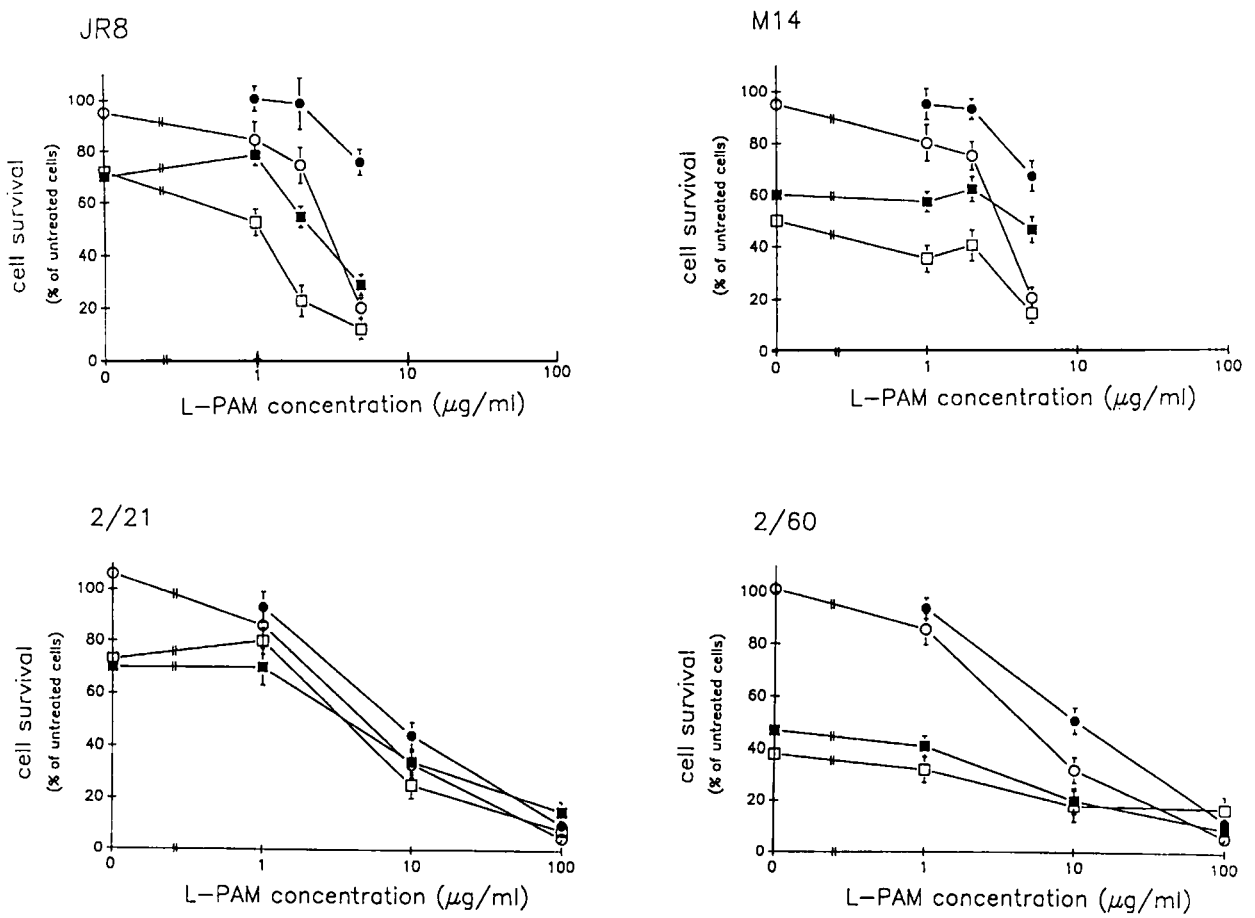

Figure 2. Combined effect of HPT (1 h at 42°C), EA (30 μg/ml in JR8, M14 and 2/21 and 6 μg/ml in 2/60) and L-PAM. , L-PAM at 37°C; , L-PAM at 42°C; , 3 h preincubation with EA and 1 h incubation with L-PAM plus EA at 37°C; , 3 h preincubation with EA and 1 h incubation with L-PAM plus EA at 42°C. Points represent the mean ± SD (bars) of at least three independent experiments.

potentiation of the L-PAM effect, at least at the intermediate concentration, was observed when the cytotoxic drug was used in combination with EA under hyperthermic conditions (synergistic index 2.37 at L-PAM 2 μg/ml). In contrast, in M14, 2/21 and 2/60 cell lines EA did not affect L-PAM cytotoxicity under normothermic or hyperthermic conditions.

The potential of TPT as a modulator of L-PAM cytotoxicity was studied in JR8 and M14 cell lines at two different concentrations. As shown in Figure 3, TPT at a subtoxic concentration (5 ng/ml) enhanced the cytotoxic effect of L-PAM at 37°C (synergistic index 2 at L-PAM 5 μg/ml) and 42°C (synergistic index 1.73 at L-PAM 2 μg/ml) in the JR8 but not in the M14 cell line. When used at a concentration (10 ng/ml) which reduced cell growth by about 60% in both cell lines, TPT did not potentiate L-PAM cytotoxicity under normothermic conditions in JR8 or in M14 cells. However, under hyperthermic conditions an enhancement of the effect produced by the two lowest concentrations of L-PAM was observed (with 10 ng/ml TPT) in the M14 cell line (synergistic indices 2.5 at L-PAM 1 μg/ml, and 1.7 at L-PAM 2 μg/ml). In 2/21 and 2/60, 10 ng/ml TPT, which induced by itself a 30–40% reduction of cell growth, did not modify the cytotoxic effect of L-PAM at 37 and 42°C.

In an attempt to explain the different patterns of interaction between L-PAM and modulators observed in the four cell lines, we examined the expression of target enzymes and other cellular factors (HSP60, HSP70, GSTπ, topoisomerase I and H-MDR1) potentially involved in cellular response to these physical and chemical agents (Figure 4). The results of densitometric analysis are reported in Table 2. No significant differences in *hsp60* mRNA expression were found among cell lines. A mark-

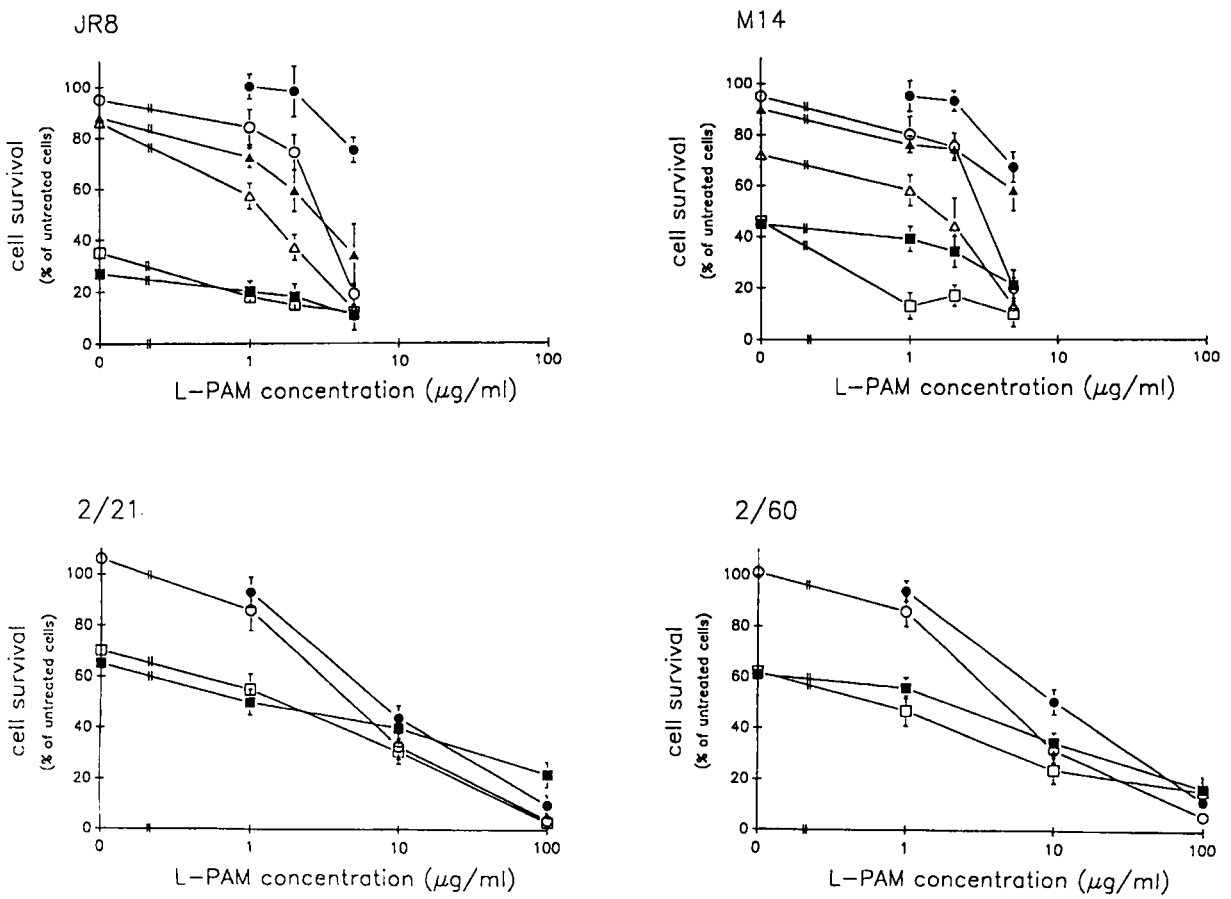

Figure 3. Combined effect of HPT (1 h at 42°C), TPT (5 ng/ml and 10 ng/ml for 24) and L-PAM. (,) L-PAM alone; (,) L-PAM (1 h) followed by 5 ng/ml TPT (24 h); (,) L-PAM (1 h) followed 10 ng/ml TPT (24 h); filled symbols are treatment at 37°C and open symbols are at 42°C (1 h simultaneously to L-PAM exposure). Points represent the mean ± SD (bars) of at least three independent experiments.

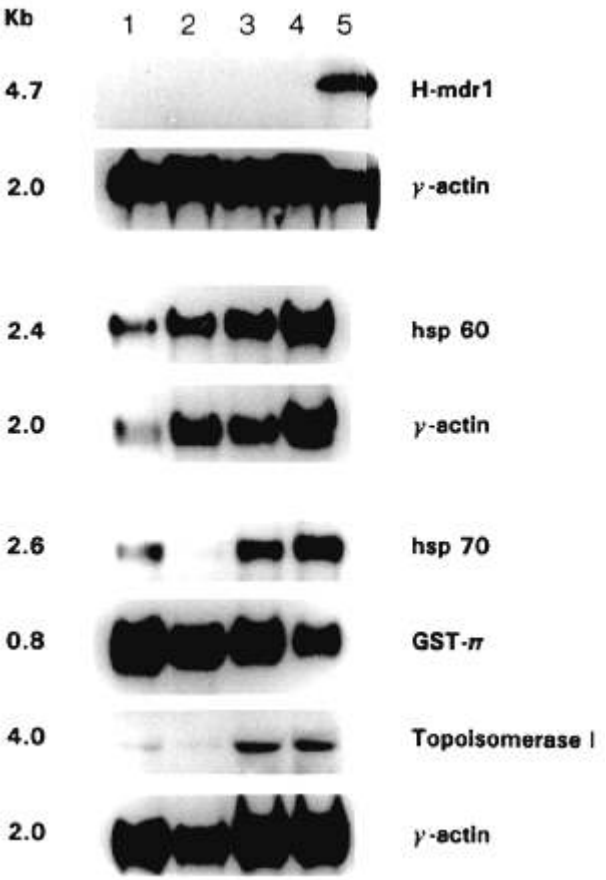

Figure 4. Expression of several putative resistance factors (*hsp60*, *hsp70*, GSTπ, Topo 1) in JR8, M14, 2/21 and 2/60 and LoVo/DX cells (lanes 1, 2, 3, 4 and 5, respectively). Total (20 μg) RNA was fractionated on a 1% agarose–formaldehyde gel, transferred to a nylon membrane and hybridized with the indicated human probes. LoVo/DX, a human colon cancer cell line selected for resistance to doxorubicin (DX) overexpressing *mdr1* gene, was used as a reference cell line.

Table 2. mRNA expression of putative resistance factors[a]

	JR8	M14	2/21	2/60
hsp60	1.2	0.7	1.7	1
hsp70	1	0.3	2.6	3.7
GSTπ	5.2	5.7	2.6	0.8
Topo I	2.5	1.9	8.9	6.5

[a] The levels of the transcrips have been normalized to γ-actin gene expression.

edly low (4- to 15-fold) *hsp70* mRNA expression was observed in M14 and JR8 which showed a high potentiation of L-PAM toxicity by HPT. In the two clones 2/21 and 2/60, which showed the highest *hsp70* mRNA level, the interaction between L-PAM and heat was moderate. Substantial differences were also observed in GSTπ expression. Relevant differences were found in topoisomerase I expression; in particular, the cell lines resistant to TPT (2/21 and 2/60) showed a level of mRNA for the enzyme three and four times higher than that found in the sensitive lines (JR8 and M14). Finally, no detectable mRNA H-*mdr1* expression was found in any of tested melanoma cell lines.

Discussion

The human melanoma cell lines examined in this study showed a modest sensitivity to L-PAM and were not affected in their growth by HPT (1 h at 42°C). However, in combination experiments, HPT was effective, although to a different extent, in increasing L-PAM cytotoxicity in all the cell lines. Specifically, the greatest enhancement was observed in JR8 and M14 cell lines, which showed lower basal levels of HSP70 than the other two cell lines. This finding suggests an involvement of HSP70, a protein known to facilitate protein folding and intracellular translocation[28] in cellular response to combined treatment.

Enhancement of L-PAM cytotoxicity by HPT has already been documented in established cell lines[14,29] as well as in primary cultures of melanoma.[12,15] Such potentiation has been ascribed to the effect exerted by heat on plasma membrane fluidity,[14] with a consequent increase of intracellular drug accumulation, or on DNA–adduct metabolism, with a reduction of DNA lesion removal.[15] Moreover, on the basis of evidence[30] that heat induces alterations in the chromatin structure, it is conceivable that the accessibility of L-PAM, a non-site-specific DNA-damaging agent, to some critical sites of the chromatin is the event determinant for its cytotoxic effect rather than cellular defense mechanisms. This interpretation is supported by the observation that minor groove binders bearing the alkylating function of L-PAM are characterized by a markedly increased cytotoxic potency.[31]

Emphasis on the use of biochemical modulators of GSH metabolism derives from the finding that GSH and related enzymes play a critical role in detoxification processes[7,8] and melanoma cell biology.[32] EA is an inhibitor of GST,[33] the enzyme which catalyzes the conjugation reaction between GSH and different kinds of drugs. In tested cell lines, a somewhat different susceptibility to EA was

observed as a function of GSTπ mRNA expression. In particular, the 2/60 cell line, which showed a low level of enzyme expression, was also the most sensitive to the treatment with EA alone. In combination experiments, a potentiation of L-PAM cytotoxicity by EA was observed only in the JR8 cell line, which showed a relatively high level of GSTπ. On the contrary, no potentiation was observed in M14 cells, which exhibited a basal level of GSTπ comparable to JR8. No interaction was observed in 2/21 and 2/60, the two clones with the lowest GSTπ expression. The efficacy of EA as an enhancer of L-PAM activity in melanoma cells has been previously reported by Hansson *et al.*,[33] who found that EA, at non-toxic concentrations, was able to increase the cytotoxicity of L-PAM in RPMI 8322 cells approximately 2-fold and that this enhancement was caused, at least in part, by an increased formation of L-PAM–DNA adducts. In our study, only the expression of the π isoform of GST was measured, since a high level of the enzyme has been related to resistance to L-PAM.[34] However, EA was shown to inhibit to a different extent several isoforms of GST (μ, ε, π), which may be important in determining tumor cell sensitivity to L-PAM.[33] Thus, a tentative explanation for the variable potentiation ability of EA in the different melanoma cell lines could be a different profile of expression of different GST isoenzymes.

Based on several lines of evidence supporting a role of topoisomerase I in DNA repair,[35–37] TPT has been used as a potential modulator of the cytotoxic activity of alkylating agents. TPT itself caused cytotoxic effects; however, no precise correlation has been found between cell sensitivity to TPT and expression of the target enzyme, as already observed in a large panel of human cell lines.[36] In combination experiments, a 24 h incubation with subtoxic concentrations of TPT following exposure to the alkylating agent potentiated L-PAM cytotoxicity in JR8 cells. A toxic concentration of TPT enhanced L-PAM activity in M14 cells, but only under hyperthermic conditions. It can be hypothesized that the enhancement of L-PAM cytotoxicity by TPT is due to the inhibition of repair of L-PAM-induced DNA lesions. This assumption is sustained by the evidence previously obtained by Kim *et al.*,[38] who demonstrated a significant enhancement of radiation response of human carcinoma cells only when TPT was given after and not before irradiation, thus suggesting that the drug inhibits the post-radiation repair processes. A potentiation of cytotoxic activity of melphalan by TPT was not found in 2/21 and 2/60 cell lines. Since these cell lines exhibited a high level of topoisomerase I expression, it is possible that a partial inhibition of enzyme function is ineffective in impairing processes involved in repair of DNA lesions caused by the alkylating agent. In contrast, in JR8 and M14, the inhibition of the already low basal levels of the enzyme could be effective in interfering with its function during repair processes. If confirmed in other cell systems, these findings may have pharmacological implications.

In conclusion, results from our study indicate that the four melanoma cell lines exhibited a variable expression of some putative mechanisms of resistance and thus potential targets of modulation. The level of expression of a cellular target was not *per se* predictive of the inhibitor ability to modulate cell response to L-PAM in different cell lines. This lack of correlation could be explained by considering that drug resistance is often associated with mechanisms that can differently contribute to drug resistance in individual tumors. As a consequence, it is unlikely that a single modulation agent is effective in all tumors.

Conclusion

The four melanoma cell lines examined exhibited a variable expression of HSP70, GSTπ and topoisomerase I, which are putative mechanisms of resistance and thus potential targets of modulation. The level of expression of these cellular targets was not *per se* predictive of the inhibitor ability to modulate cell response to L-PAM in different cell lines. The sensitization of only one cell line (JR8) to the cytotoxic effect of melphalan by EA and TPT supports an involvement of GST and DNA topoisomerase in cell defense and response to the alkylating agent. However, the variable potentiation of the cytotoxic effects of melphalan achieved in different cell systems suggests that factors other than the level of expression of the modulation target are responsible of such potentiation in individual tumors. As a consequence, it is unlikely that a single modulation agent is effective in all tumors.

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