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Dechlorination of L-phenylalanine mustard by sensitive and resistant tumor cells and its relationship to intracellular glutathione content*

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Recently, we reported in preliminary form [1] that L-phenylalanine mustard (L-PAM) resistant tumor cells can be completely sensitized to L-PAM by decreasing the intracellular concentration of the principal non-protein thiol of the cell, glutathione. As an extension of these studies, we report here that the resistance of these tumor cells to L-PAM is accompanied by intracellular conversion of L-PAM to its non-cytotoxic derivative 4-[bis(2-hydroxyethyl) amino]-L-phenylalanine (dihydroxy L-PAM) and that such detoxification is related to the intracellular concentration of glutathione.

Methods

The methods used in tumor transplantation, *in vitro* growth of murine L1210 leukemia cells, evaluation of drug cytotoxicity, and cellular transport studies have been described previously in detail [1, 2].

Dechlorination of L-PAM by sensitive and resistant tumor cells. Radioactively labeled L-PAM (1 mg/ml) was converted to its non-cytotoxic derivative, dihydroxy L-PAM, by hydrolysis in 0.1 N NAOH for 60 min at 60° [3] and served as a standard for thin-layer chromatographic studies. Optimal separation of L-PAM ($R_f = 0.93$) from dihydroxy L-PAM ($R_f = 0.5$) was achieved on MN 300 cellulose in a solvent system consisting of isopropyl alcohol–formic acid–water (65 : 1 : 34, v/v).

* A preliminary account of this work has been published [*Proc. Am. Ass. Cancer Res.* **22**, 237 (1981)].

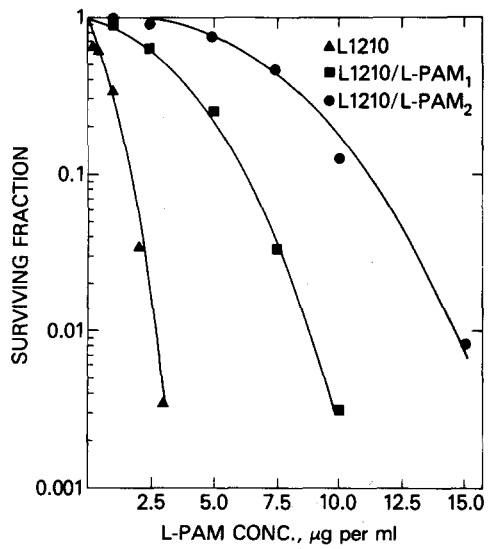

Fig. 1. Cytotoxicity of L-PAM towards L-PAM sensitive and resistant murine L1210 leukemia cells in medium containing amino acids. L1210 (▲), L1210/L-PAM_1 (■) and L1210/L-PAM_2 (●) cells (2.5×10^5/ml) were exposed to L-PAM for 48 hr at 37°. Cell survival was assessed following growth of surviving cells in soft-nutrient agar for 2 weeks [5].

Intracellular dihydroxy L-PAM was identified utilizing a procedure which did not involve washing cells in aqueous buffers following exposure to radioactive L-PAM. This was accomplished by layering 40 ml of cells (2.5×10^5 cells/ml) in medium with labeled L-PAM onto a 10-ml cushion of Versilube F-50 silicone oil and pelleting the cells by centrifugation for 30 min at 12,000 *g*. The radioactive supernatant fraction and the silicone oil were removed by aspiration, the inside of the tubes was swabbed to remove any residual radioactivity, and the cell pellets were lysed in 500 μl of distilled water. Aliquots (100 μl) were applied to MN 300 cellulose, and L-PAM and dihydroxy L-PAM were then separated by thin-layer chromatography in isopropyl alcohol–formic acid–water (65 : 1 : 34, v/v). The plates were dried, and 1.0-cm sections were removed and placed in scintillation vials for determination of radioactivity.

Intracellular glutathione concentrations were determined by the method of Griffith [4] as described previously [1].

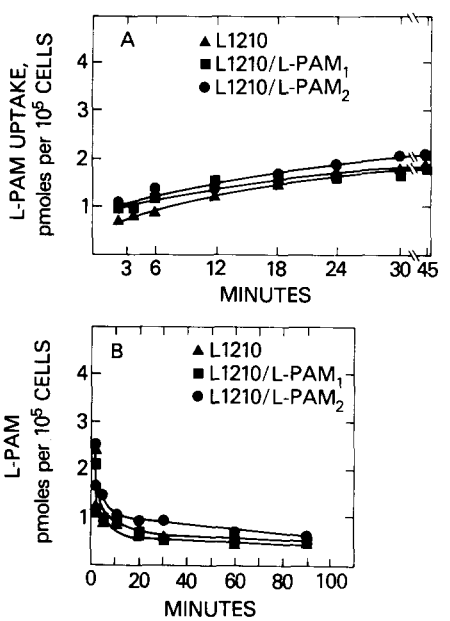

Fig. 2. Uptake and exodus of L-PAM by L-PAM sensitive and resistant murine L1210 leukemia cells in medium with amino acids. Panel A: L1210 (▲), L1210/L-PAM_1 (■) and L1210/L-PAM_2 (●) cells (2.5×10^5/ml) were exposed to [^{14}C]-L-PAM (2.5 μg/ml) for the indicated times. Data are corrected for cellular adsorption of L-PAM [2] and differences in the intracellular water space [6]: L1210 (0.63 μl/10^6 cells); L1210/L-PAM_1 (0.66 μl/10^6 cells); and L1210/L-PAM_2 (0.90 μl/10^6 cells). Panel B: L1210 (▲), L1210/L-PAM_1 (■) and L1210/L-PAM_2 (●) cells (2.5×10^5/ml) were exposed to [^{14}C]-L-PAM (2.5 μg/ml) for 45 min. Cells were then harvested by centrifugation, the resulting cell pellets were resuspended in fresh non-radioactive medium, and L-PAM exodus was monitored for 90 min.

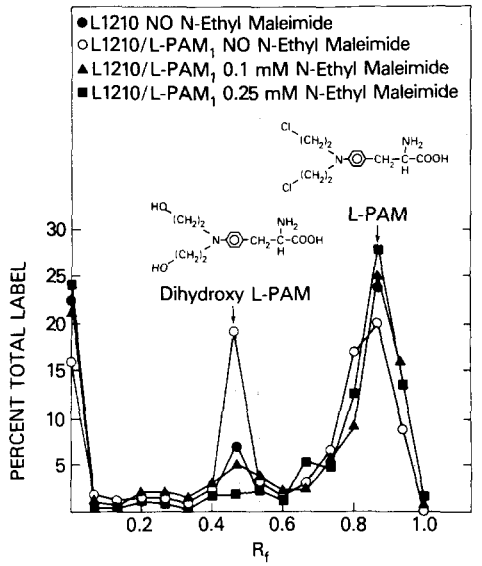

Fig. 3. Dechlorination of L-PAM by L-PAM sensitive and resistant murine L1210 leukemia cells and its inhibition by *N*-ethylmaleimide. L1210 (●) and L1210/L-PAM$_1$ (○) cells (2.5×10^5/ml) were exposed to [^{14}C]-L-PAM (1.25 μg/ml) for 3 hr. L-PAM and dihydroxy L-PAM were separated as described in Methods. L-PAM$_1$ cells were treated for 5 min with 0.1 mM (▲) or 0.25 mM (■) *N*-ethylmaleimide, prior to exposure to L-PAM.

Results

Cytotoxicity of L-PAM toward sensitive and resistant murine L1210 leukemia cells. The cytotoxicity of L-PAM (LD_{37}) toward murine L1210 leukemia cells exposed to the drug in growth medium containing amino acids ranged from 0.8 μg/ml for the parent sensitive tumor cell to 4.0 μg/ml for L1210/L-PAM$_1$ and 8.2 μg/ml for L1210/L-PAM$_2$ (Fig. 1). The absence of amino acids in the exposure medium considerably increased the cytotoxicity of L-PAM toward all three tumor cells although the differences in the degree of resistance did not change (data not shown). The results are in agreement with previously published results which indicate that L-PAM cytotoxicity is reduced by amino acids [2].

Transport and exodus of differentially cytotoxic concentrations of L-PAM by sensitive and resistant tumor cells. The cellular accumulation of a differentially cytotoxic dose of L-PAM by sensitive and resistant tumor cells is illustrated in Fig. 2A. Little difference was observed in the net uptake of a dose of L-PAM which is minimally cytotoxic in medium containing amino acids to L1210/L-PAM$_1$, non-cytotoxic to L1210/L-PAM$_2$, but which produces a 1.5 log decrease in the surviving fraction of L-PAM sensitive tumor cells.

The accumulation of a differentially cytotoxic concentration of L-PAM by L1210, L1210/L-PAM$_1$ and L1210/L-PAM$_2$ in medium devoid of amino acids was also similar (data not shown) and suggests that these three murine tumor cells, although varying substantially in their susceptibility to L-PAM, accumulate similar amounts of drug. Similarly, no differences were observed in L-PAM exodus from sensitive and resistant tumor cells preloaded with a differentially cytotoxic concentration of L-PAM (Fig. 2B).

Dechlorination of L-PAM by sensitive and resistant tumor cells. L1210/L-PAM$_1$ tumor cells dechlorinated 2 to 2.5 times more L-PAM than did sensitive tumor cells (Fig. 3), and this detoxification was inhibited completely by a brief 5-min treatment of tumor cells with the sulfhydryl reagent *N*-ethylmaleimide prior to L-PAM exposure (Fig. 3). A positive correlation existed between the intracellular concentration of glutathione and the sensitivity of murine tumor cells to L-PAM (Fig. 4A). Increases in the glutathione content of these cells were accompanied by linear increases in the LD_{37} values of L-PAM. A positive correlation also existed between the intracellular concentration of glutathione and dihydroxy L-PAM (Fig. 4B). The latter relationship indicates that increases in intracellular glutathione are accompanied by increased cellular content of dechlorinated L-PAM.

Discussion

The results of the present study indicate that the accumulation and retention of differentially cytotoxic concentrations of L-PAM by sensitive and resistant tumor cells are equivalent and suggest that classical transport-related parameters cannot account for resistance of these cells to

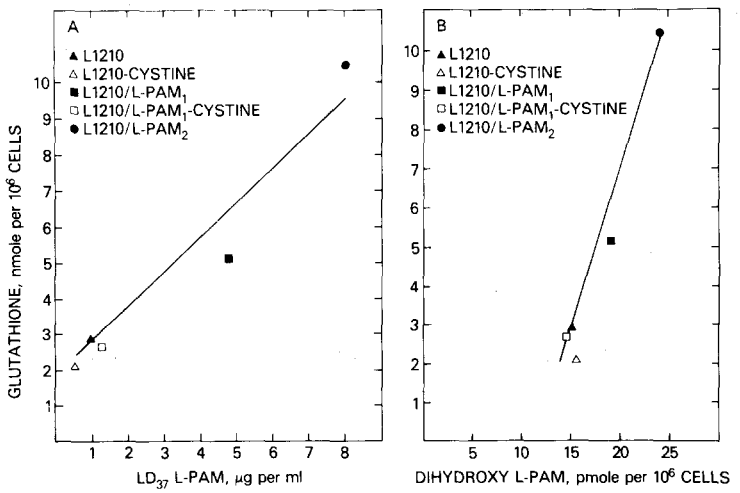

Fig. 4. Correlation between sensitivity to L-PAM, the intracellular content of glutathione and dihydroxy L-PAM. Cytotoxicity data was derived from a 48-hr exposure of cells to L-PAM in growth medium followed by growth of surviving cells in soft-nutrient agar [5]. Glutathione and dihydroxy L-PAM analyses were performed as described in Methods. L1210-cystine (△) and L1210/L-PAM$_1$-cystine (□) were grown in RPMI 1630 medium with a reduced concentration [1] of cystine for >10 passages.

L-PAM. These results are in agreement with the observations of Parsons *et al.* [7] using human melanoma cells. However, our results are not in agreement with a report [8] which indicated that L-PAM sensitive cells transport more L-PAM than do resistant cells. However, differences in the mechanism of resistance to L-PAM may exist in different cell types. Our results do indicate that L-PAM resistant cells convert to 2 to 2.5 times more L-PAM to its non-cytotoxic derivative 4-[bis(2-hydroxyethyl)amino]-L-phenylalanine than do L-PAM sensitive cells and that this dechlorination is inhibited by the sulfhydryl reagent *N*-ethylmaleimide. This detoxification of L-PAM positively correlates with the intracellular concentration of glutathione, the principal non-protein thiol of the cell.

Several studies have demonstrated that cells sensitive to certain alkylating agents generally have a lower cellular content of protein-free thiols [9–13] or a lower ratio of protein-free to protein-bound thiol [14] than do cells which have acquired resistance to these antineoplastic drugs. The results described here, and previously [1], clearly implicate glutathione as being the critical determinant in L-PAM cytotoxicity. The precise mechanism by which glutathione reduces the cytotoxic potency of L-PAM is not clearly understood at the present time. However, it is possible that direct interaction of L-PAM with glutathione and glutathione-*S*-transferase, an enzyme which participates in the dehalogenation of many electrophilic substrates [15], results in dechlorination of L-PAM and a loss of cytotoxicity. Alternatively, the higher concentration of glutathione present in L-PAM resistant cells may either result in protection from L-PAM of a cellular site critical for the expression of L-PAM cytotoxicity or glutathione may aid in displacing L-PAM which is loosely bound to cellular macromolecules during the period immediately following drug exposure. Finally, the possibility exists that glutathione somehow reduces L-PAM cytotoxicity by altering the nature of interstrand, intrastrand or DNA-protein cross-links induced by the drug [16–18]. Our observation that L-PAM resistant cells can be completely sensitized to the drug by lowering the intracellular concentration of glutathione [1] provides an important means to investigate and determine the critical cellular site(s) with which L-PAM must interact for its cytotoxicity to be expressed.

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REFERENCES

1. K. Suzukake, B. J. Petro and D. T. Vistica, *Biochem. Pharmac.* **31**, 121 (1982).
2. D. T. Vistica, J. N. Toal and M. Rabinovitz, *Biochem. Pharmac.* **27**, 2865 (1978).
3. R. L. Furner, L. B. Mellett, R. K. Brown and G. Duncan, *Drug Metab. Dispos.* **4**, 577 (1976).
4. O. W. Griffith, *Analyt. Biochem.* **106**, 207 (1980).
5. M. Chu and G. A. Fischer, *Biochem. Pharmac.* **17**, 753 (1968).
6. R. M. Wohlheuter, R. Marz, J. C. Graff and P. G. W. Plagemann, *J. cell. Physiol.* **89**, 605 (1976).
7. P. G. Parsons, F. B. Carter, L. Morrison and R. Mary, *Cancer Res.* **41**, 1525 (1981).
8. W. R. Redwood and M. Colvin, *Cancer Res.* **40**, 1144 (1980).
9. I. Hirono, *Gann* **52**, 39 (1961).
10. I. Hirono, H. Kachi and A. Ohashi, *Gann* **53**, 73 (1962).
11. T. A. Connors, *Eur. J. Cancer* **2**, 293 (1966).
12. C. R. Ball, T. A. Connors, J. A. Double, V. Ujhazy and M. E. Whisson, *Int. J. Cancer* **1**, 319 (1966).
13. R. W. Poynter, *Biochem. Pharmac.* **19**, 1387 (1970).
14. G. Calcutt and T. A. Connors, *Biochem. Pharmac.* **12**, 839 (1963).
15. W. B. Jakoby, *Adv. Enzymol.* **46**, 383 (1978).
16. K. W. Kohn, in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Eds. A. C. Sartorelli, J. S. Lazo and J. R. Bertino), p. 3. Academic Press, New York (1981).
17. W. E. Ross, R. A. G. Ewig and K. W. Kohn, *Cancer Res.* **38**, 1502 (1978).
18. L. A. Zwelling, S. Michaels, H. Schwartz, P. P. Dobson and K. W. Kohn, *Cancer Res.* **41**, 640 (1981).

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Design and testing of potential activators for hydrolytic enzymes

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Inborn errors of metabolism can arise either from a total lack of an enzyme or from a reduced enzymatic activity that results from the synthesis of an impaired enzyme or a failure to synthesize sufficient enzyme [1]. There are only a few approaches to the treatment of such enzyme insufficiency diseases, and their therapy with drugs has been a particularly intractable problem. In favorable cases, such as galactosemia [1], control of the diet may be effective but in many other cases, such as the lysosomal storage diseases [2], the accumulated metabolite is synthesized internally and cannot be controlled by regulating the diet. In such cases, there is still [3] no effective treatment