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The effect of L-amino acid oxidase on activity of melphalan against an intracranial xenograft

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Abstract We have previously shown that diet restriction-induced depletion of large neutral amino acids (LNAAs) in murine plasma to 46% of control significantly enhances intracranial delivery of melphalan without enhancing delivery to other organs. Studies have now been conducted to determine whether more substantial LNAA depletion could further enhance intracranial delivery of melphalan. Treatment with L-amino acid oxidase (LOX) significantly depleted murine plasma LNAAs: phenylalanine, leucine, and tyrosine (> 95%); methionine (83%); isoleucine (70%); and valine (46%). Experiments evaluating the intracellular uptake of melphalan and high-pressure liquid chromatography quantitation of melphalan metabolites revealed, however, that melphalan is rapidly degraded in the presence of LOX, and that the timing of the administration of melphalan following the use of LOX to deplete LNAAs is crucial. Conditions were found under which LOX-mediated degradation of melphalan was minimized and LNAA depletion was maximized, resulting in a potentiation of the antitumor effect of melphalan on human glioma xenografts in nude mice. Such potentiation could not be obtained using diet restriction alone.

Key words Melphalan · L-Amino acid oxidase · Large neutral amino acids

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Introduction

Melphalan, a bifunctional alkylating agent [1], is a nitrogen mustard derivative of L-phenylalanine. Melphalan has been found to be useful in the treatment of a number of tumors, including multiple myeloma, breast and ovarian carcinoma, and medulloblastoma [7, 14, 20, 23]. Melphalan's utility in the treatment of central nervous system tumors, such as medulloblastoma, however, may be limited by its poor rate of entry into the brain [11].

The chemotherapeutic treatment of central nervous system tumors has been frustrated by the difficulty of delivering agents across the blood–brain barrier (BBB). Efforts to overcome the BBB have centered on two approaches: discovering new agents that can cross the BBB more easily, or discovering ways to improve the

delivery of existing agents. The delivery of substances across membrane barriers, including the BBB, occurs by a number of discrete processes: bulk flow, passive diffusion, facilitated transport, and vesicular transport. Techniques to increase bulk flow by disruption of the BBB have met with relatively little success, while passive diffusion has been enhanced by the use of substances with increased lipophilicity. Vesicular transport is relatively trivial across the BBB. Therefore, facilitated transport, if it could be enhanced, holds the most promise for increasing the delivery of an existing drug into the brain.

Melphalan exhibits a low rate of passive diffusion across the BBB due to its large degree of ionization at physiological pH. Melphalan does appear, however, to be transported—albeit poorly—across the BBB by the facilitated transport system that conveys the large neutral amino acids (LNAAs). This is probably due to the structural relationship of melphalan to phenylalanine [4, 11, 12, 25]. LNAAs have a comparatively high affinity for this transport system [3, 15–19, 24] relative to similar systems in other membranes. Unfortunately, melphalan's affinity for the system is low compared with the LNAAs with which it must compete for transport across the BBB. Since LNAAs have a relatively high plasma concentration, melphalan influx into the brain is limited. The work of Groothuis et al. [13] lends credence to the theory that a significant increase in the influx of melphalan might be effected through a depletion of the plasma LNAAs.

The advantages and limitations of various methods of lowering the plasma levels of LNAAs warrant consideration. Groothuis et al. [13] gave a protein-free diet to mice to lower LNAAs to 46% of control values [10], which increased intracerebral transport of radiolabelled melphalan with K_I (blood-to-tissue transfer constant) ratios for diet to control of 1.3 in brain tumors and 1.8–3.1 in tumor-free brain. An increase in melphalan antineoplastic activity against intracranial tumors was, however, not seen. Moreover, the poor clinical applicability of this method and the desire for increased depletion of the LNAAs led to consideration of new techniques. Hormones—such as epinephrine, insulin, and human growth hormone—only modestly lower the levels of LNAAs [5, 8, 21]. On the other hand, L-amino acid oxidase (LOX)—an amino acid:oxygen oxidoreductase with a molecular mass of 140 000 that is purified from the venom of the *Crotalus adamanteus* (rattlesnake)— oxidizes amino acids, particularly LNAAs, to their respective keto acids [22, 26, 27]. LOX would be expected to greatly lower plasma LNAA levels.

The goal of this project was to determine the efficacy of LOX for depleting plasma levels of LNAAs and for enhancing subsequent melphalan activity against human glioma xenografts growing intracranially in athymic nude mice.

Materials and methods

Animals

Male or female athymic BALB/c mice (*nu/nu* genotype, 6 weeks or older) were used for all studies and were maintained as described previously [2].

Xenograft transplantation and tumor line

D-54 MG is the Duke University subline of A-172 derived from a malignant glioma by Giard et al. [9]. It was grown in cell culture and in athymic nude mice as previously described [6].

Radiochemicals

[^{14}C]Melphalan (melphalan(chloroethyl-1,2-^{14}C); 50 mCi/mmol) was obtained from Moravek Biochemicals, (Brea, Calif.) and was stored at −20°C to minimize decomposition by self-radiolysis. It was packaged by the manufacturer in methanol, which was evaporated before the experiment. The residue was resolubilized in 10 µl dimethylsulfoxide per 100 µl of original solution and was then brought to a volume of 100 µl with normal saline immediately prior to injection. Radiochemical purity was greater than 95%. [^{14}C]Leucine was purchased from New England Nuclear as NEC-279 Leucine L-[^{14}C(U)] with a specific activity of 13.0 GBq/mmol (at a concentration of 3.7 GBq/ml = 0.28 µmol/ml = 0.037 mg/ml).

LOX preparation

Crotalus adamanteus venom was purchased from the Miami Serpentarium. From the venom, LOX was purified and crystallized by the procedure of Wellner and Meister [27]. The preparations of LOX were 20 units/mg where a unit is defined by the amount of LOX required to oxidize 1.0 µmol of leucine per minute determined as described below.

Quantitation of LOX-mediated depletion of plasma LNAAs

Groups of four to six animals were injected i.v. via the tail vein with LOX at doses of 100–400 µg and killed at intervals of 0.5 to 24 h later. Blood samples were obtained via cardiac puncture, the samples were centrifuged (12,000 rpm), and the plasma was frozen. The plasma was subsequently deproteinized with the addition of 50% 5-sulfosalicyclic acid and, following removal of protein by centrifugation, amino acids in the supernatant were stabilized by addition of 1 *N* LiOH to neutrality. The LNAA levels were measured on a Beckmann amino acid analyzer.

Quantitation of plasma LOX activity

Groups of four to six animals were injected and killed as above. Blood samples were similarly obtained and centrifuged, and the plasma was separated. A 100-µl assay solution was prepared consisting of a final concentration of 0.4 *M* Tris-HCl at a pH of 7.5, 1 m*M* [^{14}C]leucine with 0.8 µCi/ml, and 40 µl of LOX-containing solution (pure LOX or plasma). The assay solution was incubated for 10 min at 37°C, and the reaction was halted with 100 µl of 20% trichloroacetic acid. After the solution was cooled on ice for 5 min, it was

microfuged at 12,000 rpm for 1 min, and 180 µl of supernatant was placed on a 2.5-cm Bio-Rad Dowex 50W-X8 (200–400 mesh hydrogen form) cation exchange resin column. The column was washed with 3.82 ml deionized water into a test tube, 2 ml was removed, and radioactivity was determined by liquid scintillation counting. Plasma LOX samples were obtained from whole blood by centrifugation at 12,000 rpm for 30 s; the plasma was removed and kept at 4°C until the assay was run. Although initial calibration experiments were performed with LOX from Sigma Chemicals (St. Louis, Mo.), most of the studies reported used LOX purified as previously described [27] and crystallized twice.

Kinetic analysis of melphalan/LOX interaction

The ability of LOX to degrade various amino acids and L-melphalan was determined in reaction mixtures containing 100 ml glycylglycine buffer (pH 7.5, 0.3 m*M* NADH, 2 m*M* α-ketoglutarate, 0.5 m*M* ADP, 20 IU glutamate dehydrogenase; Sigma Chemicals) and L-amino acid substrate or melphalan at concentrations ranging from 0.1 to 16 m*M*. The final volume was 1.0 ml, and reactions were carried out at 37°C. In this system, LOX converts LNAAs or melphalan to the corresponding α-keto acid and ammonia; the ammonia then reacts stoichiometrically with α-ketoglutarate and NADH in a reaction catalyzed by glutamate dehydrogenase. The oxidation of NADH to NAD^+ is accompanied by a decrease in OD_{340}, which is monitored and used to determine the amount of ammonia made during the reaction ($\Delta OD_{340} = 6.2$ indicates the formation of 1 µmol product). Rates of reaction were linear with time, and kinetic constants (K_m and V_{max}) were determined graphically using double reciprocal (Lineweaver-Burk) plots.

In vivo melphalan administration

Melphalan, provided courtesy of the Burroughs Wellcome Co. (Research Triangle Park, N.C.), was administered in all experiments as a single i.p. injection in 17% dimethyl sulfoxide at a dose of 71 or 36.5 mg/m^2 (23.7 or 12.2 mg/kg), which corresponds to 1.0 LD_{10} (dose lethal to 10% of treated animals) and 0.5 LD_{10}, respectively [27].

HPLC quantitation of LOX-mediated metabolism of melphalan

A Brownlee C18 reverse-phase column was used on a Hewlett Packard 1090 high-pressure liquid chromatograph connected to a Kratos Analytic Instruments Spectroflow 783 programmable absorbance detector at a wavelength of 260 nm and a range of 0.1 mV. Each run consisted of a sample of 10 µl injected via syringe. For each run, 60 fractions were collected by a Gilson Model 203 microfraction collector with 0.5 min/fraction into polypropylene Omnivials (Wheaton Company, Millville, Ohio). Two elution fluids were used in combination: 0.1 *M* ammonium acetate (pH 4.1) and 0.1 *M* ammonium acetate with 70% methanol (pH 4.1). The best results were achieved with a 30:70 combination of the fluids (net 49% methanol) at a flow rate of 1 ml/min for the first 15 min and then 1.5 ml/min after 15.5 min. The fractions collected by time were then counted with 2 ml scintillation fluid on a Beckman LS8100 scintillation counter. The samples consisted of 50 µ*M* [^{14}C]melphalan in Dulbecco's phosphate-buffered saline with or without the addition of LOX (233 µg/ml). Samples were incubated at 37°C for the various sample times: 0, 5, 15, and 30 min. Samples were stored at −20°C after incubation was completed, and they were thawed for injection into the high-pressure liquid chromatography (HPLC) column.

Evaluation of LOX modulation of melphalan toxicity

Groups of six mice each were treated with LOX i.v. at doses of 100 or 400 µg followed 2 h later by melphalan at doses of 0.5 or 1.0 LD_{10}. Animals were weighed daily and monitored for survival.

Evaluation of LOX modulation of melphalan activity against intracranial tumors

Groups of ten randomly assigned mice were treated on day 6 post-tumor implantation with LOX at dosages of 100 to 300 µg or saline i.v. via the tail vein followed at variable intervals by melphalan administered at doses of 1.0 or 0.5 LD_{10}. Additionally, certain experiments also restricted animal access to food for 18 h followed by a 6-h exposure to protein-free chow. One group of ten animals served as a control and received drug vehicle. Assessment of response was the comparison of median survival time between treated and control groups. Statistical significance was assessed by the Wilcoxon rank sum test as described previously [6].

Results

LOX-mediated depletion of murine plasma LNAAs

Substantial depletion of plasma LNAAs was seen as early as 0.5 h following i.v. injection of 100 µg LOX. The potential benefit of increasing the dose to 200–400 µg was evaluated at 2 and 4 h following treatment. Maximal depletion of LNAAs was observed at 2 h with 300 µg LOX, with the following percentages of depletion: phenylalanine > 95%, leucine > 95%, tyrosine > 95%, methionine 83%, isoleucine 70%, and valine 46% (Fig. 1). No significant depletion of histidine was noted. Use of 400 µg LOX did not appreciably increase the depletion of the LNAAs at 2 or 4 h.

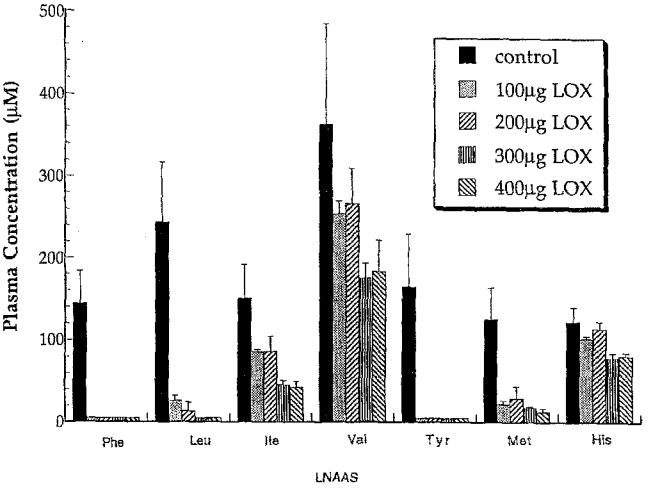

Fig. 1 Concentration of LNAAs in murine plasma 2 h following i.v. administration of LOX. At 4 h, depletion was not significantly greater (values are means, *bars* ± SD)

Murine plasma LOX level

Measurement of LOX levels (based on LOX activity) in murine plasma following i.v. administration of 100–300 μg LOX revealed persistence of activity for up to 18 h following treatment (Fig. 2).

Kinetic analysis of melphalan/LOX interaction

The similarity between the structures of melphalan and the LNAA phenylalanine generated concern that LOX could metabolize melphalan. Analysis of the interaction of LOX and melphalan revealed that melphalan was indeed degraded with a K_m of 0.30 mM and a V_{max} of 11 μmol/min mg compared with 0.13 mM and 33 μmol/min mg for phenylalanine, indicating that melphalan is a substrate of LOX (Table 1).

HPLC quantitation of LOX-mediated metabolism of melphalan

The levels of melphalan and various metabolites as measured by HPLC at various incubation times with or without the presence of 575 μg/ml of LOX are shown in Fig. 3. The solutions exposed to LOX demonstrated a significant loss of melphalan over time, indicating the metabolism of melphalan by LOX.

LOX modulation of melphalan toxicity

A comparison of 0.5 and 1.0 LD_{10} melphalan alone and in combination with 100 μg of LOX showed no increase in toxicity with the addition of LOX (Table 2, experiment 1). A study comparing 1.0 LD_{10} of melphalan alone and in combination with 400 μg LOX (experiment 2) showed a minimal increase in toxicity but no increase in weight loss.

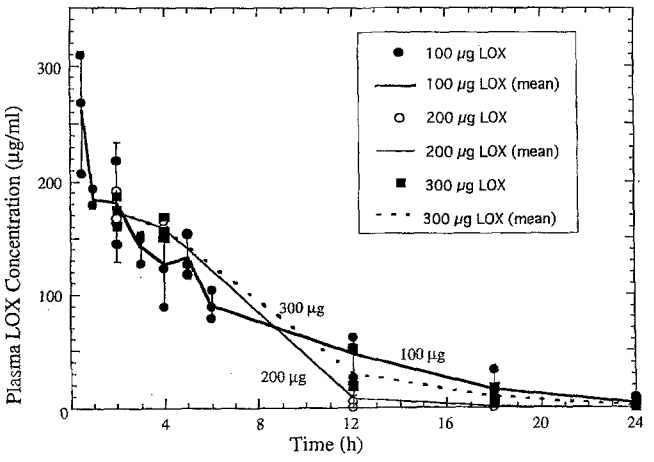

Fig. 2 LOX concentration in murine plasma at various times following i.v. administration of LOX (values are means, *bars* ± SD)

Table 1 Kinetic analysis of effects of melphalan/LOX interactions on LNAAs in athymic mice (*NA* not applicable)

Substrate	K_m (mM)	V_{max} (μmol/min mg)	$(V_{max})/(K_m)$
L-Alanine	NA	0	0
L-Leucine	0.6	100	167
L-Isoleucine	2.05	33	16
L-Valine	19	3	0.2
L-Methionine	0.47	70	150
L-Phenylalanine	0.13	3	10
L-Tryptophan	0.22	3	14
L-Tyrosine	0.13	7	54
Melphalan	0.30	11	37

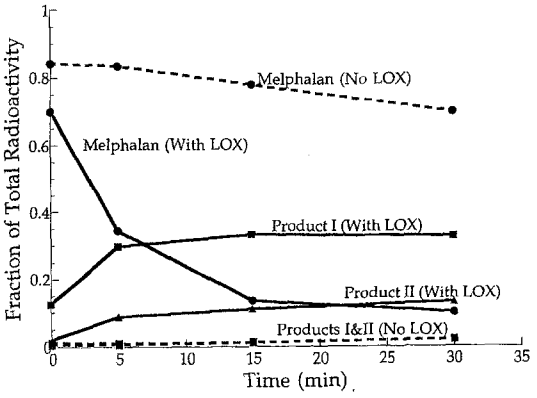

Fig. 3 HPLC measurement of melphalan and metabolites incubated in vitro in the presence or absence of LOX (50 μM melphalan, 11.5 mg/ml LOX, pH 7.4, 37°C)

Table 2 LOX modulation of melphalan toxicity in athymic mice. LOX was administered i.v. 2 h before the i.p. administration of melphalan (six animals/group)

Experiment	Melphalan dosage (LD_{10})	LOX dosage (μg)	Mean nadir weight loss (%)	Deaths
1	1.0	0	31.8	0/6
	0.5	0	16.6	0/6
	0	100	1.1	0/6
	1.0	100	19.8	0/6
	0.5	100	16.3	0/6
2	1.0	0	14.6	0/6
	0	400	0.0	0/6
	1.0	400	12.5	2/6

LOX modulation of melphalan activity

Various regimens combining LOX and melphalan were tested to compare the median survival of mice with D-54 MG human glioma intracranial xenografts.

Table 3 LOX modulation of melphalan activity against D-54 MG xenografts growing intracranially in athymic mice. Melphalan was given i.p.; LOX was administered i.v. In experiments 1 and 2, the LOX was given 2 h prior to melphalan administration. In experiments 3 through 5, LOX was given 24 h prior to melphalan administration; an 18-h fast with a 6-h protein-free diet was added to the regimen for some groups

Experiment	Melphalan dosage (LD_{10})	LOX dosage (μg)	Median survival (days)	% Increase in survival (%)
1	0	0	19.5	0
	0	100	22	12.8
	0.5	0	26.5*	35.9
	0.5	100	27*	38.5
	1.0	0	34*	74.3
	1.0	100	33*	69.2
2	0	0	20	0
	0	300	20.5	2.5
	0.5	0	31*	55
	0.5	300	30*	50
3	0	0	18	0
	0.5	0	32*	77.8
	0.5	300	32*	77.8
	0.5	0 + diet	33*	83.3
	0.5	300 + diet	34*	88.9
4	0	0	21	0
	0.5	0	33.5*	59.5
	0.5	200 + diet	41*,**	90.4
5	0	0	18	0
	0.5	0	27*	50
	0.5	200 + diet	30*,**	66.7

*$P < 0.05$ compared with controls; **$P < 0.05$ compared with melphalan alone

Neither a 100-μg nor a 300-μg dose of LOX administered i.v. 2 h before the administration of a 0.5-LD_{10} dose of melphalan resulted in any change in median survival of mice treated bearing intracranial tumors (Table 3, experiments 1, 2). This was not surprising since in vitro studies had demonstrated that melphalan was a substrate for LOX. However, a modification of the regimen using LOX 24 h prior to the administration of melphalan, together with a protein-free diet for 12 h before the melphalan was given, led to positive results (experiments 3–5). A statistically significant increase in the median survival of the tumor-bearing mice was achieved as compared with melphalan alone when 200 μg LOX was given by this modified regimen (experiments 4 and 5). Dietary restriction plus melphalan was never successful in increasing the median survival of tumor-bearing mice as compared with treatment using melphalan alone.

Discussion

The broad spectrum of melphalan activity, coupled [7, 14, 20, 23] with its facilitated transport across the LNAA pathway of the BBB [11], provided an incentive to increase intracranial delivery of melphalan by decreasing the plasma concentrations of competing LNAAs. Although our initial approach exploiting depletion of plasma LNAAs induced by diet restriction was successful in increasing delivery of melphalan to intracranial tumor and normal brain [13], we did not find an increase in the antineoplastic activity of melphalan against intracranial tumors. Accordingly, further depletion of LNAAs using a clinically exploitable pharmacological approach appeared desirable. The ability of LOX to oxidize LNAAs without producing toxicity [22, 26, 27] suggested that combination therapy with LOX plus melphalan might increase the therapeutic index of this alkylating agent. However, our in vitro studies made it clear that melphalan was a substrate for LOX and that combination therapy with melphalan plus LOX was likely to be unsuccessful if melphalan was administered at a time when murine plasma LOX levels were still appreciable. This was confirmed by our in vivo studies (intracranial tumor experiments 1–3) and suggested that melphalan must be administered at a time when the LOX levels had declined but the LNAA levels had not increased to normal values.

Manipulation of the timing of LOX and melphalan administration made it possible to identify a dosage of LOX that could be used in conjunction with an overnight fast and protein-free diet to deplete the levels of the LNAAs while in theory minimizing the level of LOX present when melphalan was administered. The purpose of the protein-free diet was to deplete and prevent repletion of the LNAAs during the 12–24-h period before the administration of melphalan. It was then possible to demonstrate a statistically significant increase in the survival of mice bearing intracranial tumors. This increased survival is presumably a measure of the ability of the regimen with LOX and protein-free diet to increase the delivery of melphalan across the BBB, since administering LOX and a protein-free diet produced no increase in length of survival when given alone.

The increased survival of mice with intracranial brain tumors, presumably secondary to increased melphalan delivery across the BBB, suggests that this may be an intriguing therapeutic modality. Even more provocative results may be seen, however, by further depleting the LNAAs and limiting the LOX activity that is present when melphalan is administered. This goal might be achieved by a number of methods, including finding a window of time during which the LNAA depletion continues while little LOX activity is present, or preventing LOX/melphalan interaction by depleting the plasma LNAAs using an external column in which a patient's plasma would be exposed to matrix-bound LOX before melphalan was administered. Increased transport across the BBB of a drug based on a particular LNAA, such as melphalan, by eliminating

the competing amino acids, may have promise for increasing the utility of other drugs in treating central nervous system diseases.

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