Effect of L-Dopa Methylester and Glutathione Depletion on Murine B16BL6 Melanoma Growth In Vitro

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The cytotoxic and growth-inhibitory effect of levodopa methylester (LDME) in murine B16BL6 (BL6) melanoma cells after glutathione (GSH) depletion was studied in vitro. Pretreatment of BL6 cells with 50 μM buthionine sulfoximine (BSO) depleted GSH content by nearly 90% and enhanced the growth-inhibitory effect of even a minimally cytotoxic concentration of LDME. Radiothymidine incorporation into BL6 cells significantly increased compared to untreated controls during the first 4 h of exposure to 0.2 mM LDME. However, pretreatment with BSO prevented this LDME-induced increase in radiothymidine incorporation.

Because the percentage of cells in S-phase of the cell cycle was not altered, these results suggest that BSO exposure may be inhibiting unscheduled DNA synthesis, which could contribute to the cytotoxic effects of LDME. In addition, spectrophotometric studies indicated that in a cell-free system, GSH scavenged dopaquinone produced by the tyrosinase-mediated oxidation of LDME, presumably by formation of glutathionyl dopa. Thus, enhancement of LDME cytotoxicity by BSO may also involve depleting the amount of GSH available for the nucleophilic addition to the quinone. *J Invest Dermatol* 97:1073–1077, 1991

Malignant melanoma is a tumor that is inherently refractory to chemotherapy. During oxidative reactions involving quinone production, pigmented melanoma cells utilize the amino acids, tyrosine and L-dopa, to synthesize melanin [1]. This biochemistry pathway is unique to pigmented cells, and has been suggested as a selective target for the chemotherapy of melanoma [2,3].

Wick et al [4] reported that L-dopa was selectively cytotoxic toward pigmented melanoma cells in vitro. Later studies [5,6] demonstrated that the water-soluble L-dopa methylester (LDME), and other L-dopa analogs, have significant antimelanoma activity both in vitro and in vivo [7,8]. At high concentrations (1–2 mM), L-dopa and LDME inhibit DNA polymerase activity and DNA synthesis in vitro [9]. This appears to be dependent on the conversion of L-dopa to dopaquinone, via the melanoma-specific enzyme tyrosinase [9]; however, the production of active oxygen species via autoxidation of L-dopa outside of the cell may also contribute to cytotoxicity in vitro [10].

Previous studies [6,11] have suggested that agents that alter the redox cycling of L-dopa between the quinol and quinone forms may alter cytotoxicity. Thus, intracellular radical scavengers may play a critical role in catechol cytotoxicity. Glutathione (GSH) is a sulfhydryl tripeptide important in the detoxification of drugs that exert cytotoxicity via a quinone intermediate. GSH forms a conjugate with dopaquinone in melanoma cells [12] and plays a key role in the regulation of melanin production [13]. Early studies [14,15] have also shown that the addition of glutathione to melanoma cells in culture leads to S-cysteylinyl dopa formation, although cysteylinyl dopa can also form in the absence of glutathionyl dopa. In addition, cysteylinyl dopa is cytotoxic to melanoma cells [7] and is excreted from pigmented cells, and it has been suggested that the formation of cysteylinyl dopa via glutathionyl dopa may be a mechanism of detoxication of dopaquinone in pigmented cells [16]. Because of the various roles GSH may play in the metabolism of dopaquinone in melanoma cells, we investigated the growth-inhibitory effects of LDME under conditions of GSH depletion in B16-BL6 melanoma cells. We found that at minimally cytotoxic concentrations, LDME induces radiothymidine incorporation into BL6 cells along with a cytostatic effect on growth. In addition, depletion of GSH content by exposure to buthionine sulfoximine (BSO) significantly enhances both the cytotoxic and cytostatic effects of LDME, but inhibits the LDME-induced increase in radiothymidine incorporation.

**MATERIALS AND METHODS**

**Culture Conditions** The highly invasive and metastatic BL6 melanoma isolated by Dr. Ian Hart [17] was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Salt Lake City, UT), sodium pyruvate, non-essential amino acids, L-glutamine, twofold vitamin solution (Gibco, Grand Island, NY), 5 μg/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Cells were subcultured weekly at a split ratio of 1:4 and were maintained at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were harvested using 0.1 mM ethyleneglycol-bis-(β-amino-ethylster)-N,N’-tetraacetic acid (EGTA) after washing the monolayer twice with Ca2+- and Mg2+-free Tyrodes’ balanced salt solution (CMF).

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**Abbreviations:**
BL6: B16BL6 melanoma
BSO: buthionine sulfoximine
CMF: Ca++- and Mg2+-free Tyrodes’ balanced salt solution
EGTA: ethyleneglycol-bis-(β-amino-ethylster)-N,N’-tetraacetic acid
LDME: L-dopa methylester
L-dopa: L-dihydroxyphenylalanine

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Growth Assays  BL6 cells were seeded into six-well culture plates (Falcon) at a density of 10^5 cells per well. After 48 h, appropriate wells were exposed to 50 μM BSO for 24 h to deplete GSH, 0–4 mM LDME for 8 h, or BSO followed by LDME. The cells were washed twice with CMF and allowed to incubate for an additional 16 h after LDME exposure. Tumor cells were then harvested using 0.1 mM EGTA, and the number of viable cells was determined by trypan blue exclusion.

Glutathione Measurements  Total glutathione content was measured as described by Adams et al [18], with minor modifications. Briefly, 10^6 cells were centrifuged at 100 × g, and the pellet was lysed by sonication in 1.0 ml of 10 mM dithionitrobenzene. To measure total GSH, 50 μl of this sample was added to a cuvette containing 50 μl dithionitrobenzene, 0.5 U glutathione reductase, 0.1 mM potassium phosphate buffer (pH 7.5), and 220 nmol NADPH in a total reaction volume of 1.0 ml. The change in absorbance was monitored spectrophotometrically at 412 nm for 5 min.

Radiothyridine Incorporation  DNA synthesis was determined by measuring incorporation of ^3H-thymidine into the acid-precipitable fraction of viable cells. BL6 cells were seeded into six-well plates and allowed to grow for 48 h. The cells were then exposed to either 50 μM BSO for 24 h, 0.2 mM LDME for 1–8 h, or BSO followed by LDME. The monolayers were washed twice with CMF, and immediately pulsed with 0.3 μCi/ml ^3H-thymidine (New England Nuclear, 50 Ci/mmol) for 1 h. Excess radiolabel was removed after rinsing the monolayer 3 times with complete medium containing 0.3 mM unlabeled thymidine. The cells were harvested with 0.1 mM EGTA, and an aliquot of cell suspension was used to determine viability by trypan blue exclusion. The acid-precipitable fraction was extracted with 0.5 ml of 10% trichloroacetic acid overnight, washed with CMF, and digested in 0.5 ml 1 N potassium hydroxide for 2 h at room temperature. Aliquots of cell extract were counted for 2 min on a Packard scintillation counter. The results are expressed as cpm/10^6 viable cells.

Autoradiographic Measurement of S-Phase Population  The percentage of cells undergoing DNA replication was determined by autoradiography, according to the method of Doolittle et al [19]. BL6 melanoma cells were grown on coverslips in six-well culture plates and exposed to either 50 μM BSO for 24 h, 0.2 mM LDME for 4 h, or BSO followed by LDME. Cells were then incubated with complete medium containing 0.3 μCi/ml ^3H-thymidine for 1 h. The monolayer was rinsed 3 times with CMF and the cells were fixed with absolute methanol for 10 min. The coverslips were dried for 30 min to remove excess methanol and mounted on microscope slides. Slides were then dipped in Ilford K.5 emulsion (Polysciences, Inc.) and stored in complete darkness for 21 d. After exposure, the slides were developed with Kodak Dektol developer, fixed with Kodak fixative, and stained with Mayer’s haematoxylin and eosin stains. Cells in S-phase label heavily and can be easily distinguished from those not in S-phase [19]. The cells were scored by counting the percentage of cells in S-phase in four randomly selected fields on each slide. Necrotic or morphologically altered cells were not scored to eliminate the acutely cytotoxic affects of the drug treatments.

Tyrosinase-Catalyzed Dopaquinone Formation from LDME  The oxidation of LDME by tyrosinase was measured as described by Kern et al [20], where dopaquinone formation is monitored at the absorbance maximum of 464 nm. The conversion of LDME to dopaquinone was determined spectrophotometrically in a reaction mixture consisting of 50 units mushroom tyrosinase and 50 μg LDME in 0.1 M potassium phosphate buffer (pH 6.5). Because sulfhydrils, including GSH, can react with quinones by nucleophilic addition to form dopaquinone [1,12,13,14], we determined the effect of GSH and cysteine on the conversion of LDME to dopaquinone by tyrosinase.

Statistical Analysis  Significant differences between treatment groups in the cytotoxicity assays, radiothyridine incorporation studies, and autorigaradiographic experiments were determined by analysis of variance and Fisher’s protected least-significant difference test. GSH measurements were analyzed by Student t test.

RESULTS  The concentration of BSO required to achieve maximum depletion of intracellular GSH in BL6 cells with minimal cytotoxicity was determined in preliminary experiments. It was determined that 50 μM BSO depleted nearly 90% of the total intracellular GSH content in BL6 cells within 24 h. In addition, when BSO was removed, GSH content remained suppressed by 50% for up to 8 h (data not shown).

The effect of LDME on the growth of BL6 cells after GSH depletion is presented in Fig 1. The concentration of LDME that inhibits growth of BL6 cells by 50% (IC₅₀) is greater than 3.2 mM. Pretreatment with 50 μM BSO significantly lowered the IC₅₀ of LDME to 1.9 ± 0.2 mM (p < 0.05). BSO also enhanced the growth-inhibitory effects of a minimally effective concentration of LDME (Fig 2). LDME alone, at a concentration of 0.2 mM, caused a modest 11% inhibition of cell growth, although this effect was delayed several hours after the LDME was removed. This concentration of LDME is termed “growth-inhibitory” because it does not result in an immediate decrease in cell viability as determined by trypan blue exclusion. Similarly, 50 μM BSO alone caused about 11% decrease in cell growth, but this decrease was not apparent until at least 8 h after the BSO was removed from the cells. However, the combination of BSO and LDME resulted in a 49% decrease in the viable cell count at the 96-h time point, indicating nearly complete inhibition of cell growth.

Because the autooxidation of LDME may generate reactive oxygen species [21], the effect of LDME exposure on GSH content was investigated. Exposure of BL6 cells to 0.2 mM LDME for 8 h did not significantly alter total intracellular GSH content (Table 1, experiment 1). As expected, BSO pretreatment alone depleted 81–86% of total intracellular GSH content. In addition, the combination of BSO followed by LDME exposure depleted GSH content 38% more than BSO alone.

High concentrations of LDME (2.4–4.8 mM) have been reported to inhibit DNA synthesis [9]. However, during the first 4 h of exposure to 0.2 mM LDME, which inhibits growth, the uptake
of radiolabeled thymidine into BL6 cells is increased compared to untreated cells (Fig 3). In contrast, BL6 cells pretreated with 50 μM BSO followed by exposure to 0.2 mM LDME did not show a significant increase in radiothymidine incorporation, compared to untreated controls. Cells that were pretreated with BSO alone did not differ in radiothymidine incorporation, until 8 h after the BSO was removed, which does not account for the decrease in radiothymidine incorporation seen in the combined BSO and LDME treatments.

The effects of the various treatments on the percentage of cells in the DNA-replicative phase of the cell cycle are shown in Table II. Neither BSO nor LDME alone, nor in combination, altered the percentage of cells in S-phase, which ranged from 27.1–28.8% of the total population. The values for S-phase in these experiments are in agreement with previously reported values [22].

The rate of putative dopaquinone formation by tyrosinase is shown in Fig 4. A preliminary experiment determined that in a cell-free system containing mushroom tyrosinase, LDME is rapidly converted to products with absorption maximums of approximately 346 and 464 nm (data not shown). These values correlate closely with reported absorption maximums for dopachrome and dopaquinone, respectively [20]. As is shown in Fig 4, putative quinone formation under these conditions is maximum within 3 min, which is consistent with a previous study [20]. When GSH is added to the reaction mixture, the absorbance at 464 nm is abolished. Similar results are observed when cysteine is added to the reaction mixture (data not shown), suggesting that sulfhydrals can scavenge dopaquinone produced by the tyrosinase-mediated oxidation of LDME. Similar conclusions have been reported with L-dopa in a previous study [14].

**DISCUSSION**

Depletion of intracellular GSH content enhances the chemotherapeutic response of several tumor cells to a variety of anticancer drugs [23–25]. In this report, we demonstrate that depletion of intracellular GSH by BSO pretreatment augments the cytotoxic and growth-inhibitory activities of LDME on BL6 melanoma cells. LDME and BSO individually cause only about 11% growth inhibition, yet the combination of BSO and LDME caused approximately 49% inhibition of growth. Thus, it can be concluded that the growth-inhibitory combination of BSO and LDME is synergistic.

At the cytostatic concentration of 0.2 mM LDME used in this study, decreased growth of BL6 cells is preceded by an increase in radiothymidine incorporation into viable cells. This increased radiothyminidine incorporation during LDME treatment could be explained by several mechanisms: 1) normal DNA replication was

**Table I.** Effect of BSO and LDME on Total Intracellular Glutathione Content in BL6 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glutathione (nmol/million cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>44.3 ± 8.2</td>
</tr>
<tr>
<td>50 μM BSO</td>
<td>6.4 ± 1.1</td>
</tr>
<tr>
<td>0.2 mM LDME</td>
<td>41.2 ± 11.4</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>33.5 ± 8.9</td>
</tr>
<tr>
<td>50 μM BSO</td>
<td>6.3 ± 4.0</td>
</tr>
<tr>
<td>50 μM BSO + 0.2 mM LDME</td>
<td>3.9 ± 1.0 b</td>
</tr>
</tbody>
</table>

* Results are expressed as the mean ± SD of three samples. Combined BSO and LDME treatments were measured in independent experiments using independent controls.

* Differs from untreated controls (p < 0.05).

* Differs from BSO alone (p < 0.05).

**Table II.** Effect of BSO and 0.2 mM LDME on the Percentage of S-Phase Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent S-Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>28.2 ± 6.0</td>
</tr>
<tr>
<td>50 μM BSO</td>
<td>28.8 ± 7.9</td>
</tr>
<tr>
<td>0.2 mM LDME</td>
<td>28.5 ± 2.9</td>
</tr>
<tr>
<td>50 μM BSO + 0.2 mM LDME</td>
<td>27.1 ± 5.5</td>
</tr>
</tbody>
</table>

* All values represent the mean ± SD of three independent samples. Each sample consisted of four randomly selected subsamples.
increased after LDME treatment; 2) unscheduled DNA synthesis was induced by LDME; or 3) the intracellular thymidine pools were altered. The autoradiographic results (Table II) suggest that the percentage of total viable cells in normal DNA replication was not increased by LDME exposure. Because the thymidine pool status (thymidine triphosphate and deoxythymidine triphosphate) was not measured in this study, no conclusions can be drawn about this explanation. However, because quinones are capable of causing DNA damage [26], it is likely that low concentrations of LDME may induce unscheduled DNA synthesis, and this may explain the initial increase in radiotylidium incorporation into BL6 cells during LDME exposure. The findings of Lai et al. [27], who demonstrated that BSO inhibited cisplatin-induced DNA repair in human ovarian cancer cells, also directly support this conclusion.

Typically, the augmentation of cytotoxic drug activity by BSO pretreatment is attributed to decreased detoxification of reactive oxygen species, resulting in enhanced oxidative damage. Catechols may exert cytotoxic effects on melanoma cells via generation of both hydrogen peroxide and cytotoxic quinones [21]. In addition, the conversion of L-dopa to dopaquinone in melanoma cells involves the generation of superoxide radicals, which may contribute to cytotoxicity [28]. Inoue et al. [21] have suggested that in pigmented melanoma cells, cytotoxicity of L-dopa analogues in vitro is due to both the exogenous production of hydrogen peroxide and the tyrosinase-mediated oxidation of the quinols to quinones. In contrast, Kable et al. [8] reported that the sensitivity of human melanoma cells to L-dopa did not correlate with tyrosinase activity, and that BSO enhancement of L-dopa cytotoxicity in human melanoma did not correlate with intracellular sulfhydryl levels. However, our results show that the combination of BSO and LDME depletes BL6 GSH content significantly more than BSO treatment alone, suggesting that GSH levels may indeed be important to the cytotoxicity of LDME. We have found that the addition of exogenous catalase (up to 100 µg/ml) does not significantly alter the growth-inhibitory effects of 0.2 mM LDME, nor does it alter the augmentation of 0.2 mM LDME-mediated growth inhibition by BSO (data not shown). We conclude that in BL6 melanoma cells growth-inhibitory effects of 0.2 mM LDME are not mediated solely by exogenous hydrogen peroxide production. However, we cannot exclude the possibility that exogenous hydrogen peroxide may be involved in the cytotoxicity of higher concentrations of LDME in vitro, or that LDME generates other active oxygen species that are cytotoxic to melanoma cells. The results seen with the radiotylidium incorporation study suggest that LDME is having an intracellular effect, which also argues that the cytotoxicity of LDME is not mediated solely by the autooxidation of LDME outside the cell.

The instability of dopaquinone precludes easily monitoring its production in intact cells. Nonetheless, it is generally accepted that L-dopa and LDME are converted to dopaquinone in pigmented melanoma cells, that GSH and cysteine conjugate with dopaquinone to form glutathionylidopa and cysteinylidopa, respectively [12]. GSH and cysteine may scavenge dopaquinone in a cell-free system (Fig. 4), resulting in the formation of glutathionylidopa and cysteinylidopa, which are known to occur in melanocytic cells [12,13]. Studies by Prezioso et al. [29] showed that inhibition of tyrosinase activity by phenylthiourea blocks the growth-inhibitory activity of the dopamine analog, 3,4-dihydroxybenzylamine. In addition Prezioso et al. [29] suggested that the growth-inhibitory effects of BSO appear to correlate with tyrosinase levels in human melanoma cells. In experiments using a colony-formation assay (not shown), we have found that the IC50 for LDME in nonpigmented B16A melanoma cells (provided by K. Hefferton, Radiation Oncology, Wayne State University, Detroit, MI) is not statistically different from that in pigmented BL6 cells (114.9 and 131.7 µg/ml, respectively). Thus, we have concluded that the cytotoxicity of LDME in this system does not closely correlate with pigmentation. However, this does not exclude the possibility that GSH depletion may lead to decreased conversion of dopaquinone to glutathionylidopa and cysteinylidopa, which may ultimately enhance the cytotoxicity of LDME.

Considering the potential for L-dopa analogues in the chemotherapy of melanoma, further studies are needed to fully understand both the mechanism of cytotoxicity and the metabolic fate of these compounds in pigmented cells. Although several mechanisms may be involved, this study demonstrates that GSH depletion enhances LDME cytotoxicity, and that this combination may be useful in the treatment of malignant melanoma.

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REFERENCES


