Levodopa and Dopamine Analogs as DNA Polymerase Inhibitors and Antitumor Agents in Human Melanoma

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ABSTRACT

Levodopa and dopamine are naturally occurring catecholamines with antitumor activity in several experimental tumor systems. Previous studies suggested that their cytotoxic effect was related in part to their inhibitory effect upon DNA polymerase. We have examined the effects of levodopa, dopamine, levodopa methyl ester, norepinephrine, and the analog 3,4-dihydroxybenzylamine upon human and murine melanoma cells. When exponentially growing cells were exposed to these drugs, a characteristic inhibition of thymidine incorporation was observed with much less inhibition of either uridine or leucine incorporation. In order to ascertain that inhibition was occurring at the level of DNA synthesis, we examined the effects of the drugs upon the incorporation of thymidine triphosphate by permeabilized melanoma cells. When melanoma cells were permeabilized by lysolecithin, thereby permitting the direct incorporation of labeled thymidine triphosphate, a similar inhibition of incorporation was observed. Dopamine at a concentration of 4.8 μM caused a 50% reduction in incorporation of label. These results suggested that inhibition did occur at the level of DNA synthesis. In the presence of the melanocyte-specific oxidase, tyrosinase, these derivatives are potent inhibitors of isolated DNA polymerase α with 50% inhibitory concentrations between 1 and 10 μM. The inhibition could be completely prevented by the presence of reducing agents such as dithiothreitol (1.0 mM). The quinols themselves were not inhibitors of DNA polymerase. Dopamine analogs represent an interesting class of antitumor agents with inhibitory activity for DNA polymerase.

INTRODUCTION

Levodopa, dopamine, and related compounds have been shown to be novel antitumor agents with significant activity in several experimental tumor systems (17–20, 23). The initial studies with these agents focused upon their interaction with melanoma cells where a specific enzyme, tyrosinase, was present; tyrosinase is capable of oxidizing these quinols to the corresponding quinones. It was shown that inhibition is qualitatively similar among various cell lines but quantitatively greater in cells possessing special oxidative enzymes. Although these agents exhibited a variety of pharmacological actions, straightforward chemical modification permitted the preparation of derivatives with enhanced therapeutic effect, e.g., 3,4-dihydroxybenzylamine (22). The inhibition of DNA polymerase was postulated as being one site of action that was based upon the ability to quinols to act as sulfhydryl reagents (18).

In order to elucidate the mechanism of action of these drugs, we have studied the effects upon DNA precursor incorporation into human and murine melanoma cells. Examination of the interaction of tyrosinase-generated quinones and isolated DNA polymerase α, as a melanoma cell model, lends support to the hypothesis that the inhibition of DNA synthesis under these conditions is a property of catecholamines.

MATERIALS AND METHODS

Chemicals. All drugs used in this study were reagent grade and were obtained from Sigma Chemical Company, St. Louis, Mo. Radiolabeled [methyl-3H]thymidine, [5-3H]uridine, L-[4,5-3H]leucine, and [methyl-3H]TTP were from the New England Nuclear, Boston, Mass. All other materials were reagent grade and were used without further purification.

The quinones were generated in situ, as described below. Alternatively, quinone free of enzyme was prepared by incubating mushroom tyrosinase (0.5 mg/ml) with 1.0 mM quinol in potassium phosphate buffer, pH 6.8, for 30 min at 25°. The reactions were followed spectrophotometrically by monitoring the absorbance peak at 385 nm. The tyrosinase could then be separated from the quinone by filtration through an Amicon CF25 filter. The quinones were used immediately after preparation.

In Vitro Assays. The origin and maintenance of the human melanoma line has been described (22). Briefly, the cells were maintained as monolayers in McCoy's Medium 5A supplemented with 10% fetal calf serum, 100 μg streptomycin per ml, and 100 units penicillin per ml in an atmosphere of 5% CO2 humidified air at 37°.

The techniques for radiolabeled precursor incorporations have been described (17). Cells were plated in Linbro multiwell tissue culture trays. Exponentially growing cultures were aspirated and washed, and 1 ml of serum-free medium containing 2-μCi/ml amounts of either [3H]thymidine (specific activity, 2 Ci/mmol), [5-3H]uridine (specific activity, 25 Ci/mmol), or [3H]leucine (specific activity, 41 Ci/mmol) and drugs were added. After 60 min at 37°, the medium was removed, cells were washed once with 0.9% NaCl solution, and 1 ml of 10% trichloroacetic acid was added. The precipitate was washed 3 times with 0.9% NaCl solution, and 0.5 ml of 1 N KOH was added. After digestion at 37° for 4 hr, a portion was added to scintillation fluid and then counted. Values are expressed as percentage of inhibition as compared to controls and represent mean ± S.E. for triplicate samples.

Permeabilized Cell Studies. In order to permit the incorporation of radioactively labeled TTP, human melanoma cells were permeabilized by treatment with lysolecithin (9). Expo-
nentially growing human melanoma cells (5 x 10^5) were plated in 60-mm plastic Petri dishes and allowed to grow for 48 hr. At this time, the medium was removed, and the cells were treated with lysolecithin (75 µg/ml for 2 min at 4°). Greater than 90% of the cells were permeabilized by this treatment, as indicated with lysolecithin (75 µg/ml for 2 min at 4°). Greater than 90% of the cells were permeabilized by this treatment, as indicated by trypan blue inclusion. A solution (1.5 ml) containing 35 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 80 mM KCl, 0.75 mM CaCl₂, 4 mM MgCl₂, 50 mM sucrose, 7.5 mM KH₂PO₄, 20 mM phosphoenolpyruvate, 1.25 mM ATP, 0.1 mM CTP and UTP, and 0.25 mM [³H]TTP (0.4 Ci/mmole), dATP, dGTP, and dGTP was added, and the cells were incubated for 30 min at 37°. The cells were then dislodged by trituration, filtered onto a Whatman GF/C glass filter, washed with 10% trichloroacetic acid, dried, and counted.

Enzyme Assays. DNA polymerase α (calf thymus) was obtained from Miles Laboratory, Elkhart, Ind. For the assay (3, 7), a final volume of 250 µl contained 50 mM KCl; 40 mM potassium phosphate (pH 7.4); 2 mM MgCl₂; 80 µM dATP, dCTP, and dGTP; 7 mM TTP; calf thymus DNA (50 µg); 0.2 to 0.8 µCi [³H]TTP; and sufficient enzyme to yield 2 to 3 pmol of tritiated thymidine incorporated into DNA after 30 min of incubation at 37°. The reactions were terminated by cooling to 0° and adding 0.1 ml of 0.1 M sodium pyrophosphate containing heat-activated sperm, DNA, and 5 ml of 5% trichloroacetic acid. The precipitate was filtered on a Whatman GF/C filter, washed with trichloroacetic acid (5%), dried, and digested with 0.5 ml Protosol (New England Nuclear). The filters were added to scintillation fluid and counted. Under the conditions of the DNA polymerase assay, tyrosinase reactions proceeded at essentially control rates.

RESULTS

Precursor Incorporation Studies. Chart 1 outlines the results of our studies of the effects of dopamine upon the incorporation of radiolabeled thymidine, uridine, or leucine incorporation by human melanoma cells. Following a 60-min exposure, the inhibition of thymidine incorporation is the most sensitive index of drug effect with lesser effects observed upon uridine and leucine incorporation. Levodopa and the analog 3,4-dihydroxybenzylamine exhibited similar dose-response curves for inhibition (Table 1).

Chart 2 describes the time course for the effect of drug upon the inhibition of thymidine incorporation. For both dopamine and 3,4-dihydroxybenzylamine at 1.0 mM, the onset of inhibition is rapid with DNA synthesis as measured by thymidine incorporation completely suppressed following 15 min of exposure. In this experiment, the analog is somewhat more effective than dopamine. Similar results were obtained with labeled deoxycytidine, deoxyuridine, deoxyadenosine, and deoxyguanosine.

Permeabilized Cell Studies. Thymidine is not a direct precursor for DNA synthesis but rather must be phosphorylated sequentially to TTP; therefore, inhibition of thymidine incorporation is not equivalent to inhibition of DNA synthesis. Cells are normally not permeable to nucleotides; in order to confirm that the inhibition of thymidine incorporation observed above represented inhibition at the DNA synthesis level, we utilized the cell permeabilization technique reported by Miller et al. (9) which permits the incorporation of labeled nucleoside triphosphates. These data are summarized in Table 2.

Levodopa and DNA Polymerase Inhibition

**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>% of inhibition^a</th>
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<tbody>
<tr>
<td><strong>Thymidine</strong></td>
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<tr>
<td>Levodopa</td>
<td>11</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzylamine</td>
<td>90</td>
</tr>
<tr>
<td>Levodopa methyl ester</td>
<td>35</td>
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<tr>
<td><strong>Uridine</strong></td>
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<tr>
<td>Levodopa</td>
<td>35</td>
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<td>3,4-Dihydroxybenzylamine</td>
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^a Values represent mean for 5 determinations at drug concentrations of 0.5, 1.0, and 2.0 mM. Cells were in exponential growth with control values (cpm/10^5 cells) for thymidine, uridine, and leucine of 1300, 3000, and 310, respectively. For these experiments, drug and radiolabeled precursor were added simultaneously and incubated for 60 min.

In the permeabilized cells, the quinol forms of levodopa, dopamine, or 3,4-dihydroxybenzylamine are no longer able to inhibit thymidine incorporation. This might be attributed to either the inability of permeabilized cells to oxidize the quinol to the quinone or to the presence of back-diffusion of the latter.

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**Chart 1.** Effects of dopamine upon thymidine (●), leucine (□), and uridine (○) incorporation. Control cells incorporated (per 10^5 cells) 5500 cpm thymidine, 8589 cpm uridine, and 2400 cpm leucine. These results of our studies showed that levodopa and 3,4-dihydroxybenzylamine upon the incorporation of thymidine, uridine, and leucine by human melanoma cells.

**Chart 2.** Time course of inhibition of thymidine incorporation by human melanoma cells. ○, dopamine; ●, 1.0 mM, □, 1.0 mM, 3,4-dihydroxybenzylamine; ●, control. Radiolabeled thymidine was added at time 0 (2 µCi/ml). After 30 min of incubation at 37°, drug was added, and sample was assayed at the indicated times. Points, mean for triplicate determinations; bars, S.E. Each experiment was repeated 3 times. Similar results were obtained with deoxycytidine, deoxyadenosine, and deoxyguanosine.
in the presence of a large excess of quinol in the medium. The inhibition could be restored either by preforming the quinone from 3,4-dihydroxybenzylamine or by generating the quinone in situ by the addition of exogenous tyrosinase. Under either of these conditions, an effective inhibition of the incorporation of TTP into DNA was restored. These results confirm that the quinone form is the inhibitory specie and that inhibition does occur at the level of DNA synthesis.

**Enzyme Studies.** In order to test the hypothesis that DNA polymerase is a site of action of these drugs, we examined the effects of levodopa, dopamine, and 3,4-dihydroxybenzylamine upon isolated mammalian DNA polymerase $\alpha$. At concentrations of $10^{-3}$ M, there was no inhibitory effect. We attributed the absence of inhibition to the lack of quinone generation in the reaction medium and elected again to mimic the in vitro case of the melanoma cell by the addition of tyrosinase to the DNA polymerase medium. Control experiments indicated that the presence of tyrosinase did not inhibit the activity of DNA polymerase.

Under these conditions, a prompt and highly potent inhibition of DNA synthesis was observed. In Chart 3, a dose-response curve exists for each agent. At concentrations greater than $10^{-5}$ M, the enzyme is completely inhibited. This inhibition in turn could be prevented by the addition of free sulfhydryl groups such as 1 mM dithiothreitol, which is a potent inhibitor of tyrosinase.

Table 3 describes the results of the effect of preincubating each of the components of the DNA polymerase assay separately with drug. Preincubating either DNA, deoxynucleotides, or both with drug did not significantly affect the inhibition caused by $10 \mu M$ 3,4-dihydroxybenzylamine. Preincubation of the enzyme, however, increased the inhibition 10-fold. These results suggest that inhibition is directed toward the enzyme rather than the template or bases.

Chart 4 shows the time course of inhibition of DNA polymerase by tyrosinase. 3,4-Dihydroxybenzylamine ($10^{-3}$ M) was added to reactions at time 0, while tyrosinase was added at 15 min. Control had inhibitor without tyrosinase. In Chart 4, a dose-response curve exists for each agent. At concentrations greater than $10^{-6}$ M, the enzyme is completely inhibited.
Levodopa and DNA Polymerase Inhibition

Levodopa and dopamine analogs are capable of inhibiting the growth of a variety of experimental tumors, and simple chemical modifications can enhance activity. The major effect upon macromolecule biosynthesis is the inhibition of thymidine incorporation, which occurs rapidly and is reduced to very low levels. Previous structure activity studies suggested that a likely site of action might be the inhibition of DNA synthesis and that oxidation to the quinone was essential for activity. The present study provides direct evidence in support of these concepts since the inhibition of TTP incorporation into permeabilized cells confirms that inhibition occurs at the level of DNA synthesis itself.

The enzymes responsible for replication of DNA in mammalian cells has been proposed to be the α-class of DNA polymerase (1, 11, 13–15). The enzyme-catalyzed polymerization of deoxynucleotides is markedly inhibited by the tyrosinase-induced oxidation of the quinols with inhibitory concentrations in the μM range. Graham et al. (4) have reported similar results upon DNA polymerase. A difference in the 2 studies, however, is the markedly diminished activity of levodopa oxidation products reported previously. Since the true quinone, as mentioned above, of levodopa is a short-lived intermediate, dopachrome, an indole derivative, was actually measured. Our technique with the quinone generated in situ permits the evaluation of short-lived intermediates such as dopa quinone itself. The fact that each of our drugs has similar inhibitory concentrations under these conditions supports the concept of a common quinone intermediate as the active species. Interestingly, inhibition can occur at μM concentrations rather than at the high concentrations required for inhibition in vivo. Since inhibition occurs only in the presence of tyrosinase-generated or chemically preformed quinone, it is apparent that the oxidized form is the active specie.

The exact nature of the inactivation of the enzyme is not clear. It is possible that crucial cysteine residues are oxidized to cystine or that sulfhydryl groups are alkylated by the quinone intermediates, thereby taking advantage of the very high oxidation-reduction potential between levodopa quinone and levodopa (± 0.37 V) (16). This potential is among the highest known in biological systems and implies that the quinones generated will be potent oxidizing agents and capable of activating enzymes that are sensitive to oxidative conditions. Since pigmented melanomas are generally characterized by having tyrosinase in a highly active form, they should be particularly sensitive to these agents in their reduced form; in fact, these observations have been made both experimentally and clinically (2, 5, 12). Other systems such as neuroblastoma and leukemias have also been shown to be sensitive to the action of quinols; however, the enzymatic mechanism of action is less clear (17, 20).

Although the results presented here confirm that the quinone oxidation products are capable of the inhibition of DNA polymerase activity directly, it is still possible that the oxidations in the intact cell are mediated through additional electron carriers. Recently, an extensive review of superoxide radical in cancer has suggested that the radical may be important in the effect of anticancer drugs with quinone properties (10). It is possible that the tumor selectivity characteristic of these quinols is the result of 2 factors: (a) the ability of these compounds to be oxidized by tyrosinase present in melanoma cells; and (b) the relative inability of tumor cells to decompose the radical oxides formed as a consequence of the oxidation.

Finally, the phenomenon described here may have relevance to the understanding of dopaminergic and noradrenergic effects, since dopamine is capable of exerting major intracellular biochemical effects. In addition to exploring further effects upon the potential antitumor effects of these agents, it is interesting to speculate that these biochemical inhibitory effects may be related to other neuropharmacological actions of these agents.

REFERENCES


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