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INHIBITION OF RIBONUCLEOTIDE REDUCTASE BY ANTITUMOR AGENTS RELATED TO LEVODOPA AND DOPAMINE*

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Abstract—Using partially purified enzyme from L1210 cells, dihydroxybenzene derivatives related structurally to dopamine were shown to reversibly inactivate ribonucleotide reductase. A structure–activity analysis revealed that derivatives with side-chains, which contain a negatively-charged group, had significantly reduced inhibitory activity. The ability of these compounds to inhibit ribonucleotide reductase was dependent on the hydroxyl groups being in the ortho position and did not correlate with free radical inhibitory activity. A kinetic analysis by the method of Lineweaver–Burk indicated that the inhibition of ribonucleotide reductase by the derivative 3,4-dihydroxybenzylamine was competitive with the reducing substrate dithioerythritol. This analog, in combination with hydroxyurea, gave synergistic inhibition or ribonucleotide reductase, suggesting different sites of action. Using Tween 80-treated L1210 cells, it was found that these drugs had an immediate inhibitory effect on ribonucleotide reductase activity in intact, reversibly permeabilized cells. Furthermore, although these drugs had no immediate effect on DNA polymerase, in permeabilized L1210 cells (when the cells were preincubated with the dihydroxybenzene derivatives for 1 hr prior to permeabilization), there was significant inhibition of DNA polymerase activity. The two key enzymes for DNA synthesis appear to be sequentially inhibited by these analogs, with the reduced form (quinol) inhibiting ribonucleotide reductase and the oxidized form (quinone) inhibiting DNA polymerase.

Dihydroxybenzene derivatives related structurally to levodopa and dopamine have been shown to be effective antitumor agents in a variety of experimental systems [1–3]. We have also shown that dopamine is capable of inhibiting melanoma growth in patients with advanced disease [4].

Our initial *in vitro* studies with these drugs in pigmented and nonpigmented cells indicated that a characteristic feature of the ortho quinols at the biochemical level was their ability to selectively inhibit DNA synthesis with relatively little effect on RNA or protein synthesis [5, 6]. Recently, we have reported a mechanistic study with various analogs of dopamine, which showed that, in the presence of an oxidizing enzyme (tyrosinase), the quinone form of these derivatives irreversibly inhibits isolated DNA polymerase, while the reduced form (quinol) has no effect on this enzyme [7].

We also examined the effect of several dihydroxybenzene derivatives on DNA polymerase activity in permeabilized cells of a deeply pigmented melanoma. While all of the drugs selected inhibited DNA synthesis in intact cells, three of the derivatives had no direct effect on DNA polymerase activity in permeabilized cells, and two agents had only a limited effect against this enzyme. Furthermore, all of these drugs are highly effective and selective inhibitors of DNA synthesis in non-tyrosinase-containing cells (e.g. L1210 and P388 lymphocytic leukemias [1]).

Since our previous results indicate that these drugs do not alter the activity of either RNA or DNA templates [7, 8], then these additional observations might be explained by either inhibition at an earlier step in DNA synthesis or, alternatively, by the presence of an oxidase other than tyrosinase in non-melanoma derived cells. Support for an alternative site of action has recently been reported by Elford *et al.* [9] who have shown that reduced polyhydroxybenzene derivatives can inhibit isolated ribonucleotide reductase. Ribonucleotide reductase is considered to be the key enzyme in the *de novo* synthesis of deoxynucleoside triphosphates, since the rate-limiting step which it catalyzes is the first step unique to DNA synthesis. Furthermore, its end-products, dNTPs, which regulate ribonucleotide reductase by feedback inhibition [10], also affect, inhibit, or stimulate DNA synthesis, perhaps by interacting with a DNA polymerase regulatory protein [11]. An enzyme which plays such a critical role in DNA replication would be an ideal site of action for a chemotherapeutic agent designed to control

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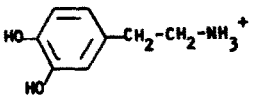
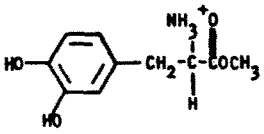
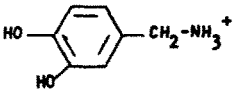
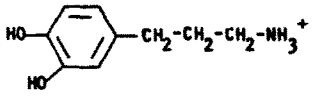
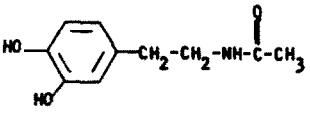
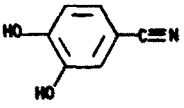
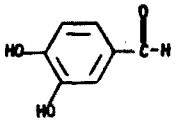
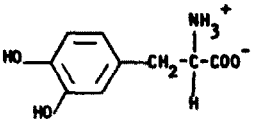
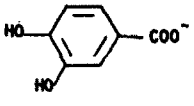
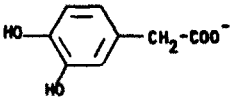
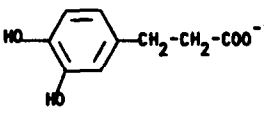
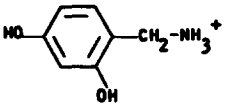
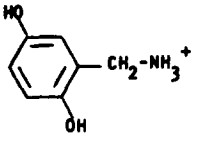
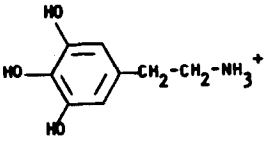
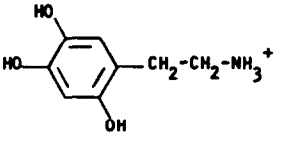
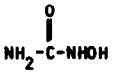
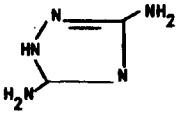
Table 1. Inhibition of partially purified ribonucleotide reductase from L1210 cells[a]

Compound	Structure	IC_{50} (μM)[b]
Dopamine		90
L-dopa methyl ester		245
3,4-dihydroxybenzyl-amine		108
3,4-dihydroxyphenyl-propylamine		92
N-acetyl dopamine		90
3,4-dihydroxybenzo-nitrile		87
3,4-dihydroxybenz-aldehyde		51
Levodopa		2,700
3,4-dihydroxybenzoic acid		1,700
3,4-dihydroxyphenyl-acetic acid		1,600

3,4-dihydroxyhydrocinnamic acid		1,300
2,4-dihydroxybenzylamine		>2,000
2,5-dihydroxybenzylamine		>2,000
5-hydroxydopamine		179
6-hydroxydopamine		>2,000
Hydroxyurea		99
Guanazole		1,900

[a] The enzyme was partially purified as described in the Methods. CDP reductase activity was determined by the method of Steeper and Steuart [18].

[b] Values represent the concentration causing 50% inhibition of enzyme activity as determined by Hill plot analysis (cf. Fig. 1).

cell growth. However, at present, only one such agent, hydroxyurea (HU*), is in clinical use [12], while other potential agents are either too toxic to be effective in man (e.g. 5-hydroxy-2-formylpyridine thiosemicarbazone [13]) or lack sufficient potency (e.g. guanazole [14]).

* Abbreviations: HU, hydroxyurea; DTE, dithioerythritol; 3,4-DHBA, 3,4-dihydroxybenzylamine; IC_{50}, concentration resulting in 50% inhibition of enzyme activity; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

In this study, we have been able to demonstrate a direct effect by the ortho dihydroxy quinol drugs on ribonucleotide reductase *in vitro* using a permeabilized cell technique. Furthermore, a mechanism of action for the inhibition of ribonucleotide reductase by these compounds is presented based on an analysis of the partially purified enzyme from L1210 cells. Studies with permeabilized L1210 cells suggest an interrelationship between the inhibition of ribonucleotide reductase and DNA polymerase by these drugs *in vitro*.

MATERIALS AND METHODS

Radiolabeled [^{3}H]dTTP, [^{3}H]thymidine, and [^{14}C] CDP were purchased from the New England Nuclear Corp., Boston, MA. Unlabeled deoxynucleoside and deoxy- and ribonucleotides were obtained from P-L Biochemicals, Milwaukee, WI. 3,4-Dihydroxybenzylamine was prepared from the parent nitrile by catalytic hydrogenation as described previously [7]. All other drugs were prepared synthetically and fully characterized [2].

The origin and maintenance of L1210 lymphocyte leukemia cells have been described [1]. Briefly, the cells were maintained in a suspension of Eagle's minimum essential medium containing 15% fetal bovine serum, 100 μg/ml of streptomycin, and 100 units/ml of penicillin in a 5% CO_2 humidified air incubator at 37°. The medium was also supplemented with 50 μM 2-mercaptoethanol [15].

Permeabilized cell technique. The assay for DNA polymerase activity in intact permeabilized L1210 cells was performed as described by Miller *et al.* [16]. Briefly, exponentially growing L1210 cells were washed two times and resuspended at 1×10^8 cells/ml in a buffered solution [16] containing 250 μg/ml of lysolecithin. The cells were placed on ice for 1 min. Permeabilized cells (1×10^7) were added to the DNA synthesis mixture containing, in a total volume of 300 μl, the following: 150 mM sucrose; 80 mM KCl; 35 mM Hepes (pH, 7.4); 5 mM potassium phosphate (pH, 7.4); 5 mM $MgCl_2$; 0.5 mM $CaCl_2$; 20 mM phosphoenolpyruvate; 1.25 mM ATP; 0.1 mM CTP, GTP, and UTP; 50 μM dATP, dGTP, and dCTP; and dTTP (8 μCi [^{3}H]dTTP). The mixture was incubated at 37° for 30 min and processed as described previously.

The CDP ribonucleotide reductase assay was performed essentially as described by Hards and Wright [17]. Exponentially growing L1210 cells were harvested by centrifugation and resuspended at 1×10^7 cells/ml in a buffered solution containing 50 mM Hepes (pH 7.2); 2 mM DTE; 280 mM sucrose; and 0.85% Tween 80. The cells were incubated at room temperature for 25 min. The permeabilized cells were then washed and resuspended in the permeabilization buffer at 5×10^7 cells/ml. Permeabilized cells (1×10^7) were then added to the reaction solution to give, at a final concentration: 50 mM Hepes (pH, 7.2); 2 mM ATP; 8 mM $MgCl_2$; 6 mM DTE; 0.4 mM CDP (20 μCi); 0.67% Tween 80; and 0.187 mM sucrose in a volume of 300 μl. The cells were incubated for 30 min at 37°, and the reaction was terminated by boiling for 5 min. Snake venom (*Crotalus adamanteus*), 2 mg in 200 μl of 0.1 M Hepes (pH 8.0) and 10 mM $MgCl_2$, was added, and the mixture was incubated for 2 hr at 37° to convert nucleotides to nucleosides. The reaction was stopped by boiling. Water (0.5 ml) was added, and precipitable material was removed by centrifugation. Deoxycytidine was then isolated, using an anion exchange resin AG 1-X8 (Biorad), as described by Steeper and Steuart [18], and modified by Cory and Whitford [19].

Partial purification of ribonucleotide reductase. L1210 leukemia cells were carried in BDF_1 male mice for 7 days. The cells (*ca.* 1×10^{10}) were harvested from the ascites fluid by centrifugation and washed twice. The cells were resuspended in 1.5 vol. of 20 mM Tris (pH 8.6) and 1 mM DTE. The cell extract was prepared by homogenizing the cells with a Potter–Elvehjem homogenizer. The extract was centrifuged at 3000 rpm for 10 min to remove the bulk of insoluble material. Following centrifugation at 100,000 *g* for 90 min, enzyme activity appeared in the supernatant fraction. Streptomycin sulfate (20%, w/v) was added to the crude extract to give a final concentration of 1%, and the solution was stirred for 30 min. The precipitate, which included RNA (an inhibitor of reductase), was removed by centrifugation at 20,000 *g* for 20 min. Ammonium sulfate was added slowly to the supernatant fluid recovered from the previous step to a final concentration of 0.243 g/ml and stirred for 30 min. The precipitate was collected by centrifugation at 20,000 *g* for 30 min. The pellet was dissolved with 3–4 ml of 10 mM Tris (pH 8.0) and 1 mM DTE, and dialyzed overnight against the same buffer. The dialysate was stored at −70°.

Ribonucleotide reductase enzyme assay. CDP reductase was assayed by the method of Steeper and Steuart [18], as modified by Cory and Whitford [19]. The standard assay mixture contained, in a final volume of 0.2 ml, the following: 2 mM ATP; 2 mM $MgCl_2$; 5 mM DTE; 0.2 mM [^{14}C]CDP (0.1 μCi); 8 mM potassium phosphate (pH 7.0); and a specified amount of partially purified enzyme.

RESULTS

The effects of several analogs of levodopa and dopamine on ribonucleotide reductase activity are presented in Table 1. The ortho dihydroxy analogs with substituted side-chains, which have either no charge or a positive charge at a pH of 7.6, have inhibitory activity against this enzyme similar to that of hydroxyurea. Those derivatives with a negatively-charged side-chain, as well as the parent compound levodopa (a zwitterion), exhibited much less inhibitory activity and were similar in potency to guanazole, a known inhibitor of ribonucleotide reductase. The inhibitory activity is not a general property of dihydroxybenzene compounds, since the meta derivative, 2,4-dihydroxybenzylamine, and the para derivative, 2,5-dihydroxybenzylamine, have no inhibitory activity against ribonucleotide reductase. The results with the two trihydroxy derivatives suggest that the para arrangement may affect the inhibitory activity, resulting from the ortho positioning. Thus, while 5-hydroxydopamine has virtually the same inhibitory effect as dopamine, 6-hydroxydopamine [which has hydroxy groups in the ortho and para arrangement (Table 1)] has no inhibitory activity against ribonucleotide reductase.

The results shown in Table 1 were determined by a Hill-type plot analysis, as described previously [7]. Atkinson *et al.* [20] and Loftfield and Eigner [21] have demonstrated that partially purified enzyme extracts and even more complicated biological systems [21] are amenable to Hill-type plot analysis. The results for HU and 3,4-DHBA (Fig. 1) and for the other analogs (data not shown) reveal a constant slope of 1 over the entire range of inhibitor concentration, which suggests complete inhibition [21].

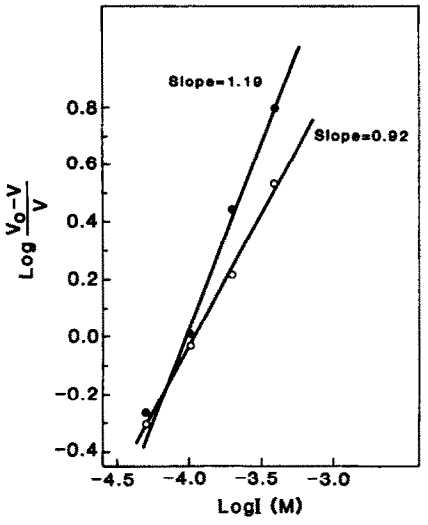


Fig. 1. Hill-type plot of the inhibition of ribonucleotide reductase by hydroxyurea (HU) (—●—) and 3,4-dihydroxybenzylamine (3,4-DHBA) (—○—). V_0 is the uninhibited reaction rate, V is the inhibited rate, and I is the concentration of inhibitor.

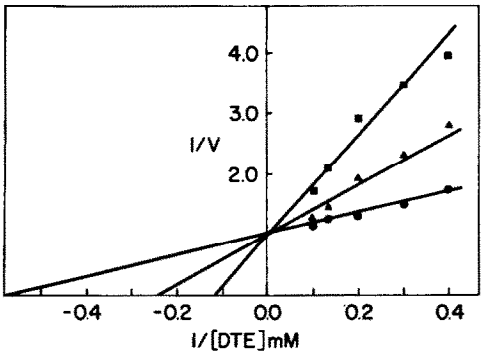


Fig. 3. A double-reciprocal plot of velocity (nmoles dCDP formed/30 min) versus DTE concentrations (mM) at two fixed levels of 3,4-DHBA. The standard assay conditions described in Methods were used, except that the DTE concentration was varied in the absence (—●—) or presence of 80 μM (—▲—) or 180 μM (—■—) 3,4-DHBA.

Figure 2 shows an Ackermann–Potter plot of enzyme activity versus enzyme concentration for two doses of 3,4-DHBA. The results, which show the dilution effect usually seen with ribonucleotide reductase [19, 22], indicate that the enzyme activity in the presence of 3,4-DHBA remains the same relative to the uninhibited activity at all enzyme concentrations. This result suggests a reversible inhibitory reaction [23] which, therefore, can be treated by conventional Michaelis–Menten kinetics. A Lineweaver–Burk kinetic analysis was used to examine the relationship between the dihydroxybenzene derivative 3,4-DHBA and the two ribonucleotide reductase substrates, ribonucleoside diphosphate (CDP) and the reducing agent, DTE.

Figure 3 shows a typical double-reciprocal plot of the effect of 3,4-DHBA on ribonucleotide reductase activity in the presence of various concentrations of DTE. The results show that the inhibition of ribonucleotide reductase by 3,4-DHBA was competitive with DTE, i.e. the inhibitory activity was abolished at high DTE concentrations. A similar kinetic analysis (not shown) indicated a partial competitive relationship between hydroxyurea and the dithiol substrate, which is in agreement with the findings of Moore [24]. 3,4-DHBA was not competitive toward the nucleotide diphosphate substrate (data not shown).

The results of the kinetic analysis suggested that HU and 3,4-DHBA do not inhibit ribonucleotide reductase by the same mechanism. We therefore

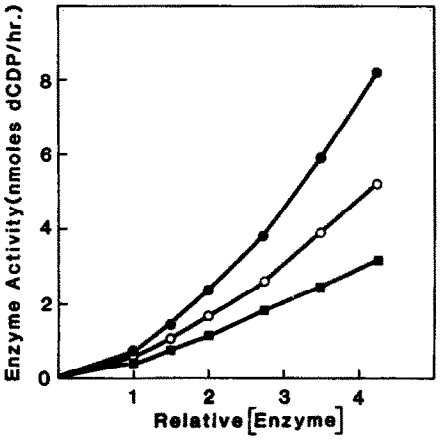


Fig. 2. Plot of ribonucleotide reductase activity versus relative enzyme concentration in the presence and absence of 3,4-DHBA. Various amounts of partially purified ribonucleotide reductase were incubated in the standard assay mixture without drug (—■—) or with drug, 0.1 mM (—○—) or 0.2 mM (—■—). Enzyme activity was determined as described in Methods.

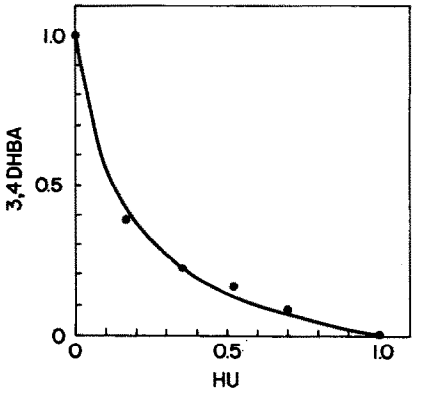


Fig. 4. An isobologram showing the synergistic inhibition of ribonucleotide reductase resulting from the combination of hydroxyurea and 3,4-DHBA. The endpoint was 60% inhibition, as determined by Hill plot analysis [21], and the concentration of 3,4-DHBA or hydroxyurea alone required to give this level of effect was 0.22 mM or 0.17 mM respectively. The data were analyzed by the method of Elion *et al.* [25].

Table 2. Requirements for CDP reduction in permeabilized L1210 cells

Component omitted	% Activity*
None	100
$MgCl_2$	28
DTE†	89
ATP	14

* The control activity was 198 pmoles/30 min/1 × 10^7 cells.
† Following permeabilization, the cells were resuspended in buffer without DTE.

examined the type of inhibitory interaction (synergistic, additive, or antagonistic) which results from combining these two inhibitors. The isobologram presented in Fig. 4 shows the nature of interaction of hydroxyurea and 3,4-DHBA on ribonucleotide reductase activity [25]. The concave appearance of this isobol suggests that a synergistic inhibition of the partially purified enzyme occurs with this combination. This result agrees with the suggestion that 3,4-DHBA does not act at the same site as HU.

The final phase of this study utilized permeabilized cells to examine directly the effects of the various inhibitors on ribonucleotide reductase activity in intact cells. The requirements for the reduction of CDP in permeabilized L1210 cells are given in Table 2. The addition of DTE had a small, but consistent, stimulatory effect on CDP reduction by the permeabilized L1210 cells. The modest stimulation of CDP reduction by DTE would suggest, as discussed by Kucera and Pauleus [26], that the reaction utilizes preferentially the endogenous reducing system involving thioredoxin and/or glutaredoxin. Mercaptoethanol, present in the suspension media [15], was not capable of substituting for the dithiol, DTE. The reaction was linear for 45 min, and the amount of CDP reduced increased linearly up to at least 2 × 10^7 cells/assay (data not shown).

The inhibitory effect of several dihydroxybenzene compounds on ribonucleotide reductase activity in permeabilized cells is given in Table 3. The enzyme activity in permeabilized cells was somewhat less sensitive to these inhibitors than was found with the isolated enzyme (Table 1), although the order of potencies remained the same. One possibility to account for the reduced sensitivity of RNR in permeabilized cells as compared to the partially purified enzyme may be related to the involvement of different reducing substrates. As mentioned previously, the thioredoxin system is apparently operating very efficiently in the permeabilized cells, and the dihydroxybenzene compounds may not be able to compete with this reducing system as effectively as against DTE used in the enzyme assay.

Preincubation had no effect, which is consistent with a reversible inhibitor (Fig. 2), where the degree of inhibition is concentration dependent and time independent [27]. Table 3 also shows the results of a study of the inhibition of DNA polymerase using permeabilized cells. As expected, there was no immediate effect of any of the dihydroxy derivatives or HU on polymerase activity. However, when the

Table 3. Effect of analogs of levodopa and hydroxyurea on ribonucleotide reductase and DNA polymerase activity in permeabilized L1210 cells*

	Ribonucleotide reductase activity				DNA polymerase activity			
	Without preincubation		With preincubation†		Without preincubation		With preincubation†	
	0.5 mM	1.0 mM	0.5 mM	1.0 mM	0.5 mM	1.0 mM	0.5 mM	1.0 mM
Levodopa	88	89	98	94	105		71	64
Levodopa methyl ester	82	56	103	51	103		72	53
Dopamine	51	30	65	31	107		86	55
3,4-Dihydroxybenzylamine	8	51	104	43	106		61	34
Hydroxyurea	69	53	67	51	116		66	54

* CDP ribonucleotide reductase activity and DNA polymerase activity in permeabilized cells were determined as described in Methods.
† L1210 cells were preincubated prior to permeabilization for 1 hr in the presence of the indicated concentration of drug.

cells were preincubated for 1 hr with these drugs, inhibition of DNA polymerase was observed. Preincubation of partially purified DNA polymerase in the presence of the reduced form of the dihydroxybenzene derivatives or HU did not affect the enzyme activity.

DISCUSSION

Ribonucleotide reductase catalyzes the one-step direct replacement of the 2'-OH group of the nucleoside diphosphate with a sulfhydryl hydrogen derived from the enzyme [28]. This reaction requires the presence of a tyrosyl free radical near the active site [29]. The resulting disulfide is, in turn, reduced in mammalian cells by thioredoxin which ultimately receives its electrons from NADPH [28].

The analysis of a number of different derivatives of levodopa and dopamine has revealed two aspects of the structure–activity relationships involved in the inhibition of ribonucleotide reductase. The side-chain moiety, while apparently playing no direct role in the inhibition of ribonucleotide reductase, does affect the inhibitory activity of the dihydroxy derivatives (Table 1). The side-chain may affect some other factors, such as site recognition or binding of the drug to the enzyme. The second structure–activity relationship involves the influence of the position of the hydroxyl groups on inhibitory activity.

It has been suggested previously [9] that the polyhydroxybenzene compounds may inhibit ribonucleotide reductase by the same mechanism as HU, which acts by destroying the tyrosine free radical required for catalytic activity [30]. Caldwell and Ihrig [31], using the inhibition of the polymerization of methyl methacrylate as a measure of the ability of phenols to act as free radical inhibitors, found the para quinol to be a somewhat stronger inhibitor than ortho dihydroxybenzene. They also found 1,2,4-trihydroxybenzene to be a more efficient free radical inhibitor than 1,2,3-trihydroxybenzene (pyrogallol). Based on these results, we find no correlation between free radical inhibition and the ability to inhibit ribonucleotide reductase by the di- and trihydroxybenzene derivatives of dopamine. Instead, it appears that it is the ortho arrangement which is required for inhibition of this enzyme. Furthermore, the results with 6-hydroxydopamine suggest that the para arrangement has a negative effect on the inhibition of ribonucleotide reductase.

A second argument against an inhibitory mechanism involving free radicals comes from our finding that a synergistic inhibition occurs with the combination of 3,4-DHBA and hydroxyurea which, as discussed by Grindey *et al.* [32], suggests that these two drugs are acting at two independent sites. From the results of our kinetic analysis, the ortho dihydroxybenzene compounds appear to inhibit this enzyme by blocking the transfer of electrons from the hydrogen donor to the disulfide, a process which is required to regenerate the active sulfhydryl groups.

Using the permeabilized cell technique, we have now been able to demonstrate that these drugs can inhibit, sequentially, two enzymes central to DNA synthesis; ribonucleotide reductase by an immediate reaction, and DNA polymerase by a slower reaction process. While the inhibition of ribonucleotide reductase involves the reduced form of the dihydroxybenzene derivatives, inhibition of DNA polymerase requires activation of these drugs to the quinone, or oxidized, form [7]. L1210 cells may possess an oxidase capable of oxidizing ortho quinols to the quinone. One interesting possibility is that ribonucleotide reductase, as an oxoreductase, may oxidize the reduced derivatives to the quinone, a process which at the same time inhibits its ability to reduce nucleoside diphosphates. An alternative possibility is suggested by recent studies [33, 34] which showed that hydroxyurea can inhibit thymidylate synthetase *in situ*, although it has no inhibitory effects on this enzyme activity in crude soluble extracts. Reddy and Pardee [35] argue that this result provides evidence for allosteric interaction within the multienzyme complex called the "replitase", responsible for *de novo* DNA biosynthesis.

This proposal could not only explain our results with the dihydroxybenzene derivatives but also our findings that HU can inhibit DNA polymerase in permeabilized cells while having no effect on the isolated enzyme. We are presently investigating whether the dihydroxybenzene compounds can inhibit thymidylate synthetase as well as several other DNA synthesizing enzymes, since the inhibition of DNA synthesis by these drugs may reflect the inactivation of a number of DNA biosynthetic enzymes.

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