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EFFECT OF IRON-CHELATING AGENTS ON INHIBITORS OF RIBONUCLEOTIDE REDUCTASE*

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Abstract—Ribonucleotide reductase from Ehrlich tumor cells is inhibited by hydroxyurea (HU), guanazole (GZ), pyrazolo-imidazole (IMPY), 1-formylisoquinoline thiosemicarbazone (IQ), 4-methyl-5-aminoisoquinoline thiosemicarbazone (MAIQ), and the dialdehydes of inosine and 5′-deoxyinosine. In the presence of EDTA, desferal, or 8-hydroxyquinoline (8-HQ) at a concentration that did not affect reductase activity, HU, GZ and IMPY more effectively inhibited the reductase. EDTA, desferal, or 8-HQ in combination with the nucleoside dialdehyde derivatives did not affect the inhibition caused by these compounds. The combination of these iron-chelating agents with MAIQ and IQ, however, reversed the inhibition caused by MAIQ and IQ. These data indicate that the inhibitors of the non-heme iron-containing component of mammalian ribonucleotide reductase can be subclassified, based on their response to the presence of iron-chelating agents.

Hydroxyurea, guanazole, pyrazolo-imidazole (IMPY), and the thiosemicarbazones have been shown to be relatively specific inhibitors of ribonucleotide reductase [1–4]. The possibility that iron plays a role in the inhibition by these compounds has been suggested by the results of various groups. Moore [5] had shown earlier that the inhibition of ribonucleotide reductase from rat tumor cells by hydroxyurea was partially reversed by ferrous ions. Brockman *et al.* [6] reported that the combinations of desferal and hydroxyurea, guanazole, or pyridine-2-aldehyde thiosemicarbazone potentiated the inhibition of DNA synthesis in intact tumor cells produced by hydroxyurea, guanazole, or the thiosemicarbazone alone. Exogenous iron reversed the inhibition by hydroxyurea and guanazole but only partially reversed the inhibition of DNA synthesis by the thiosemicarbazone. Sartorelli *et al.* [7] have suggested that the [iron–thiosemicarbazone] complex is the species that actually inhibits ribonucleotide reductase. Petering's group, working with the metal complexes of the thiosemicarbazones, found that the metal complexes were more inhibitory to the mammalian reductase than was the free ligand [8]. We had reported previously that the ribonucleotide reductase from Ehrlich tumor cells consisted of two non-identical components [9], each of which could be specifically inhibited [10]. We were able to show that hydroxyurea, guanazole, IMPY, and the thiosemicarbazones [4-methyl-5-aminoisoquinoline (MAIQ) and 1-formylisoquinoline (IQ) all inhibit/inactivate the same component of the ribonucleotide reductase from the Ehrlich tumor cells [10–12].

In this study, we present data to show that, although hydroxyurea, guanazole, IMPY, and the thiosemicarbazones each inhibit the same component of the enzyme, these compounds can be further subclassified as inhibitors of the non-heme iron component, based on their responses to the presence of iron-chelating agents.

METHODS AND MATERIALS

Preparation of the enzyme fraction. Ehrlich ascites tumor cells were taken from female mice (ICR) 7 days after transplantation. Ribonucleotide reductase was purified from these cells as described previously [13]. The ammonium sulfate fraction (0–40% saturation) was dialyzed extensively prior to use.

Enzyme assays. CDP reductase was assayed by the method of Steeper and Steuart [14] using [^{14}C]CDP as substrate and snake venom (*Crotalus atrox*) instead of apyrase and alkaline phosphatase to hydrolyze the substrate and product to cytidine and deoxycytidine respectively. The assay mixture contained in a final volume of 0.150 ml: [^{14}C]CDP (0.05 μCi, 0.05 mM); dithioerythritol (6 mM); magnesium acetate (4 mM); ATP (2 mM); and the enzyme fraction. All assays were carried out in triplicate at 37° for 30 min.

Materials. [^{14}C]CDP (453 mCi/mmole) was purchased from the New England Nuclear Corp., Boston, MA. The biochemicals used in these studies were purchased from the Sigma Chemical Co., St. Louis, MO. The dialdehyde derivative of inosine (Inox, NSC 118994), 4-methyl-5-aminoisoquinoline thiosemicarbazone (MAIQ, NSC 246112), 1-formylisoquinoline thiosemicarbazone (IQ, NSC 92188), pyrazolo-imidazole (IMPY, NSC 51143), and guanazole (GZ, NSC 1895) were obtained from the Drug Synthesis and Chemistry Branch, National

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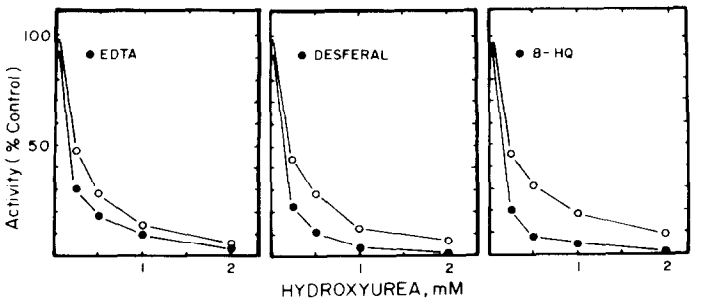

Fig. 1. Effect of EDTA, desferal, or 8-hydroxyquinoline on inhibition of ribonucleotide reductase by hydroxyurea. EDTA (0.167 mM), desferal (0.1 or 0.2 mM), or 8-hydroxyquinoline (8-HQ, 0.5 or 1.0 mM) was added in combination with various concentrations of hydroxyurea to the enzyme. The reaction was started by the addition of substrate. The reactions were carried out for 30 min at 37°. All assays were done in triplicate. Key: Open circles (○) show the percent activity remaining in the presence of hydroxyurea alone; closed circles (●) show the percent activity remaining in the presence of the combinations of hydroxyurea plus the iron-chelating agents. The control CDP reductase activity was 2.28 nmoles·(30 min)$^{-1}$·(mg protein)$^{-1}$.

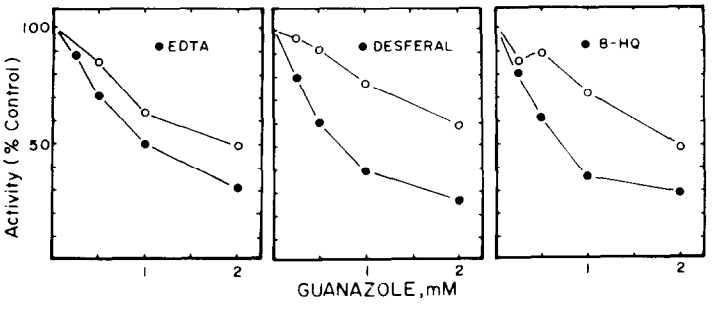

Fig. 2. Effect of EDTA, desferal, or 8-hydroxyquinoline on inhibition of ribonucleotide reductase by guanazole. EDTA (0.167 mM), desferal (0.1 or 0.2 mM), or 8-hydroxyquinoline (8-HQ, 0.5 or 1.0 mM) was added in combination with various concentrations of guanazole to the enzyme. Key: Open circles (○) show the percent activity remaining in the presence of guanazole alone; closed circles (●) show the percent activity remaining in the presence of the combinations of guanazole plus the iron-chelating agents. The reactions were set up as described in the legend to Fig. 1. The control CDP reductase activity was 2.32 nmoles·(30 min)$^{-1}$·(mg protein)$^{-1}$.

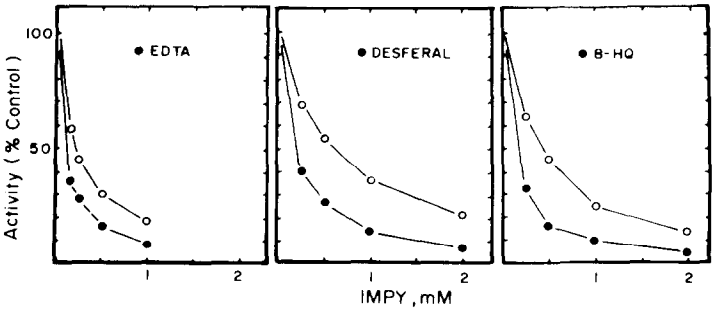

Fig. 3. Effect of EDTA, desferal, or 8-hydroxyquinoline on inhibition of ribonucleotide reductase by IMPY. EDTA (0.167 mM), desferal (0.1 or 0.2 mM), or 8-hydroxyquinoline (8-HQ, 0.5 or 1.0 mM) was added in combination with various concentrations of IMPY to the enzyme. Key: Open circles (○) show the percent activity remaining in the presence of IMPY alone; closed circles (●) show the percent activity remaining in the presence of IMPY plus the iron-chelating agents. The reactions were set up as described in the legend to Fig. 1. The control CDP reductase activity was 1.85 nmoles·(30 min)$^{-1}$·(mg protein)$^{-1}$.

Table 1. Effect of $Fe(NH_4)_2(SO_4)_2$ on inhibition of reductase by IMPY

Sample	CDP reductase activity [nmoles·(30 min)$^{-1}$·(mg protein)$^{-1}$]
Control*	2.42
+ Fe^{2+} (5 μM)	2.18 (90)†
+ IMPY (1 mM)	0.68 (28)
+ IMPY (1 mM) + Fe^{2+} (5 μM)	1.52 (63)
+ IMPY (0.25 mM)	1.43 (59)
+ IMPY (0.25 mM) + Fe^{2+} (5 μM)	2.13 (88)

* The 0–40% ammonium sulfate fraction was used as the source of the enzyme. The solution of $Fe(NH_4)_2(SO_4)_2$ was made up just before use. All assays were carried out in triplicate; the assays agreed within 3 per cent.

† Numbers in parentheses are percentages of control values.

Cancer Institute, through the assistance of Dr. Leonard Kedda. The dialdehyde derivative of 5′-deoxyinosine (5′-deoxyinox) was synthesized in this laboratory as described previously [15]. The mice were purchased from the Lab Supply Co., Indianapolis, IN.

RESULTS

Effects of iron-chelating agents on inhibition of reductase by hydroxyurea, guanazole, and IMPY. As seen in Fig. 1, the addition of EDTA, desferal, or 8-hydroxyquinoline to the reaction mixtures containing hydroxyurea increased the inhibition of reductase activity. The concentrations of EDTA (0.167 mM), desferal (0.1 or 0.2 mM), or 8-hydroxyquinoline (0.5 or 1.0 mM) used in these studies did not affect reductase activity by themselves. There was essentially no difference in the response to any of the concentrations of iron-chelating agents used. In Fig. 2 similar data are shown for guanazole. EDTA, desferal, and 8-hydroxyquinoline all potentiated the inhibition of reductase activity by guanazole. In Fig. 3, the effects of the iron-chelating agents on IMPY inhibition of reductase activity are shown. Again, these chelators markedly enhanced the inhibition of the reductase by IMPY. When low concentrations of iron (5 μM) were used in reaction mixtures containing IMPY (1 mM), the inhibition by IMPY was reduced markedly. These data are shown in Table 1.

Effects of iron-chelating agents on inhibition of reductase by MAIQ and IQ. As seen in Fig. 4, when EDTA, desferal, or 8-hydroxyquinoline was added in combination with MAIQ, the inhibitory effects of MAIQ were reduced. When similar experiments were carried out with IQ (Fig. 5), again it was observed that the inhibition by the thiosemicarbazone was eliminated by the presence of the iron-chelating agents.

Attempt to reverse inactivation of MAIQ-treated reductase. Ribonucleotide reductase was incubated with various concentrations of MAIQ (5–50 μM) for 1 hr on ice, followed by chromatography on Sephadex G-50. As seen in Fig. 6, although each of the concentrations would be sufficient to completely inhibit the reductase activity if added directly to the assay mixture, passage of the reductase/MAIQ solution through the Sephadex G-50 columns removed enough MAIQ for reductase activity to recover to some extent, even in the reductase preparation incubated with 50 μM MAIQ. Addition of desferal to the enzyme after passage through Sephadex G-50 did not restore reductase activity. On the other hand, the addition of ferrous ions to the reductase preparation after incubation with 50 μM MAIQ and pas-

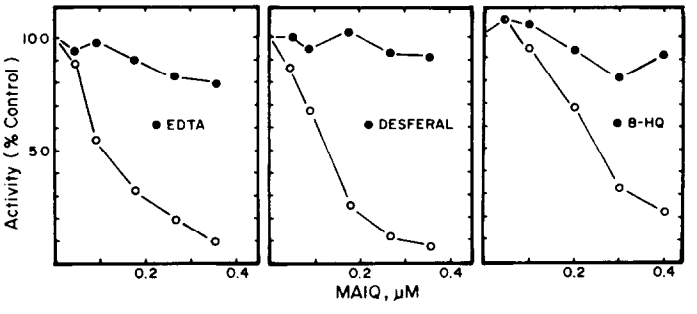

Fig. 4. Effect of EDTA, desferal, or 8-hydroxyquinoline on inhibition of ribonucleotide reductase by MAIQ. EDTA (0.167 mM), desferal (0.1 or 0.2 mM), or 8-hydroxyquinoline (8-HQ, 0.5 or 1.0 mM) was added to the enzyme in combination with various concentrations of MAIQ. Key: Open circles (○) show the percent activity remaining in the presence of MAIQ alone; closed circles (●) show the percent activity remaining in the presence of MAIQ plus the iron-chelating agents. The reactions were set up as described in the legend to Fig. 1. The control CDP reductase activity was 2.32 nmoles·(30 min)$^{-1}$·(mg protein)$^{-1}$.

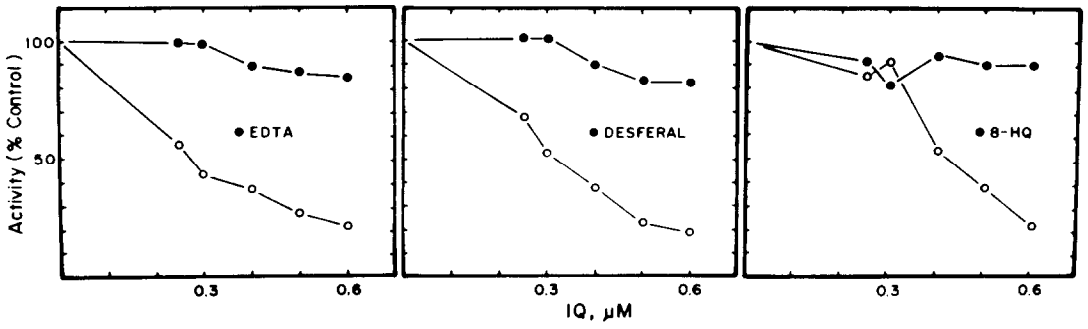

Fig. 5. Effect of EDTA, desferal, or 8-hydroxyquinoline on inhibition of ribonucleotide reductase by IQ. EDTA (0.167 mM), desferal (0.1 or 0.2 mM), or 8-hydroxyquinoline (8-HQ, 0.5 or 1 mM) was added to the enzyme in combination with various concentrations of IQ. Key: open circles (○) show the percent activity remaining in the presence of IQ alone; closed circles (●) show the percent activity remaining in the presence of IQ plus the iron-chelating agents. The reactions were set up as described in the legend to Fig. 1. The control CDP reductase activity was 2.28 nmoles·(30 min)$^{-1}$·(mg proteins)$^{-1}$.

sage through Sephadex stimulated reductase activity (Table 2).

Effect of iron-chelating agents on inhibition of reductase by Inox and 5′-deoxyinox. Inox and 5′-deoxyinox have both been shown to be inhibitors of ribonucleotide reductase activity [15, 16]. When EDTA, desferal, or 8-hydroxyquinoline was added to assay mixtures containing Inox or 5′-deoxyinox, these chelators neither enhanced nor decreased the inhibition caused by these dialdehyde derivatives.

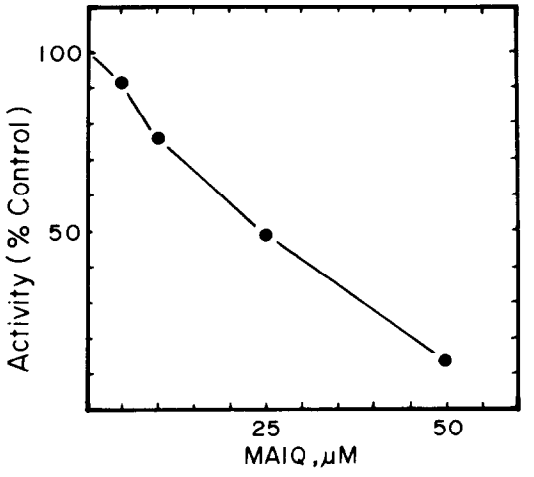

Fig. 6. Effect of MAIQ concentration on ribonucleotide reductase. The enzyme preparation (1 ml) was incubated on ice for 1 hr with the various concentrations of MAIQ shown in the legend. The samples were then passed over Sephadex G-50 columns (2.25 cm) and eluted with Tris–HCl buffer (0.02 M, pH 7) containing 1 mM dithioerythritol. The two peak rubes (1.5 ml/fraction) of the void-volume peak were combined and concentrated to 1 ml in an Amicon B-15 Minicon. The controls containing no inhibitor were carried through the same procedures. The enzyme assays were set up as described previously in Methods and Materials. All assays were carried out in triplicate. The control CDP reductase activity was 1.0 nmoles·(30 min)$^{-1}$·(mg protein)$^{-1}$.

DISCUSSION

The ribonucleotide reductase from Ehrlich tumor cells has been shown to consist of two, non-identical components, both of which are required for enzymatic activity [9]. One of the components contains non-heme iron and the other component has effector-binding site(s), analogous to the B2 and B1 proteins, respectively, of the ribonucleotide reductase from *Escherichia coli* [17, 18]. By separating the components, it was possible to categorize the known reductase inhibitors as inhibitors/inactivators of the non-heme iron component or the effector-binding site component [10, 11]. In the current study, the effects of various iron-chelating agents in combination with the reductase inhibitors were determined. The inhibition of reductase activity by Inox or 5′-deoxyinox was not altered by the iron-chelating agents, EDTA, desferal, or 8-hydroxyquinoline. This was expected since Inox and 5′-deoxyinox interact with and thereby inactivate the component of ribonucleotide reductase that contains the effector-binding sites but they do not interact with the component containing the non-heme iron site. Data are presented, however, which show that these iron-chelating agents have divergent effects on the inhibition of reductase by hydroxyurea, guanazole and IMPY as one group and MAIQ and IQ as the other group. Each of these compounds (hydroxyurea, guanazole, IMPY, MAIQ and IQ) complex iron to varying degrees. It was reported previously that the inhibition of reductase by hydroxyurea could be partially reversed by ferrous ions [5]. Moore interpreted the data to suggest that hydroxyurea was binding the free exogenous iron that was required for the activation of rat tumor reductase. The ribonucleotide reductase from the Ehrlich tumor cells, however, does not require and is not activated by exogenous iron. The lack of a requirement for exogenous iron also has been reported for purified mammalian reductase from Molt-4F cells [19]. This indicates that perhaps the hydroxyurea, guanazole and IMPY complex with the iron on the reductase component and the iron-chelating agents bind the endogenous iron in the enzyme preparation. Attemps to determine the endogenous iron concentrations by chemical

Table 2. Effect of ferrous ions on MAIQ-treated ribonucleotide reductase

Enzyme preparation*	CDP reductase activity [nmoles·(30 min)$^{-1}$·(mg protein)$^{-1}$]	% Control
Control	1.00	100
+ Fe^{2+} (6.67 μM)	0.92	92
MAIQ-treated	0.16	16
+ Fe^{2+} (6.67 μM)	0.39	39

* The enzyme fraction (0–40% ammonium sulfate fraction) was incubated in the presence and absence of MAIQ (50 μM) for 1 hr on ice. The samples were then passed over Sephadex G-50 columns. The two fractions from each column, containing the maximum absorbance at 280 nm, were pooled, concentrated in Amicon B-15 Minicon, and assayed for CDP reductase activity. All assays were carried out in triplicate; the assays agreed within 3 per cent.

assays in our enzyme preparations and reagents, however, have shown that the concentration of endogenous iron is less than 3 μM, which was the limit of the sensitivity of the bathophenanthroline method that we used [20]. This low level of endogenous iron tends to rule out a mechanism by which the iron-chelating agents remove the endogenous iron through chelation, leaving hydroxyurea, guanazole or IMPY to interact with the enzyme-bound iron, since the concentrations of these compounds used were greatly in excess of the iron which was present. The mechanism of inhibition of reductase by hydroxyurea, guanazole or IMPY, however, must involve iron. Moore [5] and Brockman *et al.* [3] demonstrated iron involvement with hydroxyurea and guanazole. In the present experiments, inhibition of reductase by IMPY (1 mM) was partially reversed by Fe^{2+} at concentrations as low as 5 μM (Table 1). It is possible that there is a unique iron chemistry involved, between the iron-chelating agents, the reductase inhibitors and the iron-site on the enzyme, that is responsible for the observations.

Regarding the thiosemicarbazones, Sartorelli's group had previously suggested that the [thiosemicarbazone–iron] complex is the species that interacts with ribonucleotide reductase [7]. Saryan *et al.* [8] reported that the metal complexes of the thiosemicarbazones were three to six times more potent than the free thiosemicarbazone [14] as inhibitors of reductase. It also had been shown that the thiosemicarbazone–Fe complexes could exist in both the Fe(III) and Fe(II) forms and that under the conditions used for ribonucleotide reductase assay, in which 6 mM dithiothreitol is utilized, the Fe(II) thiosemicarbazone was the predominant species [21]. The results of our studies suggest that the [thiosemicarbazone–Fe] complex is the actual inhibitory species. This conclusion is based on the observations that EDTA, 8-hydroxyquinoline and desferal, which are strong iron-chelating agents, essentially reverse the inhibitory actions of MAIQ and IQ.

Our data further show that incubation of the enzyme with high concentrations of MAIQ (50 μM) results in an inactive enzyme species. When desferal was added to the MAIQ-treated enzyme after Sephadex G-50 chromatography, no restoration of enzyme activity occurred. The addition of ferrous ions (6.67 μM) to this MAIQ-inactivated preparation, however, partially restored enzyme activity suggesting that, at high concentrations of MAIQ, iron is removed from the non-heme iron component.

Based on the results of the studies presented here, inhibitors of ribonucleotide reductase which are specific for the component containing non-heme iron can be further subclassified by the response of the inhibitors to the presence of iron-chelating agents. These data suggest that the therapeutic index of the antitumor agents hydroxyurea, guanazole and IMPY might be greatly improved by a protocol in which desferal is included.

Studies in intact tumor cells and tumor-bearing mice are currently underway to test these approaches.

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