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Mode of Inhibition of Tumor Cell Ribonucleotide Reductase by 2,3-Dihydro-1*H*-pyrazolo[2,3-*A*]imidazole (NSC 51143)[1]

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ABSTRACT

2,3-Dihydro-1*H*-pyrazolo[2,3-*a*]imidazole (NSC 51143; IMPY) inhibits partially purified ribonucleotide reductase from Ehrlich tumor cells. Both cytidine 5′-diphosphate and adenosine 5′-diphosphate reductase activities were inhibited by IMPY, although adenosine 5′-diphosphate reductase activity was inhibited to a greater extent than was cytidine 5′-diphosphate reductase activity at all concentraions of IMPY studied. The inhibition of the intact enzyme by IMPY could be reversed by the addition of the exogenous non-heme iron-containing subunit (tris(hydroxymethyl)aminomethane fraction) but not by the addition of the effector-binding subunit. Further, the inhibition of the intact enzyme or the tris(hydroxymethyl)aminomethane fraction by IMPY could be reversed by the addition of 6 μM $Fe(NH_4)_2(SO_4)_2$, and the inhibition of IMPY could be potentiated by 0.167 mM ethylenediaminetetraacetic acid. These results demonstrate that IMPY inhibits tumor cell nucleotide reductase by interaction with the iron of the non-heme iron-containing subunit.

INTRODUCTION

IMPY[3] has been shown to be a relatively potent and selective inhibitor of DNA synthesis without affecting RNA or protein synthesis (8). The inhibition of DNA synthesis was reversed if IMPY was removed from the culture medium. The specificity of the selective inhibition of DNA synthesis suggested that the site of action of IMPY was at the ribonucleotide reductase step. It was shown that, in fact, ribonucleotide reductase activity from HEp-2 cells was inhibited by IMPY (2).

Recently, we have separated ribonucleotide reductase into 2 non-identical components (4) and have been able to characterize the known reductase inhibitors with regard to their specificity for inhibiting or inactivating the individual components (3). We have been able to further characterize these components, which we originally referred to as the Tris and dye fractions, as the non-heme iron-containing subunit, and the effector-binding subunit respectively (6). With this system, we have been able to determine the subunit with which IMPY interacts and, from that, determine the nature of the inhibition of tumor cell ribonucleotide reductase by IMPY.

MATERIALS AND METHODS

Purification of Tumor Cell Ribonucleotide Reductase and Separation into Components. Ribonucleotide reductase was partially purified through the ammonium sulfate step from Ehrlich tumor cells as described previously (5). The AMS fraction was used in many of these studies. The AMS fraction was used to prepare the Tris and dye fractions on blue dextran-Sepharose as described previously, except that the dye fraction was eluted from the column with 0.25 M NaCl in 0.02 M Tris-HCl, pH 7.0, containing 1 mM dithioerythritol (4).

Enzyme Assays. CDP reductase activity was assayed by the method of Steeper and Steuart (12), utilizing [^{14}C]CDP as substrate and snake venom (*Crotalus atrox*) instead of apyrase and alkaline phosphatases to generate the nucleosides. ADP reductase activity was assayed by the method of Cory, *et al.* (7), utilizing [^{3}H]ADP as substrate. The assay mixtures were as described previously (3), with a final reaction volume of 150 μl. All assays were carried out in triplicate. The variability in the triplicate assays was routinely <5%. The reductase reactions were carried out at 37° for 30 min. Aliquots (0.5 ml) of the column effluent were taken for counting in Amersham ACS scintillation fluid in minivials.

Inhibition Studies with IMPY. The effects of IMPY on ribonucleotide reductase activity were determined under 2 different sets of conditions. In certain series of experiments, the IMPY was added at various concentrations to the assay mixtures, and the reactions were carried out immediately. In these cases, the IMPY was added to the enzyme, and the reaction was started by the addition of the "substrate mixture," consisting of the substrate, effector, and dithioerythritol. In the other type of experiments carried out, IMPY was incubated with the intact enzyme or the Tris fraction for 1 hr on ice. The mixtures were then passed over Sephadex G-50 columns. The protein peak eluting in the void volume was concentrated by ultrafiltration in Amicon B-15 Minicon concentrators. Control samples, which were not treated with IMPY, were carried through the same procedures.

Materials. IMPY was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., through the assistance of Dr. Leonard H. Kedda. The labeled substrates [^{14}C]CDP and [^{3}H]ADP, were purchased from New England Nuclear, Boston, Mass. The biochemicals used in these studies were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Effect of IMPY on CDP and ADP Reductase Activities. The effect of various concentrations of IMPY on the CDP and ADP reductase activities in the partially purified AMS fraction was studied. In these experiments, IMPY, at the final concentrations

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[3] The abbreviations used are: IMPY, 2,3-dihydro-1*H*-pyrazolo[2,3-*a*]imidazole (NSC 51143); AMS fraction, the 20 to 40% $(NH_4)_2SO_4$ protein fraction containing the ribonucleotide reductase activity.

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indicated, was added directly to the reductase assay mixture. There was no preincubation with IMPY before the start of the reactions. These results are shown in Chart 1. IMPY was an effective inhibitor of ribonucleotide reductase activity. As shown in Chart 2, there was a time dependence for the inhibition of reductase activity by IMPY. Incubation of the AMS fraction with 0.5 mM IMPY (the final concentration of IMPY during the reductase assay was 0.2 mM) resulted in an increase in the percentage of inhibition. Without incubation, this concentration of IMPY caused 43% inhibition; while after 4 hr of incubation on ice, 73% inhibition was observed.

Identification of Subunit Inhibited by IMPY. We had previously shown that the reductase inhibitors could be categorized as Tris-fraction inhibitors or dye-fraction inhibitors (3). Experiments were carried out to define the subunit inhibited by IMPY. In this experiment, the enzyme fraction was incubated with IMPY (2.72 mM) for 1 hr. After removal of excess IMPY by molecular exclusion chromatography on Sephadex G-50, the activity in the sample treated with IMPY was determined and compared with a control sample which had been carried through the same steps. The results of these experiments are shown in Table 1. The IMPY-treated enzyme, after passage over Sephadex G-50, had only 18% of the activity of the control sample. However, the addition of exogenous Tris fraction stimulated reductase activity to 66% of control. The addition of dye fraction had no effect on restoring reductase activity to the IMPY-treated sample.

Effect of Fe^{2+} on Inhibition by IMPY. Since the Tris fraction, which contains the non-heme iron (6), was the component inhibited by IMPY in the intact enzyme, the effects of Fe^{2+} on the inhibition by IMPY were studied. IMPY and ferrous ions, at the concentrations indicated, were added directly to the enzyme, and the reaction was started immediately by the addition of substrate. As seen in Chart 3, the addition of low concentrations of Fe^{2+} (6.67 and 20 μM) to reaction mixtures partially reversed the inhibition caused by IMPY. Higher concentrations of Fe^{2+} were inhibitory to the reductase activity even in the absence of IMPY. To further demonstrate the role of ferrous

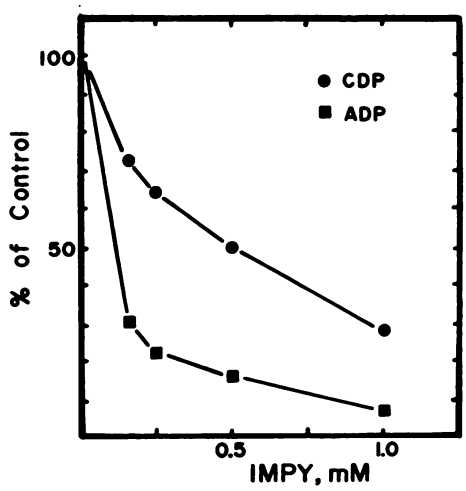

Chart 1. Comparison of the effect of IMPY on CDP and ADP reductase activities. CDP and ADP reductase activities were determined in the presence of the concentrations of IMPY indicated. The AMS fraction was the source of reductase activity. The control CDP and ADP reductase activities were 2.14 and 1.02 nmol product per 30 min per mg protein. The assays were run in triplicate.

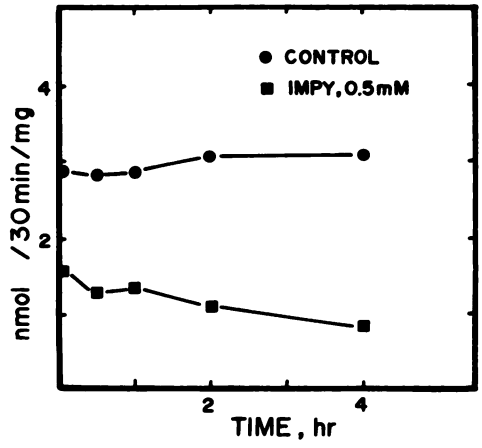

Chart 2. Effect of incubation on inhibition of CDP reductase activity by IMPY. The AMS fraction was incubated on ice in the presence (0.5 mM) and absence of IMPY. Aliquots (50 μl) were taken at the intervals indicated and assayed for reductase activity. In the reaction mixture, the concentration of IMPY was 0.167 mM. The assays were run in triplicate.

Table 1

Effect of Tris fraction on inhibition of intact enzyme by IMPY

Aliquots (1.0 ml) of the AMS fraction were incubated on ice in the presence and absence of IMPY (2.72 mM, final concentration) for 1 hr. The control and IMPY-treated samples were passed through Sephadex G-50 columns (25 x 2 cm) which were equilibrated with 0.02 M Tris-HCl, pH 7.0, containing 1 mM dithioerythritol to remove excess IMPY. The protein peak eluting in the void volume was concentrated in an Amicon B-15 Minicon. The concentrated AMS fraction control and AMS fraction /IMPY enzyme samples had protein concentrations of 8.7 and 8.4 mg/ml, respectively. Aliquots (50 μl) were used for the reductase assays. The Tris fraction (25 μl) which was added had a protein concentration of 11.2 mg/ml. The Tris fraction alone had no residual reductase activity. The final reaction volume was 150 μl, and all assays were carried out in triplicate.

Sample	CDP reductase (nmol/30 min/mg protein)	% of control
AMS fraction control	2.05	100
+ Tris fraction	2.22	108
AMS fraction-IMPY	0.37	18
+ Tris fraction	1.36	66

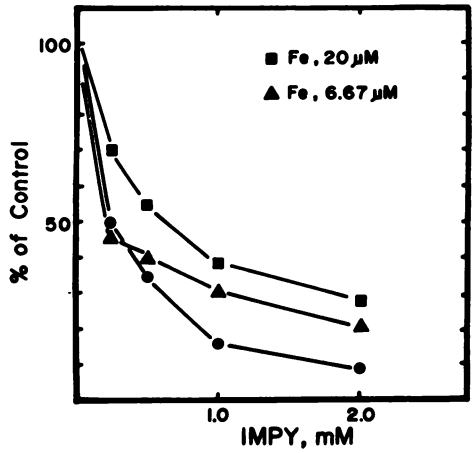

Chart 3. Effect of exogenous iron on inhibition of reductase activity by IMPY. $Fe(NH_4)_2(SO_4)_2$, at as final concentration of 6.67 or 20 μM, was added to reaction mixtures containing IMPY at the various concentrations indicated. The $Fe(NH_4)_2(SO_4)_2$ solution was made up fresh just before addition to the assay mixtures. The CDP reductase activity in the control was 1.51 nmol product per 30 min per mg protein. These assays were run in triplicate. ●, reductase activity measured at various concentrations of IMPY and reported as the percentage of control values.

ions in reversing the inhibition by IMPY, the Tris fraction was incubated with IMPY (3 mM final concentration) for 1 hr on ice. This sample and the control were passed over Sephadex G-50 columns to remove the excess (or unbound) IMPY. As seen in Table 2, the addition of Fe^{2+} to the Tris fraction caused a marked reversal of the IMPY-induced inhibition.

Effect of EDTA on Inhibition of Reductase Activity by IMPY. Since low concentrations of Fe^{2+} could reverse the inhibition of the intact enzyme or the Tris fraction caused by IMPY, the effect of EDTA was studied. IMPY and EDTA, at the final concentrations indicated, were added to the reaction mixtures without preincubation. As seen in Chart 4, EDTA, at a concentration (0.167 mM) which itself had no effect on reductase activity, resulted in a significant increase in the inhibition caused by IMPY. In an attempt to determine whether the marked difference in inhibition of ADP and CDP reductase activities by IMPY was related to the effects of endogenous iron, a comparison of the Fe^{2+} and EDTA effects on IMPY inhibition was made. For these studies, IMPY, Fe^{2+}, and EDTA were added to the reaction mixtures at the final concentration indicated. As seen in Table 3, there were slight differences in the effects of both exogenous Fe^{2+} and EDTA on the CDP and ADP reductase activities in the control. The addition of 5 μM exogenous Fe^{2+} caused 10% inhibition of CDP reductase activity in this AMS fraction preparation and 28% inhibition of ADP reductase activity. EDTA caused a slight activation of CDP reductase activity and a 15% decrease in ADP reductase activity. At both concentrations of IMPY used in this experiment, ADP reductase was inhibited to a greater extent than was CDP reductase. For CDP reductase activity, 5 μM Fe^{2+} caused a reversal of IMPY inhibition. The reversal of inhibition by Fe^{2+} was not apparent for ADP reductase activity. Further, the effects of EDTA were greater for CDP reductase activity than for ADP reductase activity.

Table 2

Effect of IMPY on Tris fraction

Aliquots (0.9 ml) of the Tris fraction were incubated in the presence (3.0 mM) and absence of IMPY for 1 hr on ice. The Tris fraction contained protein (11.2 mg/ml). The samples were then passed over Sephadex G-50 columns (25 × 2 cm) and concentrated as indicated in Table 1. Aliquots (50 μl) of the control (7.1 mg/ml) and IMPY-treated (6.7 mg/ml) Tris fractions were then assayed for reductase activity by adding exogenous dye fraction (50 μl, 6.4 mg/ml) to the reaction mixture. The Tris and dye fractions alone had no residual reductase activity. The assays were carried out in triplicate in a final reaction volume of 150 μl.

Sample	CDP reductase (nmol/30 min/mg ptrotein)	% of control
Tris fraction	1.59	100
+ Fe^{2+}, 6.67 μM	1.67	105
+ Fe^{2+}, 13.34 μM	1.58	100
Tris fraction-IMPY	0.50	31
+ Fe^{2+}, 6.67 μM	1.26	79
+ Fe^{2+}, 13.34 μM	1.46	92

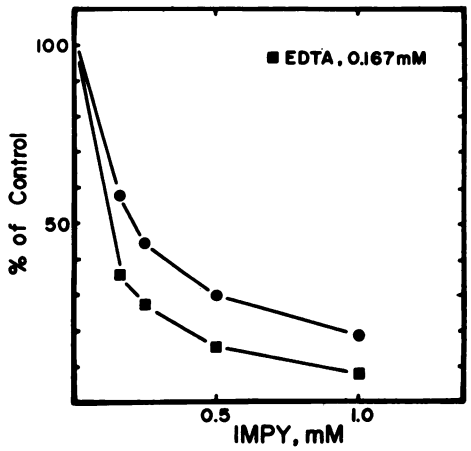

Chart 4. Effect of EDTA on inhibition of reductase by IMPY. EDTA at a final concentration of 0.167 mM in the reaction mixture was added to assay tubes containing the indicated concentrations of IMPY. The assays were run in triplicate. ●, reductase activity measured at various concentrations of IMPY and reported as the percentage of control values. The CDP reductase activity in the control was 1.32 nmol product per 30 min per mg protein.

Table 3

Comparison of EDTA and Fe^{2+} effects on inhibition of CDP and ADP reductase by IMPY

The CDP and ADP reductase activities were determined, as indicated in "Materials and Methods," under the conditions shown below. The activities were determined in the same experiment utilizing the same AMS fraction.

Sample	Reductase activity (nmol/30 min/mg protein) CDP	ADP
Control	2.00	0.82
+ Fe^{2+}, 5 μM	1.80	0.59
+ EDTA, 0.167 mM	2.16	0.70
IMPY, 0.25 mM	1.18	0.26
+ FE^{2+}, 5 μM	1.58	0.20
+ EDTA, 0.167 mM	0.78	0.20
IMPY, 1 mM	0.56	0.05
+ FE^{2+}, 5 μM	1.13	0.07
+ EDTA, 0.167 mM	0.19	0.04

Table 4

Effect of combination of IMPY and EDTA on reductase activity in tumor cell extracts

Ehrlich tumor cells were incubated in culture as described previously (3). EDTA (0.1 mM), IMPY (0.2 mM), and a combination of EDTA and IMPY were added to the appropriate culture flasks. The cells (approximately 8×10^7) were then incubated at 37° for 2.5 hr. The cells were collected by centrifugation, and the cell pellet was homogenized in 2 ml of 0.02 M Tris-HCl, pH 7, containing 1 mM dithioerythritol. The homogenate was centrifuged for 1 hr at 30,000 × *g*. The supernatant fluids were assayed for CDP reductase activity.

Source of extract	CDP reductase (nmol/30 min/mg protein)	% of control
Control	0.087	100
IMPY-treated, 0.20 mM	0.100	115
EDTA-treated, 0.10 mM	0.095	109
IMPY + EDTA	0.038	44

Effect of Combination of IMPY and EDTA on Reductase Activity on Tumor Cell Extracts. Since it was observed that EDTA could potentiate the inhibition by IMPY in partially purified preparations of tumor cell ribonucleotide reductase, it was important to determine whether EDTA would have an effect on the inhibition of reductase activity by IMPY in intact cells. The results of this experiment are shown in Table 4. Clearly, the treatment of intact tumor cells with the combination of IMPY and EDTA produced a synergistic effect on the inhibition of reductase activity as measured in the crude extracts prepared from these cells.

DISCUSSION

IMPY has been shown to be an effective inhibitor of DNA synthesis (8) and, as a result, an agent which could be used to

synchronize cell populations (1). The mechanism by which DNA synthesis is inhibited had been reported to be due to the inhibition of ribonucleotide reductase activity (2). The data that we report here confirm that IMPY is an inhibitor of partially purified ribonucleotide reductase. The data also tend to indicate that IMPY does not need to be metabolized to generate an inhibitor of reductase, since IMPY effectively inhibits both the AMS fraction and the more highly purified Tris fraction. These current data do not indicate whether the known metabolites of IMPY (9, 10) are also inhibitors of reductase activity. Contrary to a previous report (11), we found that ADP reductase activity was inhibited to a greater extent than was CDP reductase activity at all concentrations of IMPY studied. Since we have been able to separate the ribonucleotide reductase into 2 components (4), it has been possible to characterize various known inhibitors of ribonucleotide reductase with respect to the enzyme component with which they interact (3). Using this approach, it was shown that IMPY inactivated the Tris fraction which contains the non-heme iron. The inhibition of the reductase activity by IMPY was partially reversed by low concentrations of exogenous Fe^{2+}. This was shown for both the intact enzyme (AMS fraction) and the isolated Tris fraction. Further, the inhibition by IMPY could be potentiated by the addition of EDTA. Presumably, the effect of the addition of exogenous Fe^{2+} is to reverse the binding of IMPY to the enzyme at the non-heme iron site. Since such low concentrations of Fe^{2+} will partially reverse the inhibitory effects of IMPY, the effect of EDTA is viewed as the result of the binding of low levels of endogenous Fe^{2+} in the enzyme preparations. The results of the studies in which the enzyme was incubated with IMPY, followed by gel filtration to remove excess or unbound IMPY, showed that the effect of IMPY on the enzyme could persist *in vivo* even after the excess IMPY was excreted. Whether the effect of IMPY is to bind tightly to the enzyme causing the inhibition (Table 1) or whether IMPY removes the non-heme iron cannot be decided from these experiments. In either case, the addition of ferrous ions could presumably reverse the loss of enzyme activity.

That these effects may be important in the therapeutic use of IMPY was demonstrated by the results of the experiments carried out with intact cells. Phase I trials have shown that one of the reported side effects of IMPY is hemolysis (13). With the hemolysis would come the dumping of iron which in turn could interact with IMPY to reverse the inhibitory effects at the ribonucleotide reductase site. These data suggest that the variability in objective responses observed with the use of IMPY might be related to the pools of endogenous iron in the tumor tissues which would, in effect, reduce the effectiveness of IMPY as a ribonucleotide reductase inhibitor. It is possible that IMPY might be a more useful drug if given in combination with an iron-chelating agent such as EDTA or Desferal. Experiments are in progress in cells in culture and in tumor-bearing mice to test whether such combinations could improve the therapeutic index of IMPY.

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