Evaluation of Combinations of Drugs That Inhibit Ehrlich Tumor Cell Ribonucleotide Reductase

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ABSTRACT

The nature of the inhibition of Ehrlich tumor cell ribonucleotide reductase by combinations of agents directed at the non-heme iron-containing component and the effector-binding component was studied with the use of isobolograms. From these studies, it was determined that the combinations of pyrazoloimidazole (IMPY) and dialdehyde of inosine, IMPY and deoxyguanosine triphosphate (dTTP), IMPY and deoxyadenosine triphosphate (dATP), and IMPY and deoxythymidine triphosphate (dTTP) gave synergistic inhibition of cytidine diphosphate reductase. The combination of dATP and dTTP also gave synergistic inhibition. The combinations of hydroxyurea and IMPY, 4-methyl-5-aminoisoquinoline thiosemicarbazone (MAIQ) and IMPY, and dialdehyde of inosine and diphosphate derivative of 5'-deoxyinosine gave antagonistic inhibition. Other combinations utilizing MAIQ and dATP, MAIQ and dGTP, MAIQ and dTTP, hydroxyurea and dGTP, and hydroxyurea and dTTP gave inhibition which was additive.

INTRODUCTION

Ribonucleotide reductase offers a unique metabolic site which holds excellent promise as a target of antineoplastic agents. With the separation of the ribonucleotide reductase from Ehrlich tumor cells into its 2 components (5), it was possible to show that each of the protein components (the non-heme iron-containing component and the effector-binding component) could be specifically and independently inhibited (3). With this, the various known ribonucleotide reductase inhibitors could be categorized with respect to whether they were inhibitors of the non-heme iron-containing component or the effector-binding component (8). These are summarized in Table 1. Since each component could be inhibited and inactivated independently, it was possible to study combinations of ribonucleotide reductase inhibitors to determine if synergism could be obtained by such an approach.

In this report, we present data which show that certain combinations of reductase inhibitors do result in the synergistic inhibition of tumor cell ribonucleotide reductase.

MATERIALS AND METHODS

Preparation and Assay of Ribonucleotide Reductase. Ribonucleotide reductase was partially purified from Ehrlich tumor cells through the ammonium sulfate step as described previously (6). CDP reductase was assayed by the method of Steeper and Steuart (15) except that snake venom (Crotalus atrox) was used in place of apyrase and alkaline phosphatase. The following reaction mixture was contained in a final volume of 0.15 ml: [14C]CDP (0.05 μCi, 7.5 nmol); diithioerythritol (900 nmol); magnesium acetate (600 nmol); ATP (300 nmol); inhibitor or inhibitors; and the enzyme preparation. The enzyme assays were run in the presence and absence of various concentrations of the single inhibitor and combinations of inhibitors. All assays were carried out in triplicate except for the controls which were run in quadruplicate. The reductase reactions were carried out for 30 min at 37°. The snake venom reactions were carried out for 1 hr at 37°. The standard deviations for enzyme assays were usually ±2% of the activity determined. The data were analyzed by the method of Elion et al. (9).

Protein Concentrations. Protein concentrations were estimated by the method of Lowry et al. (13) utilizing bovine serum albumin as standard.

Materials. [14C]CDP (453 mCi/mmol) was purchased from New England Nuclear, Boston, Mass. The nucleotides and other biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. MAIQ, Inox, and IMPY were obtained from the Drug and Synthesis Branch, Division of Cancer Treatment, National Cancer Institute, through the assistance of Dr. Leonard H. Kedda. 5'-Deoxyinosine was synthesized in this laboratory (7).

The Ehrlich tumor cells were grown in female mice (ICR). The mice were purchased from Laboratory Supply Company, Indianapolis, Ind.

RESULTS

Combinations of Ribonucleotide Reductase Inhibitors Which Give Synergistic Inhibition

Combinations of a Tris Fraction and a Dye Fraction Inhibitor. As seen from the isobolograms shown in Chart 1, combinations of ribonucleotide reductase inhibitors consisting of a Tris fraction inhibitor and a Dye fraction inhibitor resulted in synergistic inhibition of CDP reductase activity. These combinations included: IMPY and Inox; IMPY and dGTP, IMPY and dATP; and IMPY and dTTP. In the latter 2 combinations, EDTA (0.167 mM) was included in the reaction mixtures. EDTA alone had no effect on CDP reductase activity.

1 This research was supported by Grant CA27398 from the USPHS, National Cancer Institute, and the Phi Beta Psi Sorority.
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Received October 31, 1980; accepted January 26, 1981.
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Table 1

Inhibitors of the individual components of ribonucleotide reductase

<table>
<thead>
<tr>
<th>Tris fraction inhibitors (non-heme iron subunit)</th>
<th>Dye fraction inhibitors (effector-binding subunit)</th>
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</thead>
<tbody>
<tr>
<td>HU</td>
<td>dATP</td>
</tr>
<tr>
<td>Guanazole</td>
<td>dTTP</td>
</tr>
<tr>
<td>IMPY</td>
<td>dGTP</td>
</tr>
<tr>
<td>MAIQ</td>
<td>Inox</td>
</tr>
<tr>
<td>1-Formyllsorolone thiosemicarbazone</td>
<td>5'-DeoxyInox</td>
</tr>
<tr>
<td></td>
<td>Pyridoxal phosphate</td>
</tr>
</tbody>
</table>

Combinations of Dye Fraction Inhibitors or Tris Fraction Inhibitors. Several combinations of reductase inhibitors directed at the same component (either the dye fraction or Tris fraction) gave inhibition which was additive. These combinations included: dGTP and dTTP; dATP and dTTP; dATP and Inox; and HU and MAIQ. The isobolograms for these combinations are shown in Chart 4.

Combinations of Ribonucleotide Reductase Inhibitors Which Give Antagonistic Inhibition

Combinations of Tris Fraction Inhibitors or Dye Fraction Inhibitors. The combinations of HU and IMPY, MAIQ and IMPY, and Inox and 5'-DeoxyInox each gave antagonistic inhibition of CDP reductase activity. The isobolograms for these combinations are shown in Chart 5.

Combinations of a Tris Fraction and a Dye Fraction Inhibitor. Several combinations of reductase inhibitors consisting of a Tris fraction and a Dye fraction inhibitor gave inhibition which was antagonistic. These combinations included: HU and 5'-DeoxyInox; HU and dATP; MAIQ and Inox; and MAIQ and 5'-DeoxyInox. These data are given in Chart 6.

Effect of EDTA on Combinations of Ribonucleotide Reductase Inhibitors

We have reported previously that iron-chelating agents potentiated the inhibition of CDP reductase by HU, guanazole, and IMPY (4, 8). With several combinations consisting of reductase inhibitors directed at the individual components, EDTA was included with the Tris fraction and Dye fraction inhibitors. The effect of EDTA on the nature of the inhibition by these combinations is summarized in Table 2. The concentration of EDTA (0.167 mM) used in these combinations had no effect on the reductase alone. In the case of the combination of IMPY and Inox, the presence of EDTA caused a shift in inhibition from synergism to antagonism while, in the cases of HU and dTTP and HU and dGTP combinations, EDTA shifted the inhibition from additive to antagonistic. On the other hand, for the combination of HU and IMPY, the presence of EDTA caused a shift in the nature of the inhibition from antagonism to additive.

Chart 1. Isobolograms of combinations of reductase inhibitors which give synergistic inhibition. For the combination of IMPY and Inox, the control enzymatic activity was 3.2 nmol deoxycytidine formed per 30 min per mg protein. The concentrations of IMPY or Inox alone required to give 70% inhibition were 0.96 and 1.93 mM, respectively. For the combination of IMPY and dGTP, the control activity was 2.6 nmol/30 min/mg protein while the concentrations of IMPY and dGTP alone required to give 70% inhibition were 0.96 and 0.027 mM, respectively. For the combination of IMPY and dATP in the presence of EDTA (0.167 mM), the control activity was 2.8 nmol/30 min/mg protein. The concentrations of IMPY or dATP alone required to give 70% inhibition were 0.21 and 0.020 mM, respectively.

Chart 2. Isobolograms of combination of reductase inhibitors which give synergistic inhibition. For the combination of dATP and dGTP, the control activity in the absence of inhibition was 3.4 nmol deoxycytidine per 30 min per mg protein. The concentrations of dATP or dGTP alone required to give 70% inhibition were 0.030 and 0.013 mM, respectively.
Ribonucleotide Reductase Inhibition

Chart 3. Isobolograms of combinations of reductase inhibitors which give additive inhibition. For the combination of MAIQ and dATP, the control activity in the absence of inhibitors was 3.0 nmol deoxycytidine per 30 min per mg protein. The concentrations of MAIQ and dATP alone required to give 65% inhibition were 0.19 \( \mu M \) and 0.024 mM, respectively. For the combination of MAIQ and dGTP, the control activity was 2.3 nmol/30 min/mg protein. The concentrations of MAIQ and dGTP alone required to give 60% inhibition were 0.20 \( \mu M \) and 0.015 mM, respectively. For the combination of MAIQ and dTTP, the control activity was 2.5 nmol/30 min/mg protein. The concentrations of MAIQ or dTTP alone required to give 70% inhibition were 0.20 \( \mu M \) and 0.015 mM, respectively. For the combination of HU and dGTP, the control activity was 3.2 nmol/30 min/mg protein. The concentrations of HU or dGTP alone required to give 70% inhibition were 0.8 and 0.017 mM, respectively. For the combination of HU and dTTP, the control activity was 2.9 nmol/30 min/mg protein. The concentrations of HU or dTTP alone required for 60% inhibition were 0.65 and 0.017 mM, respectively.

DISCUSSION

With the finding that ribonucleotide reductase from Ehrlich tumor cells consisted of 2 nonidentical components which were both required for enzymatic activity (5), it was possible to demonstrate that each component could be specifically and independently inhibited (3). While combination chemotherapy has been studied for many drug combinations involving agents directed at different metabolic sites with appropriate theoretical approaches (1, 11, 12, 14, 16), the ribonucleotide reductase enzyme provided a metabolic site at which drugs in combination could be targeted to the nonidentical components of this enzyme. It was possible to study combinations of ribonucleotide reductase inhibitors (Table 1) to determine the nature of the inhibition produced by these drug combinations, i.e., synergistic, additive, or antagonistic.

With a combination of drugs consisting of an agent directed at the non-heme iron component and an agent directed at the effector-binding component, it was to be expected, based on

The concentrations of dGTP and dTTP alone required to give 60% inhibition were 0.016 and 0.019 mM, respectively. For the combination of dATP and dTTP, the control activity was 1.8 nmol/30 min/mg protein. The concentrations of dATP and dTTP alone required to give 55% inhibition were 0.020 and 0.023 mM, respectively. For the combination of dATP and Inox, the control activity was 3.1 nmol/30 min/mg protein. The concentrations of dATP and Inox alone required to give 60% inhibition were 0.016 mM and 1.75 mM, respectively. For the combination HU and MAIQ, the control activity was 2.3 nmol/30 min/mg protein. The concentrations of HU and MAIQ alone required to give 50% inhibition were 0.7 mM and 0.22 \( \mu M \), respectively.
Table 2

<table>
<thead>
<tr>
<th>Combination</th>
<th>EDTA (0.167 mM)</th>
<th>Nature of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMPY and Inox</td>
<td></td>
<td>Synergism</td>
</tr>
<tr>
<td>IMPY and Inox</td>
<td>+</td>
<td>Antagonism</td>
</tr>
<tr>
<td>HU and dTTP</td>
<td></td>
<td>Additive</td>
</tr>
<tr>
<td>HU and dTTP</td>
<td>+</td>
<td>Antagonism</td>
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<tr>
<td>HU and dGTP</td>
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<td>Additive</td>
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<tr>
<td>HU and dGTP</td>
<td>+</td>
<td>Antagonism</td>
</tr>
<tr>
<td>HU and IMPY</td>
<td></td>
<td>Antagonism</td>
</tr>
<tr>
<td>HU and IMPY</td>
<td>+</td>
<td>Additive</td>
</tr>
</tbody>
</table>

The theoretical treatment of Grindey et al. (11), that 2 drugs acting independently on the same enzyme should produce synergistic inhibition. From the data obtained and analyzed by the use of isobolograms (9), it was observed that the combinations of IMPY and Inox, IMPY and dGTP, IMPY and dATP, and IMPY and dTTP produced synergistic inhibition of ribonucleotide reductase. Another synergistic combination consisting of dATP and dGTP was observed even though both of these compounds are inhibitors of the effector-binding subunit. The explanation for the observed synergistic effect by the combination of dATP and dGTP may be related to the multiplicity of sites for the various effectors of ribonucleotide reductase, as has been determined in the case of the enzyme from Escherichia coli (2), although not yet demonstrated for the mammalian enzyme.

Antagonism was observed with the combinations of agents specific for the same subunit of ribonucleotide reductase. The combinations of HU and IMPY and MAIQ and IMPY which are directed at the non-heme iron component caused antagonistic inhibition of reductase. Likewise, the combination of Inox and 5'-Deoxyinox also resulted in antagonism. These results were anticipated based on the fact that the agents of these particular combinations were specific inactivators of the same component. On the other hand, several combinations of inhibitors, consisting of a non-heme iron subunit inhibitor and an effector-binding subunit inhibitor, also gave antagonistic inhibition of reductase. These included the combinations of HU and 5'-Deoxyinox, MAIQ and 5'-Deoxyinox, MAIQ and Inox, and HU and dATP. These results were not anticipated based on the specific and independent inhibition of each component, and an explanation for these data is not obvious. From earlier studies with the combination of MAIQ and Inox, data were obtained which appeared to be consistent with the inhibitors acting by summation (3). However, by isobologram analysis which re-

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Chart 5: Isobolograms of combinations of reductase inhibitors which give antagonistic inhibition. For the combination of HU and IMPY, the control activity was 3.0 nmol deoxycytidine per 30 min per mg protein. The concentrations of HU and IMPY alone required to give 70% inhibition were 0.20 and 0.25 mM, respectively. For the combination of MAIQ and IMPY, the control activity was 2.1 nmol/30 min/mg protein. The concentrations of MAIQ and IMPY alone required to give 50% inhibition were 0.21 μM and 0.68 mM, respectively. For the combination of Inox and 5'-Deoxyinox (5'-dINOX), the control activity was 3.2 nmol/30 min/mg protein. The concentrations of Inox and 5'-Deoxyinox alone required to give 70% inhibition were 2.3 and 2.1 mM, respectively.

Chart 6: Isobolograms of combinations of reductase inhibitors which give antagonistic inhibition. For the combination of HU and 5'-Deoxyinox (5'-dINOX), the control activity was 3.2 nmol deoxycytidine per 30 min per mg protein. The concentrations of HU and 5'-Deoxyinox alone required to give 60% inhibition were 0.48 mM and 1.8 mM, respectively. For the combination of HU and dATP, the control activity was 3.0 nmol/30 min/mg protein. The concentrations of HU and dATP alone required to give 65% inhibition were 0.73 and 0.016 mM, respectively. For the combination of MAIQ and Inox, the control activity was 1.5 nmol/30 min/mg protein. The concentrations of MAIQ and Inox alone required to give 65% inhibition were 0.18 μM and 1.66 mM, respectively. For the combination of MAIQ and 5'-Deoxyinox, the control activity was 2.7 nmol/30 min/mg protein. The concentrations of MAIQ and 5'-Deoxyinox alone required to give 50% inhibition were 0.26 μM and 1.35 mM, respectively.

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quired far more concentration data points, this combination of MAIQ and Inox gave inhibition that was antagonistic. While the analysis of combinations by isobolograms requires much more data, it does appear to provide a more general view of the type of inhibition obtained by combinations over that of Webb (16). The third category of inhibition observed with the various combinations was that of additive inhibition. These combinations included drugs directed at each of the components and drugs directed at the same component of reductase. The former combinations included MAIQ and dATP, MAIQ and dGTP, MAIQ and dTTP, HU and dGTP, and HU and dTTP, while combinations of the latter type included dGTP and dTTP, dATP and dTTP, dATP and Inox, and HU and MAIQ. The additive nature of the observed inhibitions indicates that each of the compounds of these combinations interacts at sites independently of the other.

The results of these studies indicate that it is not possible to predict a priori the nature of the interactions which will result from combinations of drugs. This was shown quite clearly in the studies with EDTA. Since EDTA and other iron-chelating agents potentiate the inhibition of HU, IMPY, and guanazole (4, 8), it was anticipated that a combination of reductase inhibitors including EDTA would provide synergistic inhibition. As seen in Chart 1 and Table 2, even the effects of adding EDTA to a combination of reductase inhibitors could not be predicted. These studies do suggest, however, that IMPY might prove to be a useful agent in combination with an appropriate inhibitor of the effector-binding subunit to provide synergistic inhibition of ribonucleotide reductase. On the other hand, MAIQ and HU did not appear to be useful components of combinations to achieve a synergistic response. However, as discussed by Grindey et al. (10), the nature of the interactions of combinations of drugs obtained in vitro may not be the nature of the interaction to be obtained in vivo.

Studies are currently under way to determine the efficacy of this approach to combination chemotherapy in the in vivo situation.

REFERENCES
Evaluation of Combinations of Drugs That Inhibit Ehrlich Tumor Cell Ribonucleotide Reductase

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Cancer Res 1981;41:1637-1641.

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