Inhibition of DNA Ligase from Human Thymocytes and Normal or Leukemic Lymphocytes by Antileukemic Drugs

Jean-Claude David, Thérèse Bassez, Micheline Bonhommet, and Régine Rusquet

Laboratoire de Biochimie du Développement, La Centre National de la Recherche Scientifique n 256, Université de Rennes I, Campus de Beaulieu, 35042, Rennes Cedex, France

ABSTRACT

Human DNA ligase was purified from both normal and leukemic peripheral lymphocytes and normal thymocytes. The activity of the purified enzymes was assayed in the presence of several widely used antileukemic drugs. Melphalan and prednisone at 5 mM had no effect. Carmustine, chlorambucil, and cyclophosphamide were more effective at inhibiting the enzyme from leukemic cells, whereas Adriamycin and vinblastine and their derivatives were stronger inhibitors of the enzyme from normal cells. Vincristine and etoposide inhibited DNA ligase from thymocytes and normal lymphocytes with a low Ki but were totally ineffective on the enzyme from leukemic cells.

The three classes of intercalating anthracyclines, Vicia alkaloids, and podophyllotoxin derivatives, were the only drugs found to markedly inhibit DNA ligases from normal cells. Less substituted molecules of the Vicia alkaloids and podophyllotoxin classes were the more active inhibitors, whereas in the intercalating anthracycline group, it was the more substituted compounds. The clinical consequences of these observations are discussed with respect to the role of DNA ligase in DNA replication and repair.

INTRODUCTION

Antitumoral chemotherapy essentially began about 40 years ago with the discovery of nitrogen mustard (16, 17). The first temporary remissions were obtained with the antimitabolite, aminopterine in 1948 (11) and complete remissions with the closely related compound methotrexate (22). In 1956, a third class of antitumoral agents was described by Farber et al. (11) with actinomycin and the related compound methotrexate (22). At present, all the antitumor substances are classified into 5 groups: (a) electrophilic agents; (b) inhibitors of DNA synthesis; (c) DNA interacting substances; (d) hormones of the mitotic spindle; and (e) hormones (21). With the possible exception of the last group, all these substances may, in fact, interfere with DNA metabolism. Several of these compounds are known to induce DNA breaks (35, 41, 42) thereby rendering DNA enzymatic studies of great interest. Among the enzymes involved in DNA replication or repair, DNA ligases have received little attention (8).

The existence of enzymes capable of joining DNA fragments was proposed more than 20 years ago for prokaryotic cells (19, 30). Polynucleotide ligases were first described in 1967 (14, 15). Their physiological importance has been assessed in prokaryotes by the inability of Escherichia coli mutants defective in these enzymes to repair their DNA (9, 40). However, in eukaryotes, the association of DNA ligase with replication or repair has been known for just a few years (44). The presence of active DNA ligase in lymphoid and myeloid tissues (26) as well as the stimulation of its activity during rat liver regeneration (44) or lymphocyte division (43) suggest that it may have an important function in proliferative states such as leukemia. However, at the present time, DNA ligase activity has not been studied in the different types of leukemias (8). In this report, we describe investigations on the effects of antileukemic drugs on DNA ligase activity purified from normal thymocytes and lymphocytes and from acute myeloblastic leukemia lymphoblasts.

MATERIALS AND METHODS

Drugs

Adriamycin was provided by Roger Bellon Laboratories, Paris, France. AD32 was a gift of Dr. Israel, University of Tennessee Medical School, Knoxville, TN. m-AMSA and o-AMSA were kind gifts of Professor B. Baguley, University of Auckland, New Zealand; ara-C and cyclophosphamide, from Sigma. Carmustine and etoposide, from Bristol Laboratories, Paris; chlorambucil, from Techni-Pharma Laboratories, Monaco; cyclophosphamide, from Laboratories Lucien, Paris. DMCOOH, DMCOOK, DMCONH2 (1-p-carboxyamidophenyl-3,3-dimethyltriazene), MMCONH2, generous gifts of Dr. R. Lassiani, University of Trieste; daunorubicin and methotrexate, from Specia, Paris; melphalan, Welcome Laboratories, Paris; prednisone, from Roussel Uclaf, Paris; vinblastine, vincristine, and vindesine, from Eli Lilly, Saint Cloud, France. Kinetoplast DNA and pBR322 were gifts from Dr. M. Duguet, Paris, France.

Preparation of Cells

Thymuses were obtained from 3 different normal cases of thoracic surgery (Case 1, female age 15 months; Case 2, male age 30 months; Case 3, male age 4 months). Thymuses were received little attention (8).

1 This work was supported by a Grant from Institut National de la Santé et de la Recherche Médicale No. 833003 and Federation Nationale des Centres de Lutte contre le Cancer.

2 To whom requests for reprints should be addressed.

Received 7/2/84; revised 12/11/84; accepted 1/9/85.
EFFECT OF ANTILEUKEMIC DRUGS ON DNA LIGASE

Case 3, male 10 years) from the Department of Thoracic Surgery, Hôpital Pontchaillou, Rennes, France. Thymocytes were separated after gentle hand homogenizing in RPMI (Eurobio, Paris), separated from RBC on Ficoll gradients and washed 3 times for 15 min at 1200 rpm in a Jouan refrigerated centrifuge at 4 °C. The thymuses were kept no longer than 1 h in ice before processing).

Leukemic WBC were obtained from the Centre Anticancéreux, Hôpital Pontchaillou, Rennes, France. Three cases were selected: Case 1, acute myeloblastic leukemia M2, female, 54 years (77% blasts); Case 2, chronic myelogenous leukemia, male, 62 years (85% blasts); and Case 3, chronic lymphocytic leukemia, female, 40 years (90% blasts). WBC were separated from heparinized blood by centrifugation on Ficoll gradients (Flobio, Paris) for 15 min at 1400 rpm and washed 3 times in RPMI for 10 min at 1200 rpm at 4 °C. Normal WBC were obtained from the Centre de Transfusion de Rennes and purified in the same manner.

DNA Ligase Purification

DNA ligase was purified separately from the 3 types of thymocytes and the 3 types of leukemic lymphocytes. DNA ligase purification was basically as described previously (6) with the following modifications. Thymocytes (20 g) were suspended in 200 ml of TEM buffer and leukemic lymphocytes (4.5 × 10⁹ cells) in 100 ml of TEM. The suspension was homogenized for 1 min in a Sorvall Omni Mixer, centrifuged for 60 min at 140,000 × g, and the supernatant dialyzed for 16 h against 2 changes of TEM buffer containing 20% glycerol (TEMG buffer). This extract was adsorbed onto a PE₁₁₁ phosphocellulose column equilibrated at pH 7.2. DNA ligase was eluted by 0.3 M KCl and further purified using, consecutively, Sephadex G 150, Sephadex CM 50, and DNA cellulose chromatography as described (6). The sedimentation coefficients of the enzymes from thymocytes, normal lymphocytes, and lymphoblasts were, respectively, 5 ± 0.5s, 5 ± 0.6s, and 5.2 ± 0.6s (SD) and were not significantly different. The homogeneity of the enzymes was checked by polyacrylamide gel electrophoresis and found to be greater than 95% for all the purified enzymes (Fig. 1). No significant differences in the migration mobilities were observed between the enzymes from the different sources. In each case, detectable activity was found at the band level.

Enzyme Assays

DNA ligase activity was assayed routinely using a modification of the method described by Modrich and Lehman (34).

Preparation of [³H]Poly[d(A-T)]₂

The incubation mixture contained in 1 ml: 50 mM Tris HCl pH 8.6, 20 mM dithiothreitol, 18 µM dTTP (Sigma), 18 µM dATP (Sigma), 4 absorbance units of poly[d(A-T)]₂, (Boehringer), 5 units of DNA polymerase I, large fragment (New England Nuclear), 1.5 µM [³H]dTTP (78 Ci/mmol; New England Nuclear), and 0.1 mg of bovine serum albumin (Sigma). After a plateau of incorporation was reached, the reaction was stopped by 250 µl of 5 M NaCl followed by a 20-min incubation at 70 °C. The solution was dialyzed against 50 volumes of a solution containing 100 mM NaCl and 0.1 mM EDTA; 200-µl aliquots were stored at −80 °C until further use.

DNA Ligase Assay

Each assay (0.1 ml) contained 25 mM Tris-HCl (pH 8), 4 mM MgCl₂, 1 mM ATP, 0.3 mM [³H]dATP (78 Ci/mm; New England Nuclear), 0.1 mg of bovine serum albumin, 5 mM dithiothreitol, and 0 to 40 µl of DNA ligase solution.

After 30 min incubation at 37 °C, the assays were heated in a boiling...
EFFECT OF ANTILEUKEMIC DRUGS ON DNA LIGASE

water bath for 3 min, and 10 units of exonuclease III (New England Nuclear) were added. After incubation for 30 min at 37 °C, the samples were precipitated with trichloroacetic acid (5% final concentration) filtered through Whatman GFC filters, and counted. One unit of DNA ligase activity is defined as the amount converting 1 nmol of d(A-T)n to an exonuclease III-resistant form in 30 min under the assay conditions.

All the kinetics studies were repeated using a second independent enzyme test according to a modification (5) of the method described by Olivera (37). The substrate was prepared as follows. One mm oligo-(dT)12-18 (P. L. Biochemicals) in 50 mm Tris, pH 7.6, was incubated for 30 min at 37 °C with E. coli alkaline phosphatase (10 units/ml). The reaction was stopped by the addition of 0.1 volume of 20 mm KH2PO4. This mixture was adjusted to pH 10.5 with 1 N NaOH and kept for 15 min in a boiling water bath. After adjusting the pH to 7.6 with 1 N HCl, the extract was subjected to centrifugation at 10,000 rpm for 10 min. The supernatant containing 200 μM dephosphorylated oligo(dT)12-18 was used for 5'-32P labeling in the presence of 60 mm Tris-HCl, pH 7.6, 6 mm MgCl2, 6 mm 2-mercaptoethanol, 300 μM [γ-32P]ATP (20 Ci/mmol), and 15 units per ml of T₄ injected E. coli 5'-polynucleotide kinase (P. L. Biochemicals) at 37 °C until the plateau of incorporation was reached. The resulting [S'-32P]oligo(dT)12-18 was stored at −20 °C until use. For the determination of ligase activity, each assay (0.3 ml) contained 6 μM [32P]oligo(dT)12-18, 6 μM polydeoxyadenylate (P. L. Biochemicals), 25 mm Tris-HCl (pH 7.6), 6 μM MgCl2, 1 mm ATP, 2.5 mm dithiothreitol, bovine serum albumin (31 μg/ml), and 100 μl of enzyme solution. The samples were incubated at 37 °C for 30 min. After the addition of 1 unit of alkaline phosphatase and dilution with 300 μl of ice-cold water, they were incubated for 30 min at 80 °C. The samples were precipitated with trichloroacetic acid, 5% final concentration, filtered through Millipore filters, and counted. One unit of DNA ligase is defined as the amount which renders 1 nmol of [5'-32P]oligo(dT) resistant to alkaline phosphatase.

The resulting [S'-32P]oligo(dT)12-18 was stored at −20 °C until use. For the determination of ligase activity, each assay (0.3 ml) contained 6 μM [32P]oligo(dT)12-18, 6 μM polydeoxyadenylate (P. L. Biochemicals), 25 mm Tris-HCl (pH 7.6), 6 μM MgCl2, 1 mm ATP, 2.5 mm dithiothreitol, bovine serum albumin (31 μg/ml), and 100 μl of enzyme solution. The samples were incubated at 37 °C for 30 min. After the addition of 1 unit of alkaline phosphatase and dilution with 300 μl of ice-cold water, they were incubated for 30 min at 80 °C. The samples were precipitated with trichloroacetic acid, 5% final concentration, filtered through Millipore filters, and counted. One unit of DNA ligase is defined as the amount which renders 1 nmol of [5'-32P]oligo(dT) resistant to alkaline phosphatase.

Controls for Other Enzyme Activities

DNA Polymerase α and β Activities. The polymerases activities were assayed on purified ligases using activated calf thymus DNA in the presence of dATP, dCTP, dGTP, and [3H]dTTTP (7.1 Ci/mmol; New England Nuclear). The α and β activities were separately assayed according to the method of Carre et al. (5).

DNA Topoisomerases. ATP-independent relaxation of supercoiled DNA was assayed on the purified ligase preparations according to the method of Duquet et al. (10). ATP-dependent catenation of pBR322 DNA form I was assayed as described by Liu (23) and ATP-dependent decatenation was assayed in a similar manner with the exception that the DNA substrate was kinetoplast DNA. Controls made with the enzymes from the 3 sources reveal that there was neither DNA polymerases α or β nor topoisomerases I or II activities copurified with the ligase preparation.

RESULTS

Inhibition of DNA Ligase Activity by Antileukemic Drugs. Thirteen widely used and 8 experimental antileukemic drugs were tested on the activity of purified DNA ligases from normal thymocytes and normal and leukemic peripheral lymphoblasts. Cyclophosphamide, melphalan, prednisone, ara-C, and methotrexate (0.5 mm) were without effect on ligase activity from either type of cell (Table 1). Carmustine was slightly inhibitory, reducing the ligase activity of leukemic cells to 80% of the control. Chlorambucil and the experimental drugs DMCOOH and MMCONH2 were more potent inhibitors of the blast enzyme, with residual activities representing 40, 50, and 28% of the control of the 2 other experimental drugs. m-AMSA was found to be totally ineffective in inhibiting the different enzymes. However, o-AMSA inhibited the ligase activity from thymocytes, lymphocytes, and blasts to 40, 42, and 45% of the control values, respectively. Unexpectedly, daunorubicin, Adriamycin, and AD32 were more potent inhibitors of DNA ligase from the normal cell types.

Thymocyte ligase was almost totally inhibited (25, 10, and 5% of control values), whereas the enzyme from normal lymphocytes displayed activities of 45, 25, and 18% of control values for the 3 drugs, respectively. The enzyme derived from blast cells was much less inhibited with residual activities of 72, 50, and 56%. Podophyllotoxin, etoposide, and the Vicia alkaloids were also more effective in inhibiting ligase from normal cells, although vinblastine and vindesine were less effective.

For these reasons, kinetic studies were performed for each effective drug on DNA ligase activity. As depicted in Chart 1, Adriamycin, etoposide, and vincristine were effective inhibitors of ligase at a dose of 0.1 mm in thymocytes and normal lymphocytes while having little (Adriamycin) or no apparent effect on the enzyme extracted from blasts. Only chlorambucil was more effective at inhibiting ligase from leukemic cells.

Effects of Antileukemic Drugs on Mixed DNA Ligases. When the thymic and lymphocytic ligases were mixed in equal
EFFECT OF ANTILEUKEMIC DRUGS ON DNA LIGASE

Chart 1. Dependence of DNA ligase activity on inhibitor concentration. Purified enzymes (about 0.05 unit in 20 µl; 0.4 to 0.8 µg of protein) were mixed with different concentrations of the drugs. The enzyme assays were performed as described under "Materials and Methods." Ordinate, residual activity in percentage of control; abscissa, final drug concentration. Activity of the enzyme from leukemic cells ( ), normal lymphocytes ( ) and normal thymocytes ( ). A to D, the effects of adriamycin, chlorambucil, etoposide, and vincristine, respectively.

Table 2
Effect of antileukemic drugs on the activity of DNA ligase either from specific origin or mixed together

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Thymocytes</th>
<th>Lymphocytes</th>
<th>Blasts</th>
<th>T + L</th>
<th>T + B</th>
<th>L + B</th>
<th>T + L + B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>10</td>
<td>25</td>
<td>50</td>
<td>0</td>
<td>37</td>
<td>41</td>
<td>25</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>70</td>
<td>100</td>
<td>40</td>
<td>80</td>
<td>50</td>
<td>51</td>
<td>45</td>
</tr>
<tr>
<td>Etoposide</td>
<td>25</td>
<td>10</td>
<td>100</td>
<td>10</td>
<td>62</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Vincristine</td>
<td>15</td>
<td>10</td>
<td>100</td>
<td>5</td>
<td>70</td>
<td>65</td>
<td>45</td>
</tr>
<tr>
<td>MMCONH₂</td>
<td>100</td>
<td>100</td>
<td>28</td>
<td>100</td>
<td>55</td>
<td>62</td>
<td>57</td>
</tr>
</tbody>
</table>

* T, thymocytes; L, lymphocytes; B, blasts.

amounts, inhibition by Adriamycin, etoposide, and vincristine was similar to that found for the individual enzymes with complete inhibition in the case of Adriamycin (Table 2).

The mixing of enzyme from normal cells with that of blasts resulted in no further inhibition by the 3 drugs. Chlorambucil and the experimental antileukemic drug MMCONH₂ were more potent inhibitors of blast rather than normal cell-derived ligase with no further inhibition when assayed on a mixture of enzyme from normal and blast cells.

Kinetics of DNA Inhibition. Kinetic studies were performed using drugs effective in inhibiting DNA ligase at doses below 0.5 mm. The results for chlorambucil, etoposide, and vincristine are presented in Chart 2. With respect to poly[d(A-T)]ₙ substrate, the Kₘ were, respectively, 4 x 10⁻⁷, 2 x 10⁻⁷, and 10⁻⁷ M for ligase purified from thymocytes, normal, and lymphoblasts. Although all the observed inhibition mechanisms were competitive, the K were different. All the studied mechanisms of inhibition are competitive (Table 3). Although etoposide, podophyllotoxin, and vincristine failed to inhibit ligase from blasts, they did reduce that of normal cells. The thymocyte-extracted ligase was inhibited with a Kₘ of 10⁻⁵ and 5 x 10⁻⁵ M, respectively, for etoposide and vincristine. The 3 drugs, daunorubicin, Adriamycin, and AD32, inhibited with low Kₘ (10⁻⁵, 7 x 10⁻⁶, and 5 x 10⁻⁶ M, respectively), the enzyme from thymocytes, medium Kₘ (2 x 10⁻⁵, 2 x 10⁻⁵, and 10⁻⁵ M, respectively), the enzyme extracted from lymphocyte, and high Kₘ (5 x 10⁻⁴, 10⁻⁴, and 2 x 10⁻⁴ M, respectively), the enzyme extracted from blasts. K were very high for the other compounds studied (chlorambucil, vinblastine, vindesine, o-AMSA, and DMCOOK).

DISCUSSION

For several years, DNA, rather than RNA or proteins, has been considered an important target for carcinogenicity (4) and the
critical cellular event which leads to mutagenicity seems to be a semiconservative DNA synthesis on a damaged template (29). DNA synthesis, either replication or repair, requires several enzymes such as polymerases (36), DNA glycosylases, endonucleases (25), and others (24). Among DNA-metabolizing enzymes, DNA ligases (46) are of special interest. Although some functions of these enzymes are known in prokaryotic systems (20), there is a lack of information concerning eukaryotic DNA ligases. Increased activity of DNA ligase has been found in monkey kidney cells after irradiation (31) or pretreatment with mitogenic drugs (33) and after treatment of human fibroblasts by carcinogenic drugs (32).

To our knowledge, this is the first time that DNA ligase has been purified to such homogeneity from different human lymphoid sources. Ligase activity was found in thymocytes, normal lymphocytes, acute myeloblastic, chronic myelogenous, and chronic lymphocytic leukemia peripheral blasts. Whatever the origin of the enzyme, the sedimentation coefficient was always the same (5S). This is in good agreement with the existence of a unique DNA ligase in normal thymus and blood after birth in birds (7).

Relationships between Structure and Effects of the Drugs.
The action of antileukemic drugs depended on the source of ligase being tested. Melphalan and prednisone had no inhibitory effect at up to 5 mM. The other alkylating agent chlorambucil was a potent inhibitor of ligase from thymocytes and blasts.

Among the miscellaneous drugs studied, the inhibitors of nucleic acid synthesis, ara-C and methotrexate, have virtually no effect on the enzyme activity. m-AMSA and o-AMSA deserve special mention, since only o-AMSA appeared to be equally active on the enzyme from the 3 different sources. The nitrosoureas, carmustine and cyclophosphamide, were almost inactive. Podophyllotoxin and to a greater extent its derivative etoposide were more effective inhibitors of the ligase from thymocytes and lymphocytes. This indicates an important effect of the sugar moiety of etoposide. Other poisons of the mitotic spindle, such

![Diagram](chart1.png)

**Chart 2.** Kinetics of inhibition of DNA ligases by chlorambucil, etoposide, and vincristine. About 0.05 unit is incubated under conditions of initial velocity with increasing amounts of poly(dA-T)ₙ in the absence or in the presence of constant amounts of inhibitors. 1/V is expressed in 1/nmol [3H]poly(dA-T)ₙ resistant to exonuclease III in 30 min and 1/S in 1/poly(dA-T)ₙ in 10⁻⁷ M⁻¹. Chlorambucil: A, thymic enzyme; B, leukemic enzyme. Etoposide: A, thymic enzyme; B, lymphocyte enzyme. Vincristine: A, thymic enzyme; B, lymphocyte enzyme.
EFFECT OF ANTILEUKEMIC DRUGS ON DNA LIGASE

As vincristine, vinblastine, and vindesine have a comparable effect, possibly enhanced by the presence of a -CH₃ group. Intercalating anthracyclines are also of great interest.

The inhibition of the activity of ligase from normal cells by intercalating anthracyclines appear to be enhanced by the presence of a lateral chain on C-13 of the drug molecule. Although based upon just a few representatives of each family of drugs, these observations may stimulate further structure-function studies (Chart 3).

When enzymes purified from different sources were mixed together, drug inhibition in the presence of blast-extracted enzyme was not increased. This raised a major question about the structural and catalytic similarity of the 3 types of enzymes. At present, it appears that several biochemical properties are identical. All 3 enzymes have a sedimentation coefficient close to 5S, their cofactors and temperature optimas are identical. However, these observations do not exclude structural differences. The possibility that closely related enzymes like DNA polymerases and topoisomerases copurify with ligases seems unlikely, since no such activities have been detected in the purified ligase preparations. This observation is important since the activity of topoisomerases is affected by some antileukemic drugs. m-AMSA but not o-AMSA has recently been shown to induce (28) topoisomerase II. In the present study, the effect of o-AMSA and the absence of an effect of m-AMSA on DNA ligase is in agreement with the absence of interference between ligase and topoisomerase. The concomitant involvement of several DNA enzymatic systems points out the study of DNA breaks under different conditions. Using relaxation methods, it was found that DNA is not damaged in blasts from acute myeloblastic leukemia in resting conditions.⁴

All except the experimental drugs used in this study are currently used alone or in combination (47) for the treatment of leukemias. These agents have been found to affect DNA metabolism. Carmustine is supposed to carbamoylate cellular macromolecules (3), and this process could be responsible for the inhibition of ligation of X-ray-induced DNA strand breaks (1, 13). Chlorambucil determines sister chromatid exchange in human lymphocytes (41), and cyclophosphamide induces DNA repair in human leukocytes (35). Adriamycin has formerly been reported to be a noninducer of DNA repair synthesis (38), but more recent work shows that it causes DNA-protein cross-links and DNA single- and double-strand breaks (42). Vincristine determines sister chromatid exchange in human lymphocytes (42) and has been reported not to induce (2) or to induce poorly, DNA repair synthesis (40). Etoposide has been shown to cause single-strand breaks rapidly repaired after drug removal (27, 48).

The major antileukemic present therapeutic includes the drugs studied in this report either alone or in combination (i.e., ara C + anthracycline in acute myeloblastic leukemia, prednisone plus Vicia, or ara-C + etoposide alkaloids in acute lymphoblastic leukemia). The fact that some antileukemic agents like vincristine and etoposide are inhibitors of DNA ligase from normal lymphoid cells without affecting the enzyme purified from leukemic source, and taking into account that this enzyme is required for both replication (36) and repair (9, 39), might be a crucial factor for the chemotherapy of leukemia.


Chart 3. Molecular structures of the antileukemic drugs.
REFERENCES

Inhibition of DNA Ligase from Human Thymocytes and Normal or Leukemic Lymphocytes by Antileukemic Drugs

Jean-Claude David, Thérèse Bassez, Micheline Bonhommet, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/45/5/2177

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.