Inhibition of thymidylate synthase by pergularine, tylophorinidine and deoxytubulosine

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The activity of thymidylate synthase (TS) purified in our laboratory from Lactobacillus leichmannii was inhibited by pergularine (PGL) and tylophorinidine (TPD) and deoxytubulosine (DTB) isolated from the Indian medicinal plants Pergularia pallida and Alangium lamarckii respectively. Cytotoxicity studies showed that cell growth of L. leichmannii was inhibited (IC50 = 40-45 µM) by all the three alkaloids, the concentrations > 80-90 µM resulting in complete loss of the enzyme activity. K values of the enzyme calculated from Lineweaver-Burk and Dixon plots for PGL, TPD and DTB were 10x10^(-5), 9x10^(-5) and 7x10^(-5) M respectively. These are typed as 'non-competitive' inhibitors of TS. All the three alkaloids inhibited (IC50 = 50 µM) the elevated TS activity of leukocytes in cancer patients with clinically diagnosed chronic myelocytic leukemia (n=10), acute lymphocytic leukemia (n=8) and metastatic solid tumours (n=3).

Thymidylate synthase (TS) (CH3THF: dUMP C-methyltransferase, EC 2.1.1.45) is ubiquitous and is of prime importance as it provides precursors for DNA synthesis through de novo pathway, thymidylate production being the critical rate-limiting step for DNA replication. TS levels are highly elevated in chemically or virally transformed cells and malignant cells. These two important factors therefore make the enzyme a key target for cancer chemotherapy. Particularly rich sources of this enzyme are the methotrexate (MTX) and dichloroMTX-resistant bacterial strains Lactobacillus casei, Streptococcus faecium and vitamin B12-supplemented Lactobacillus leichmannii. TS has long been studied as a target for antiproliferative agents due to its role in DNA synthesis and its rich mechanistic features. Compounds that mimic its substrate (dUMP) and cofactor (CH3THF) have been studied as potential inhibitors of TS. Amongst these, 5-fluorouracil (5-FU) and MTX are the well known competitive inhibitors of TS. However, there are other inhibitors that do not mimic either the substrate or cofactor. Anti-TS drugs 'dissimilar' to the substrate and cofactor are less likely to have the side effects that are produced by the nucleotide and folate mimics. We report here the inhibitory action of the phenanthroindolizidine alkaloids pergularine (PGL) and tylophorinidine (TPD) and the β-carboline-benzoquinolizidine alkaloid deoxytubulosine (DTB) (Fig. 1) isolated from the Indian medicinal plants Pergularia pallida (N.O. Asclepiadaceae) and Alangium lamarckii (N.O. Angiaceae) respectively on TS purified from B12-supplemented L. leichmannii.

Materials and Methods

All the chemicals used were of AR grade. Vitamin B12, ethylenediaminetetraacetic acid (EDTA) (disodium salt), (dl)-L-tetrahydrofolate [(dl)-L-THF], formaldehyde, 2-mercaptoethanol, RNase A, DNase I, DEAE-cellulose and dextran (av. MW, 100,000-200,000) were from Sigma Chemical Company, St. Louis, Missouri, USA; hydroxyapatite from Bio-Rad Laboratories, Richmond, California, USA; 5-[3H]dUMP (ammonium salt, 10.6 Ci/m mole) from Radiochemical Centre, Amersham, UK; Sephadex G-100 from Pharmacia Fine Chemicals, Uppsala, Sweden; activated charcoal and naphthalene
(scintillant grade) from E. Merck, Dermstadt, Germany; vitamin B	extsubscript{12} assay medium from HiMedia Laboratories Pvt Ltd, Bombay, India were used. The chemotherapeutic drugs used in leukemia treatment were daunomycin, arabinofuranosylcytosine (Ara-C), 6-thioguanine (6-TG), cyclophosphamide (CPA), vincristine (VNC), adriamycin, L-asparaginase, MTX and prednisolone which were all standard products marketed by reputed pharmaceutical firms.

**Isolation and characterization of PGL, TPD and DTB**

PGL and TPD were isolated from the air-dried roots of *P. pallida* and characterized\textsuperscript{18}. DTB was isolated\textsuperscript{19} from the crude extracts of the flowers of *A. lamarckii* and identified\textsuperscript{22}.

**Purification and assay of TS**

TS was purified from the crude extracts of B	extsubscript{12}-supplemented *L. leichmannii* (ATCC 7830) as described earlier\textsuperscript{14}. TS was assayed\textsuperscript{23} by [\textsuperscript{3}H] release method using the assay mix (buffer B)\textsuperscript{24} in a total volume of 265 μl reaction mixture. The enzyme protein was measured by the method of Lowry et al.\textsuperscript{25} after removal of 2-mercaptoethanol by evaporation\textsuperscript{14}.

Patients with chronic myelocytic leukemia (CML) and acute lymphocytic leukemia (ALL) and metastatic solid tumours (MST) (breast, uterus and colon cancers) selected for the study were all from the Tata Memorial Hospital (Chemotherapy Department) and BARC Hospital, Mumbai. Normal healthy donors selected were employees of BARC.

**Isolation and preparation of human leukocytes and their lysates**

Blood (10 ml) from normal healthy donors and cancer patients collected in anticoagulant medium (1 ml) of 1.5% EDTA containing 0.7% NaCl, were treated with 3% dextran (Sigma) containing 0.15 M NaCl (1:2, v/v). These were processed within 30 min for the isolation of leukocytes and their lysate preparation\textsuperscript{26}. All procedures were carried out at 4°C.

**Results and Discussion**

**Cytotoxicity of PGL, TPD and DTB**

The cytotoxic effects of PGL, TPD and DTB on the growth of *L. leichmannii* are shown in Fig. 2. Inset shows the growth inhibition curves in presence of 50
μM of all the three alkaloids. The three alkaloids showed approximately the same IC₅₀ value of 40-45 μM and increasing concentrations up to 100 μM resulted in the growth inhibition of the cells up to 75-85%. Thymidylate synthase provides precursors for DNA biosynthesis through de novo pathway, production of thymidylate being the rate-limiting step for DNA synthesis, and hence a key target for cancer chemotherapy. Therefore, inhibition of TS by PGL, TPD and DTB was examined.

**Inhibition of TS by PGL, TPD and DTB**

Time course of inhibition of thymidylate synthase by PGL, TPD and DTB is presented in Fig. 3. The inhibition was found to be in the order DTB > TPD > PGL. The three alkaloids tightly bind to TS during the ternary complex formation with the substrate dUMP and cofactor CH₂THF²⁰,²¹ and inhibit the enzyme activity. IC₅₀ values for PGL, TPD and DTB were 45 μM, 42 μM and 40 μM respectively. Alkaloid concentrations of 80-90 μM resulted in total loss of the enzyme activity, thus suggesting that both classes of the alkaloids were potential antitumour compounds.

**Inhibition constants**

Lineweaver-Burk plots of initial velocity versus the specified variable substrate concentrations at a series of

The inhibitor was preincubated at 30°C for 10 min prior to the substrate addition. Slope of each inhibited reaction is plotted against inhibitor (I) in the inset. Values presented in the plots were the average of two independent experiments].

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**Fig. 3—Time course of inhibition of thymidylate synthase from L. leichmannii** for PGL, TPD and DTB. The TS assays were conducted in a total volume of 265 μl reaction mixtures in the presence of: (—O—), no alkaloid; (—X—), 5 μM PGL/TPD/DTB and (—□—), 10 μM; (—Δ—), 20 μM; (——), 30 μM of alkaloid. Values presented are the average of two independent experiments].

**Fig. 4—Inhibition of LITS by (A), PGL; (B), TPD and (C), DTB. | Lineweaver-Burk double reciprocal plots and the replot (inset) of LITS inhibition by the three inhibitors are shown. Reaction rates were followed by measuring the [³H] released of 5-[³H]dUMP. Unless specified otherwise, reactions were initiated by the addition of 10-15 μg enzyme to 250 μl assay mix. For Kᵢ determination, the reaction components were essentially same as described in text in a total volume of 265 μl with the specified variable substrate dUMP concentrations at different fixed concentrations of the inhibitors. (—O—), no inhibitor; (—X—), 5 μM inhibitor; (—□—), 10 μM; (—Δ—), 20 μM; (——), 30 μM. The inhibitor was preincubated at 30°C for 10 min prior to the substrate addition. Slope of each inhibited reaction is plotted against inhibitor (I) in the inset. Values presented in the plots were the average of two independent experiments].
Inhibition of cell growth and thymidylate synthase activity of *Lactobacillus leichmannii*.

IC\(_{50}\) values of growth of *L. leichmannii* were calculated as described in the growth inhibition experiments in the text (see Fig. 2). The \(K_i\) values of LITS for different inhibitor alkaloids were calculated from the initial velocity experiments as described in the text. These values were derived from Lineweaver-Burk and Dixon plots as described.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Growth inhibition IC(_{50}) ((\mu M))</th>
<th>(K_i) (M)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>PGL</td>
<td>45</td>
<td>10 x 10(^{-6})</td>
<td>This study</td>
</tr>
<tr>
<td>TPD</td>
<td>43</td>
<td>9 x 10(^{-6})</td>
<td>This study</td>
</tr>
<tr>
<td>DTB</td>
<td>40</td>
<td>7 x 10(^{-6})</td>
<td>This study</td>
</tr>
<tr>
<td>FdUMP</td>
<td>---</td>
<td>6.7 x 10(^{-6})</td>
<td>(39)</td>
</tr>
<tr>
<td>DTMP</td>
<td>---</td>
<td>2.2 x 10(^{-6})</td>
<td>(39)</td>
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<tr>
<td>MTX</td>
<td>---</td>
<td>5 x 10(^{-6})</td>
<td>(39)</td>
</tr>
<tr>
<td>FGlu(_4)</td>
<td>---</td>
<td>3.3 x 10(^{-5})</td>
<td>(40)</td>
</tr>
</tbody>
</table>

FGlu\(_4\), folyltriglutamate (pyrovoyltetraglutamate, unreduced form)

IC\(_{50}\) values of cell growth inhibition of *L. leichmannii* and the \(K_i\) values of *L. leichmannii* TS (LITS) for the three alkaloids in comparison with the classical competitive inhibitors 5-FdUMP and MTX. Evidence obtained from dialysis experiments together with the Sephadex G-100 chromatographic data clearly revealed that the alkaloids are tightly bound to thymidylate synthase and that the binding was irreversible\(^{26,29}\).

**Inhibition of TS in leukocytes**

Levels of human leukocyte TS (HITS) in cancer patients with CML, ALL and metastatic solid tumours that include breast, uterine and colon cancers (B, C and D respectively) compared to normal healthy controls (A) are shown in Fig. 6. TS levels were elevated 65-fold in CML patients (n=10) and 35-fold in ALL patients (n=8) when compared with normal healthy control subjects (n=12) whose baseline activity was negligibly low. TS levels of the leukocytes of patients with the metastatic solid tumours (n=3) were 8-fold elevated. HITS activity of untreated patients selected for the study was higher compared to that of the patients responding to chemotherapy, and a good correlation between the leukemic leukocyte count, blast cell value of myeloblasts/lymphoblasts and the enzyme activity has been recorded\(^{26,31}\) thus clearly revealing the biochemical strategy of the tumour marker enzyme activity that accurately monitors status of the disease and the clinical diagnosis of the hematologic malignancy. About 50% of the HITS activity was inhibited by PGL, TPD and DTB at 50 \(\mu M\) concentrations in the three types of cancers under study (Fig. 6). These results show that PGL, TPD and DTB...
inhibit the activity of thymidylate synthase and could serve as potential anticancer agents.

In rapidly proliferating cell populations, adequate supplies of thymidylate are critical to DNA synthesis, and TS inhibition had been examined for application in cancer therapy.7,8,32 TS-inhibiting agents are the most effective compounds with either potential antimicrobial or antitumour action or both.31 TS inhibitor analogs have structural similarity to either the substrate dUMP or the cofactor CH₂THF and as a result, by virtue of their affinity towards TS binding sites, their accumulation in the cells is favoured. Recently, some compounds non-structurally related to substrate or cofactor but with a fairly good affinity towards the TS binding sites have been synthesized.32,33 The design of such TS inhibiting anticancer drugs have resulted in a wealth of biochemical and structural data together with some proposed catalytic mechanisms.34 Antifolate TS inhibitors such as naphthalimide, benzoquinazoline and dideazafolate compounds have been extensively investigated for their potential application as anticancer agents.5,35,39

Studies on 5-FdUMP showed that this inhibitor in the presence of cofactor CH₂THF forms a covalent linkage with TS during the ternary complex formation and since the dissociation constant of the complex is lower than the practical concentrations of the enzyme, binding is stoichiometric and the inhibitor is, in effect, an active site titrant.39 It would therefore be inferred that LITS homodimer has two active binding sites, one per each subunit49,40 and it is implicated that two moles of each of the inhibitor alkaloid possibly bind per mole enzyme. Furthermore, our results showed that LITS binding to the phenanthroindolizidine inhibitor alkaloids PGL and TPD and the β-carboline-benzoquinazidine inhibitor alkaloid DTB is very tight through a possible covalent bond and is irreversible.28,29 2,6-Diaminobenz[c]indole type and 2,4-diamino-5-substituted- pyrrole[2,3-d]pyrimidine type inhibitors of TS and dihydrofolate reductase (DHFR) respectively are some such nonclassical noncompetitive inhibitors41,42. Alkaloid binding to LITS could be quite similar to that of LITS. Human TS has been purified from the cell lines of CCRF-CEM human lymphocytic leukemia43 and from the blast cells of patients with acute myelocytic leukemia (AML)44 and partially characterized. Lockshin et al. showed that human leukemic TS (Mr, 66,000) is a homodimer and binds 2 moles of FdUMP per mole enzyme.45
Our findings on the highly elevated human leukemic leukocyte TS activity as measured by the [3H] release assay are in corroboration with those reported by Silber et al. In human leukemias, blood plasma (leukocyte fraction) TS and the blood serum thymidine kinase (TK) levels serve as markers in the progression of malignancy.

Although some physico-chemical properties of the phenanthroindolizidine alkaloids have been elucidated, very little is known about their cytotoxic and antitumour activity. Of these alkaloids, most important are tylophorine, tylocrebrine, cytoprneurine and a congener of tylophoridine reportedly having antitumour activity in different species. Inhibition of DNA synthesis by tylocrebrine and cytoprneurine has been demonstrated by two groups of investigators independently. Some of the chemical and spectral properties of emetine alkaloids have been extensively reviewed. However, information on their biochemical and biological properties is scanty. Considerable interest in the emetine alkaloids such as tubulosine has been evoked with the discovery of their antileukemic and amebicidal activities that are mainly associated with the transfer reaction in protein biosynthesis. Biological properties other than antitumour activity of the Antiangium emetine alkaloids and demethylcephaeline that are of pharmacologic and clinical significance are their antiplatelet aggregation, antihypotensive and sedative action. Common structural determinants of biological activity have been pointed out for both phenanthroindolizidine and the emetine alkaloids.

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