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Preclinical report

β-Carboline-benzoquinolizidine alkaloid deoxytubulosine inhibits thymidylate synthase activity in leukemic leukocytes from patients with chronic myeloblastic leukemia and acute lymphoblastic leukemia

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Precursor 2′-deoxythymidine 5′-monophosphate for DNA biosynthesis is supplied by thymidylate synthase (TS) (EC 2.1.1.45) through a *de novo* pathway and the enzyme levels are elevated in malignancy. TS is therefore a key target for cancer chemotherapy. Human leukocyte TS levels in patients with chronic myeloblastic leukemia (CML) and acute lymphoblastic leukemia (ALL) are highly elevated (66- and 33-fold, respectively) compared to the low baseline activity of normal healthy controls. Preliminary screening tests for the antitumor activity of the β-carboline-benzoquinolizidine alkaloid deoxytubulosine (DTB) (isolated from the Indian medicinal plant *Alanguim lamarckii*) were performed employing *in vitro* inhibition studies on the leukemic leukocyte TS as the probe enzyme. Enzyme activity of the leukemic leukocytes was potently inhibited by DTB (IC_{50}=50 μM) in both CML and ALL. The emetine alkaloid DTB was assessed for its biochemical and biological evaluation for the first time as a potential antileukemic agent. [© 1998 Lippincott Williams & Wilkins.]

Key words: Acute lymphoblastic leukemia, antitumor compound, chronic myeloblastic leukemia, deoxytubulosine, inhibition, thymidylate synthase.

Introduction

Thymidylate synthase (TS) (EC 2.1.1.45) is ubiquitous and is a crucial enzyme that plays a pivotal role in supplying an essential precursor for the DNA synthesis which accompanies cell proliferation. The biosynthesis of 2′-deoxythymidine 5′-monophosphate (dTMP; thymidylate) from 2′-deoxyuridine 5′-monophosphate (dUMP) is accomplished through a *de novo* pathway.[1] The enzyme reductively methylates dUMP to dTMP in the presence of cofactor 5,10-methylenetetrahydrofolate (CH_2-THF) which is concomitantly oxidized to dihydrofolate (DHF) that is enzymatically reduced back to THF by dihydrofolate reductase (DHFR).[2,3] Both the enzymes (i.e. TS and DHFR) thus participate in a 'thymidylate cycle' and have been considered attractive targets for the development of anticancer chemotherapeutic agents.[4–7] Chemically or virally transformed cells, rapidly dividing cells and malignant cells have highly elevated levels of TS.[8–10] Prolonged or repeated treatment of tumor cells growing *in vitro* or bacterial cells with drugs such as methotrexate (MTX) or 5-fluorouracil (5-FU) results in resistant clones due to gene amplification with the resultant elevation in these enzyme levels.[6] MTX-resistant strains of *Lactobacillus casei*, *Streptococcus faecium* and B_{12}-supplemented *Lactobacillus leichmannii* are particularly rich sources of TS.[11–16] Classical competitive TS inhibitors such as 5-FU and MTX have long been considered most effective antiproliferative agents. However, anti-TS drugs 'dissimilar' to the substrate and cofactor remain attractive as these are less likely to have side effects that are caused by the nucleotide and folate mimics.[17] We have for the first time assessed the β-carboline-benzoquinolizidine alkaloid deoxytubulosine (DTB) (Figure 1) isolated from the Indian medicinal plant *Alangium lamarckii* (NO Alangiaceae)[18] for its biological and biochemical evaluation as an anti-TS inhibitor compound. We report here on the potential antitumor activity of this unique alkaloid of the

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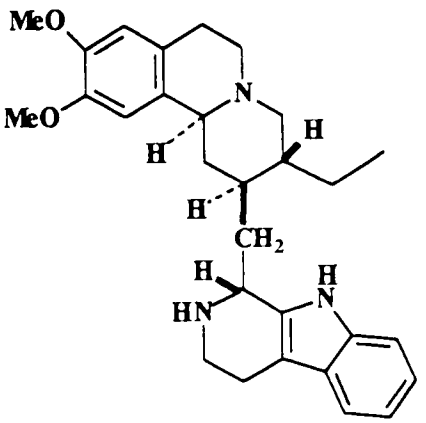

Figure 1. Molecular structure of the β-carboline-benzoquinolizidine alkaloid deoxytubulosine (DTB).

emetine family against chronic myeloblastic leukemia (CML) and acute lymphoblastic leukemia (ALL).

Materials and methods

Chemicals and biochemicals

All the chemicals and biochemicals employed in the present study were of analytical reagent grade. 5-[^{3}H]dUMP (ammonium salt, 10.6 Ci/mmol) was purchased from Radiochemical Centre (Amersham, UK). Dextran (average MW, 100 000–200 000), (*dl*)-L-tetrahydrofolate [(*dl*)-L-THF], formaldehyde (HCHO), dUMP (sodium salt), 2-mercaptoethanol and ethylenediaminotetraacetic acid (EDTA) (disodium salt) were procured from Sigma (St Louis, MO). Activated charcoal and naphthalene (scintillant grade) were products of Merck (Darmstadt, Germany). Chemotherapeutic drugs used for CML and ALL treatments were daunomycin (DNM), arabinofuranosylcytosine (Ara-C), 6-thioguanine (6-TG), cyclophosphamide (CPA), vincristine (VNC), adriamycin (ADM), L-asparginase (ASP), MTX and prednisolone (PDS), which were all standard products marketed by reputed pharmaceutical firms and were employed for therapeutic treatment as indicated in the drug regimens (Table 1).

Isolation and characterization of DTB

DTB was isolated from the crude extracts of the flowers of *A. lamarckii* essentially as described by Venkatachalam *et al.*,[18] and its identity was established through physical and spectral means.[19]

CML and ALL patients

CML and ALL patients selected for the study were from the Tata Memorial Hospital (Chemotherapy Department) and BARC Hospital, Bombay. Normal healthy contols were all employee donors of BARC.

Isolation and preparation of human leukocytes and their lysates

Isolation of erythrocyte and platelet-free human leukocytes was essentially according to the method of Bertino *et al.*[20] with minor modifications. Blood (10 ml) from leukemic patients and normal healthy donors was collected into the anticoagulant medium (1 ml) of 1.5% EDTA containing 0.7% NaCl and the blood samples were treated with 3% dextran containg 0.15 M NaCl (1:2, v/v). Samples were processed within 30 min after collection for the isolation of leukocytes and their lysate preparation.

Protein assay

Protein measurement of the lysates was according to the method of Lowry *et al.*[21]

TS assay

Enzyme assay of human leukocyte TS (HTS) was essentially according to the method of Lomax and Greenberg[22] with the assay mix (buffer B) as described by Wahba and Friedkin[23] in a total volume of 265 μl reaction mixture. The reaction mixture components were 275 μM (*dl*)-L-THF, 13 mM each of HCHO and $MgCl_2$, 40 μM dUMP, 75 μM 2-mercaptoethanol, 100 μM 5-[^{3}H]dUMP (sp. act. 6×10^6 c.p.m./μmol) and 10–15 μg of the leukocyte lysate enzyme in 50 μM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 20 mM 2-mercaptoethanol (buffer A). TS reactions were carried out at 30 C as described[16] and 100 μl aliquots of the filtrates were counted in Bray's solution[24] on an LKB 1217 Rackbeta (Finland) liquid scintillation counter for [^{3}H]HO released, which was taken as a measure of the rate of dTMP produced enzymatically. Unless otherwise specified, enzyme specific activity units were expessed as c.p.m. $\times 10^6$ of ^{3}H released/mg protein/h at 30 C.

Table 1. Inhibition of human leukocyte TS by DTB in CML and ALL

Normal/ leukemic	Sex/age (years)	Therapy	Leukocyte count (/ml)	Blast value (%)	TS activity (U/mg protein/h)	
					Control	+DTB
Normal						
1	M, 51	–	5000	4	0.80	0.42
2	M, 47	–	9000	5	0.75	0.39
3	M, 48	–	8000	3	0.26	0.14
4	M, 30	–	6000	4	0.00	–
5	F, 50	–	8000	3	0.62	0.33
6	M, 45	–	7000	5	0.18	0.11
7	M, 26	–	8000	4	0.05	0.03
8	F, 24	–	6000	3	0.00	–
9	M, 38	–	6000	4	0.00	–
10	F, 30	–	7000	5	0.00	–
CML						
1	M, 25	–	110000	75	20.06	10.47
2	M, 14	Ara-C, 6-TG and DNM	40000	15	12.24	6.39
3	F, 16	–	125000	80	24.48	12.74
4	M, 30	Ara-C, CPA and VNC	58000	25	14.72	7.69
5	M, 18	DNM and Ara-C	92000	63	17.92	9.37
6	F, 18	–	105000	92	19.76	10.28
7	M, 35	Ara-C, CPA and VNC	70000	45	16.02	8.32
8	M, 12	–	91000	60	17.88	9.30
ALL						
1	M, 10	ADM, Ara-C and ASP	79000	45	6.26	3.26
2	M, 14	–	105000	75	8.24	4.30
3	F, 4	–	130000	90	11.41	5.94
4	M, 32	ADM, MTX, VNC and PDS	84000	62	9.48	4.94
5	M, 5	ADM, MTX, VNC and PDS	82000	51	7.12	3.71
6	F, 28	–	109000	80	10.62	5.52

Human leukocytes were prepared as described in the text. Activity of the disease in leukemic patients was determined from the percent of the blast cells (myeloblasts and lymphoblasts of CML and ALL, respectively). Percentage of blast cells was recorded from the bone marrow (obtained by sternal puncture and aspiration) of the leukemic patients, whereas the blast values of the normal healthy subjects were those of the peripheral blood. Percent blast cells was determined by the routine standard light microscopic examination of Giemsa-stained smears. Therapeutic regimens employed for the CML patients were: Ara-C, 100 mg/m^2/day; 6-TG, 100 mg/m^2/day; CPA, 100 mg/m^2/day; VNC, 1.4 mg/m^2. Therapeutic regimens of ALL patients were: ADM, 20 mg/m^2; Ara-C, 100 mg/m^2/day; ASP, 6000 IU/m^2/day; intrathecal MTX, 10 mg/m^2; VNC, 1.4 mg/m^2; PDS, 40 mg/m^2/day. TS activity of the leukocytes was measured by the ^{3}H release assay as described in Methods. Specific activity units expressed were ^{3}H released c.p.m. $\times 10^3$/mg protein/h at 30 C. Reaction mixtures with the added inhibitor alkaloid consisted of 50 μM DTB.

Results

Elevated levels of TS in leukemic leukocytes and their response to drug treatment

TS levels of leukemic leukocytes of human blood observed in the leukemic patients with the clinical diagnosis of CML and ALL, and the enzyme inhibition profiles obtained in the presence of the inhibitor alkaloid DTB are shown in Table 1. Baseline leukocyte TS activity of normal healthy controls has been observed to be too low to be measured by the sensitive ^{3}H-release assay. In fact, it can be clearly seen that the normal controls nos 4, 8, 9 and 10 did not exhibit leukocyte TS activity. Results obtained from the CML (n=8) and ALL (n=6) patients presented in Table 1 clearly show that the levels of leukemic leukocyte TS activity are markedly pronounced, and are highly elevated to 66- and 33-fold in CML and ALL patients, respectively, compared to those of the healthy control subjects (n=10). It can be clearly observed that four fresh CML patients (nos 1, 3, 6 and 8), prior to chemotherapy before hospitalization in the chemotherapy ward, displayed much higher leukocyte dTMP synthase activity in comparison with the rest of the four patients (nos 2, 4, 5 and 7) who underwent chemotherapy after hospitalization. Antileukemic drugs used for therapy of these four CML patients were Ara-C, 6-TG, DNM, CPA and VNC with the drug regimens as indicated (see footnote to Table 1). It can also be observed that three ALL patients (nos 2, 3 and

6) prior to chemotherapy showed relatively higher leukocyte enzyme activity as compared to the remaining three patients (nos 1, 4 and 5) who underwent therapeutic treatment with antileukemic drugs such as ADM, Ara-C, ASP, MTX, VNC and PDS as indicated in the drug regimens (see Table 1).

Correlation betweeen TS levels, leukocyte count and blast values in CML and ALL

Our results also clearly show that there is a good correlation between the leukemic leukocyte count and the respective TS levels and between the myeloblast and lymphoblast values and the respective enzyme profiles as is recorded in Table 1. Thus our results show a good correlation between the clinical diagnosis of the hematologic malignancy and the biochemical strategy of the tumor marker enzyme activity of the leukemic leukocyte TS.

TS inhibition by DTB in leukemic leukocytes

In vitro studies on the enzyme inhibition with the leukemic leukocytes of both CML and ALL patients further showed that about 50% of the TS activity is inhibited in the presence of DTB at 50 μM concentrations, indicating that the IC_{50} value of leukemic leukocyte TS for DTB is 50 μM. Thus our results from the DTB inhibition studies unequivocally show that this alkaloid is a powerful anti-TS inhibitor compound and therefore is strongly suggestive as a potential antileukemic agent.

Cytotoxicity studies

Cytotoxicity experiments conducted with vitamin B_{12}-supplemented cells of *L. leichmannii*, a rich source of TS, revealed an IC_{50} value of 40 μM for DTB; DTB concentrations above 50 μM were lethal to the cells. TS activity of these cells was potently inhibited by DTB (IC_{50}=40 μM); inhibition kinetics showed that TS has a K_i value of 7×10^{-6} M for DTB, which is comparable to that of the fluoropyrimidine TS inhibitor FdUMP.[25,26]

Discussion

Accumulating evidence shows that TS activity is highly elevated in rapidly dividing cells and proliferative malignant cells.[2,8,10] Expression of the TS gene is cell cycle associated and high in the S phase. High TS and DHFR levels are found in tissues with high cell turnover such as malignant tumors. High levels of these enzymes prior to treatment, possibly due to gene amplification, may be responsible for innate 5-FU and MTX resistance.[5,6,27] However, data on baseline levels of TS in human tissues and malignant tumors are scarce,[28] mainly because the enzyme activity is either too low to be measured or there is no measurable activity, as can be seen in the present studies on the normal leukocytes. Very few attempts were made to isolate TS from human tumors, presumably because of its low levels in human tissues and difficulties in stabilizing the mammalian enzyme.[29,30] Most of the mechanistic studies on TS have therfore been performed with the stable enzyme isolated from MTX-resistant bacterial strains of *L. casei* and *S. faecium*,[11-14] and extensively reviewed.[31]

Present studies show that the enzyme levels of TS are highly elevated in the leukemic leukocytes of human blood in CML and ALL. These findings are in corroboration with those reported by Silber *et al.*[32] TS is a unique enzyme that evokes interest as it is involved in the *de novo* pathway of production of thymidylate which is an essential precursor required in DNA replication and repair. TS synthesis is highly induced and its levels elevated in proliferative cells. Hence the enzyme is a key target in cancer chemotherapy. Classical inhibitors like 5-FU and MTX are considered most effective antiproliferative agents by virtue of their competing with either the nucleotide substrate or the folate cofactor competitively. Anti-TS drugs that do not mimic the substrate or cofactor but compete with them non-competitively are believed to be the better alternative inhibitors (compared to the competitive inhibitor drugs) as they have the least side effects that are otherwise caused by the nucleotide and folate mimics.[17] Human leukemic cell lines and bacterial thymidylate synthases are homodimers with two active binding sites that bind 2 mol of fluoropyrimidine (5-FdUMP) per 1 mol enzyme.[13,28,33,34] DTB is a novel non-classical non-competitive anti-LITS drug that has potential anticancer activity.[35] Based on the evidence that 2 mol of the alkaloid bind per 1 mol LITS through a tight and irreversible covalent linkage, it is implicated that human leukemic leukocyte TS too possibly binds 2 mol of DTB per 1 mol enzyme. These results will be communicated elsewhere. There is evidence to show that DTB stongly binds to DNA.[18] Whether the RNA associated with TS[13-16,36] has affinity in binding to DTB is not clearly understood and this needs further investigations.

Amongst various other biochemical and biological properties of the emetine alkaloids that are of

pharmacologic and therapeutic value are their antihypotensive, antiplatelet aggregation and sedative activities.[37-39] Interestingly, these alkaloids have also been demonstrated to display antitumor and amebicidal activities, whereby they selectively inhibit the transfer reaction in protein biosynthesis.[40,41] We have demonstrated oncostatic (this study) as well as cytostatic (results of which will be communicated elsewhere) activities of DTB. Emerging frontiers of the biochemistry of alkaloids and the myriad naturally occurring phytochemicals in general will unravel in understanding the mechanistic features of their antitumor activity that is of invaluable pharmacologic significance. Curcumin is one such novel phytochemical, the chemopreventive activity of which has been demonstrated in our laboratories.[42] *Alangium* alkaloid DTB is of a high order of potency as an antitumor agent and has to undergo extensive tests using other parameters of carcinogenesis.

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