Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 1105–1110

BIOORGANIC & MEDICINAL CHEMISTRY

Dihydrofolate Reductase and Cell Growth Activity Inhibition by the β-Carboline-benzoquinolizidine Plant Alkaloid Deoxytubulosine from *Alangium lamarckii*: Its Potential as an Antimicrobial and Anticancer Agent

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Received 16 September 1998; accepted 30 November 1998

Abstract—β-Carboline-benzoquinolizidine plant alkaloid deoxytubulosine (DTB) was evaluated and assessed for the first time for its biochemical and biological activity employing the biomarker dihydrofolate reductase (DHFR) (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) as the probe enzyme, a key target in cancer chemotherapy. DHFR, employed in the present investigations was purified from *Lactobacillus leichmannii*. DTB, isolated from the Indian medicinal plant *Alangium lamarckii* was demonstrated to exhibit potent cytotoxicity. The alkaloid potently inhibited the cell growth of *L. leichmannii* and the cellular enzyme activity of DHFR ($IC_{50}=40$ and 30 μM for the cell growth and enzyme inhibitions, respectively). DTB concentrations >75 μM resulted in a total loss of the DHFR activity, thus suggesting that the β-carboline-benzoquinolizidine plant alkaloid is a promising potential antitumor agent. Our results are also suggestive of its potential antimicrobial activity. DTB binding to DHFR appears to be slow and reversible. Inhibition kinetics revealed that DHFR has a K_i value of 5×10^{-6} M for DTB and that the enzyme inhibition is a simple linear 'non-competitive' type.

Introduction

Dihydrofolate reductase (DHFR) is a strategic enzyme that is considered an attractive target for the development of anticancer chemotherapeutic agents.[1–4] DHFR (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) in the presence of coenzyme NADPH as follows: $DHF + NADPH + H^+ \rightarrow THF + NADP^+$.[5,6] The enzyme ($M_r \sim 20{,}000$) is ubiquitous and is of prime significance as it is of considerable pharmacological interest because of its key metabolic position in the formation of THF that is essential in 1-carbon metabolism. Coupled with thymidylate synthase (TS), it is directly involved in thymidylate (dTMP) production through de novo pathway.[7,8] DHFR is therefore pivotal in providing purines and pyrimidine precursors for the biosynthesis of DNA, RNA and amino acids at various stages. Gene amplification results in the overproduction of DHFR in the chemically or virally transformed cells and tumor cells.[5] Direct involvement in DNA replication, and significant elevation of the enzyme levels in malignant cells and the antifolate methotrexate (MTX)-resistant cells[9–14] make DHFR a key target for both antimicrobial and anticancer drug design in cancer chemotherapy. *Pneumocystis carinii* and *Toxoplasma gondii* are the widely prevalent life-threatening pathogenic bacteria which are a major cause of mortality in AIDS patients; antifolates are demonstrated to be the most effective agents against these pathogenic microbes.[15–19] The main objective of the present investigation is the biochemical and biological evaluation and assessment of a hitherto unreported class of the β-carboline-benzoquinolizidine alkaloid deoxytubulosine (DTB) (Fig. 1) isolated from the flowers of the Indian medicinal plant *Alangium lamarckii* (Alangiaceae)[20] employing DHFR purified from the vitamin B_{12}-supplemented cells of *Lactobacillus leichmannii* as the probe enzyme. The chemico–biological interactions between DHFR and DTB, and the synchronous cytotoxic and anti-DHFR activities of this unique alkaloid of the emetine family are reported in the present study.

Results

Inhibition of cell growth by DTB

Figure 2 shows results of the cytotoxicity experiments carried out on DTB with the vitamin B_{12}-supplemented

Key words: *Alangium lamarckii*; deoxytubulosine; *Lactobacillus leichmannii*; cytotoxicity; dihydrofolate reductase; inhibition.
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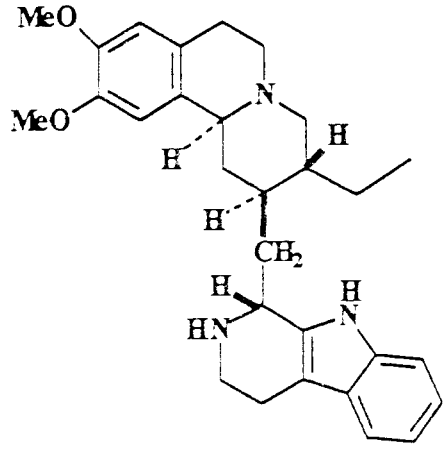
0968-0896/99/$ - see front matter
PII: S0968-0896(98)00262-4



Deoxytubulosine (DTB)

Figure 1. Molecular structure of the β-carboline-benzoquinolizidine plant alkaloid, deoxytubulosine.

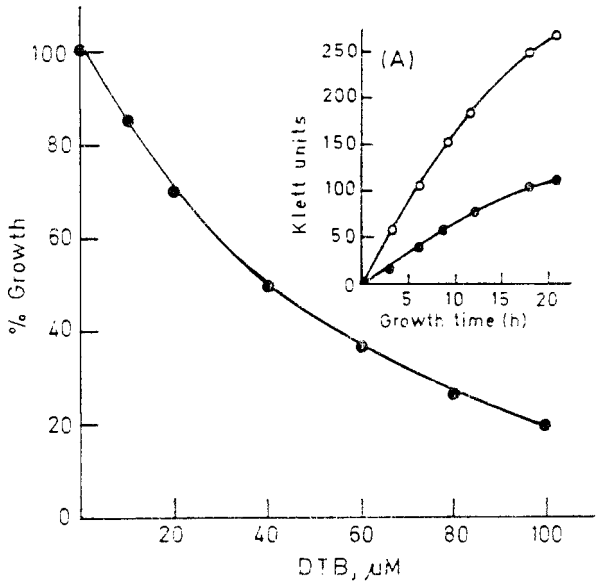


Figure 2. Inhibiton of cell growth of *L. leichmannii* by the *Alangium* alkaloid DTB. The microbial cells were grown in vitamin B_{12} assay growth medium as described.[48] Growth response with time at 30°C was recorded by measuring increase in turbidity during the 18 h growth period prior to the stationary phase as described in the Experimental section. Klett units measured at 18 h cell growth time in the absence of the inhibitor alkaloid (255 KU) [inset (A)] was taken as 100% growth activity. Inset (A) illustrates the exponential growth curves of *L. leichmannii* in the absence (O), and in the presence (●) of 50 μM DTB.

cells of *L. leichmannii*. The inset of Figure 2(A) presents the growth inhibition curves of the bacterial cells obtained in the presence of 50 μM concentrations of the plant alkaloid. The growth curves illustrated from our results clearly reveal that the cell growth is progressively inhibited in the presence of increasing concentrations of DTB. Inhibition curve shows that the cells have an IC_{50} value of 40 μM for DTB. Growth inhibition up to 80–85% of the initial growth activity was recorded with the further addition of increasing concentrations of DTB up to 100 μM in the growth medium. Alkaloid concentrations >150–175 μM were observed to be lethal to the cells. Thus, our results unequivocally show that the plant alkaloid is potently cytotoxic. DHFR is a crucial enzyme that couples with TS and provides precursors for DNA synthesis through de novo pathway. Thymidylate production being the rate-limiting step for DNA synthesis, both these enzymes are the key targets in cancer chemotherapy. DHFR purified from *L. leichmannii* was therefore next examined for its inhibition by DTB.

Inhibition of DHFR by DTB

Figure 3 depicts the results obtained on the inhibition of *L. leichmannii* DHFR activity by DTB, the bacteriostatic effects of which have been previously demonstrated by us with the same *L. leichmannii* cells as aforementioned. Our results clearly revealed that potent inhibition of the microbial enzyme activity by the plant alkaloid is affected by a slow binding (see below) of DTB to DHFR during the ternary complex formation of the enzyme with the inhibitor alkaloid in the presence of the cofactor NADPH. IC_{50} value of DHFR for DTB was calculated to be 30 μM. DTB concentrations >75 μM in the reaction assay mix resulted in a complete loss of the enzyme activity thus explicitly suggesting that the β-carboline-benzoquinolizidine group of alkaloids have a high potential as antitumor compounds. It is of interest to note that the IC_{50} value of the bacterial cells for DTB observed in the cytotoxicity studies (Fig. 2) was approximately the same as that obtained in the bacterial enzyme inhibition studies on DHFR viz., 30–40 μM.

Inhibition constant of DHFR for DTB

Figure 4 delineates DHFR inhibition kinetics employing the Dixon plot for the K_i determination using two different fixed substrate concentrations of DHF (0.57 and 1.13 μM) and graded variable concentrations of DTB (5–30 μM). Results presented in the figure reveal that *L. leichmannii* DHFR has a K_i value of 5×10^{-6} M for DTB. Thus, β-carboline-benzoquinolizidine plant alkaloid appears to be a potent inhibitor of the enzyme. Inhibition, as displayed by the Dixon plot, is a simple linear 'non-competitive' type.

DTB binding studies

Evidence from gel filtration (Sephadex G-100, 1×40 cm column) studies further supported by the dialysis experiments clearly indicated that the *Alangium* alkaloid DTB binding to the *L. leichmannii* enzyme DHFR during the ternary complex formation, is slow and reversible (untabulated results).

Comparative K_i values

Table 1 shows the K_i values of *L. leichmannii* DHFR for the nonclassical non-competitive β-carboline-benzoquinolizidine class of alkaloid DTB (present study) and the

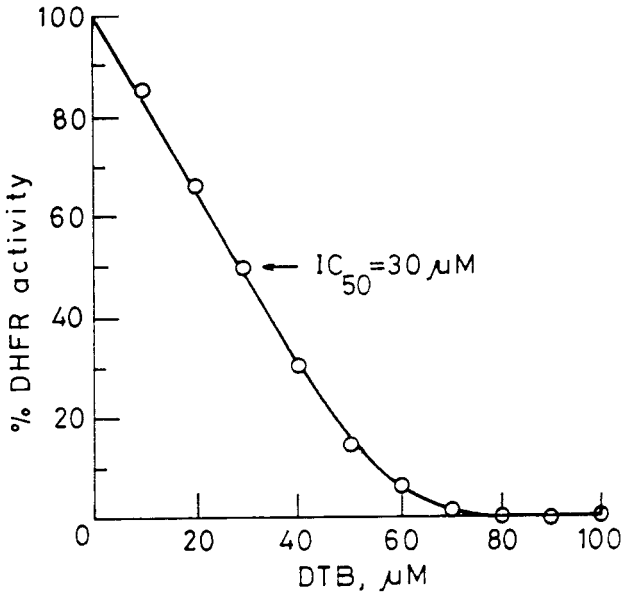


Figure 3. *L. leichmannii* dihydrofolate reductase inhibition by the β-carboline-benzoquinolizidine alkaloid DTB. The DHFR assays[50] were carried out in a total volume of 1 ml reaction mixture in the presence of designated concentrations (10–100 μM) of the plant alkaloid as described in the text. The control specific activity of the enzyme measured in the absence of the alkaloid in the assay mix was taken as 100% activity.

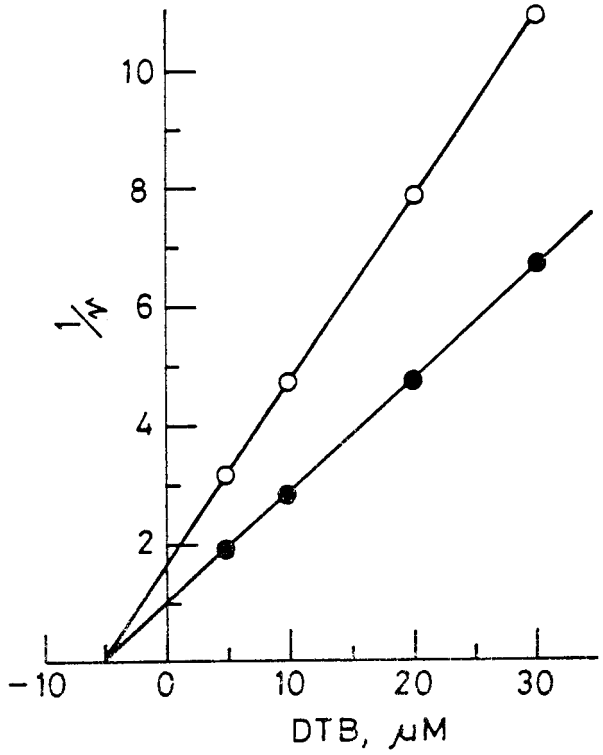


Figure 4. Dixon plot showing noncompetitive inhibition of DHFR by DTB. Reaction velocities of *L. leichmannii* DHFR inhibition were studied at two substrate concentrations of DHF. The inhibitor alkaloid DTB was preincubated at 30°C for 10 min with the DHFR assay mix prior to the substrate addition. The enzyme assays[50] were performed in a total volume of 1 ml reaction mixture as described in the Experimental section. The DHF concentrations employed were 0.57 μM (O); 1.13 μM (●). Values presented in the plots were the average of two independent experiments carried out.

phenanthroindolizidine class of alkaloids pergularinine (PGL) and tylophorinidine (TPD)[21] as compared to certain well-documented classical competitive DHFR inhibitors. It is of interest to note that the K_i values calculated for DTB along with those of PGL and TPD (viz., 9×10^{-6} M and 7×10^{-6} M, respectively) fall within a proximate range of that reported for the classical folate analogue 1-deazamethotrexate (DMTX),[22] which is one of the most potent classical competitive inhibitors of DHFR (Table 1). It is also of interest to note that the K_i value for the unreduced cofactor viz., NADP is identical to that of DTB reported in this study. Our observations thus show that β-carboline-benzoquinolizidine class of *Alangium* alkaloids are the most effective and powerful inhibitors of DHFR. Our results unequivocally show that these plant alkaloids which are considered powerful anti-TS[23–25] and anti-DHFR compounds, have a high potential as anticancer agents. Thus, the emetine group of *Alangium* alkaloids such as DTB (this study), like the *Pergularia* phenanthroindolizidine alkaloids namely PGL and TPD,[21] are relatively of high order of potency as antitumor compounds. The increasing order of inhibition potency of the plant alkaloids with respect to DHFR activity was recorded to be in the following sequential order: DTB > TPD > PGL.

Discussion

Tubulosine and the related bases are comprised of a top isoquinoline portion of monoterpenoidal origin and a lower pendant indole system originating from tryptophan.[26] While the indole portion of deoxytubulosine molecule plays an active role in the binding site of TS,[23] it is possibly both the indole as well as the isoquinoline portions of the alkaloid that could take part at the active binding sites of the DHFR enzyme molecule resulting in increased inhibition. Documented evidence shows that DHFR binds to N atom/NH_2 groups of the inhibitor analogues or nonanalogues by a strong hydrogen bonding.[27] Binding of the purine analogues such as TMP in the 'folate mode' would enable the N1-nitrogen of the purine, upon protonation, in conjunction with the 2-amino moiety, to participate in the salt bridge with Glu 32 and Wat 403, similar to TMP binding with *Escherichia coli* and *P. carinii* reductases.[15] Benzoquinolizidine moiety of the plant alkaloid could therefore have affinity with the *L. leichmannii* DHFR for a strong hydrogen-bonding around the active site domains of the amino acid residues at the regions of the three N atoms. The 9,10-methoxy groups of DTB could be close to the Leu 28, Phe 31 and the side chain of Ile 50 as is the case with the *E. coli* DHFR bound to TMP with its 3′- and 4′-methoxy groups.[28] Furthermore, our results unequivocally suggest that DTB binding to DHFR is slow and reversible, a situation similar to that of pyridoxal 5′-phosphate (PLP) binding to *L. leichmannii* TS.[29] *L. leichmannii* DHFR has a single reactive sulfhydryl group close to the active center of the enzyme molecule (Narasimha Rao, unpublished results). It is

Table 1. K_i values of *L. leichmannii* DHFR for DTB as compared to various other slow, tight-binding and slow-binding inhibitors

DHFR inhibitor	Enzyme source	K_i (M)	Reference
DTB	*L. leichmannii*	5×10^{-6}	This study
PGL	*L. leichmannii*	9×10^{-6}	21
TPD	*L. leichmannii*	7×10^{-6}	21
MTX	*L. leichmannii*	6.7×10^{-11}	30
FA (unreduced)	*L. leichmannii*	1.4×10^{-5}	30
DMTX	*S. faecium*	5.8×10^{-7}	22
TMP	*S. faecium*	9.6×10^{-10}	22
PM	Ehrlich ascites	1.7×10^{-8}	31
NADP (unreduced)	Ehrlich ascites	4.9×10^{-6}	31

The K_i values of DHFR for various inhibitors were calculated from the initial velocity experiments as described. MTX, 7,8-dihydroMTX, aminopterin, methyl 4-amino-4-deoxy-10-methylpteroate and TMP behave as slow, tight-binding inhibitors, whereas DMTX and 1-deaza-4-amino-4-deoxypteroate function as slow-binding DHFR inhibitors,[22] similar to the inhibitor plant alkaloids PGL, TPD[21] and DTB (this study). DTB, deoxytubulosine; PGL, pergularinine; TPD, tylophorinidine; MTX, methotrexate; FA, folic acid, DMTX, 1-deaza-methotrexate; TMP, trimethoprim; PM, pyrimethamine; NADP, β-nicotinamide adenine dinucleotide phosphate.

therefore proposed that possibly 1 mol of DTB binds per mol of this enzyme.

Evidence from the present investigations clearly shows that the β-carboline-benzoquinolizidine plant alkaloid DTB is potently cytotoxic and powerfully inhibits the enzymatic activity of DHFR. Coupled with TS, DHFR provides thymidylate for DNA biosynthesis through de novo pathway. The DHFR levels, like those of TS, are highly elevated in human malignancy. These two enzymes are therefore key targets for cancer chemotherapy. Extensive investigations on the chemico–biological interactions of certain plant alkaloids with the related crucial enzyme TS have been recently reported from this laboratory.[4,23–25] Classical competitive inhibitor drugs such as MTX, trimethoprim (TMP), pyrimethamine (PM) and others[14,30–36] (see Table 1) have been particularly streamlined in the earlier investigations. However, anti-DHFR drugs 'dissimilar' to the substrate or cofactor remain highly attractive because they are less likely to have the side effects that are produced by the folate or the pyridine nucleotide mimics.[37] Inhibitor alkaloid DTB, which is a substrate/cofactor nonanalogue of DHFR, is therefore believed to cause the least side effects from a therapeutic point of view. With a low K_i value for DTB, the β-carboline-benzoquinolizidine alkaloids appear to have a potential as both antimicrobial and anticancer agents. DHFR and TS levels are markedly pronounced in human malignancy and both the enzymes are potently inhibited by the β-carboline-benzoquinolizidine and the phenanthroindolizidine plant alkaloids. These results have been communicated separately elsewhere.[4,21,25,38]

Amongst the natural products, alkaloids taxonomically enjoy only limited distribution. Within the broad spectrum of medicinally important plant alkaloids, there is a slight preponderance of those containing the indole nucleus as the basic unit. Biochemical and biological properties of emetine plant alkaloids, especially those of the *Alangium* family, are of paramount significance from the standpoint of their unique therapeutic value as anti-infective and anticancer agents. Alkaloids isolated from *A. lamarckii* have antihypotensive and antiplatelet aggregation activities,[39,40] whereas the alkaloids of *A. chinense* and *A. vitiense* reportedly display sedative and antileukemic activities, respectively.[41,42] Tubulosine exhibits amebicidal activity.[43] From the view point of structural considerations, emetine, an ipecac alkaloid, and cycloheximide, a glutarimide antibiotic, are potent amebicides and their mechanism of action is based on the inhibition of transfer reaction in protein synthesis.[44–46] Synergistic DHFR inhibitory and bacteriostatic effects of DTB from *Alangium* on the *L. leichmannii* cells unequivocally are suggestive of its high potential as a powerful and most effective anticancer and antimicrobial agent. DTB, with a small K_i value comparable to that of DMTX, evidently appears to be a promising antitumor compound of high potency. Consequently, there is a dire need to investigate hitherto unexplored naturally occurring phytochemicals of biochemical and therapeutic interest. Curcumin, which was extensively studied in our laboratories, is a novel phytochemical that has an exclusive chemopreventive action against chemical carcinogenesis.[47] From our present findings, it is contended that the *Alangium* alkaloids are of high order of potency as antineoplastic agents and therefore need extensive tests employing other parameters of carcinogenesis.

Experimental

Chemicals and biochemicals

All the chemicals and biochemicals used in the present study were of analytical reagent grade. L-7,8-Dihydrofolate (DHF), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), 2-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), streptomycin sulfate, vitamin B_{12}, DEAE-cellulose and TEAE-cellulose were procured from Sigma, St. Louis, MO. Alumina-G was purchased from Woehlm Pharma, Eschwege, Germany. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, whereas vitamin B_{12} assay medium was procured from HiMedia Laboratories Pvt. Ltd., Bombay.

Isolation and characterization of DTB

The β-carboline-benzoquinolizidine alkaloid DTB was isolated from the crude extracts of the flowers of *A. lamarckii* as described by Venkatachalam et al.[20] The isolated DTB was identified on the basis of its physical and spectral data with those reported.[26]

Growth inhibition experiments

Growth inhibition experiments were performed with the subcultures of the vitamin B_{12}-supplemented *L. leichmannii* (ATCC 7830) grown in the vitamin B_{12} assay medium[48] in the presence or absence of the alkaloid by turbidimetry. Klett Summerson colorimeter using red

filter (640–700 nm) was employed to measure turbidity for the growth response.

DHFR purification

DHFR was purified from the crude extracts of B_{12}-supplemented *L. leichmannii* (ATCC 7830) essentially according to the method of Kessel and Roberts[30] with minor modifications.

Protein assay

The enzyme protein was measured by the method of Lowry et al.[49] after removal of mercaptoethanol as described elsewhere.[48]

DHFR assay

DHFR was assayed essentially according to the method of Friedkin et al.[50] with minor modifications.[51] The assay mix (buffer B) of a total volume of 1 mL reaction mixture consisted of 100 μM DHF, 50 μM NADPH, 50 mM 2-mercaptoethanol and 10–15 μg of the purified enzyme in 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 20 mM 2-mercaptoethanol (buffer A). Enzymatic reactions were carried out at 30 °C using appropriate blanks and the decrease in absorbance at 340 nm was monitored in a recording double beam Shimadzu UV 250 spectrophotometer. The readings were recorded in the initial 1 to 2 min linear phase of the reaction. Decrease in the absorbance at 340 nm was thus taken as a measure of THF produced enzymatically from DHF. The molar extinction coefficient at 340 nm (E_{340}) for this reaction is approximately 12×10^3 cm^2/mol.[51] Specific activity was expressed as μmol THF formed/h/mg protein.

Determination of the enzyme inhibition constant

Inhibition constant (K_i) of DHFR for DTB was determined employing the Dixon plots obtained from the initial velocity studies using the designated substrate and inhibitor concentrations for measuring the reaction rates.[52]

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