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Growth-inhibitory effects of 5,10-dideazatetrahydrofolic acid on variant murine L1210 and human CCRF-CEM leukemia cells with different membrane-transport characteristics for (anti)folate compounds*

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Summary. 5,10-Dideazatetrahydrofolic acid (DDATHF) is a potent inhibitor of glycinamide ribonucleotide transformylase, one of the folate-dependent key enzymes in de novo purine biosynthesis. The present report demonstrates that multiple membrane-transport routes may be involved in the cellular uptake of DDATHF. These routes include the classic reduced folate carrier and a membrane-associated folate-binding protein (mFBP). The role of an mFBP in the uptake of DDATHF was suggested from observations that (a) the mFBP showed a very high binding affinity for DDATHF, (b) murine and human leukemia cells expressing an mFBP were highly sensitive to growth inhibition by DDATHF, and (c) protection against this growth inhibition could be achieved using folic acid rather than reduced folate compounds.

Introduction

The role of inhibitors of folate metabolism with targets other than dihydrofolate reductase in the treatment of neoplastic diseases is an area of considerable current interest [8, 10, 22, 23]. 5,10-Dideazatetrahydrofolic acid (DDATHF) is a new antifolate acting as a potent inhibitor of glycinamide ribonucleotide transformylase (GAR-TFase), one of the folate-dependent key enzymes in purine biosynthesis de novo [3, 4, 24]. Recent studies [6, 25, 29] have demonstrated that membrane transport of DDATHF can proceed via the classic low-capacity/high-affinity carrier system for reduced folate compounds (RF carrier), which is also used by the antifolate methotrexate (MTX). In the present study we investigated the question as to whether other transport routes could also contribute to the cytotoxic effects of DDATHF. In particular, we tested the growth-inhibitory effects of DDATHF in variant murine L1210 and human CCRF-CEM leukemia cells [13, 14] expressing a membrane-associated folate-binding protein (mFBP) as an alternative folate-internalizing system in comparison with parental cells expressing the RF carrier.

Materials and methods

Materials. RPMI-1640 medium (with and without folic acid) and (non) dialyzed fetal calf serum were obtained from Gibco (Grand Island, N. Y.). [^{3} H]-Folic acid (specific activity, 35 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif.) and purified prior to use as described elsewhere [13, 14]. DDATHF was generously donated by Dr. G. B. Grindey (Lilly Research Laboratories, Indianapolis Ind., USA). Unlabeled methotrexate (MTX) was a gift from Pharmachemie (Haarlem, The Netherlands). 10-Ethyl-10-deazaaminopterin (10-EdAM) was kindly provided by Ciba-Geigy (Basel, Switzerland). *d*, 1-5-Formyltetrahydrofolate (5-formyl-THF), folic acid, *d*,1-5-methyltetrahydrofolate (5-methyl-THF) and hypoxanthine were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell lines. Murine leukemic L1210 cells and human leukemic CCRF-CEM cells, both expressing the RF carrier [12, 28], were used as controls. CEM/MTX is a subline of CCRF-CEM that shows approximately 200-fold resistance to MTX due to impaired transport via the RF carrier [14]. CEM-7A is a subclone of CCRF-CEM exhibiting 30-fold over-expression of the RF carrier [15], and CEM-FBP is a subline of CEM/MTX that lacks the RF carrier but expresses high levels of an mFBP [14]. L1210-B73 is a subline of L1210 expressing both the RF carrier and an mFBP [13, 18]. For growth-inhibition studies, all cell lines were maintained in folate-free RPMI-1640 medium (1×10^5 cells/ml) supplemented with 10% dialyzed fetal calf serum and 1 nM 5-formyl-THF as the sole folate source (unless otherwise indicated).

Results and discussion

Recent studies [25, 29] have demonstrated that the RF carrier in L1210 and CCRF-CEM cells exhibits an affinity for DDATHF (K_m, 1–3 μM) comparable with that of reduced folate compounds such as 5-formyl-THF/5-methyl-

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Table 1. Relative affinity of the FBP from CEM-FBP cells and L1210-B73 cells for (anti)folate compounds

Compound	Relative affinitiy[a]	
	CEM-FBP	L1210-B73
Folic acid	1	1
5-Methyl-THF	0.33	0.50
5-Formyl-THF	0.09	0.19
MTX	0.008	0.03
10-EdAM	0.009	0.03
DDATHF	0.92	0.95

[a] CEM-FBP and L1210-B73 cells (3×10^6/ml in HEPES buffered saline, pH 7.4 [8]) were incubated for 10 min at 4° C with 100 pmol [^{3}H]-folic acid in the absence or presence of increasing concentrations of (anti)folate compound. The inverse molar ratio of compound required to displace 50% of [^{3}H]-folic acid from the FBP is depicted as relative affinity. Results represent the mean of 3 separate experiments (SE <15%)

Table 2. Inhibition of the growth of human leukemia cells by DDATHF

Addition	IC_{50} (nM)		
	CCRF-CEM (RF carrier)	CEM/MTX (defective RF carrier)	CEM-7A (RF carrier overproducer)
1nM 5-formyl-THF	10.6±3.1	80±13	2.2±0.2
20 nM 5-formyl-THF	51 ±17	76±12	27 ±8
20 nM folic acid	9 ±1.2	ND	2.2±0.3
100 μM hypoxanthine	>50 μM	>50 μM	>50 μM

Duration of exposure of cells to DDATHF, 72 h. Results represent the mean of 4 separate experiments (± SD). ND, Not determined. IC_{50} is defined as the concentration of drug required to inhibit cell growth by 50% of the control value

THF and antifolate compounds such as MTX and 10-EdAM [28]. A typical feature of the RF carrier is its poor affinity for folic acid (K_m, 200–400 μM), whereas mFBPs usually exhibit a high affinity for folic acid (K_d, 0.1–1 nM) [21]. Table 1 shows the affinity of the mFBP expressed by L1210-B73 and CEM-FBP cells for a number of (anti)folate compounds relative to the affinity for folic acid. The mFBP exhibited high affinity for 5-formyl-THF and 5-methyl-THF but poor affinity for two folate-based inhibitors of DHFR: MTX and 10-EdAM. Interestingly, the mFBP was found to have a very high affinity for DDATHF that was almost equivalent to that of folic acid.

To establish the extent to which the RF carrier or the mFBP play a role in membrane transport of DDATHF, growth-inhibition studies were performed in the absence or presence of 20 nM folic acid or 5-formyl-THF. In DDATHF transport via the mFBP, high-affinity binding of folic acid can provide protection from growth inhibition. Likewise, since the RF carrier shows high affinity for 5-formyl-THF but not for folic acid, 5-formyl-THF has been used to prevent growth inhibition by DDATHF at the level of this carrier. Growth-inhibition studies in human leukemic CEM cells that have a defective RF carrier (CEM/MTX) or over-express the RF carrier (CEM-7A) support previous observations [25, 29] that the RF carrier is an important route for cellular uptake of DDATHF

Table 3. Inhibition by DDATHF of the growth of murine and human leukemic cells expressing the carrier and/or FBP

Addition	IC_{50} (nM)			
	CCRF-CEM (RF)	CEM-FBP (FBP)	L1210 (RF)	L1210-B73 (RF + FBP)
1 nM 5-formyl-THF	10.6± 3.1	1.6±0.2	9.2±2.5	4.1±1.5
20 nM 5-formyl-THF	51 ±17	5.1±1.1	76 ±6	42 ±18
20 nM folic acid	9 ± 1.2	93 ±8	9.1±2.4	88 ±14
100 μM hypoxanthine	>50 μM	>50 μM	>50 μM	>50 μM

Duration of exposure of cells to DDATHF, 72 h

(Table 2). The sensitivity of CEM-7A cells to DDATHF was 5-fold that of parental CCRF-CEM cells, most likely due to an increased overall transport of DDATHF via the RF carrier in CEM-7A cells. Impaired transport via the RF carrier could account for the resistance of CEM/MTX cells to DDATHF. However, it should be noted that the degree of resistance to DDATHF (8-fold) is significantly lower than that for MTX (200-fold) [14]. Except in CEM/MTX cells, 20 nM 5-formyl-THF provided protection against inhibition of the growth of CCRF-CEM andd CEM-7A cells by DDATHF (Table 2). The addition of 20 nM folic acid had no protective effect, which is compatible with the low affinity of the RF carrier for folic acid as compared with 5-formyl-THF.

Table 3 shows the growth-inhibitory effects of DDATHF on CEM and L1210 cells expressing the RF carrier and/or mFBP. The sensitivity of CEM-FBP cells to DDATHF was 6- to 7-fold that of the parental cells, whereas that of L1210-B73 cells was 2.2-fold that of parental L1210 cells. Protection against inhibition of the growth of CEM-FBP cells was provided to some extent by 5-formyl-THF (3.2-fold), but significantly higher protection was obtained following the addition of 20 nM folic acid (58-fold). This is consistent with the relative affinity of the mFBP for folic acid and 5-formyl-THF as compared with DDATHF (Table 1). In L1210-B73 cells, uptake of DDATHF can proceed via the RF carrier as well as via the mFBP. This can be concluded from the observation that both 5-formyl-THF (mainly via the RF carrier and, to some extent, via the mFBP) and folic acid (via the mFBP) were effective in protecting L1210-B73 cells from growth inhibition by DDATHF. In all cases, CEM and L1210 variants were protected from DDATHF concentrations of <50 μM by the addition of 100 μM hypoxanthine (Tables 2, 3), which emphasizes that inhibition of purine biosynthesis is the primary site of action of DDATHF [4, 24].

We have recently demonstrated [16–18] that the mFBP from CEM-FBP and L1210-B73 cells also exhibits very high affinity (comparable with folic acid) for two quinazoline folate-based inhibitors of thymidylate synthase, N^{10}-propargyl-5,8-dideazafolic acid (CB3717) and 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid. Consequently, the mFBP was found to play an important

role in the uptake of these compounds by CEM-FBP [16, 17] and L1210-B73 cells [18]. In addition, Henderson and Strauss [11] have shown that homofolate, a folate-based inhibitor of de novo purine/pyrimidine biosynthesis, can be transported via the mFBP, which suggests that multiple transport routes can be important for the uptake of different antifolate compounds.

In summary, the present study showed that the novel antifolate 5,10-dideazatetrahydrofolate (DDATHF), an inhibitor of one of the folate-dependent enzymes in de novo purine biosynthesis (GAR-TFase), can enter murine and human leukemia cells via multiple transport routes: the RF carrier or an mFBP. In this respect, DDATHF is an interesting compound since it is one of a series of antifolates (other than the classic antifolate MTX) for which both the RF carrier and the mFBP show high affinity. Based on the recent identification of mFBPs both in a number of tumor cells [9, 21, 27] and in normal cells and tissues [1, 2, 5, 7, 9, 19–21, 26], these results could have therapeutic implications for either the sensitivity of tumor cells to DDATHF or the toxicity of this substance to normal cells.

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