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Further studies on the interaction of nonpolyglutamatable aminopterin analogs with dihydrofolate reductase and the reduced folate carrier as determinants of *in vitro* antitumor activity

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Abstract

Thirteen structural analogs of the potent nonpolyglutamatable dihydrofolate reductase inhibitor N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) with modifications in the side chain, the *para*-aminobenzoyl moiety, or the 9,10-bridge were evaluated for the ability to inhibit human recombinant dihydrofolate reductase (DHFR), to utilize the reduced folate carrier (RFC) for influx, and to inhibit the growth of CCRF-CEM human leukemia cells in culture. In spectrophotometric assays of the kinetics of the reduction of dihydrofolate by DHFR in the presence of NADPH, these compounds had K_i values ranging from 0.2 to 1.3 pM, and thus were not greatly different in potency from the parent drug PT523. By comparison, the K_i values of aminopterin (AMT), methotrexate (MTX), and 10-ethyl-10-deazaaminopterin (EDX) were 3.7, 4.8, and 11 pM. In assays of competitive inhibition of [^{3}H]MTX influx into CCRF-CEM cells, the K_i values ranged from 0.21 to 7.3 μM, as compared with 0.71, 5.4, and 1.1 μM for PT523, AMT, and EDX. The K_t for MTX was also re-analyzed and found to be 4.7 μM, in better agreement with the literature than our previously reported value of 7.1 μM. The IC_{50} values of these compounds as inhibitors of the growth of CCRF-CEM cells after 72 hr of drug exposure ranged from 0.53 to 55 nM, and were qualitatively consistent with the other results.

Keywords: N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) and analogs; Dihydrofolate reductase; Reduced folate carrier

1. Introduction

N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) is a structural analogue of the classical anticancer drug AMT in which the γ-terminal region of the glutamate residue is modified. Originally synthesized in our laboratory in 1988 [1,2], PT523 has since been studied by several groups [3–8] with a view to defining its mode of action and exploring its therapeutic potential in comparison with newer folate antagonists developed by pharmaceutical companies as cancer drugs. As with AMT and other classical antifolates containing a 2,4-diaminopyrimidine moiety, the proximal target of PT523 is DHFR, and its cellular uptake relies on active transport by the RFC. Because of the substitution of N^{δ}-hemiphthaloyl-L-ornithine for L-glutamic acid as the side chain, PT523 is not converted to long-chain polyglutamates by FPGS. Thus, a critical feature that sets this drug apart from AMT and other classical antifolates is that the only way the FPGS level of a tumor can influence sensitivity to PT523 is through qualitative or quantitative differences in endogenous reduced folate polyglutamate pools, rather than through differences in the ability of the tumor to convert the drug to γ-polyglutamyl conjugates. The presumptive clinical relevance of this model rests on the common view that certain types of tumors with low FPGS activity (e.g. soft-tissue sarcomas) are relatively resistant to antifolates that depend upon polyglutamation to express their full range of biochemical effects [9–11]. Accordingly,

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Abbreviations: AMT, aminopterin; MTX, methotrexate; [^{3}H]MTX, [3′,5′,7-^{3}H]-MTX; FPGS, γ-folylpolyglutamate synthetase; DHFR, dihydrofolate reductase; RFC, reduced folate carrier; EDX, edatrexate, 10-ethyl-10-deazaaminopterin; PDX, 10-propargyl-10-deazaaminopterin.

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it was proposed that a preferred clinical application for PT523 might be one in which there is less FPGS activity in a tumor than in dose-limiting host tissues [3,12]. By the same token, it was also postulated that restoration of depleted endogenous folate pools with leucovorin (LV) might be less efficient in a tumor with low FPGS activity than in normal host tissues with higher FPGS activity. A relevant observation in this regard is that, in comparison with MTX and several other polyglutamatable classical antifolates, the growth of cultured tumor cells in the presence of PT523 is relatively insensitive to stepwise increases in the amount of LV in the medium [8].

In parallel with our preclinical studies and an impending Phase I clinical trial of PT523, a library of second-generation analogs was synthesized in our laboratory with the aim of delineating the optimal structural features for DHFR binding and cellular influx via the RFC system [13–17]. Data on the ability of a series of analogs of PT523 modified at N^5 and/or N^8 to inhibit the binding of [^{3}H]MTX to the RFC of CCRF-CEM lymphoblastic leukemia cells, along with data on their ability to bind to purified recombinant human DHFR and inhibit the growth of CCRF-CEM cells in culture, were published recently [18]. In the present paper, we report the results of similar experiments using thirteen additional PT523 analogs modified in the side chain, the *para*-aminobenzoyl ring, and the 9,10-bridge region as opposed to the B-ring (i.e. the pyrazine moiety). With minor differences, preliminary results for some of the compounds in this group were also disclosed recently [19]. The chemical structures of PT523 and the analogs that are the subject of this paper are given in Fig. 1.

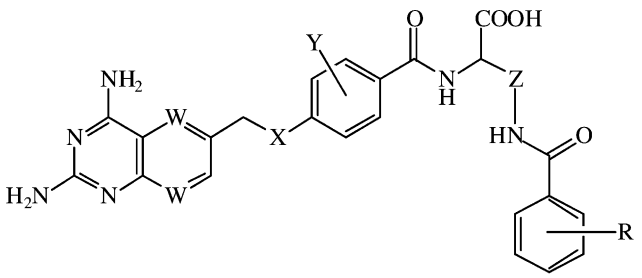
2. Materials and methods

2.1. Drugs and radiochemicals

PT523 and its side chain analogs (**1**–**13**, Fig. 1) were synthesized at the Dana-Farber Cancer Institute as described [1,2,13–17]. [^{3}H]MTX, specific activity 23 Ci/mmol, was purchased from Moravek Biochemicals, and was determined to have >99% radiochemical purity by HPLC. Tritium analysis by liquid scintillation counting (LSC) was performed in Redi-Safe scintillation fluid (Fisher Scientific) using an LKB-Pharmacia instrument.

2.2. Biochemical and biological assays

Test compounds were checked for purity by reversed-phase HPLC on a C_{18} silica gel analytical column as described [13–17], and were re-purified on a milligram



	R	W	X	Y	Z
PT523	2-COOH	N	NH	H	$CH_2CH_2CH_2$
1	3-COOH	"	"	"	"
2	4-COOH	"	"	"	"
3	2-COOH	"	"	"	CH_2
4	"	"	"	"	CH_2CH_2
5	"	"	"	"	$CH_2CH_2CH_2CH_2$
6	"	"	"	"	CH_2SCH_2
7 (α-D,L)	"	"	"	"	$CH_2CH_2SCH_2$
8	"	"	"	2,3-(CH=CH)$_2$	$CH_2CH_2CH_2$
9	"	"	CH_2	"	"
10	"	"	CHMe *(R,S)*	"	"
11	"	"	CHEt *(R,S)*	"	"
12	"	"	CHPg *(R,S)*	"	"
13	"	CH	CH_2	"	"

Fig. 1. Chemical structures of PT523 analogs modified in the amino acid side chain, the *para*-aminobenzoyl moiety, or the 9,10-bridge.

scale whenever necessary. Procedures for measuring the activity of PT523 analogs as DHFR inhibitors and as competitive inhibitors of RFC-mediated [^{3}H]MTX influx in CCRF-CEM human leukemia cells have been published previously [15,18], and were used essentially without change except for inclusion of 6 mg/L of bovine serum albumin in the DHFR assay buffer in order to minimize time-dependent changes in the activity of the enzyme. For the best results, especially in the case of the inhibitors with the lowest K_i, it was important to add the inhibitor to the cuvette in amounts that gave closely spaced concentrations at the upper end of the concentration range, affording more robust data for least-squares calculation in the Henderson-type plot of $I_t/1-(V_i/V_o)$ vs. V_o/V_i. Thus, the final concentrations of inhibitor in a typical set of titrations were 0, 10, 20, 30, 40, 45, 49, 50, 51, and 52 nM. In some cases it was also helpful to double the concentration of the dihydrofolate and NADPH co-substrates to 100 and 120 μM, respectively. This allowed a more accurate assessment of the K_i for the three most potent competitive inhibitors in Table 1. Near the inhibitor–enzyme equivalence point for these compounds, the reactions were so slow that accurate rate determinations were not possible except at the higher substrate concentrations.

3. Results and discussion

3.1. Positional change of the phthaloyl COOH group

As shown in Table 1, PT523 analogs in which the aromatic COOH group was moved from the *ortho* position to the *meta* or *para* position of the N^{δ}-aroyl moiety are essentially equivalent to PT523 in terms of DHFR inhibition. However, when their K_i values as inhibitors of [^{3}H]MTX influx were compared, the potency of the *meta* isomer (**1**) was almost 2-fold lower relative to PT523, whereas that of the *para* isomer (**2**) was decreased 10-fold. Thus, the effect of moving the COOH was much greater on RFC-mediated influx than on DHFR inhibition. We had reported previously that the potency of the *meta* isomer as an inhibitor of CCRF-CEM cell growth during continuous exposure for 72 hr was decreased 5-fold relative to PT523, while that of the *para* isomer was decreased 37-fold [14]. The decreased efficiency of these compounds as substrates for the RFC is qualitatively consistent with this result, and, taken together with the relatively small differences in their K_i values against DHFR, suggests that the antiproliferative activity of these positional isomers of PT523 is related, in part, to their ability to saturate the RFC. It must be kept in

Table 1
Dihydrofolate reductase inhibition, reduced folate carrier interaction, and *in vitro* antitumor activity of PT523 analogues

Compound[a]	Key modification[a]	DHFR (K_i, pM)[b]	RFC influx (K_i, μM)[c]	Cell growth IC_{50} (nM)[d]
PT523[e]	–	0.30 ± 0.036	0.71 ± 0.12	1.5 ± 0.39
1	R = 3-COOH	0.20 ± 0.060	1.2 ± 0.12	7.5 ± 0.47
2	R = 4-COOH	0.21 ± 0.087	7.3 ± 0.40	55 ± 20
3	Z = CH_2	1.3 ± 0.12	2.4 ± 0.12	5.1 ± 0.58
4	Z = CH_2CH_2	0.68 ± 0.12	0.70 ± 0.18	5.5 ± 1.6
5	Z = $CH_2CH_2CH_2CH_2$	0.70 ± 0.28	1.2 ± 0.09	2.9 ± 0.97
6	Z = CH_2SCH_2	0.24 ± 0.08	2.4 ± 0.27	4.4 ± 1.1
7[f]	Z = $CH_2CH_2SCH_2$	0.47 ± 0.10	1.9 ± 0.28	5.0 ± 0.56
8	Y = 2,3-(CH=CH)$_2$	0.21 ± 0.03	1.8 ± 0.25	1.2 ± 0.22
9	X = CH_2	0.35 ± 0.06	0.41 ± 0.05	0.53 ± 0.07
10[g]	X = CHMe	0.50 ± 0.10	0.49 ± 0.09	0.63 ± 0.08
11[g]	X = CHEt	0.62 ± 0.10[h]	0.52 ± 0.06	1.2 ± 0.25
12[g]	X = CHPg[i]	0.60 ± 0.02[h]	0.33 ± 0.03	1.3 ± 0.35
13	W = CH, X = CH_2	0.21 ± 0.05[h]	0.21 ± 0.08	0.69 ± 0.04[h]
Reference compounds				
AMT	X = NH (Glu side chain)	3.7 ± 0.35	5.4 ± 0.09	4.4 ± 0.1
MTX	X = NMe (Glu side chain)	4.8 ± 0.45	4.7 ± 1.3[j]	14 ± 2.6
EDX[g]	X = CHEt (Glu side chain)	11 ± 0.9	1.1 ± 0.34	1.6 ± 0.26

[a] See Fig. 1 for structures of numbered compounds and definitions of R and W–Z.
[b] Assays used purified human recombinant DHFR and were performed essentially as reported earlier [20] with minor modifications. Results are means ± SD (N ≥ 3).
[c] K_i values were determined as described [13], and refer to inhibition of [^{3}H]MTX influx into CCRF-CEM leukemic lymphoblasts over a period of 60 sec. Results are means ± SD (N ≥ 3). The K_i of AMT as an inhibitor of [^{3}H]MTX influx is from Ref. [13].
[d] CCRF-CEM cells were exposed to drug for 72 hr as described [19]; values are means ± SD (N ≥ 3). Growth data for PT523 and **1**–**11** are from Ref. [19]; those for **12** and **13** are from Ref. [17].
[e] For PT523, W = N, X = NH, Y = H, Z = $(CH_2)_3$, R = 2-COOH.
[f] α-D,L mixture.
[g] 10*R*, 10*S* mixture.
[h] DHFR data for **11**–**13** from Ref. [17].
[i] Pg = propargyl.
[j] K_i for [^{3}H]MTX influx was determined as described previously [13] but a new series of assays with freshly obtained CCRF-CEM cells (American Type Culture Collection) gave a lower K_i than the 7.1 ± 0.72 μM value reported earlier [18].

mind, however, that differences in antiproliferative activity among PT523 analogs are probably a better reflection of differences in steady-state accumulation inside the cell, rather than merely differences in the kinetics of unidirectional influx. Because direct measurement of steady-state drug accumulation for every compound in the series would have been prohibitively laborious in the absence of these compounds in radiolabeled form, such experiments were not done.

3.2. Chain length changes in the amino acid moiety

Given that the side chain in PT523 contains three CH_2 groups whereas glutamic acid contains only two, it was of interest to consider whether the N^{δ}-hemiphthaloyl-L-ornithine side chain has the optimal length for interaction with DHFR and the RFC. Thus, we examined the activity of five analogs differing only in the length of the side chain. As documented in Table 1, stepwise deletion of one, and then two CH_2 groups in compounds **4** and **3**, respectively, led to a 2- to 4-fold increase in K_i against DHFR; the addition of an extra CH_2 group in **5** led to a 2.5-fold increase in K_i against DHFR.

In contrast, the only change well tolerated for RFC substrate activity appeared to be the deletion of one CH_2 group.

It was suggested previously, on the basis of an analysis of the ^{1}H NMR spectrum of the ternary complex of human DHFR with PT523 and NADPH in solution, that the bound PT523 can induce a change in the protein conformation allowing the phenyl ring of the N^{δ}-hemiphthaloyl moiety to interact hydrophobically with a proline residue in the distal region of the active site [20]. This conclusion was also supported independently by X-ray crystallography [21]. In the DHFR–MTX–NADPH complex, this interaction is absent, probably accounting for the lower K_i of PT523 as compared with MTX. The results in Table 1 suggest that three CH_2 groups in the side chain provide an optimal opportunity for induction of this conformational change in the protein.

When $CH_2CH_2CH_2$ was replaced by CH_2SCH_2, as in **6**, the K_i against DHFR remained essentially the same. The racemic $CH_2CH_2SCH_2$ analog **7** had a K_i value 2-fold higher than that of the CH_2SCH_2 analog **6**. The IC_{50} of D,L-MTX has been reported to be twice that of L-MTX [22], in keeping with the expectation that when two enantiomers with very different binding affinities are present, the quantitative contribution of the weaker inhibitor should be practically zero. This principle is also applicable when K_i values from a Henderson-type plot are considered. Thus, if one assumes that the effect of racemization on DHFR inhibition by a chain-lengthened analog such as **7** is similar to that of MTX, we would predict that the K_i of the L-enantiomer of **7** should be one-half of the value determined for the D,L-mixture, making it approximately equipotent with **6** and slightly more potent than PT523.

When the abilities of **6** and **7** to inhibit [^{3}H]MTX influx were compared, the K_i of both analogs was increased 2- to 3-fold relative to PT523. Thus, in contrast to DHFR inhibition, which appeared to be relatively unaffected by this modification, replacement of CH_2 by S in both **6** and **7** was unfavorable for influx relative to PT523.

When the antiproliferative activity of the five PT523 analogs modified in the length of the amino acid moiety was examined, a quantitative correlation with the results of either the DHFR or influx assays was difficult to establish across the entire group because the data for growth inhibition had larger standard deviations in most cases than the influx or DHFR data. Nonetheless, some qualitative trends were discernible. For example, the 3.4-fold greater potency of PT523 relative to the analog **3** with one CH_2 group in the side chain appeared consistent with the 4.3-fold difference in DHFR binding and the 3.4-fold difference in influx between these two compounds.

3.3. Modification of the para-aminobenzoyl moiety

With regard to the remaining analogs in Table 1, it can be seen that substitution of a 4-amino-1-naphthoyl group for the *para*-aminobenzoyl group to give **8** produced a slight improvement in DHFR binding but a 2.5-fold decrease in RFC-mediated influx. As a result of these opposing effects, a statistically meaningful change in antiproliferative potency relative to PT523 was not observed.

Not surprisingly, in view of what appears to be a general feature of our compounds relative to the corresponding glutamate analogs, the K_i value of **8** against human DHFR was an order of magnitude lower than the value reported by Piper and coworkers [23] for the 4-amino-1-naphthoyl analog of AMT as an inhibitor of murine DHFR. Interestingly, the K_i of **8** as an inhibitor of [^{3}H]MTX influx into CCRF-CEM cells was not quite as low as the K_i for the AMT analog in L1210 cells [23]. However, a strict comparison of the two sets of results is difficult because they were obtained with enzyme and cells from different species.

3.4. Modification of the 9,10-bridge

In the case of the 10-deaza analogs **9**–**12**, it can be seen in Table 1 that substitution of CH_2 for NH at the 10-position resulted in only small changes in either influx or DHFR inhibition, although there was nevertheless a small improvement in cytotoxicity.

The effect of a branched-chain carbon at the 10-position on influx and DHFR binding was also examined. The 10-ethyl-10-deaza analog **11** was of particular interest in view of the extensive clinical trials performed in both the U.S. and Europe with EDX ([24]; for a review of the clinical literature on EDX, see Ref. [25]). The 10-propargyl-10-deaza analog **12** was similarly of interest because of recent evidence that the 10-propargyl-10-deaza analog of AMT [26], also known as PDX [27,28], may be a useful suc-

cessor to EDX. As shown in Table 1, there was very little difference in DHFR binding or RFC-mediated influx between the 10-ethyl and 10-propargyl analogs of PT523, and also no difference in the IC_{50} for cell growth. The 10-methyl analog (**10**) appeared to bind slightly better to DHFR than the 10-ethyl and 10-propargyl analogs, and was also somewhat more potent in the growth inhibition assay; however, its RFC-mediated influx was not statistically different. Interestingly, all the 10-alkyl-10-deaza analogs of PT523 were at least 10-fold better inhibitors of human DHFR than EDX, which in our hands had a K_i of 11 pM at pH 7.4 as compared with reported K_i values of 2.8 pM against the mouse enzyme at pH 7.3 [24] and 5.8 pM against the human enzyme at pH 6.9 [28]. The reason that our K_i for EDX against human DHFR was higher than that reported by the Sirotnak group is probably that our assay was carried out at a pH of 7.4, as was done with all of our other PT523 analogs [15]. This pH would probably not give optimal inhibition (cf. Refs. [29,30]), but was chosen as being physiologically more relevant. The 10-alkyl-10-deaza analogs of PT523 also seem to be better DHFR inhibitors than PDX, whose reported K_i against the human enzyme was 13 pM even at pH 6.9 [28].

With regard to influx, it can be seen from Table 1 that the 10-alkyl-10-deaza analogs of PT523 were more potent competitive inhibitors of the RFC than EDX with K_i values of 0.3–0.5 μM as compared with 1.1 μM for EDX. By using a DHFR binding assay to directly quantify the amount of drug in cell extracts, Sirotnak and coworkers determined the influx K_m of EDX at pH 6.9 to be 1.1 μM [28], which is the same as the K_i we obtained at the slightly higher pH using the [^{3}H]MTX influx competition assay. Interestingly, our K_i value for AMT (5.4 μM) was higher than the value they reported (1.2 μM) [28], suggesting that RFC-mediated influx may be more pH-dependent when the 10-position is occupied by nitrogen than by carbon. More efficient uptake at pH 6.9 than at pH 7.4 has likewise been observed with MTX in L1210 mouse leukemia cells [31]. Thus, our finding of a higher K_m for AMT influx in CEM cells at pH 7.4 than was obtained by the Sirotnak group at pH 6.9 was not unexpected. Even after allowance is made for the very different analytical methods used in the two studies, it appears that the ability of **11** to compete with [^{3}H]MTX for the RFC is indeed greater than that of EDX.

It was reported originally that the 10-propargyl group in PDX results in a 3.8-fold decrease in binding to purified DHFR from murine L1210 leukemia cells in comparison with EDX, along with a 1.8-fold decrease in RFC-mediated influx and a 1.4-fold decrease in cytotoxicity [26]. When these studies were repeated with human DHFR and the human CCRF-CEM cell line [28], the potency of PDX against DHFR was lower than that of EDX by a factor of two. The much smaller difference in DHFR binding that we observed between the 10-ethyl and 10-propargyl analogs when the side chain was N^{δ}-hemiphthaloyl-L-ornithine is difficult to explain. However, it is tempting to speculate that the extra binding energy conferred by the hydrophobic phenyl ring of hemiphthaloylornithine may have a leveling effect in comparison with the less tightly bound glutamate analogs (i.e. differences in K_i between reversible inhibitors become less distinguishable as their binding affinity comes closer and closer to "irreversibility").

Just as the 10-ethyl- and 10-propargyl-10-deaza analogs of PT523 were found to be similar in their ability to inhibit DHFR, the results in Table 1 revealed no differences at all in their influx and cytotoxicity. The apparent leveling effect seen with DHFR inhibitors may also pertain to inhibition of [^{3}H]MTX influx via the RFC. The data reported for EDX and PDX indicated a 3-fold decrease in RFC-mediated influx for the latter compound, along with a commensurate decrease in cytotoxicity after a 3-hr pulse [28]. Interestingly, we found the influx K_i of **12** to be 0.33 μM, which is almost identical to the value of 0.3 μM reported for PDX [28]. Thus, the effect of N^{10}-propargyl substitution on influx appears to be independent of whether the side chain is L-glutamic acid or N^{δ}-hemiphthaloyl-L-ornithine.

The IC_{50} values of **11** and **12** against CCRF-CEM cells were not substantially different from those of EDX, despite the fact that they were better DHFR inhibitors as well as better substrates in the [^{3}H]MTX influx assay (Table 1). Furthermore, it was of interest that the 3-fold difference in cytotoxicity reported for PDX vs. EDX against CCRF-CEM cells [28] was not observed in the case of **12** vs. **11**. It should be noted, however, that our cells were exposed continuously to drug for 72 hr, whereas in the reported work on EDX and PDX the cells were exposed for only 3 hr, then washed, re-plated, and allowed to grow out for 72–96 hr [28]. The short exposure time to EDX and PDX was chosen in order to be able to assess the cytotoxicity of the drugs under conditions where significant polyglutamation was not expected to occur. Not surprisingly, the IC_{50} reported for EDX under these conditions was two logs higher than our IC_{50} after 72 hr of continuous exposure, during which EDX would have been extensively polyglutamated. The lack of a difference in cytotoxicity between **11** and **12** is consistent with the fact that these compounds were nearly equipotent in the DHFR and RFC assay.

It should be noted that the 10-alkyl-10-deaza analogs in Table 1 were tested as mixtures of 10*R* and 10*S* stereomers, and that our data for EDX, as well as those reported for EDX and PDX [28], were likewise obtained with 10*R*/10*S* mixtures. In an important study of the effect of stereochemistry at the 10-position on DHFR binding, RFC-mediated influx, and cell growth inhibition by EDX, DeGraw and coworkers [32] found an approximately 3-fold difference in K_i against DHFR between the 10*R* and 10*S* isomers of this drug, along with a commensurate 3:1 ratio of IC_{50} values against L1210 cells. In contrast, binding by the 10*R* and 10*S* isomers of EDX to the RFC was the same. Because cellular free drug accumulation studies could not be done with the individual isomers, there was no way to assess whether the cytotoxicity difference

between the 10*R* and 10*S* stereomers was due only to differences in DHFR binding or also reflected differences in the level of free drug in the cytoplasm at steady state.

A chiral synthesis of the individual 10*R* and 10*S* isomers of the 10-alkyl-10-deaza analogs of PT523 does not exist at this time. However, relying on the cytotoxicity data published earlier for the 10*R* and 10*S* isomers of EDX as inhibitors of L1210 cell growth [31], we suspect that a resolved C10 isomer of one of the 10-alkyl-10-deaza analogs of PT523 will prove to be several times more active than PT523 itself, and could even be more active than the B-ring analogs [15,17]. Thus, a practical synthetic route allowing biochemical and biological evaluation of the individual 10*R* and 10*S* isomers of the 10-alkyl-10-deaza analogs of PT523 (e.g. **10**) would be of interest.

3.5. Dual modification of the B-ring and 9,10-bridge

The recent availability of **13** [19] made it possible to determine how replacement of N^5 and N^8 in **9** by carbon might affect interaction with the RFC. As shown in Table 1, the K_i of **13** as an inhibitor of [^{3}H]MTX influx into CCRF-CEM cells had a strikingly low value of 0.21 μM as compared with 0.41 μM for **9** and 0.71 μM for PT523. Indeed, the 5,8,10-trideaza compound was the best inhibitor of [^{3}H]MTX influx via the RFC of any of the PT523 analogs that we have studied to date, including those modified in the B-ring [18].

In addition to its efficient utilization of the RFC for influx, **13** was also found to be a potent DHFR inhibitor, with a K_i of 0.21 pM as compared with 0.35 pM for **9** (Table 1). It thus appears that replacement of the B-ring nitrogen atoms in **9** enhances both influx and DHFR inhibition. It was somewhat surprising, therefore, that **13** was not more active than **9** in the cell growth assay as well. Again, however, this minor inconsistency may be due to differences in the rate of efflux of non-bound drug after all of the binding sites in the cell have been saturated.

An important factor to be considered with regard to net cellular accumulation of PT523 and its analogs, and therefore their ability to arrest cell growth in comparison with other antifolates that utilize the RFC for influx, is how efficiently these compounds efflux from the cell once DHFR is saturated. Recent years have witnessed a significant appreciation of the ability of several members of the multidrug resistance protein (MRP) family of ATP-dependent pumps to mediate the efflux of antifolates from various types of cells (reviewed in Ref. [33]). However, CCRF-CEM cells have not been used in these studies, and little is actually known about the role the MRP efflux pumps may play in regulating the level of antifolate drugs in CCRF-CEM cells that have not been transfected with MDR genes or purposely selected for resistance to antifolates or other drugs. Although an assessment of the ability of PT523 and other nonpolyglutamatable analogs to serve as substrates for the various MRP pumps would be of considerable interest, such studies were outside the scope of the present work.

Overall, the lesson that may be drawn from this paper and our earlier work on B-ring analogs [18] is that substitution of L-glutamic acid for N^{δ}-hemiphthaloyl-L-ornithine in the classical DHFR inhibitors seems to invariably lead to a substantial enhancement of both DHFR inhibition and RFC-mediated influx. As a result, a dramatic increase in cytotoxic potency is observed despite the fact that these compounds, unlike the corresponding L-glutamate analogs, are not converted intracellularly to polyglutamates. While PT523 itself was chosen as the first candidate for clinical trial in this series because there was a significant amount of preclinical data already available, the library of second-generation analogs created during this research affords a rare and valuable opportunity to examine the contributions of DHFR binding and RFC-mediated influx to the pharmacodynamics of classical DHFR inhibitors in the absence of polyglutamation.

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