Biochemical Pharmacology, Vol. 31, No. 2, pp. 255–256, 1982.
Printed in Great Britain.

0006-2952/82/020255-02 $03.00/0

Effect of 11-oxahomofolate and its reduced derivatives on human dihydrofolate reductase and on human cells having different amounts of dihydrofolate reductase*

(*Received* 15 *April* 1981; *accepted* 15 *July* 1981)

One of the mechanisms of cellular resistance to the anticancer agent, methotrexate, is an increased level of dihydrofolate reductase (EC 1.5.1.3). One approach to overcome this resistance, first postulated by Misra *et al.* [1], is to design analogs that require reduction by dihydrofolate reductase to exert their cytotoxic effects. Such a compound would be able to take selective advantage of the increased levels of this enzyme in MTX-resistant cells, providing that the amount of dihydrofolate reductase is the major rate-limiting biochemical determinant of its action. Recently, our laboratories have synthesized 11-oxahomofolate which was found to be a substrate for dihydrofolate reductase from bacterial sources and which could inhibit *Streptococcus faecium* and *Lactobacillus casei* cell growth in culture [2]. We examined the behavior of this compound and its reduced derivatives on the growth of human cells which have different levels of dihydrofolate reductase (DHFR).

The cell lines in this study were human KB parental cells (KBP) and a 6500-fold methotrexate (MTX) resistant cloned subline (KB/6b) having a 40-fold increase in DHFR activity developed in this laboratory. Cells were maintained in monolayer in RPMI 1640 medium containing 100 μg kanamycin/ml and supplemented with 5% fetal calf serum. Cells were seeded in 25 cm^2 flasks, and the drugs were added after 24 hr to ensure that the cells were attached and in log phase. The cell number was determined at the time of drug additions and 48 hr later by hemocytometer counts at which time the cells had undergone two doublings. All cell numbers were determined by an average of at least two flasks for each condition. Spectral data in RPMI 1640 at 37° for 35 hr showed no decomposition of 11-oxahomofolate and approximately 31% decomposition of H_2-oxahomofolate.

Homofolate (NSC 79249) was included in the study for comparative purposes and was provided by Dr. Roy L. Kisliuk of Tufts University School of Medicine, Boston, MA. 7,8-Dihydro-homofolate (H_2-homofolate) and 7,8-dihydro-11-oxahomofolate (H_2-oxahomofolate) were synthesized by dithionite reduction according to a published procedure [3]. 1-L-5,6,7,8-Tetrahydro-11-oxahomofolate (H_4-oxahomofolate) was prepared by the enzymatic reduction of the dihydro derivative by KB/6b DHFR and purified by Sephadex 6-25 chromatography with 0.1 N ammonium bicarbonate containing 1% β-mercaptoethanol followed by lyophilization. All compounds used in cell culture were in phosphate-buffered saline and the concentrations were determined by u.v. absorption at their respective maxima [2, 4].

Table 1 shows the effects of 11-oxahomofolate and its reduced derivative on cell growth for the KBP and KB/6b cell lines. The DHFR activity in the KB/6b cell line was increased 40-fold and this was accompanied by a 6500-fold resistance to MTX. Both 11-oxahomofolate and H_2-oxahomofolate showed little cytotoxicity in these cell lines (Table 1), the ID_{50} values being > 100 μM and 100 μM, respectively, and there was no difference in their effects on the two lines. Homofolate has also been shown to be equally potent in murine cells having different levels of DHFR [5]. Several possibilities could account for the low degree of cytotoxicity of these compounds, one being poor substrate activity with human DHFR. Therefore, we next looked at their effects on purified human DHFR from the cells. Enzyme was extracted and purified as previously described [6], and the kinetic properties of the enzyme with these analogs are shown in Table 2. Kinetic constants for dihydrofolate (H_2-folate), NADPH, MTX and H_2-homofolate are included for comparison. There was no difference between the DHFR from the parental and MTX-resistant cells in K_m values for NADPH and H_2-PteGlu and K_i value for MTX. The dihydro derivatives of homofolate and 11-oxahomofolate were substrates for human DHFR with K_m values of 5.2 μM and 4.0 μM respectively. The substitution of oxygen for nitrogen at the 11 position had no effect on the K_m of H_2-homofolate but the V_{max} was decreased by a factor of 2. Non-reduced homofolate and 11-oxahomofolate did not show substrate activity with human DHFR but they competitively inhibited the reduction of H_2-PteGlu, and the K_i values are shown in Table 2. This evidence of binding to the enzyme without equivalent catalytic activity is also seen with folic acid. A second possibility for poor cytotoxicity could be due to the product of the reduction, H_4-oxahomofolate, being a non-toxic metabolite in cells. We examined the cytotoxicity of the reaction product, 1-L-H_4-oxahomofolate on both cell lines at 10 μM and observed no inhibition of cell growth. A third reason for the low degree of cytotoxicity of H_2-oxahomofolate could be diminished permeability of this compound in cells and without labeled compounds we could not evaluate this possibility.

In summary, the analogs 11-oxahomofolate and H_2-oxahomofolate show little toxicity in these human cell lines. However, they did not show cross resistance with MTX in the KB/6b cell line. The dihydro derivative is a substrate for human DHFR and the substitution of nitrogen by oxygen could still sustain the substrate activity with a

* Supported by Grants CA 27364 and CA 27101 from NCI, USPHS.

Table 1. Inhibition of cell growth, ID_{50} values*

Cell line	DHFR activity [μmoles·min^{-1}·(mg protein)$^{-1}$]	ID_{50} (μM) MTX	11-Oxahomofolate	H_2-oxahomofolate
KBP	0.007	0.01	> 100 (18%)	100
KB/6b	0.284	65	> 100 (18%)	100

* Experimental details are described in the text. The ID_{50} value is the concentration that causes 50% inhibition of cell growth. Numbers in parentheses are the percent inhibition of cell growth.

Table 2. Kinetic behavior of homofolate and 11-oxahomofolate and their reduced derivatives on human DHFR*

Enzyme source	K_m (μM)				V_{max} ratio	
					H_2-homofolate	H_2-oxahomofolate
	H_2-folate	NADPH	H_2-homofolate	H_2-oxahomofolate	H_2-folate	H_2-folate
KBP	0.7	5.9				
KB/6b	0.7	5.9	5.2	4.0	0.77	0.32
	K_i (μM)					
	MTX	Folate		Homofolate	11-Oxahomofolate	
KBP	5×10^{-6}					
KB/6b	7×10^{-6}	2.5×10^{-2}		7.5	33.0	

* Enzyme assays were at 37°, pH 7.5, in the presence of 0.15 M KCl. K_m and V_{max} values were determined by varying the concentration of each substrate with a fixed saturating concentration of NADPH (100 μM) and analysis of the data by Lineweaver–Burk plots. For V_{max} comparisons, the same enzyme preparation was used for all substrates. The K_i value for MTX was determined by the method of Cha [7, 8] as previously described [6], and the K_i values for the other compounds were determined by standard Lineweaver–Burk analysis.

decrease in reduction rate. Providing the compound and its reduced derivatives are able to be transported into cells, the results suggest that the reduced tetrahydro form may not be toxic enough to interfere with cellular metabolism. A second generation of compounds should be synthesized. Several considerations should be kept in mind in accordance with the aim of taking advantage of a high activity of DHFR for the cytotoxic effect. The tetrahydro form has to be toxic and DHFR has to be the rate-limiting enzymatic process. The binding affinity of the analog to enzyme should not be tight. Otherwise the compound will be tied up by the enzyme. Nevertheless, the lack of cytotoxicity of these non-reduced analogs on human cells ($ID_{50} > 100$ μM) and the potent activity against *S. faecium* and *L. casei* suggest that 11-oxahomofolate could be considered as an antibacterial agent. Since these organisms require folate for growth, we tested 11-oxahomofolate and H_2-oxahomofolate against a non-folate requiring bacterium, *Escherichia coli* K12, MC4100. At 100 μM, there was no inhibition of growth in minimal medium. However, these compounds may have a limited usefulness against folate requiring bacteria.

Department of Pharmacology
University of North Carolina
at Chapel Hill
Chapel Hill, NC 27514, U.S.A.

BARBARA A. DOMIN
YUNG-CHI CHENG*

Department of Biochemistry
College of Medicine
University of South Alabama
Mobile, AL 36688, U.S.A.

M. G. NAIR

REFERENCES

1. D. K. Misra, S. R. Humphreys, M. Friedkin, A. Goldin and E. J. Crawford, *Nature, Lond.* **189**, 39 (1961).
2. M. G. Nair, C. Saunders, S.-Y. Chen, R. L. Kisliuk and Y. Gaumont, *J. med. Chem.* **23**, 59 (1980).
3. M. G. Nair, S.-Y. Chen, R. L. Kisliuk, Y. Gaumont and D. Strumpf, *J. med. Chem.* **22**, 850 (1979).
4. L. Goodman, J. DeGraw, R. L. Kisliuk, M. Friedkin, E. J. Pastore, E. J. Crawford, L. T. Plante, A. Al-Nahas, J. F. Morningstar, Jr., G. Kwok, L. Wilson, E. F. Donovan and J. Ratzan, *J. Am. chem. Soc.* **86**, 308 (1964).
5. C. A. Nichol and M. T. Hakala, *Biochem. Pharmac.* **15**, 1621 (1966).
6. B. A. Domin, Y.-C. Cheng and M. T. Hakala, in *Chemistry and Biology of Pteridines* (Eds. R. L. Kisliuk and G. M. Brown), p. 395 Elsevier, North Holland, Amsterdam (1979).
7. S. Cha, *Biochem. Pharmac.* **24**, 2177 (1975).
8. S. Cha, R. R. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 2187 (1975).

* Author to whom all correspondence should be addressed.