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Transport and Inhibitory Activity of New Folate Analogues in HeLa Cells

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SUMMARY

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Evidence supporting the existence of distinct transport systems for folate and methotrexate (MTX) in HeLa cells has been presented. Folate and MTX were observed not to compete with each other in competitive transport experiments in HeLa cells. The selective inhibition of folate and/or MTX transport by folate analogues further substantiated the existence of separate transport systems. 10-Oxaaminopterin inhibited the uptake of both MTX and folate, whereas 10-thioaminopterin selectively inhibited MTX uptake. The thio and oxa analogues of folic acid effectively inhibited folate transport and had a negligible effect on MTX transport. In comparison with the inhibitory activity of MTX, the oxa and thio analogues of aminopterin had a high level of antifolate activity as determined by inhibition of the incorporation of [2-^{14}C]deoxyuridine into acid-precipitable DNA and cell growth. The potential use of 10-oxaaminopterin in the treatment of MTX-resistant forms of cancer is discussed in relation to its unique transport characteristics and its high antifolate activity.

INTRODUCTION

The number of transport systems for folate coenzymes in mammalian cell lines has been the subject of active investigation in recent years (1-5). Folic acid and 5-methyltetrahydrofolic acid do not compete in L1210 murine leukemia cells (2). The transport of 5-methyltetrahydrofolic acid is inhibited by MTX[2] in these cells, suggesting two distinct pathways for folate and reduced folates (2). In support of this view, Huennekens and Henderson (6) have shown that the MTX transport system is sensitive to sulfhydryl inhibitors like *p*-chlorophenylmercurisulfonate, whereas the folate transport system was insensitive in L1210 leukemia cells.

MTX resistance in tumor cells is associated with impaired transport of the drug as well as increased levels of dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3) (7, 8). Kessel *et al.* (9) have shown that a good correlation exists between the transport of MTX into leukemia cells *in vitro* and antitumor ac-

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[2] The abbreviations used are: MTX, methotrexate; TA, 10-thioaminopterin, *N*-{*p*-[[(2,4-diamino-4-deoxy-6-pteridinyl)methyl]thio]benzoyl}-L-glutamic acid; OA, 10-oxaaminopterin, *N*-[α-(2,4-diamino-4-deoxy-6-pteridinyl)-*p*-anisoyl]-L-glutamic acid; TFA, 10-thiofolic acid, *N*-{*p*-[[(2-amino-4-hydroxy-6-pteridinyl)methyl]thio]benzoyl}-L-glutamic acid; OFA, 10-oxafolic acid, *N*-[α-(2-amino-4-hydroxy-6-pteridinyl]-*p*-anisoyl]-L-glutamic acid.

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tivity against transplantable mouse leukemias. A decrease in transport of MTX was shown to be associated with an increase in the oncogenicity of the leukemic cells. An antimetabolite capable of entering cells via the folate as well as the reduced folates/MTX pathways could be effective in the treatment of MTX-resistant tumors and be used in combination with MTX in the treatment of nonresistant forms of cancer. Mishra *et al.* (10) suggested that analogues that can act as substrates for dihydrofolate reductase and that can interfere with tetrahydrofolate utilization would be capable of selective toxicity in MTX-resistant forms of cancer.

It was then of interest to design and test (a) potential antimetabolites capable of entering the cell solely by the folate or both the folate and reduced folates/MTX transport systems and (b) analogues that could enter the cell by the folate pathway and act as substrates for dihydrofolate reductase. We have delineated the transport characteristics of 10-thioaminopterin, 10-oxaaminopterin, 10-thiofolic acid, and 10-oxafolic acid and determined the antifolate activity of these compounds as monitored by the inhibition of DNA synthesis and cell growth. Both the thio and oxa analogues of aminopterin are powerful inhibitors of dichloromethotrexate-resistant *Lactobacillus casei* dihydrofolate reductase (11, 12). The thio and oxa analogues of folic acid were designed as substrates for dihydrofolate reductase (13, 14). Only 10-thiofolic acid has substrate activity.

MATERIALS AND METHODS

Materials. [^{3}H]Folic acid (47 Ci/mmole) and [^{3}H]MTX (250 mCi/mmole) were purchased from Amersham/Searle, and [2-^{14}C]deoxyuridine (58.1 mCi/mmole), from New England Nuclear. Folic acid was purchased from Sigma, and MTX was generously supplied by Dr. Charles Baugh, University of South Alabama. Both folic acid and MTX were repurified by DEAE-cellulose chromatography. 10-Thioaminopterin, 10-oxaaminopterin, 10-thiofolic acid, and 10-oxafolic acid were synthesized in our laboratory as previously described (11–14).

Cells. HeLa cells were purchased from Flow Laboratories and maintained in monolayer cultures. The cells were grown in Eagle's minimum essential medium containing 2.2 μM folic acid and supplemented with 10% newborn calf serum, 50 units/ml of pencillin, and 50 μg/ml of streptomycin. The pH of the medium on the monolayers was maintained with a bicarbonate buffer in tightly closed flasks or Blake bottles. The cells were tested for mycoplasma contamination by Flow Laboratories. The cultures used throughout these studies were prepared as follows. Monolayers of cells in several Blake bottles were treated with 0.25% trypsin, pooled, and reseeded into several 75-cm^2 flasks. Each flask contained approximately 2.5×10^6 cells. On the following day the medium was replaced with 25 ml of fresh medium. The cultures were then incubated at 37° for 48 hr prior to use, so that cell surfaces altered by the trypsin could regenerate to a normal state *in vitro*.

Uptake of radiolabeled folate or MTX. The transport studies were performed with cultures prepared as described above. The cell sheets were washed twice with warm minimal essential medium without serum to remove residual serum from the monolayers. The growth medium was replaced with 10 ml of minimal essential medium without serum. Radiolabeled folate or MTX was then added to the cultures, which were incubated at 37° for various time intervals as described later. At the end of the incubation period the medium was discarded and the cultures were washed twice with ice-cold phosphate-buffered NaCl to stop further incorporation of the radioisotope. The cells were harvested with a rubber policeman, pelleted by centrifugation at $150 \times g$ for 10 min, and solubilized in 0.5 ml of Unisol (Isolab) tissue solubilizer. Ten milliliters of Unisol complement were added to each sample. The radioactivity associated with the cells was determined in a Beckman (LS31SOT) scintillation spectrophotometer.

Effects of analogues on transport of

[^{3}H]folate and [^{3}H]MTX. Monolayers of HeLa cells were prepared in 75-cm^2 flasks and washed to remove the majority of the serum proteins as described above. The medium was replaced with minimal essential medium without serum. The 10-oxa and 10-thio analogues of aminopterin and folic acid were added to the cells in the desired concentration, and 0.82 μM radiolabeled folic acid or MTX was simultaneously added to each flask. As will be shown, this concentration is near or below the transport K_m (K_t) values of folate and MTX. The cultures were incubated at 37° for 10 min, harvested, and pelleted by centrifugation. The cells were solubilized in tissue solubilizer, and the amount of radioactivity associated with the cells was determined as described above.

Effects of analogues on cell growth. Monolayers of HeLa cells in 75-cm^2 flasks were incubated for 48 hr in the presence of the desired concentration of each analogue. At the end of the incubation period the cultures were treated with trypsin, and the number of cells in each flask was determined by counting with a hemocytometer.

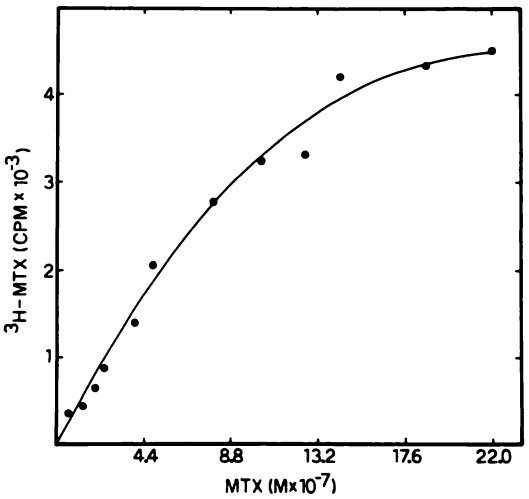

FIG. 1. *Transport of MTX into HeLa cells as a function of concentration*

Duplicate cultures of HeLa cells (2.0 × 10^7 cells) were incubated in 10 ml of minimal essential medium without serum and increasing concentrations of [^{3}H]MTX (250 mCi/mmole) for 10 min at 37°. At the end of the incubation period the cells were washed twice with ice-cold phosphate-buffered NaCl, harvested, and solubilized in Unisol tissue solubilizer. The radioactivity associated with the cells was determined by liquid scintillation spectrophotometry.

RESULTS

Radiolabeled folate and MTX (0.82 and 0.8 μM, respectively) were rapidly transported into HeLa cells in a linear fashion throughout a 25-min exposure. A 10-min incubation period was chosen as the end point for all subsequent transport studies, since incorporation of MTX and folate is linear and no detectable intracellular metabolites of MTX accumulate during this time interval (15). The rates of [^{3}H]MTX and [^{3}H]folate uptake are linear at low concentrations (Figs. 1 and 2) and reach saturation at the higher concentrations. The K_t values for folate (16.3 μM) and MTX (0.8 μM) in HeLa cells were calculated from Lineweaver-Burk plots for each substrate, with correlation coefficients of 0.93 and 0.98, respectively. The reported K_t for MTX is 3–10 μM in L1210 leukemia cells (1) and 2 μM in rabbit reticulocytes (16). The K_t for folate has been reported to be 200 μM in L1210 leukemia cells (1).

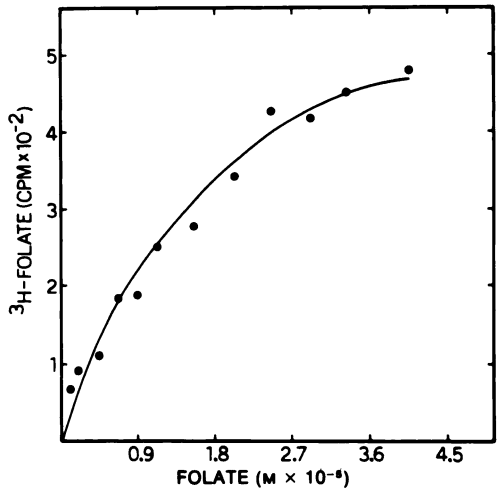

FIG. 2. *Transport of [^{3}H]folate into HeLa cells as a function of concentration*

Duplicate cultures of HeLa cells (2.0 × 10^7 cells) were incubated in 10 ml of minimal essential medium without serum and increasing concentrations of [^{3}H]folate (0.15 mCi/mmole) for 10 min at 37°. At the end of the incubation period the cells were treated as described in the legend to Fig. 1.

To determine whether HeLa cells have one or more transport systems for folic acid and MTX, monolayers of cells were incubated in the presence of a constant amount of [^{3}H]MTX and increasing concentrations of folic acid. A MTX concentration of 0.8 μM was chosen because this is its transport K_m. At the end of a 10-min incubation the amount of radioactivity associated with the cells was determined. A 100-fold increase in folic acid concentration did not affect the influx of [^{3}H]MTX into HeLa cells (Table 1). When the amount of radiolabeled folic acid remained constant (0.82 μM) and the concentration of MTX was varied over the same range, similar results were obtained. Thus MTX did not inhibit the influx of radiolabeled folic acid into HeLa cells, and vice versa. These findings suggest that folic acid and MTX enter HeLa cells by separate transport pathways.

The folate analogues used in this study (11–14) belong to the classical series of antifolates with alterations at the C^9-N^{10} bridge region, and their structures are shown in Fig. 3. The effects of these analogues on the influx of [^{3}H]folate and [^{3}H]-MTX in HeLa cells were studied to determine the pathway used by these compounds to enter the cells. Monolayers of HeLa cells were incubated in the presence of a constant amount of [^{3}H]folate and increasing concentrations of each of the compounds. After 10 min the cultures were

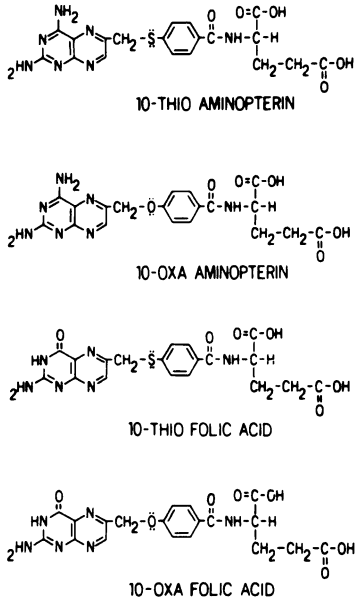

FIG. 3. *Chemical structures of folate analogues*

harvested and the amount of radioactivity associated with the cells was determined (Fig. 4A and Table 2). Three of the compounds, OA, TFA, and OFA, inhibited [^{3}H]folate uptake by 50% at concentrations as low as 6.0, 7.8, and 7.8 μM, respectively. As much as 71.0 μM TA was required to produce the same effect. Similar competitive experiments were carried out with radiolabeled MTX (Fig. 4B and Table 2). The thio and oxa analogues of aminopterin, TA and OA, competed effectively with [^{3}H]MTX, whereas the thio and oxa analogues of folic acid, TFA and OFA, were considerably less effective in inhibiting the influx of radiolabeled MTX into the cells. Both TA and OA inhibited the uptake of [^{3}H]MTX by 50% at concentrations of 2.7 and 0.74 μM, respectively. In the concentration range used in these studies the maximum inhibition of folate and MTX transport attained by the four analogues was betwen 80% and 90%.

The effects of the oxa and thio analogues of folic acid and aminopterin on the incorporation of [2-^{14}C]deoxyuridine into acid-precipitable DNA were determined. Monolayers of HeLa cells were incubated for 48 hr in the presence of [2-^{14}C]deoxyuridine, the precursor of thymidylate, and selected

TABLE 1

Effect of folate on MTX uptake in HeLa cells

Monolayers of HeLa cells (2×10^7 cells) were incubated in 10 ml minimal essential medium, without serum and containing 8.0 μM [^{3}H]MTX and the indicated concentrations of folate, for 10 min at 37°. At the end of the incubation the cells were washed with cold phosphate-buffered NaCl and processed as described in the legend to Fig. 1.

^{3}H-MTX (8.0×10^{-7} M)	folate (M$\times10^{-6}$)	CPM incorp. in 2×10^7 cells
+	--	2144
+	.7	2333
+	3.9	2291
+	20.4	2321
+	40.8	2153
+	54.4	2252
+	74.8	2231

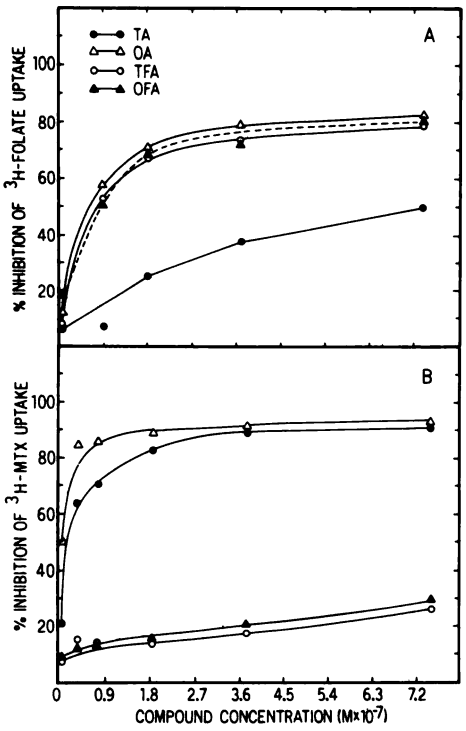

FIG. 4. *Effects of analogues on influx of [^{3}H]folate and [^{3}H]MTX into HeLa cells*

A constant amount of [^{3}H]folate (0.82 μM) or [^{3}H]MTX (0.8 μM) and the indicated concentrations of each of the four analogues were simultaneously added to monolayers of HeLa cells (2.8 × 10^7 cells). The cultures were incubated for 10 min at 37° in essential medium without serum. At the end of the incubation period the cells were washed with ice-cold phosphate-buffer NaCl and processed as described in the legend to Fig. 1. The incorporation of MTX and folate in the uninhibited controls was 3.4 × 10^3 and 2.8 × 10^3 cpm, respectively.

TABLE 2

Effects of analogues on influx of [^{3}H]folate and [^{3}H]MTX into HeLa cells

	Folate Analogs ID_{50} (M×10^6)			
	TA	OA	TFA	OFA
^{3}H-MTX	2.7	0.74	71.0	71.0
^{3}H-folate	71.0	6.0	7.8	7.8

concentrations of the analogues. At the end of the incubation period the cultures were harvested and the acid-precipitable radioactivity incorporated into DNA was determined as described in the legend to Fig. 5. The effect of MTX on cellular DNA synthesis was included in these experiments to determine the effectiveness of the four folate analogues relative to that of MTX. Both TA and OA inhibited the incorporation of [2-^{14}C]deoxyuridine into acid-precipitable counts by 50% at concentrations of 700 and 85 nM, respectively (Fig. 5 and Table 3). TFA produced the same effect at 10 μM, and OFA did not cause any significant effect. The ID_{50} values of OA and MTX are similar, indicating

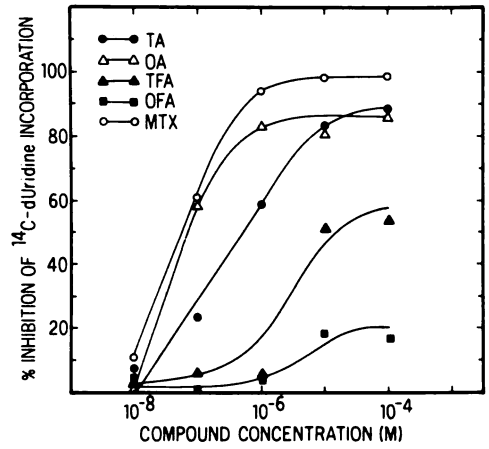

FIG. 5. *Effects of folate analogues on DNA synthesis*

[2-^{14}C]Deoxyuridine (0.25 μCi) and the indicated concentrations of each analogue were added to semiconfluent monolayers of HeLa cells (1 × 10^7 cells) and incubated at 37° for 48 hr. At the end of the incubation period the cultures were washed with ice-cold phosphate-buffered NaCl, harvested, and pelleted. The cells were resuspended in 2.5 ml of distilled water. The DNA was precipitated with 0.25 N perchloric acid and collected on 0.45-μm Millipore filters. The acid-precipitable counts were determined by liquid scintillation spectrophotometry. The incorporation of [2-^{14}C]deoxyuridine in the uninhibited controls was 1.5 × 10^5 cpm.

TABLE 3

Effects of folate analogues on DNA synthesis and cell growth

Folate Analogs	DNA Synthesis ID_{50} (M)	Cell Growth ID_{50} (M)
MTX	7.5×10^{-8}	1.0×10^{-8}
OA	8.5×10^{-8}	7.5×10^{-8}
TA	7.0×10^{-7}	1.5×10^{-6}
OFA	$>10^{-4}$	$>10^{-4}$
TFA	1.0×10^{-5}	$>10^{-4}$

that OA is as potent as MTX in inhibiting DNA synthesis.

Parallel studies were done to determine the effects of the four analogues and MTX on cell proliferation. Monolayers of HeLa cells were exposed to selected concentrations of each analogue or MTX and incubated at 37° for 48 hr. At the end of the incubation period the cells were treated with trypsin and counted with a hemocytometer. OA was as effective as MTX in inhibiting HeLa cell proliferation (Fig. 6 and Table 3). TA was also effective in inhibiting cell growth, but required a higher concentration. TFA did show some inhibitory effect on cell growth, but the maximum inhibition obtained was 45% at 100 μM. As expected, OFA did not affect cell growth.

DISCUSSION

Whether one or two transport systems exist in mammalian cells for folate and reduced folates/MTX has been the subject of recent investigation. Using mathematical models, Goldman (1) concluded that L1210 leukemia cells have one transport system. However, he left open the possibility that two transport systems with similar K_t values could be operative in these cells. By chemically modifying the receptors for MTX transport, Huennekens and Henderson (6) showed that MTX transport was sensitive to *p*-chlorophenylmercurisulfonate but that folate transport was not affected, suggesting the existence of separate transport systems. Competitive transport studies between folate and MTX in our cell system show that neither competed with the other when the concentration of MTX was kept constant and the concentration of folate was varied over 100-fold (Table 1), and vice versa. Similar observations were made by Huennekens and Henderson in L1210 murine leukemia cells (6). These results support the existence of two separate transport systems in HeLa cells. Similar competitive transport studies between the four folate analogues and radiolabeled folate or MTX show that (a) the oxa and thio analogues of folic acid use the folate pathway preferentially, (b) one of the analogues of aminopterin (TA) uses the reduced folates/MTX pathway preferentially, and (c) OA uses both pathways efficiently (Fig. 4 and Table 2). The findings we report using classical folate analogues capable of competing with either radiolabeled folate or MTX as well as with both provide strong evidence for the existence of two separate transport systems for folate and reduced folates/MTX in HeLa cells.

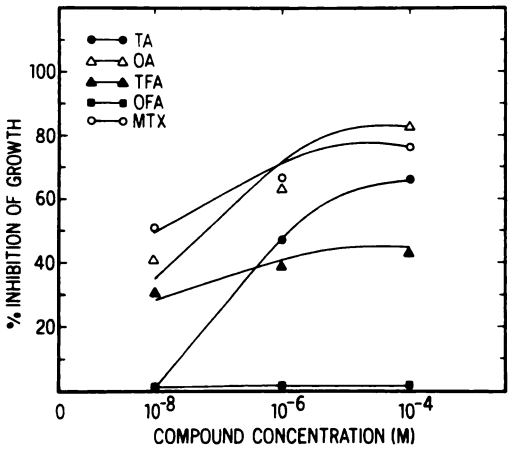

FIG. 6. *Effects of folate analogues on growth of HeLa cells*

Monolayers of cells (1×10^7 cells) were incubated for 48 hr in the presence of the indicated concentrations of each analogue. At the end of the incubation the cultures were treated with trypsin and the number of the cells in each flask was determined by counting with a hemocytometer. Percentage inhibition represents the difference in cell number between cells treated with the analogues and untreated cells at 48 hr. Each point represents the average of duplicate samples.

The MTX transport system of MTX-resistant tumor cell lines has been shown to be defective by many investigators (7, 8, 17, 18). An antimetabolite capable of entering the cell by either transport system could be used in the chemotherapy of MTX-resistant forms of cancer. Competitive experiments between OA and radiolabeled folate or MTX show that the analogue is very effective in competing with both compounds at low concentrations [ID_{50} = 0.74 μM for MTX and 6.0 μM for folate (Fig. 4 and Table 2)], demonstrating that it uses both transport systems. OA is as effective as MTX in inhibiting HeLa

cell DNA synthesis (Fig. 6), thus showing potential usefulness in the treatment of MTX-resistant forms of cancer. Although TA is biologically active (Figs. 5 and 6), it apparently offers no additional advantage over MTX, since its transport characteristics are similar to those of MTX.

Quite recently Bertino *et al.* (19) and Huennekens and Henderson (6) raised the possibility of folate depletion as an alternative approach to cancer chemotherapy. In an earlier report Kisliuk and Gaumont (20) showed that homofolic acid exerts antimetabolic activity in bacterial systems by blocking folate transport. Both TFA and OFA effectively inhibited the transport of radiolabeled folate into HeLa cells [ID_{50} = 7.8 μM for both (Fig. 4 and Table 2)]. The pteridine moiety of TFA and OFA is the same as that of folic acid, and thus their ability to share the folate transport system is understandable. TFA inhibited DNA synthesis by 50% at 10 μM, but OFA did not show any antifolate activity (Fig. 5 and Table 3). Thus both TFA and OFA have the ability to block folate transport, but only TFA has demonstratable antifolate activity. Experiments *in vitro* carried out to determine the ability of TFA to serve as a substrate of partially purified dichloromethotrexate-resistant *L. casei* dihydrofolate reductase have shown that the 7,8-dihydro form of TFA has weak substrate activity (10, 12). It is not known whether the relatively low antifolate activity observed for TFA was indeed due to the conversion *in vivo* of TFA to its tetrahydro form and subsequent interference with tetrahydrofolate utilization or resulted from a blockage of folate transport. The results obtained with OFA support the former hypothesis.

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