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Simultaneous Activity of Two Different Mechanisms of Folate Transport in Ovarian Carcinoma Cell Lines

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Abstract We investigated whether the folate receptor α-isoform (FRα), which is overexpressed on ovarian carcinoma cells, is functionally active in internalizing the physiological form of folate, 5-methyl tetrahydrofolate (THF). Six ovarian tumor cell lines, expressing different levels of FRα (COR ≫ OVCAR3 > IGROV1 > OVCAR4 > SKOV3 > OVCAR5), were maintained in folate-depleted medium and internalization of 10 nM evaluated as acid-resistant radioactivity at 0° and 37°C. The amount of 5-methyl[^{3}H]THF present in this fraction was not strictly related to the number of membrane receptors, since even cell lines with low FRα expression, e.g., OVCAR4, showed efficient internalization. Time-course studies indicated that, whereas no uptake was detected at 0°C, at 37°C the internalized fraction showed a slow and constant increase, until 4 h. At this time, the internalized radioactivity represented <50% of the total bound in COR, OVCAR3 and IGROV1 cells, whereas the other cell lines tested internalized fourfold more folate than their surface binding capacity. The incubation in the presence of a concentration (50 nM) of 5-methyl[^{3}H]THF, which best ensures receptors saturation on cells with highest FR levels (COR and OVCAR3), had slight effect on surface binding of all the tested cell lines, including IGROV1 and SKOV3. In contrast, the increase of the uptake was more pronounced, particularly in SKOV3 cells. These results, together with the accumulation curves of folic acid (FA) and 5-methylTHF at 37°C, suggested the presence of a molecule on ovarian carcinoma cells with high affinity for reduced folates, possibly a reduced folate carrier (RFC). Measurement of radioactivity present in the supernatant of IGROV1 and SKOV3 cells, subjected to hypotonic lysis and cell fractionation, further indicated that 5-methyl[^{3}H]THF was translocated to the cytosol and, despite differences in membrane levels of FRα expression this internalized fraction was similar in both cell lines. Inhibition experiments to selectively block FRα or RFC activity showed a differential sensitivity of the two pathways depending on the cell line examined. Internalization was more consistently inhibited on IGROV1 than on SKOV3 cells by treatments that disrupt FRα activity, e.g., incubation with excess FA and phosphatidylinositol specific phospholipase C, whereas Probenecid, which preferentially inhibits the carrier-mediated pathway, showed a strong inhibitory effect on both cell lines. These findings suggest that the internalization of 5-methylTHF in these tumor cells depends not only on the level of overexpressed FRα, but another transport route, with features characteristic for RFC, is functional and participates in folate uptake. J. Cell. Biochem. 65:479–491. © 1997 Wiley-Liss, Inc.

Key words: folate receptor; folate uptake; reduced folate carrier; ovarian carcinoma cells

Folate receptors (FRs), previously referred to as membrane-associated, folate-binding proteins (FBP), represent a family of glycoproteins that participate in folate binding and uptake. Four isoforms α, β, γ, and γ′, have been identified and characterized [Elwood, 1989; Lacey et al., 1989; Ratnam et al., 1989; Sadasivan et al., 1989; Shen et al., 1994]. cDNA cloning has shown that they are 70–80% identical but, due to differences in the carboxy-terminal segment, the proteins are differentially processed and can be membrane bound (α and β) [Elwood, 1989; Lacey et al., 1989; Luhrs, 1989; Sadasivan et al., 1989; Verma et al., 1992] or secreted (γ and γ′) [Shen et al., 1994, 1995]. FR isoforms have a selective tissue specificity on normal and tumor cells [Mantovani et al., 1994; Ross et al., 1994; Shen et al., 1994; Weitman et al., 1992a; Weitman et al., 1992b], exhibit different affinity and stereospecificity for folate compounds and antifolates [Wang et al., 1992], and probably play different roles in folate metabolism. The α isoform (FRα) is a 38–40 kDa, GPI-anchored protein [Lacey et al., 1989; Luhrs, 1989; Alberti et al., 1990] that binds FA with

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high affinity. The protein is present at very low levels on normal epithelial tissues, at medium levels on kidney, lung, and breast, and at high levels on placenta and choroid plexus [Weitman et al., 1992a; Weitman et al., 1992b; Mantovani et al., 1994; Ross et al., 1994]. Overexpression of FRα has been detected on epithelial tumors derived from the ovary and uterus and on tumors derived from the nervous system [Campbell et al., 1991; Coney et al., 1991; Weitman et al., 1992; Ross et al., 1994]. FRα appears to be directly involved in the uptake of the physiological form of folate present in the plasma, 5-methyl tetrahydrofolate (THF) [Kamen et al., 1988; Rothberg et al., 1990]. Several studies on cell lines of normal or tumor origin have demonstrated the simultaneous presence of FRα and another folate transport route with higher affinity for reduced folates than folic acid, the RFC, [Westerhof et al., 1991, 1995; Dixon et al., 1992] and in some cases their functional association [Kamen et al., 1991; Prasad et al., 1994]. In the same and other cell lines, it also has been reported that FRα expression levels can be modulated not only by decreasing the external folate concentration [McHugh et al., 1979; Kane et al., 1988; Jansen et al., 1989] but also by retinoids and steroids [Orr et al., 1995b]. Increased levels of FRα enhance survival of the cells in conditions of limiting folate concentrations [Dixon et al., 1992; Luhrs et al., 1992; Matsue et al., 1992; Bottero et al., 1993; Chung et al., 1993]. In addition, we showed that FRα provide a growth advantage in vivo to transfected cells [Bottero et al., 1993], suggesting a functional role for the receptor in cell growth.

To analyze the activity of FRα expressed on tumor cells, we focused on ovarian carcinomas, which overexpress this molecule both in vivo and in vitro in the absence of reported gene amplification [Foulkes et al., 1993]. For functional studies, ovarian carcinoma lines were adapted to growth in the presence of physiological folate concentrations, which are 100-fold lower than those normally present in standard medium. Previous studies indicated that in contrast with observation on other cell lines, the expression of the receptor was generally not regulated by extracellular folate concentration and receptor upmodulation was detected in only one out five cell lines [Miotti et al., 1995]. To test further the functionality of the FRα overexpressed on ovarian tumor cells, we analyzed its capacity to internalize 5-methylTHF. Using a panel of six ovarian tumor cell lines with a wide range of FRα expression levels, we found that higher levels are associated with increased folate uptake, but that inhibition of FRα activity does not completely block folate internalization. These data indicate that the net internalization of 5-methylTHF depends on the activity of both FRα and a not yet identified molecule with characteristic feature of RFC.

MATERIALS AND METHODS

Cell Lines

The following ovary carcinoma cell lines were used: COR (Dr. G. Balconi, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy); OVCAR3 (Dr. I. Pastan, NIH, Bethesda, MD); IGROV1 (Dr. J. Benard, Institute G. Roussy, Villejuif, France); OVCAR4 and OVCAR5 (Dr. R. Camalier, NCI, Frederick, MD); SKOV3 (ATCC, Rockville, MD). All cell lines were adapted for at least 2 months to growth in folate-deficient medium RPMI 1640 (GIBCO BRL-Life Technologies, Paisley, Scotland) containing 20 nM FA as described [Miotti et al., 1995]. The medium was supplemented with 5% fetal calf serum (FCS) (Sigma, St. Louis, MO), 2 mM glutamine, 100 u/ml gentalin. Before all assays, cells were grown for 4 days in medium without FA and containing 5% FCS.

Antibodies and Immunofluorescence Assays

MOv18 (IgG1) monoclonal antibody (MAb) [Miotti et al., 1987], which recognize an epitope expressed on FRα, was provided in purified form (GMP grade) by Centocor (Malvern, PA). Fluoresceinated MOv18 was prepared by incubating 1 mg of the MAb equilibrated in 0.1 M $NaHCO_3$, pH 8.5 with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (50 μg/ml final concentration) in DMSO, with shaking for 1 h at room temperature. The conjugate was separated from unbound florochrome by desalting on a 10-ml Sephadex G-25 column equilibrated in 0.1 M $NaHCO_3$, pH 8.5. Collected fractions were dialyzed against PBS. Direct immunoflorescence (IF) assay was carried out by incubating cells with fluoresceinated MOv18 (25 μg/ml) for 1 h on ice. IF analyses were carried out using the FACscan (Becton Dickinson, Sunnyvale, CA). Fluorescence units were calculated as (mean fluorescence × number of positive cells) × 10^{-3}.

Cell Accumulation of Radiolabelled Folates

Titration of [^{3}H]FA (specific activity, 32 Ci/mmol; Amersham, Little Chalfont, UK) and 5-methyl[^{3}H]THF (specific activity, 34 Ci/mmol; Moravek Biochemicals, Brea, CA) was performed in 24–48-well plates on adherent cells starting from 100 nM folate concentration in PBS containing 20 mM HEPES. After incubation for 2 h at 37°C, cells were washed twice with cold PBS, recovered, and assessed for radioactivity. Nonspecific binding was evaluated by measuring radioactivity bound to the cells in the presence of 100-molar excess of the corresponding unlabelled folate.

Internalization of 5-methyl[^{3}H]THF

Evaluation of internalized radioactivity as acid resistant fraction. Adherent cells grown in 24–48-well plates were incubated at 0°C and 37°C for different time periods in the presence of 10 or 50 nM 5-methyl[^{3}H]THF in PBS containing 20 mM HEPES. At the end of the incubation, membrane-bound acid-sensitive radioactivity was recovered by treating the cells with saline containing 40 mM acetic acid, pH 3. After two washes with cold PBS, cells were harvested and the radioactivity in the acid-resistant fraction evaluated.

Evaluation of internalized radioactivity in the cytosol. Cells grown in T-25 flasks were incubated for 2 h or 4 h in the presence of 10 nM 5-methyl[^{3}H]THF at 0°C and 37°C. After washing with cold PBS, cells were subjected to hypotonic lysis and cell fractionation [Kamen et al., 1989]. Briefly, 2 ml of buffer (10 mM Tris-HCl, pH 8, 0.02 μg/ml leupeptin, 0.24 trypsin inhibitor units/ml Aprotinin, and 1 μM cold 5-methyl-THF) were added to each flask at 0°C, and the flasks were placed at −80°C for 15 min. The freezing and thawing steps were carried out twice. After the addition of 1 ml of ice-cold buffer, samples were centrifuged for 1 h at 100,000g in a Ti65 rotor (Beckman Instruments, Palo Alto, CA) to separate the membrane fraction (pellet) from the cytosolic fraction (supernatant).

Inhibition of 5-methyl[^{3}H]THF Internalization

Cells grown in T-25 flasks for 4 days were submitted to one of the following treatments to inhibit folate uptake: (1) Incubation for 2 h at 37°C in the presence of 1 μM FA to saturate surface FRα; (2) Incubation with 30 mU/10^6 cells of recombinant phosphatidyl inositol specific phospholipase C (PI-PLC) (Oxford Glycosystem, Oxon, UK) for 1 h at 37°C. At the end of the treatment the supernatant containing released FRα was removed. The effect of PI-PLC treatment was evaluated in parallel by IF on cell suspensions using fluoresceinated MAb MOv18; (3) treatment with PI-PLC as above described and after removal of the released FRα, incubation of the cells for further 2 h at 37°C with 1 μM FA; (4) Incubation with 1 or 10 mM Probenecid (Sigma) in PBS for 30 min at 37°C. At the end of the inhibitory step, 10 nM 5-methyl[^{3}H]THF (final concentration) was added to treated or untreated cells, and they were incubated for 2 h at 37°C. Internalized 5-methyl[^{3}H]THF was evaluated as radioactivity present in the cytosolic fraction as described above.

RESULTS

5-methyl[^{3}H]THF Internalization

The capacity to internalize the physiological form of folate, i.e., 5-methylTHF, was investigated in six ovary carcinoma cell lines, which, as determined by direct IF with MOv18 MAb, express FRα at very different levels (Table I). The cell lines were previously adapted for at least 2 months to growth in medium containing a physiological folate concentration (20 nM) [Miotti et al., 1995] and further maintained for 4 days before the internalization assay in folate-depleted medium. Internalization of 10 nM 5-methyl[^{3}H]THF was tested after 4 h in cells incubated at 0°C and 37°C and evaluated as the acid-resistant fraction (see Materials and Methods). As shown in Table I, cells with low FRα expression as SKOV3 and OVCAR5 (fluor. units $\times 10^{-3} = 3.5$ and 1.6, respectively), internalized an amount of 5-methyl[^{3}H]THF (0.7–0.8 pmol/10^6 cells) similar to that detected into IGROV1 cells, which express FRα at intermediate levels (fluor. units $\times 10^{-3} = 24.1$). By contrast, OVCAR4 cells efficiently internalized 5-methyl[^{3}H]THF (2.1 pmol/10^6 cells) at levels higher than those of IGROV1 cells, which have twice the number of FR receptors. COR and OVCAR3 cells (fluor. units $\times 10^{-3} = 217.6$ and 66.9) internalized 5.8 and 3.5 pmol/10^6 cells, respectively.

Figure 1 illustrates the time-course of binding and internalization of 10 nM 5-methyl[^{3}H]THF in the six cell lines. After a 30 min of incubation at

TABLE I. 5-methyl [^{3}H]THF Internalization

Cell line	FRα expression[a] fluorescence units × 10^{-3}	5-methyl [^{3}H]THF internalized	
		pmol/10^6 cells[b]	Percent of total radioactivity[c]
COR	217.6	5.78 ± 0.77 (3)[d]	14.3 ± 3.7
OVCAR3	66.9	3.54 ± 1.61 (4)	28.6 ± 11.3
IGROV1	24.1	0.92 ± 0.15 (4)	45.8 ± 11
OVCAR4	11.6	2.15 ± 0.63 (3)	78.0 ± 2.4
SKOV3	3.5	0.81 ± 0.31 (6)	78.8 ± 10
OVCAR5	1.6	0.69 ± 0.11 (2)	75.2 ± 1.7

[a]FACS analysis with fluoresceinated MOv18 MAb.
[b]Evaluated as acid-resistant fraction after incubation at 37°C for 4 h (see Materials and Methods).
[c]Total radioactivity contained in the acid-sensitive plus acid-resistant was taken as 100%.
[d]Values are the mean (±SD) of 2–6 experiments.

0°C, the total binding became stable on all cell lines examined except in COR, which have an extremely high number of FRα and in which saturation was obtained after ~2 h of incubation. At 37°C, two different kinetics were observed: cells as COR, OVCAR3, and IGROV1 showed a rapid increase in total binding, whereas during the following period of observation until 4 h, a slow increase was observed. In parallel, the radioactivity present in the acid-resistant fraction (no more than 50% of total binding at 4 h) (see Table I), showed a slight and constant increase. In the other cell lines (OVCAR4, SKOV3, and OVCAR5) that bound <1 pmole of 5-methylTHF at 0°C, the phase of receptor saturation was less evident and the internalized radioactivity at 4 h represented >70% of the total bound 5-methyl[^{3}H]THF (see Table I). In all the tested cell lines, the radioactivity present in acid-resistant fraction at 0°C was no more than 5% of that found in the corresponding fraction at 37°C, and the difference between total binding at 37° and 0°C corresponded roughly to the internalized fraction.

The 10 nM 5-methyl[^{3}H]THF could be a non-surface-saturating concentration for cells with the highest FR expression, and as a consequence, the internalized fraction could be underestimated. So, the total binding (0°C) and uptake (37°C) were evaluated at 50 nM and compared to those at 10 nM on cells with high (COR and OVCAR3) and low (IGROV1 and SKOV3) levels of FR expression. Figure 2 shows that whereas total binding, which essentially correspond to surface binding, was only slightly affected by the fivefold increase in concentration in three of four lines tested (129, 105, and 90% in COR, OVCAR3, and IGROV1, respectively), the uptake was more pronounced (150–170%). The cell line with the lowest number of receptors, SKOV3, showed a much more relevant increase of the total bound (157%) and of internalized fraction (340%).

The above reported experiments showed that ovary carcinoma cells display a different efficiency of 5-methylTHF internalization that was not strictly dependent on the levels of membrane FRα expression and particularly in SKOV3 cells internalization seemed also to be dependent on 5-methylTHF concentration. Since two mechanisms of folate transport (FRα and RFC), which differ in their affinity for FA as compared to reduced folates and in their optimal molar concentrations, could both be present on the same cell line, we evaluated the accumulation at 37°C (surface binding plus internalization) of [^{3}H]FA and 5-methyl[^{3}H]THF (Fig. 3) as a function of their concentration. On IGROV1 cells, the FA binding was constant from 100 to 3.12 nmol/l (1.17–0.8 pmol/10^6 cells), decreasing thereafter as the concentration of the radiolabelled compound declined. On SKOV3 cells, maximum FA binding was 0.19 pmol/10^6 cells and the curve thereafter paralleled that observed with IGROV1 cells. By contrast, the accumulation of 5-methyl[^{3}H]THF was much more concentration-dependent in both cell lines. This was particularly evident on SKOV3 cells, which at 100 nM concentration accumulated an amount of 5-methyl[^{3}H]THF (3.5 pmol/10^6 cells) even higher than that of IGROV1.

To prove that the 5-methyl[^{3}H]THF sequestered in the acid resistant-fraction reaches the cytoplasm, cell fractionation was carried out on the above characterized cell lines, IGROV1 and SKOV3. After 2 h of incubation with 10 nM 5-methyl[^{3}H]THF, cells were lysed and the cyto-

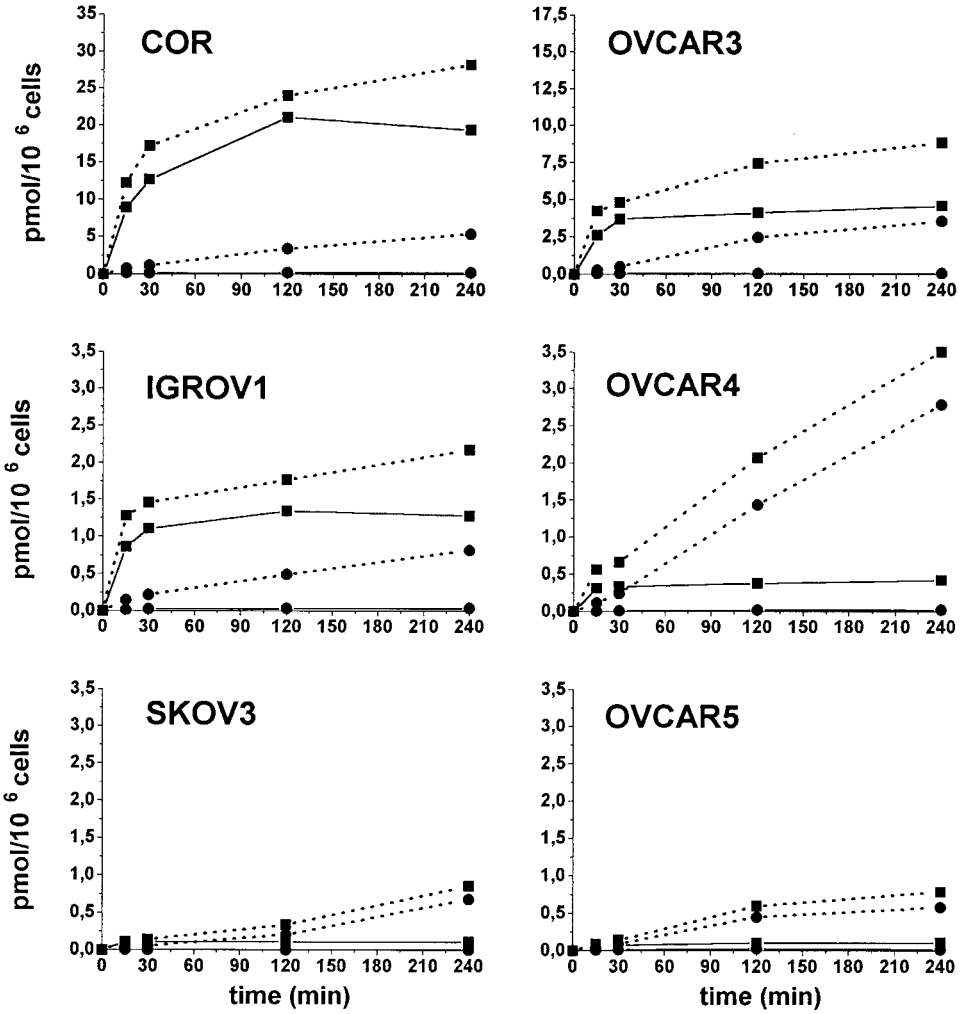

Fig. 1. Time-course of 5-methyl[^{3}H]THF binding and internalization. Ovary carcinoma cells grown for 4 days in folate-depleted medium were incubated for different periods of time at 0° (—) or 37°C (. . .) in the presence of 10 nM 5-methyl[^{3}H]THF. After washing, the radioactivity present on the cell surface was dissociated by incubation for 2 min at 0°C with 40 mM acetic acid in saline (acid-sensitive fraction = AS). After further washing, radioactivity bound to the cells (acid-resistant fraction = AR ●) was recovered. Total bound 5-methyl[^{3}H]THF (■) represents the sum of the AS and AR radioactivity. Note that different scales for *y* axes have been used: 2X (OVCAR3), 10X (IGROV1, OVCAR4, SKOV3, and OVCAR5).

solic fraction separated by ultracentrifugation. The two assays (Fig. 4) gave comparable results, confirming the presence of the folate in the cytosol of both cell lines (1 pmol/10^6 cells). The difference in surface binding was also confirmed.

Inhibition of 5-methyl[^{3}H]THF Internalization

Experiments to selectively block the FR were performed on IGROV1 and SKOV3 cells, and the effect of this blocking on surface binding and internalization of 5-methyl[^{3}H]THF was evaluated (Fig. 5).

Incubation of the cells with an excess of FA (1 μM) strongly reduced the membrane binding of 10 nM 5-methyl[^{3}H]THF on IGROV1 cells (3.6% of control), whereas on SKOV3 cells, despite the lower number of FRα expressed, the treatment was slightly less effective (15% of the control). The internalized radioactivity was 40% and 67% of the control in IGROV1 and SKOV3 cells, respectively.

PI-PLC treatment of IGROV1 cells induced a drastic reduction of FRα expression (20–30% of control), as detected by direct IF with MAb MOv18 (Fig. 6A,3). On SKOV3 cells only slight or no effect at all was observed (Fig. 6B,3). No recovery of FR expression was detected on both cell lines left at 37°C for 2 h after PI-PLC treatment (Fig. 6A,4, B,4). The reduction of

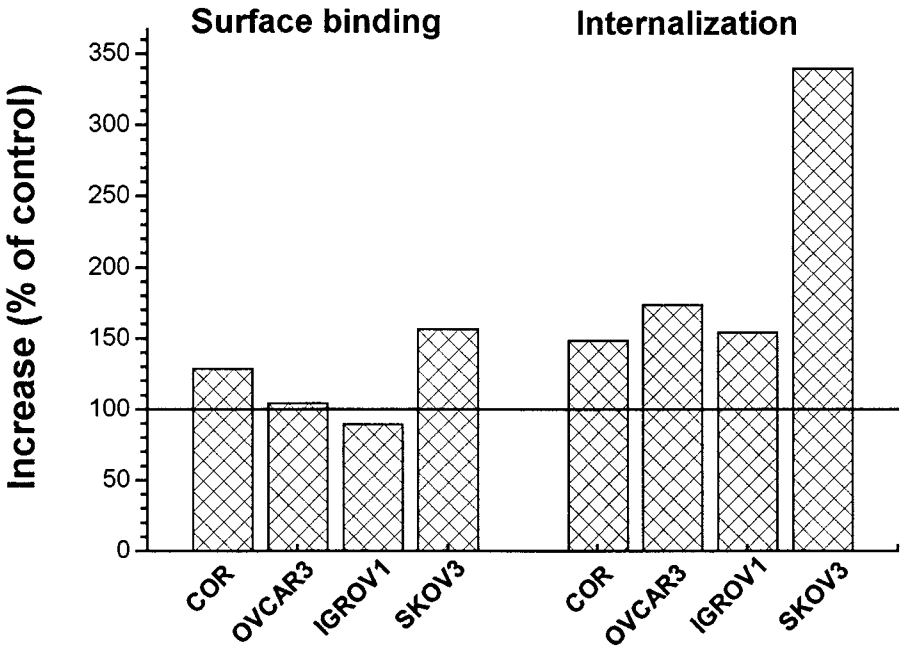

Fig. 2. Effect of 50 nM 5-methyl[^{3}H]THF concentration on surface binding and internalization. Four ovary carcinoma cell lines (COR, OVCAR3, IGROV1, and SKOV3) grown for 4 days in folate-depleted medium were incubated for 4 h at 0°C or 37°C in the presence of 10 nM or 50 nM 5-methyl[^{3}H]THF and the AS and AR fractions evaluated (see Materials and Methods). The % of increase in total surface binding (total binding at 0°C) and internalization (AR fraction at 37°C) in the presence of 50 vs. 10 nM 5-methyl[^{3}H]THF is reported.

5-methylTHF membrane binding (Fig. 5) was comparable to that detected by IF; indeed, membrane-bound radioactivity was 15% of control on treated IGROV1 cells and 80% on SKOV3 cells. As a consequence of PI-PLC treatment, the internalized radioactivity was reduced in IGROV1 cells (48% of control), on the contrary, SKOV3-treated cells showed no reduction or even an increase in the internalized fraction as compared to the control cells.

To verify whether PI-PLC treatment and cold FA could exert their inhibitory activity acting on the same molecule, an additional incubation of PI-PLC treated cells was carried out in the presence of 1 μM FA. As a consequence, 5-methyl[^{3}H]THF membrane binding and internalization were reduced on both cell lines at levels obtained with 1 μM FA alone (unshown results).

Probenecid has been used to inhibit RFC transport in vivo and in vitro [Sirotnak et al., 1980; Gewirtz et al., 1984; Kamen et al., 1991; Prasad et al., 1994; Bourke et al., 1995] and should not influence surface binding to FRα [Kamen et al., 1991]. Incubation with 10 mM Probenecid had some effect on the membrane binding of 5-methyl[^{3}H]THF, which was more pronounced on IGROV1 cells than on SKOV3 cells (60% and 73%, respectively, of control). Internalization was drastically reduced on both cell lines to 20% of control cells (Fig. 5). The effect was dose dependent; indeed in the presence of 1 mM Probenecid, the internalized fraction corresponded to 70–80% of the control (data not shown).

DISCUSSION

The present study shows that FRα, which is overexpressed in different amounts on ovary carcinoma cell lines and is active in mediating 5-methylTHF internalization, does not represent the only mechanism involved in folate uptake. Here, we report evidence suggesting that another transport route, with features characteristic for RFC, is functional in these cells and partecipate in folate uptake.

In the past, the activity of the RFC has been investigated mainly with respect to transport of methotrexate and antifolates in leukemia and carcinoma cell lines [reviewed in Sirotnak, 1985]. Only recently, from different human cDNAs libraries, highly similar cDNAs have been cloned, whose transfection restores metotrexate uptake in transport deficient cells [Moscow et al., 1995; Prasad et al., 1995; Williams et al., 1995; Wong et al., 1995]. Although the notion

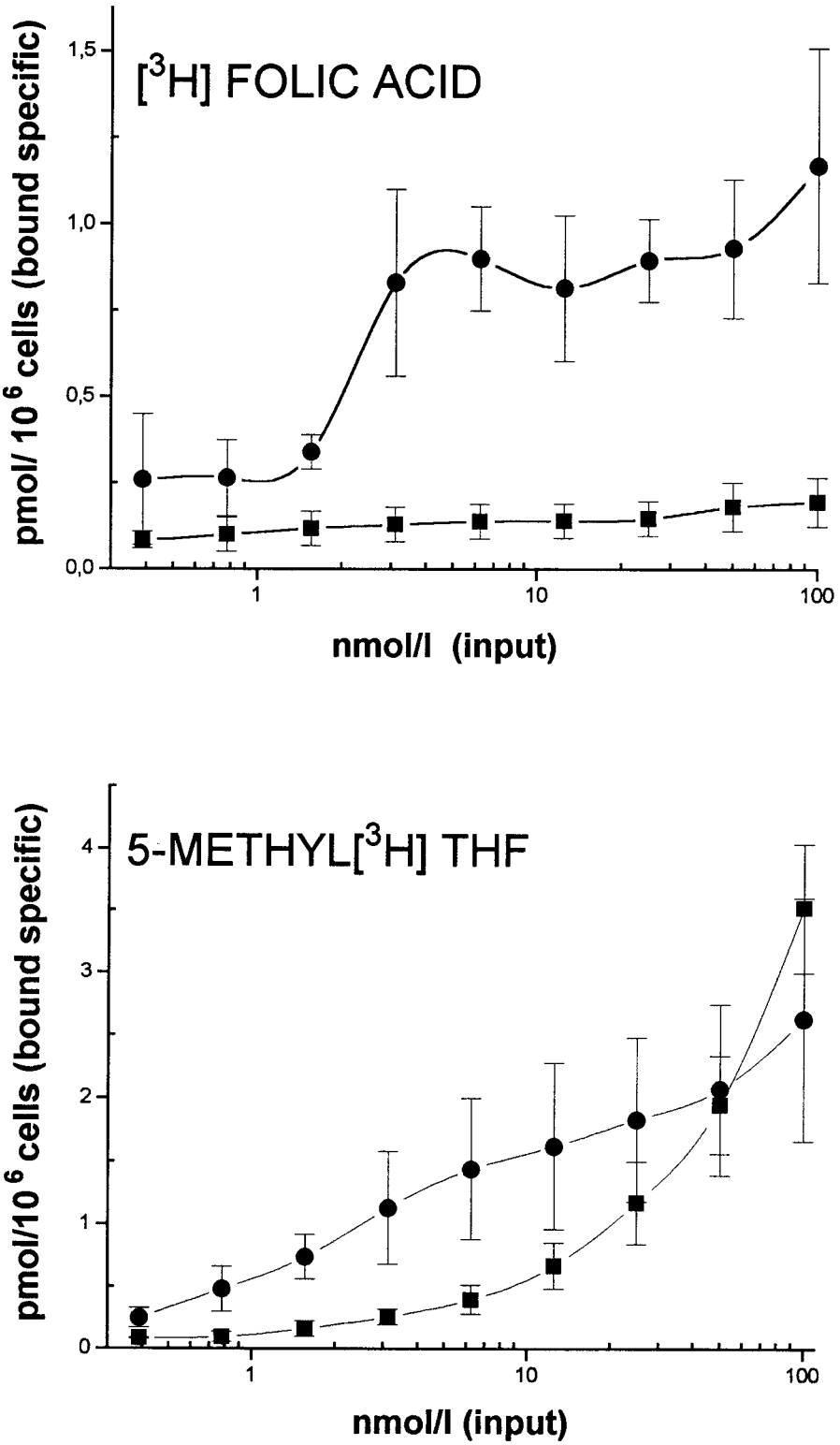

Fig. 3. Concentration-dependent accumulation of folates. IGROV1 (●) and SKOV3 (■) cells grown for 4 days in folate-depleted medium were incubated for 2 h at 37°C in the presence of serial dilutions of [^{3}H]FA and 5-methyl[^{3}H]THF. Data are the mean of two and three experiments (±SD) on SKOV3 and IGROV1 cells, respectively.

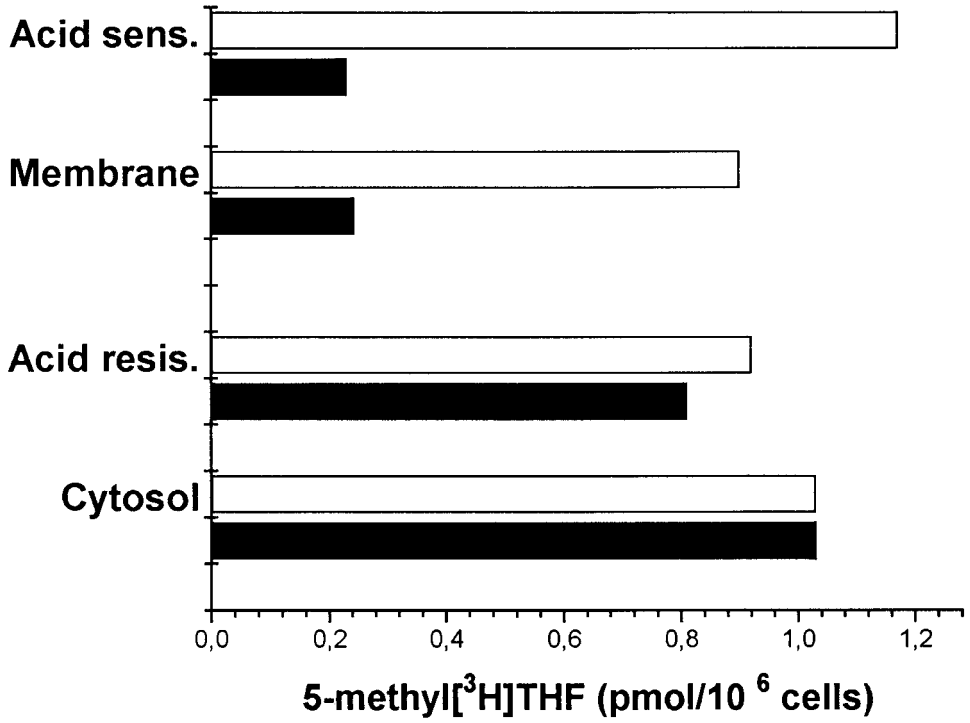

Fig. 4. Evaluation of 5-methyl[^{3}H]THF cytosolic and membrane fractions. IGROV1 (□) and SKOV3 (■) cells grown for 4 days in folate-depleted medium were incubated for 4 h in the presence of 10 nM 5-methyl[^{3}H]THF. After washing with cold PBS, cells were lysed in hypotonic buffer containing a 100-molar excess of cold 5-methylTHF plus protease inhibitors and then frozen and thawed twice at −80°C. Samples were centrifuged for 1 h at 100,000g to separate the pellet containing the membrane fraction from the supernatant containing the cytosol fraction. Data are the mean of two separate experiments and are compared to those obtained for the acid-sensitive and acid-resistant fractions.

that FR does not use the conventional coated-pit endocytotic pathway is controversial [Rothberg et al., 1990; Mayor et al., 1994; Ritter et al., 1995], a potocytosis model has been proposed for FR-mediated folate uptake. In this model [Anderson et al., 1992; Anderson, 1993], potocytosis of small molecules such as folates take place into small membrane invaginations, called caveolae, where FRα binds and concentrates the folates. After the sealing of the compartment, acidification by a proton pump, which activity has been demonstrated in JAR cells [Prasad et al., 1994], leads to the dissociation of the ligand from FRα, facilitating ligand transport in the cytoplasm via the RFC.

FRα was found to be coupled to RFC activity on MA104 (normal monkey kidney) and JAR (human choriocarcinoma) cell lines [Kamen et al., 1991; Prasad et al., 1994]. In contrast, the two folate transport systems were found to act independently of each other on a FRα-positive L1210 murine leukemia cell line [Westerhof et al., 1991] and on breast cancer cells transfected with FRα cDNA [Dixon et al., 1992]. Recently, in three carcinoma cell lines (KB, IGROV1, and MA104), which express various levels of FR, RFC activity was found to constitute the major route for anti-folate transport [Westerhof et al., 1995].

Several indirect evidences suggest the presence of RFC activity on our ovarian carcinoma cells lines: (1) two patterns of time-dependent 5-methylTHF binding and uptake were observed, in particular cell lines that showed relatively low levels of FR expression as OVCAR4, SKOV3, and OVCAR5, were efficient in folate uptake and internalized until fourfold more folate than their membrane binding capacity (Fig. 1, Table I), (2) the incubation in the presence of 50 vs. 10 nM 5-methylTHF strongly increased the uptake in SKOV3 cells (Fig. 2), (3) the slopes of the concentration-dependent folate accumulation curves indicated that at 100 nM folate concentration a molecule is active on IGROV1 and SKOV3 cells with higher affinity for reduced folates than for FA (Fig. 3), and (4) at 100 nM concentration this activity

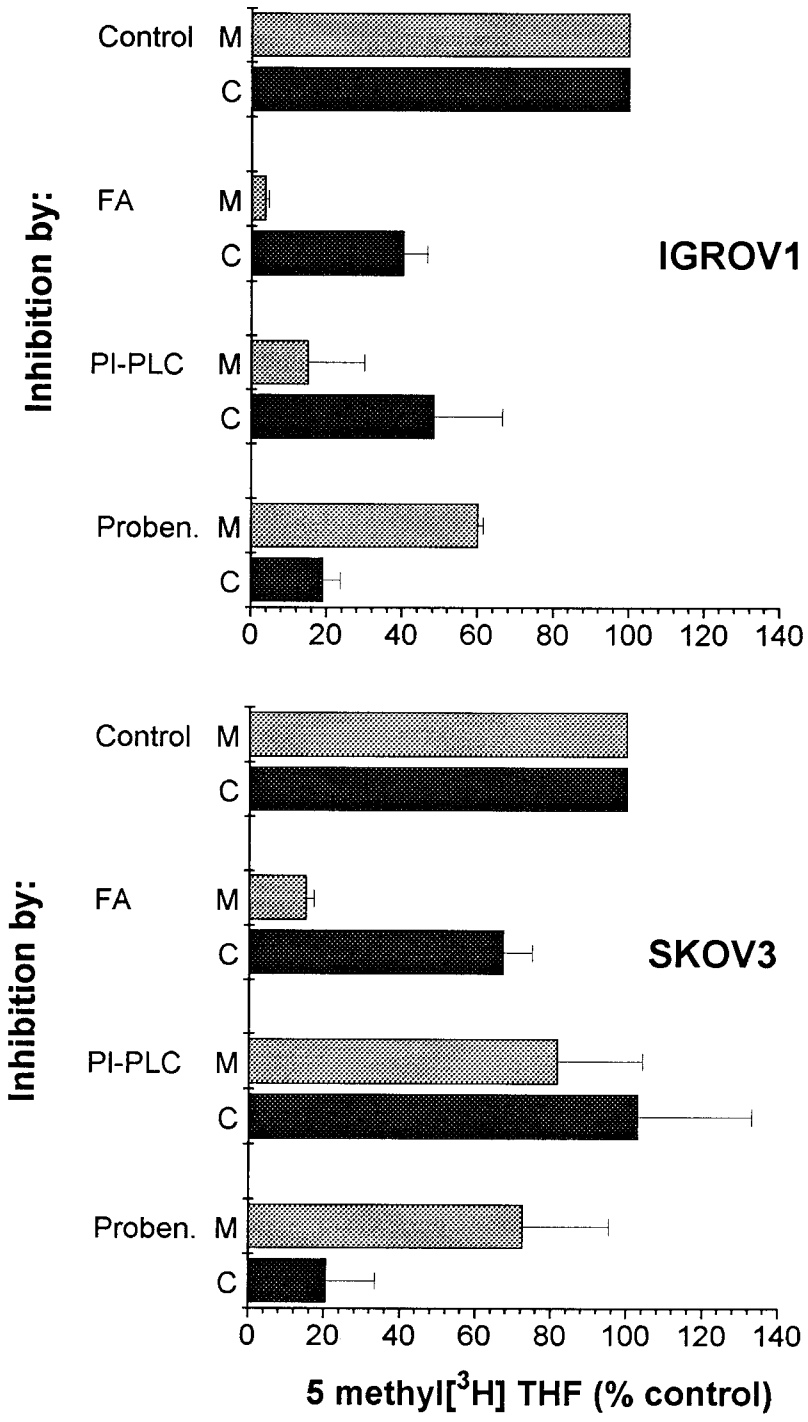

Fig. 5. Effect of different inhibitors on 5-methylTHF binding and internalization. IGROV1 and SKOV3 grown for 4 days in folate-depleted medium were alternatively incubated at 37°C in the presence of 1 μM FA for 2 h, or in the presence of PI-PLC for 1 h, or with 10 mM Probenecid for 30 min. PI-PLC only was removed before incubation with 10 mM 5-methyl[^{3}H]THF (final concentration) for 2 h at 37°C. After washing, the membrane- (M) and cytosol-associated radioactivity (C) were separated as described. Data are the mean (±SD) of 3–6 experiments.

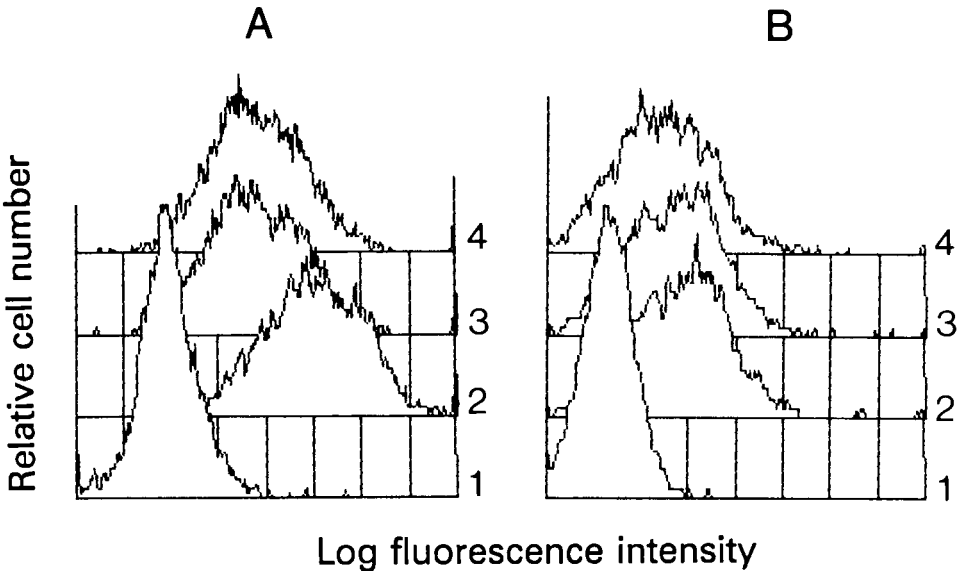

Fig. 6. FACS analysis of the effect of PI-PLC treatment on FRα expression. IGROV1 **(A)** and SKOV3 cells **(B),** treated for 1 h at 37°C with PI-PLC (3), were tested for FR expression with fluoresceinated MAb MOv18 and their fluorescence compared to that of untreated cells (2). Recovery of FR expression after PI-PLC treatment was evaluated in cells left 2 h at 37°C before IF assay (4). Control cells, not incubated with fluoresceinated MAb, are reported in profile 1.

allowed SKOV3 cells to accumulate more 5-methylTHF than observed for IGROV1 cells.

The functional activity of FR on ovarian tumor cells was demonstrated in experiments revealing decreased 5-methylTHF internalization after treatment of the cells with inhibitors of the membrane binding, such as incubation with excess FA or with PI-PLC (Fig. 5). However, the reduction in internalization (50–60%) was consistently less than expected, suggesting that some proportion of 5-methylTHF internalization proceeds via a FR-independent pathway. Whereas PI-PLC treatment left ~20% of potentially functional protein on cell surface, the complete inhibition of membrane binding obtained by FA (3.6% of control) best ensured that FR activity was completely blocked. In addition, the observed uptake in PI-PLC treated cells could not be due to recovery of the protein during the following 2 h period of incubation at 37°C, since FR expression remained unchanged (Fig. 6A4). On SKOV3 cells, which express a low level of FRα, the role of this molecule is probably less relevant. FA was effective in inhibiting the membrane binding (16% of control) and only slightly effective in blocking internalization (67% of control). Consistent with our observations in other ovarian carcinoma cell lines [Miotti et al., 1992], FR expressed on SKOV3 was poorly or not affected by PI-PLC treatment. When in further treatments reduction of expression to 50% was obtained, a parallel decrease in internalization was observed (data not shown). By contrast, when PI-PLC treatment was uneffective, internalization of 5-methylTHF was consistently increased. To explain these results, we suppose the presence of some other (GPI-linked) protein that may influence the PI-PLC sensitivity and activity of FRα.

Inhibition experiments using the organic acid Probenecid showed a consistent decrease in the internalized fraction (80%) on both IGROV1 and SKOV3 cells. Although probenecid reportedly does not interfere with 5-methylTHF membrane binding to FRα [Kamen et al., 1991], it caused a detectable binding decrease on both ovarian carcinoma cell lines, more consistently (40%) on IGROV1 cells. Since the decreased 5-methylTHF binding cannot account alone for the high inhibition of internalization observed (compare to inhibition by FA), the latter might rest in an effect of Probenecid predominantly on the RFC.

All together, these results allow us to affirm that both in IGROV1 and SKOV3 cells, a variable fraction of internalized 5-methylTHF proceeds through a FR independent route, whereas the existence and the extent of an interaction

between the two molecules cannot be determined.

The amount of internalized folate was not proportional to the FRα overexpression, particularly in the cells with the highest levels (COR and OVCAR3). On the squamous cell carcinoma cell line UM-SCC38 [Orr et al., 1995a], amplification of the FRα gene was found to be associated with a point mutation that negatively influences the binding of folate to the protein and also has a dominant negative effect on the wild-type receptor by inhibiting 5-methyl-THF internalization. However, it is unlikely that a similar phenomenon plays a role in ovarian carcinoma cells, since the overexpressed FRα is able to bind folate and the level of binding correlates with the level of detected by direct IF with anti-FR MAb. It is possible that internalization is regulated on the basis of a specific cellular requirement [Kamen et al., 1986], or that the FR are not all functionally active and do not participate equally in folate internalization. In KB cells, which have a level of FR expression analogous to COR, only 10–15% of FR was found to participate to folate internalization [Rijnboutt et al., 1996]. Noteworthly, in the same study separation of FR based on partial Triton-X (TX) 100 insolubility of GPI-anchored proteins, showed that TX-insoluble fraction, which has biochemical characteristics similar to caveolae, contain 65% of total FR, but this fraction does not participate to folate internalization. In addition, RFC was completely excluded from TX-insoluble domains. The conclusions of this study are against a caveolae-mediated folate internalization in KB cells. However, based on morphological criteria, caveolae only incidentally were detected in KB cells, and no demonstration of the presence of their structural component, caveolin [Rothberg et al., 1992] was reported. Therefore, the route of folate internalization may be, dependending on the cell line, a consequence both of the presence of structurally organized caveolae and of FR overexpression. Indeed, in other systems, overexpression of a protein has been shown to influence its membrane arrangement followed by changes in functional interactions with other proteins [Clissold, 1994].

In conclusion, our results demonstrate the simultaneous activity of two different mechanisms of folate trasport in a panel of ovary carcinoma cells. The presence of these activities in vitro suggests that these two mechanisms also operate in vivo and that their relative efficiency is relevant in determining the uptake and therapeutic effect of antifolate-based treatments of ovarian tumors.

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