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Review

Cellular aspects of folate and antifolate membrane transport★✪

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Folates — one carbon carriers — take part in the metabolism of purine, thymidylate and some amino acids. Internalization of these compounds employs several mechanisms of transport systems. Reduced folate carriers and folate receptors play the most important role in this process. The physiological role of these molecules in normal and neoplastic cells is described regarding changes in transport activity and connection of transport systems with resistance to antifolates and cancer development.

Folates act as one-carbon carriers in a set of interconversions of metabolic cycles of purine, thymidine, methionine, histidine and serine biosynthesis. Such activity makes folates essential for normal growth and maturation. Eukaryotes, however, are unable to synthesize folates, hence they require an external source for these compounds. The first step in their assimilation by cells is transport into the cytoplasm across the plasma membrane. Subsequent to cellular uptake, these compounds are converted by folylpolyglutamate synthetase (FPGS) (EC 6.3.2.12) to the polyglutamate form by the addition of several glutamic acid residues. This process is important for establishing and maintaining a folate pool in the cell, because polyglutamates are better retained in the cell and are gener-

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Abbreviations: BSP, bromosulfophthalein; FPGS, folylpolyglutamate synthetase; FR, folate receptor; GPI, glycosylphosphatidylinositol; IFC, intestine folate carrier; LV, leucovorin (folinic acid); MDR, multidrug resistance; MTX, methotrexate; OAT, organic anion transporter; RPE, retinal pigment epithelium; RFC, reduced folate carrier.

ally higher affinity substrates for folate-utilizing enzymes [1]. Folates are also essential to the mitochondrial metabolism, so they are transported into mitochondrial matrix by carrier-mediated mechanism, converted by FPGS to its polyglutamates [2], and utilized in glycine metabolism.

Disruption of folate metabolism has long been known to inhibit cell growth and has been established as a target in chemotherapy with the use analogs of these compounds, antifolates. Classical antifolates require the same mechanism as folates for cell entry and are substrates for polyglutamylation, which process can exceed the intracellular concentration and maintain inhibition of target enzymes after removal of the extracellular drug. In addition, polyglutamates are more potent inhibitors of some folate-dependent enzymes [3]. The second class, non-classical antifolates enter cells by passive diffusion without transport system and are not converted to the polyglutamates. As a result, non-classical folates are usually more weakly retained within the cells after removal of extracellular drug.

As for most anticancer drugs, cells can acquire resistance to antifolates. In most cases, this is not MDR-type resistance, but is rather connected with a reduction of polyglutamylation, changes in target enzymes and, of particularly interest for this paper, failure of cellular system of drug-transport.

FOLATE TRANSPORTERS

Multiple transport systems have been identified in the cell membrane that play a role in mediated internalization of folates. Each system utilizes a specific set of membrane proteins, that bind the transported molecule with high affinity and specificity. These systems occur in two general categories: a) membrane channels or carriers that vectorialy move molecules, b) endocytic vesicles that are internalized. These transport proteins, for maximal efficiency of transport, are often segregated from other molecules in the membrane to form domains, that are enriched in that transporter species. The transport systems can be distinguished by their preferences for various folate compounds as substrates, as well as by differences in temperature and pH dependence. The main known mechanisms for folate delivery from the extracellular space to the cytoplasm are carrier mediated or receptor initiated processes, which operate efficiently at physiological pH. The reduced folate carrier (RFC) and folate receptor (FR) function independently and exhibit distinct properties. The existence of different transport systems raises the possibility of substrate channeling to particular enzymes within the cell. However, although the FR and RFC employ diverse mechanisms of folate delivery, rate and extent of folate polyglutamylation achieved are nearly identical, irrespective of its route of entry. Thus, both systems appear to deliver folate to the same intracellular compartment [4]. There is, in addition, evidence for an influx route that operates optimally at low pH. Of particular importance is the efflux transport, directly coupled to energy metabolism, that opposes concentrative transport mediated by RFC at physiological pH.

Reduced folate carrier

The major transport system is reduced folate carrier, the bidirectional transporter for both natural reduced folates and antifolate chemotherapeutics such as methotrexate (MTX). This protein belongs to the Major Facilitator Superfamily of transport carriers predicted to consist of twelve transmembrane domains with the N- and C-termini, and the large loop between the sixth and seventh transmembrane domains, directed to the cytoplasm. It exhibits similarity with the 55 kDa human GLUT1 glucose transporter [5]. Recently in some species cloned cDNA, that was able to complement MTX transport in RFC defective cells, coded a 46–58 kDa protein [5, 6]

which contains one consensus signal for N-glycosylation. Besides, RFC possesses one or more vicinal thiol groups essential for its transport function [7] and does not contain an ATP-binding region consistent with the lack ability of this carrier to perform ATP hydrolysis. In several cells multiple RFC transcripts are identified, which are encoded by a single gene locus whereas heterogeneity results from multiple transcriptional starts and variable splicing [8, 9]. This gives the possibility to generate unique RFC molecules that exhibit tissue- or cell line-specific distribution.

Transport kinetic properties of RFC show poor affinity for folic acid (K_m 200–400 μM) as compared with reduced folate cofactors and MTX (K_m 1–10 μM) [10, 11]. RFC-mediated transport is not directly coupled to energy metabolism but the energy for the uphill transport of folates delivers the transmembrane organic anion gradient. The extent of the transmembrane folate gradient achieved is further modulated by energy status through the activity of the independent exit pumps that are highly sensitive to the energy balance of the cell. Since association with co-transport with intracellular anions occurs, a characteristic feature of this process is inhibition by organic and inorganic anions in the external medium. The RFC activity also depends on the differentiation state of the cell. During maturation of HL-60 cells, transport *via* the RFC decreased, which coincides with fewer numbers of RFC molecules per cell as result of reduced *de novo* synthesis [12, 13].

Folate receptors

A second folate transport mechanism is mediated by folate receptors, a particulate form of folate binding proteins, which are anchored to the cell membrane by glycosylphosphatidylinositol (GPI) residues. FRs have a mass of 38–40 kDa and are coded by two genes (FR α and β) with differential tissue expression [14]. The third member of this gene family, γ-type, is a secretory protein due to the lack of signal for GPI attachment [15]. There is no sequence homology between the membrane-anchored FR and membrane-spanning RFC that would suggest structural or functional similarities. Unlike RFC, FR exhibits a much greater affinity for folic acid and 5-methyltetrahydrofolate (K_d 1–10 nM), but lower for other reduced folates (K_d 10–300 nM) and have different properties in terms of energy, ion, and pH dependence. Folate binding to FR is decreased after energy depletion and in absence of chloride anion, but does not diminish until the pH falls below 5.0 [16].

The FRs are thought to be usually clustered on the cell surface and preferentially associated with uncoated membrane invaginations (caveolae) characterized by the presence of the marker protein — caveolin. Caveolin-1 tightly binds cholesterol and can oligomerize into large protein complexes functioning as a raft organizer. These microdomains (rafts) serve in the sorting of associated proteins and have been implicated in processes such as membrane trafficking, cell adhesion, providing sites for assembling cytoplasmic signaling molecules such as Src-tyrosine kinase family [17–19] and moreover are essential for the proper internalization and recycling of the FR [20]. However, there is recent evidence, that FRs are not constitutively concentrated in caveolae and in the absence of agents that promote clustering (e.g. antibodies used to immunodetection) are diffusely distributed over the plasma membrane [21]. The process of folate delivery by the FR is known as potocytosis [22] and is performed by using a pathway that is separated from the clathrin-coated pit endocytosis [20]. First, folate binds to the externally oriented receptor followed by the internalization of caveolae and formation of a compartment that is acidified by a H^+ pump. At a low pH folate dissociates from its receptor and is transported into the cell, the receptor instead returns on the cell surface [23]. The quantity of accumulated folate appears to be regulated at the stage of its release from the receptor into the cytoplasm. Folate-de-

pleted or not rapidly dividing cells take up folates only until the cytoplasm accumulates physiological levels of these compounds, and then the process is markedly inhibited, even though the FR remains functional [24]. Such regulatory abilities are observed only when the FR is targeted to caveolae, as opposed to chimeric FR targeted to clathrin-coated pits [25]. The controversial question is, whether the transported molecules reach the cytoplasm by diffusing across membrane through water-filled channels or by specific carriers which cooperate with FR. The suggestion, that transmembrane movement of the folate into the cytoplasm occurs through an anion carrier is based on the observation, that this process is sensitive to inhibitors of RFC or low temperature [26]. However, these factors also show an impairing effect on folate binding to FR, which could lead to direct inhibition of uptake *via* FR [16].

A key determinant of clustering and functional activity of GPI-anchored proteins, including FR, appears to be cholesterol-enriched lipids rafts [27]. As shown in many studies, depletion of cellular cholesterol disrupts raft domains in the cell membrane and impairs the ability of GPI-anchored proteins to: associate with these microdomains [28], to take up folates efficiently [29] and to be retained in endosomes before being recycled back to the plasma membrane [30]. Cholesterol-depleted cells have a lower number of caveolae as well as fewer receptors per caveolae, hence the rate of folate internalization is reduced.

FR expressed at sufficient levels can be a significant transport route for MTX at low blood levels (100–500 nM), hence has pharmacological and physiological importance, especially in cells with impaired RFC function [16, 31, 32]. However, FR becomes a very minor contributor to transport at higher levels of MTX (1–10 μM), when normally dominate the RFC. These effect are due to the slow net cycling rate of the FR when compared with the RFC [13].

Low pH transporter

Recently reported folate transport pathway with optimal activity at low pH has been described in intestinal cells [33, 34], in kidney [35] and in cultured L1210 cell lines lacking functional RFC as result of acquired resistance to MTX. In these cells much higher rate and extent of uptake could be achieved by decreasing the pH from 7.4 to 6.2 [36, 37]. The low pH transporter system in parental L1210 cells, in spite of comparable amounts, is more difficult to detect due to its lower activity relative to RFC.

The low pH transporter is energy-dependent, saturable and partially inhibited by ionophores. This system can mediate the influx of certain folates also at physiological pH, although at a significantly lower rate than mediated by RFC. In contrast to RFC, which is sodium independent but stimulated by removal of external anions, the low pH transporter is minimally sensitive to ionic composition. Also, none of the organic anions, that inhibit RFC, are effective in impairing low pH transporter function [37]. These data indicate that this transport route operate by a mechanism different from RFC and other anion exchangers.

The controversial question is, reported by Henderson & Strauss [36], the inability of this system to transport 5-methyltetrahydrofolate, the main physiological form of folate in the circulation of mammals, whilst the other reports presented the evidence that this folate form could be a substrate for the low pH transporter, although with different kinetics, to cause a lower V_{max} of transport [37].

The specific mechanism of folate intestinal transport has been the subject of intensive studies, however very little is known about the molecular identity of the transport system involved. The properties of intestinal folate carrier (IFC-1) are similar to the low pH transporter characteristics in terms of pH optimum and saturability when expressed in an intestinal epithelial cells, but when transferred to

Xenopus oocytes, it displays RFC properties. In addition, the open reading frame of IFC gene is identical to that of the RFC [34]. It is probable that involvement of specific posttranslational modifications or the existence of an auxiliary protein may account for such tissue- or cell line-specific distribution. Moreover, identification of several RFC splice forms raises the possibility that unique RFC molecules may be generated as a result of the use of alternative transcriptional start sites [8].

Efflux pumps

This transport route makes possible efflux of folate out of the cell. Its activity is directly linked to ATP hydrolysis and opposes influx transport, hence transmembrane folate gradient is determined by the net effect of these two independent processes. Loss of folate efflux pumps activity causes enhanced net transport that can contribute to augmented folate accumulation and decreased growth requirement for folic acid [10].

Detailed studies revealed an activity of three separate routes one of which is identified as RFC, and two others as unidirectional efflux pumps belonging to multispecific organic anion transporters, although the existence of two separate routes is controversial [38]. The major portion of efflux occurrs *via* the bidirectional influx/efflux RFC transporter, the identity of which was established by its requirement for anions in external medium and from its sensitivity to specific inhibitors. The two unidirectional routes show considerable similarities in energy-dependence and inhibitor sensitivities, but were separated due to their differential inhibition by bromosulfophthalein (BSP), probenecid, and vincristine. The postulated predominant BSP-sensitive efflux route mediate the unidirectional extrusion of MTX and – as described in L1210 cells – another anion, cholate, whereas the probenecid-sensitive route is not able to transport cholate [39, 40]. In ATP-depleted cells, the unidirectional efflux is markedly inhibited and appears to be mediated solely by the RFC route [38]. Similar unidirectional efflux pumps, have been identified for other anionic compounds including cyclic AMP and oxidized glutathione. The function of the unidirectional efflux systems is to extrude various organic anion catabolites that might otherwise become toxic if allowed to accumulate within cells. An organic anion transporter (OAT-K1) has been described in the basolateral membranes of renal tubules, where it mediates excretion of MTX. It was suggested that OAT-K1 facilitates efflux when MTX is accumulated in the cytoplasm and contributes at least in part to folinic acid (leucovorin, LV) rescue by exchanging intracellular MTX for extracellular LV [41, 42]. This transport route in the renal tubule membranes implies significant pharmacological role of OAT-K1 in the kidney.

In addition, energy-dependent unidirectional efflux pumps for large lipophilic drugs have also been identified in cells with acquired multidrug resistance, which is mediated by a membrane P-glycoprotein. Various inhibitors of this system (such as reserpine, verapamil, and quinidine) can partially reduce the efflux of MTX. The evidence for MDR-mediated antifolate efflux is also obtained from the studies in which resistance is observed after transfection with MDR proteins [43].

BIODISTRIBUTION AND IMPORTANCE OF FOLATE TRANSPORTERS

The cell membrane is rich in folate transporters in normal tissues, such as choroid plexus, thyroid, placenta and kidney, in which transport of folate is an apparent physiological process [44]. In brain, reduced folates are transported from blood into cerebrospinal fluid against a concentration gradient by choroid plexus, and moreover folic acid, which the brain cannot utilize, is removed to blood.

Thus, high expression of FR in choroid plexus helps to maintain the cerebrospinal fluid concentrations of folate within relatively narrow limits. Thus, suitable folate level prevents the occurrence of foetal neural tube defects [45]. Hence, in human placenta, folate receptors are present at high levels in an aim to maintain a concentrative maternal-to-fetal flux of the vitamin with minimal dependence on variations of maternal dietary intake [46]. In kidney, the activity of folate transporters plays an important role in conserving folate level to counteract the development of deficiency by regulating urinary folate excretion and reabsorption across the apical membrane of the proximal tubule [47]. Urinary excretion also represents the major route of elimination of antifolates such as MTX from the body. The organic anion transporter OAT-K1, expressed predominantly in renal straight tubules, participates in secretion of MTX from tubular epithelial cells into urine, preventing its potential toxicity [48]. The following organ, liver, although is a major storage site of folate, expresses a relatively low level of FR [44, 49], except a greater abundance described in pig liver [50]. The liver participates in elimination of folate compounds from systemic blood circulation to hepatocytes, from which they are then actively excreted into the bile. This process, characterized as an energy-dependent efflux, is particularly efficient for MTX and is sensitive to various bile acids e.g. taurocholate. This bile acid sensitive carrier in hepatocytes, absent in dedifferentiated hepatoma cells, differs from previously described transporters for the organic anions [51]. It was also observed the insulin-dependent MTX efflux [52].

The expression of transport molecules in cells or tissues obtained under physiological conditions, may not be as high as has been observed in established cell lines maintained *in vitro* in the folate conditioned medium [53]. Besides, several malignant cell lines express significantly higher FR or RFC levels [44]. In ovarian carcinoma overexpression of FRα is a diagnostic tumor marker [54, 55], hence these receptor may be a useful immunological or pharmacological target for the development of selective cancer therapy [56].

Taking into account the distribution of FR and RFC, some data suggests that these proteins work in a concerted manner to assure the best transport efficiency. FR is known to be present in many cell types of the neural retina, whereas RFC is only present in retinal pigment epithelium (RPE), RFC displays an apical distribution in RPE, which contrasts with the basolateral distribution of FR in these cells. This fact can possibly explain the vectorial transfer of folate across the RPE cell layer from the choroidal blood to the neural retina [57]. Some defects in RFC activity in RPE could be induced *via* oxidation of vicinal thiol groups by nitrous oxide, a molecule thought to be involved in infections and ischemic processes of the RPE and in the pathogenesis of diabetic retinopathy [7]. In kidney, a role for both pathways (RFC and FR) in maintaining suitable folate levels is showed [43], however basolateral uptake occurs primarily by the RFC [58].

CHANGES IN TRANSPORT ACTIVITY

Transport system activities could become distinct in response to external stimuli including folate availability or exposure to chemotherapeutic drugs. Quantitative and qualitative changes of transport properties make cells adapted to these variable factors.

The adaptation to gradual lowering of extracellular concentration of folates is associated with changes leading to the enhanced intracellular accumulation of folate compounds. As described in numerous studies, these changes result from: 1) RFC1 gene amplification and consequent RFC overexpression [59], 2) expression of alternatively spliced truncated RFC, that is coexpressed with the native carrier [60], 3) structural alteration of RFC increasing its transport affinity to reduced fo-

late cofactors and folic acid [61], 4) or at last increased rate of carrier translocation [62]. Cells growing *in vitro* in folate-restrictive conditions, as far as the variety of carcinoma cell lines, can also contain elevated levels of FR [53, 54, 56, 63–65]. Increased expression of these proteins is associated with rearrangement in the FR promoter region leading to production of novel transcripts with enhanced stability [64, 66]. The increase of FR expression, besides ensuring the growth of cells by its contribution to folate intake, also participates in the antifolate resistance by the internalization of folate cofactors, which would compete with inhibitor hindering its cytotoxic effect. In addition, folate accumulation is observed in cells with impaired folic acid exporter activity [10].

On the other hand, defective transport is a common mechanism of resistance to antimetabolites when cells are placed under selective pressure with antifolates that utilize RFC as the major route of entry into cells. This mechanism of resistance could not, however, eliminate the ability of cells to reach — *via* the same transport system — folate concentration sufficient for cell growth. The resistance has been attributed to changes in the expression or mutations in this protein. Decreased RFC expression was described both in tumor samples with a poor response to MTX chemotherapy [67] and in cultured neoplastic cells made resistant to antifolates [68]. Likewise, in tissues or cells expressing functionally significant levels of FR, for MTX cytotoxicity or resistance may be relevant the decreased level of FR expression [69, 70], which can occur as result of increased DNA methylation [71]. The point mutations in various locations of the RFC gene have also been associated with decreased MTX transport. These have resulted in prematurely truncated proteins [72, 73], or full-length proteins that are non-functional [74–76], have an altered substrate specificity [77] or altered stability and improper cellular localization [78]. Markedly impaired influx of MTX with relative conservation of reduced folate transport may account for the development of MTX resistance allowing tumor cells to meet their folate requirement [77]. Besides, impaired MTX transport may be due to an alteration in the mobility of the carrier with a failure to translocate its substrate across the cell membrane [79, 80]. To the uderstanding of the basic mechanisms of drug resistance could contribute the finding of a novel membrane protein SQM1, that may affect — in an unknown manner — MTX transport. Expression of SQM1 was shown to be reduced in some cell lines resistant to MTX and correlated with a diminished rate of MTX transport [81]. This finding gives new possibilities, e.g. by detecting SQM1 protein in clinical specimens, it may be possible to monitor in tumors the development of drug resistance, caused by transport failure. The ability to reverse MTX-resistance by formation SQM1-liposome is also under investigations [82].

In cells with a defective carrier protein, MTX can be transported through P-glycoprotein [83, 84], a transmembrane efflux pump, encoded by the *MDR1* gene, removing various lipophilic drugs that enter the cell by passive diffusion through the lipid bilayer. The P-glycoprotein-expressing multidrug-resistant cell lines are not usually cross-resistant to a hydrophilic MTX. However, after loss of influx transporter activity, hydrophilic compounds enter cells primarily by passive diffusion and may become P-glycoprotein substrates. Hence, a deficiency in the MTX carrier enables P-glycoprotein to confer resistance to MTX. The role of P-glycoprotein in MTX transport was reinforced by transfection with MDR proteins, which in some cells conferred a marked level of resistance to short-term (1–4 h) exposure to MTX [43]. On the other hand, lipophilic antifolates, such as trimetrexate, are more expected to cause MDR resistance [85].

TRANSPORT SYSTEMS AND ANTIFOLATE THERAPY

Folate analogs are frequently used in cancer therapy, in the treatment of patients with rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis and others. For many folate analogs, influx is a major determinant of the free drug level achieved and critical determinant of cytotoxicity. A large class of classical antifolates pass into the cell through RFC- or FR-mediated membrane transport. The efficacy of transporters in mediating internalization and cytotoxicity depends on their affinity for the folate analog, their degree of expression and the level of competing folates [86]. In this aspect, biochemical modulation of transport system may contribute to an improved efficacy of folate-based therapy in a clinical setting and hence establishment of the transporter's affinities is essential for each antifolates. Numerous results indicate that transport of MTX, an inhibitor of dihydrofolate reductase, or ZD9331, an inhibitor of thymidylate synthase, mainly proceeds *via* the RFC, while FR is important route in the uptake of inhibitors of thymidylate synthase such as CB3717, ICI-198 and the inhibitor of purine synthesis, 5,10-dideazatetrahydrofolate (DDATHF) [14, 87]. These data have many implications e.g. induced expression of FR by low folate medium could reverse the resistance to the antimetabolites, which have a high affinity for this route of entry [88]. Since antifolates compete with natural folate compounds as transport substrates, there appears the possibility of protection from growth inhibition at the level of FR or RFC transport by adding folic acid or LV respectively [89–91]. The data from clinical trials show, that LV supplementation resulted in a significant reduction in the most common side effects of MTX [92, 93]. Our data show, that addition of LV to *in vitro* culture of murine leukemic 5178Y cells gives similar protective growth-response to cytotoxic MTX activity (Fig. 1).

Modulation of antifolate transport efficacy was also described as a response to the changes in plasma membrane redox state, since free membrane SH groups are known to be essential for increased capacity of the MTX transport carrier. Results with thymocytes and thymic lymphosarcoma cells revealed that, after short-term exposure to cysteine, the rate and extent of MTX accumulation was preferentially enhanced exclusively in neoplastic thymocytes, whereas in normal cells the transport remains unchanged [94]. Similarly, other authors showed that in normal isolated hepatocytes the efficiency of MTX trans-

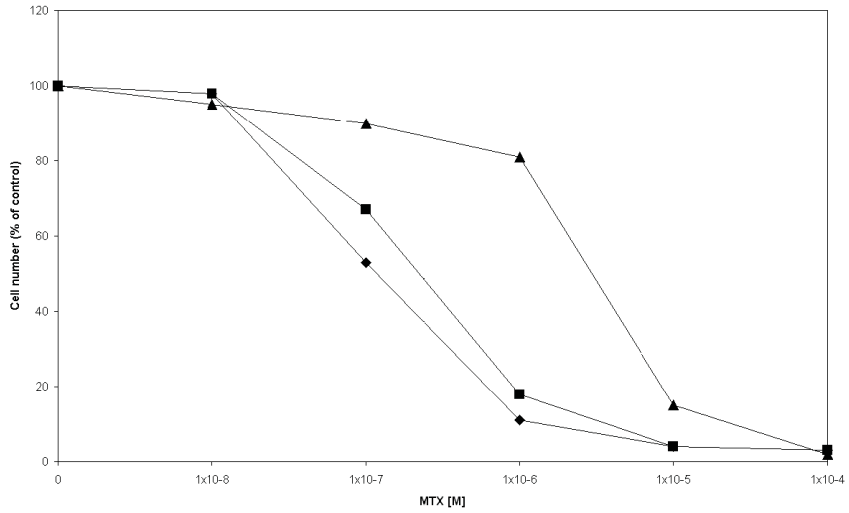

Figure 1. The effect of leucovorin (LV) supplementation on MTX toxicity *in vitro*.

Murine leukaemia 5178Y cells were growing in suspension in the Fisher medium supplemented with 8% of new calf serum at 37°C and 5% CO_2. 3×10^4cells/ml were seeded in the medium containing various concentrations of MTX and without (◆) and with LV added at concentration of 10^{-7} M (■) and 10^{-5} M (▲). After 48 h cells were counted in Neubauer camera. Results were express as % of cells cultured without drug supplementation.

port system increases in GSH-deficient cells [95].

FINAL REMARKS

The functional activity of RFC or FR is an important clinical parameter and potential prognostic factor for successful treatment, hence determination of its tissue levels might have clinical relevance for antifolate therapy. Severe undesirable side effects observed in clinical trials might be related to the presence of high folate-affinity proteins in normal cells, in addition to their increased density in many tissues under folate restriction. Instead, the use of folic acid supplementation for patients with cancer treated with antifolate therapy prevents the biochemical changes in this receptor and could reverse toxicity, but also diminishes the antitumor effects [65, 86]. Understanding of the functional characteristics of the transport of folates and folate analogs have led to the development of novel antifolate agents through rational drug design and targeted therapeutic approaches for tumors that express or lack the presence of these transport proteins. The overexpressed, by a variety of malignant cell lines, FR may offer, *via* the receptor-mediated endocytosis uptake pathway, a suitable mechanism for selective delivering of radiopharmaceuticals to tumors for diagnostic imaging and/or radiation therapy [96]. In the future a new and selective treatment will become available to more effective treatment of patients with a variety of malignancies.

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