Reduced folate carrier: biochemistry and molecular biology of the normal and methotrexate-resistant cell

GEOFFREY BOSSON
School of Applied and Molecular Sciences, Ellison Building, Northumbria University, Newcastle-upon-Tyne NE1 8ST, UK

Accepted: 21 March 2003

ABSTRACT

The cytotoxic drug methotrexate uses the reduced folate carrier for transport into the cell, where it inhibits key enzymes in nucleotide biosynthesis. Resistance to methotrexate can be achieved by altering the genetic code of the reduced folate carrier gene and thus change the structure and function of the protein. Our understanding of RFC structure and function is based on the information gained from studying the uptake of folates and antifolates in living cells and the application of molecular techniques to determine gene expression and genetic mutations. The aim of this essay is to explain the structure and function of the reduced folate carrier, review the molecular biology of the reduced folate carrier gene and the mutations and polymorphisms that can result in methotrexate resistance.

KEY WORDS: Drug resistance. Folic acid. Methotrexate. Molecular biology.

Introduction

The cellular synthesis of organic biomolecules involves either the joining of two or more complex molecules or simply the addition of atoms to existing precursor molecules. In such anabolic pathways, the sequential addition of single carbon atoms provides greater flexibility, and an almost infinite series of organic compounds can be produced.

Carbon atoms can be presented as either carbon dioxide (CO_2), methyl ($-CH_3$), methylene ($-CH_2$), methenyl ($-CH=$), formyl ($-CH=O$) or forminino ($-CH=NH$) groups. Folates act as cofactor carriers and donors of such 1-carbon units, and are especially important in the biosynthesis of adenine, guanine, thymidine and methionine, and in the metabolism of serine and histidine.

The importance of folate to humans is emphasised by deficiency-associated diseases such as megaloblastic anaemia, neural tube defects and cardiovascular disease.[1,2] While prokaryotic cells can synthesise folates, humans and other eukaryotes must obtain folates from the environment; hence their classification as vitamins.

The major route for cellular uptake of folate is the reduced folate carrier (RFC), which is therefore a pharmacological target for inhibiting cell growth. Mutations in this and other key proteins involved in folate metabolism may modify a cell's response to such drugs (e.g., methotrexate) and result in resistance.

Folates and folate metabolism

Folates are a family of molecules based on folic acid (pteroylglutamic acid, folacin). The name is derived from the Latin *folium* meaning leaf, and indicates the main dietary source as green-leafed vegetables.[3] It is also found in fruits, and is produced by the normal intestinal flora. The molecule is composed of a pterin linked to *para*-aminobenzoic acid (PABA), plus one or more glutamate residues (Figure 1).

A normal diet provides sufficient folates, in several chemical forms, to maintain health, growth and replication. Most dietary folates are polyglutamylated and must have the glutamate residues removed before the core molecule can be absorbed by the small intestine. Here, they are transported by the intestinal folate carrier (IFC-1) across the intestinal cells to the basolateral membrane, where they diffuse along a concentration gradient into the submucosa.[4]

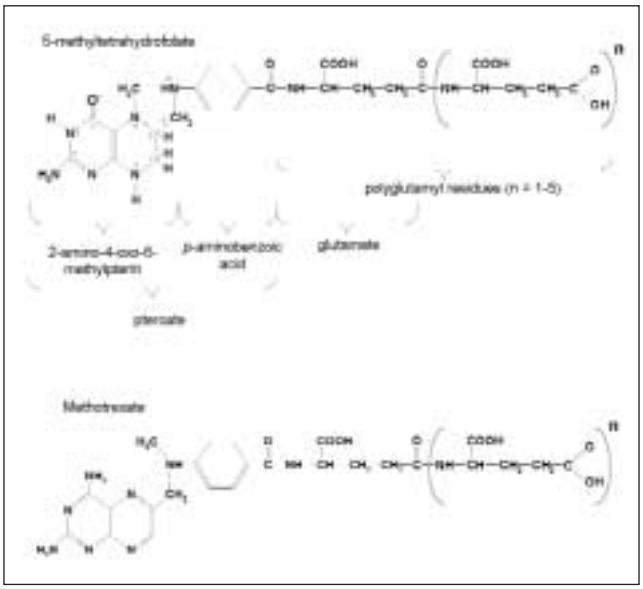

Fig. 1. Structural components of a typical folate molecule (5-methyltetrahydrofolate) and the structural similarity of the cytotoxic antifolate methotrexate.

Correspondence to: Dr G Bosson
Email: geoffrey.bosson@unn.ac.uk

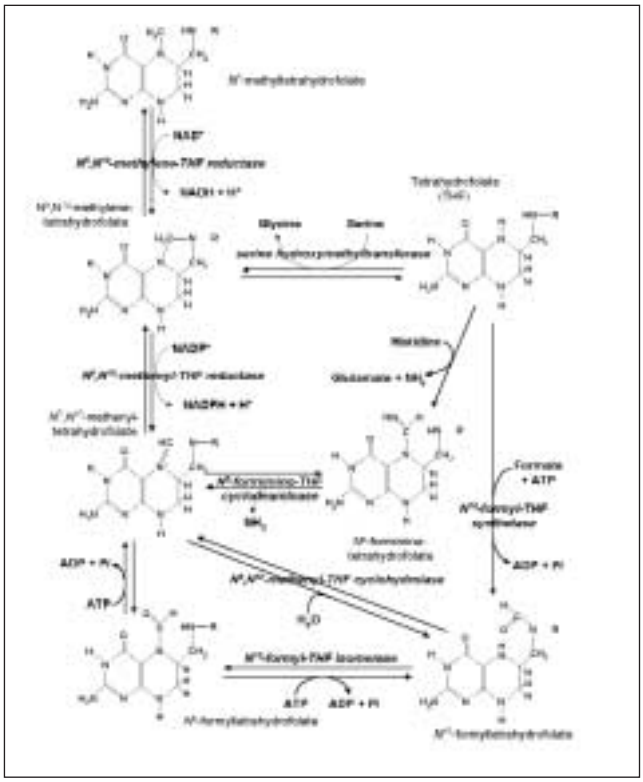

Fig. 2. Conversion of the 1-carbon residue carried by tetrahydrofolate through various oxidative states. For clarity, only the pterin residue is shown in each intermediary, the remaining PABA-glutamate residues are represented by R (modified from Voet *et al*.[3]).

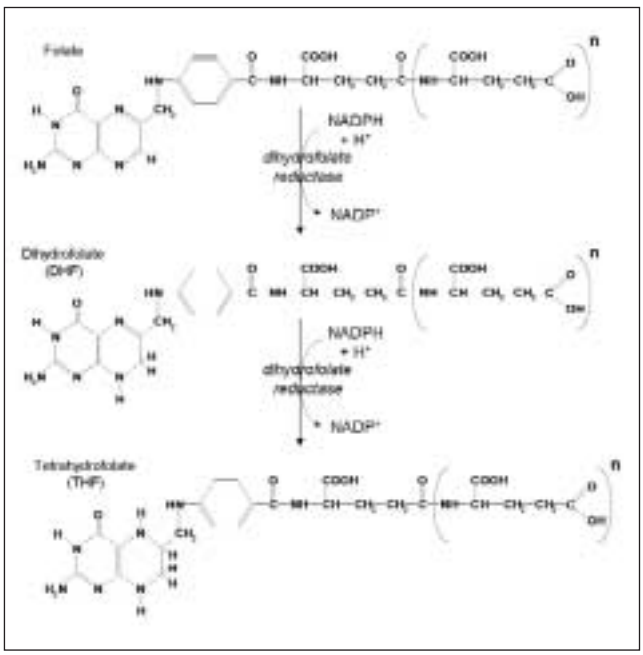

Fig. 3. Activation of folate to tetrahydrofolate (THF).

It has also been suggested that an export mechanism at the basolateral membrane actively pumps folates from the cell and thus pulls folates from the intestinal lumen to replace those moving out of the cell.[5] In either mechanism, the folates delivered into the submucosa will diffuse into the portal circulation.

On first pass through the liver, hepatocytes remove and store the majority of folates until required. Any folates that reach the kidneys are filtered and then reabsorbed along the nephron.[4] The demands of the growing fetus for folates are met by transfer from the maternal circulation via a placental folate transporter (FOLT).

The natural circulating folates, N^5-methyltetrahydrofolate (5-methylTHF) and N^5-formyl-tetrahydrofolate (5-formylTHF) (Figure 2), are taken up by almost all cells in the body. At high concentration, folates can simply diffuse into the cell, but because folates are essentially hydrophilic molecules this does not provide sufficient intracellular folate for normal cell metabolism. To provide an adequate intracellular folate concentration, active transport mechanisms must be used that can work against a concentration gradient.

Folate receptors

Folate receptors (also known as folate binding proteins [FBP]) are glycosylphosphatidylinositol membrane-bound proteins with a high affinity for folic acid (K_M 1-10 nmol/L)[6] and 5-methylTHF (K_M 1-5 nmol/L).[7] Three FBP isoforms have been identified,[8] and the genes coding for two (FRa and FRb) have been cloned.[9, 10] When folic acid or 5-methylTHF binds to a receptor, the whole complex is internalised by endocytosis against the concentration gradient.

Reduced folate carrier

The folate receptors described above have a low affinity for reduced folates (K_M 10-300 nmol/L)[8] and these must therefore be brought into the cell using a different mechanism – RFC. This bi-directional anionic exchanger is the most important of the folate uptake mechanisms, with high affinity for reduced folates.[6]

The two systems may be coordinated to provide sufficient intracellular folates,[11] although some researchers believe they act independently.[12,13] In addition, there appears to be a third mechanism, able to recognise all folates with almost equal affinity (K_M 1-5 µmol/L), which operates at low pH.[14] Although its role is poorly understood, Rajgopal *et al*.[15] described the high efficiency of IFC-1 when operating at the low pH achieved by the acidic microenvironment at the luminal surface of the intestinal cells. The third mechanism may have a similar role, or prove to be one and the same thing. Mitochondria obtain folates from the cytoplasmic pool using an independent RFC in the mitochondrial wall.[16]

Once inside the cell, folylpolyglutamate synthetase (FPGS) adds glutamate residues to the C-terminal of the existing single glutamyl residue. This increases its size and prevents its loss from the cell via the folate export pumps.[14] Folic acid must be reduced by the enzyme dihydrofolate reductase (DHFR) to activate it in a two-step process to produce the fully active tetrahydrofolate (Figure 3). The same enzyme catalyses both steps but, because the preferred substrate is dihydrofolate, the first step is slower than the second.[17] Thus, this enzyme plays a pivotal role in the activation and regeneration of folates and makes it the target for drugs such as methotrexate (MTX).

One of the THF intermediates, N^5,N^{10}-methyleneTHF, catalysed by thymidylate synthase, donates its methylene group to deoxyuridine monophosphate (dUMP) to generate

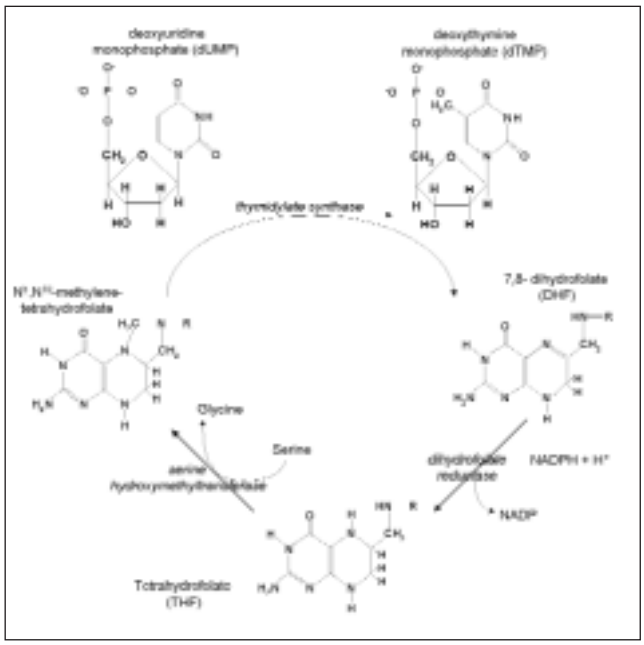

Fig. 4. Methylation of dUMP to dTMP and regeneration of dihydrofolate (DHF) to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR). For clarity, only the pterin residue is shown in each intermediary.

the cell's source of *de novo* deoxythymidylate monophosphate (dTMP). The remaining 7,8-dihydrofolate is recycled back to THF using DHFR (Figure 4).

The ability of living cells to synthesise *de novo* organic molecules is possible by adding carbon centres to each other or to existing biomolecules. It is in this role as carbon donor that tetrahydrofolate is so important, and is the result of its ability to hold the carbon in a variety of oxidative states, ranging from methyl ($-CH_3$) to methenyl ($-CH=$) groups. The 1-carbon residue can be modified from one oxidative state to another (Figure 2), which gives it great versatility in anabolic pathways.

Reduced folate carrier

The RFC is a 65 kDa transmembrane protein comprising 591 amino acid residues. In humans it exists in a highly glycosylated form with a mass of approximately 92 kDa.[18] While folate carriers are likely to be found in all higher animals, much of the data on RFC has been obtained from mouse and hamster models that show approximately 50% homology in amino acid sequence with the human protein.[19] A summary of characteristics between the three species is shown in Table 1.

Functionally, RFC is an anion exchanger that transfers hydrophilic folates across the cell membrane using the negatively charged glutamate residue of folate. Such a system requires positively charged amino acids and the arginine residues (at 133, 155 and 366 in murine cell lines) are highly conserved and likely candidates.[20] While the charge of these amino acids is important, Zhao *et al*.[20] showed that it is not the only factor when they substituted Arg-131 with histidine. Despite replacing one positively charged amino acid with another, activity was still lost.

Table 1. Characteristics of RFC from the three species most frequently used in studies

Characteristic	Human	Hamster	Mouse
Size (protein only) (inc. glycosylated attachment)	65 kDa 85 kDa	58.6 kDa	58.2 kDa
pI	9.6	9.4	9.4
Number of amino acids	591 residues	518 residues	512 residues
Location of gene	Chromosome 21	Chromosome 1	Chromosome 10
Number of alternative spliced products	18 isoforms	2 isoforms	3 isoforms

While the protein structure of RFC in all three species has a conserved potential phosphorylation site for protein kinase-C at residue 23,[21] its use has yet to be uncovered. RFC lacks the structure typical of other transmembrane transport proteins that utilise ATP, which leaves the theory that intracellular phosphate is the driving force for folate uptake. High intracellular phosphate (PO_4^{2-}) is the anion that leaves the cell along a concentration gradient. To maintain neutrality, phosphate could be carried back into the cell but the protein has an affinity for reduced folates and extracellular phosphate concentration is low.[14] Once inside the cell, folate concentration is relatively low compared to phosphate, and very little leaves the cell via the RFC under competitive conditions.

RFC structure shown in Figure 5 is based on the Kyte-Doolitle hydropathy plot[22] and the Hopp and Wood hydrophilicity plot.[16] The putative transmembrane arrangement derived from this data is accepted by the major RFC research groups;[6, 14, 20, 23, 24] however, the complete three-dimensional (3-D) structure is yet to be elucidated. Figure 5 clearly shows the arrangement of 12 α-helical transmembrane domains made up of hydrophobic amino acids. The transmembrane domains (TMD) range in size from 17-25 residues and are highly conserved between the three species studied,[25] especially the nine tryptophan residues and four of the six cysteine residues.[25]

Hydrophilic amino acids make up the four small intracellular loops, six extracellular loops, C-terminal, *N*-terminal regions and the large intracellular loop formed halfway along the sequence. Both C- and *N*-terminals of normal RFC are shown lying in the cytoplasm. This was confirmed experimentally by the transfection of a known epitope construct into the C-terminal of a K562 cell line and subsequent localisation of the translated product by immunofluorescence staining for the inserted epitope.[26] The fluorescent label was bound to the cell membrane, but only after the cell was made permeable with Triton X-100.[26] Similar studies have been performed to confirm the position of other domains in relation to the cell membrane.[27]

Human RFC is larger than rodent RFC, due to a 73-79 amino acid C-terminal extension.[25] The terminal regions are not thought to play a role in the protein's function but, like many proteins, they contain motifs recognised for membrane localisation and intracellular trafficking. The RXR (Arg-X-Arg) motif at residue 460 causes temporary retention

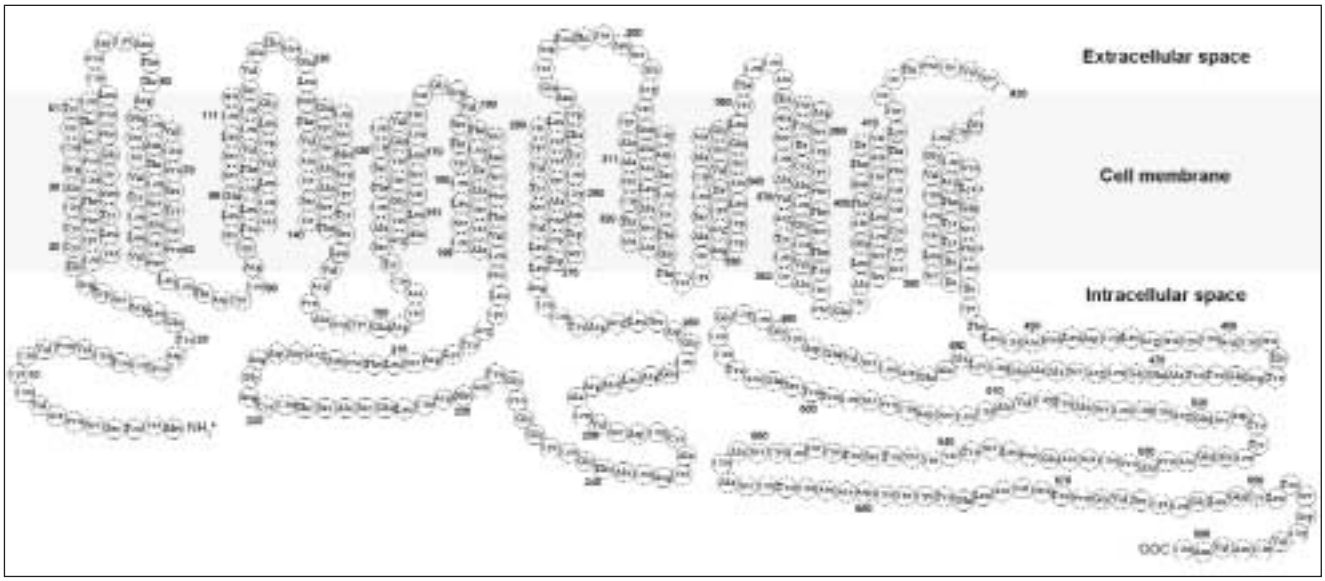

Fig. 5. Amino acid sequence and probable secondary structure producing 12 transmembrane domains of the RFC (modified from Drori *et al*.[6] and Zhao *et al*.[20]).

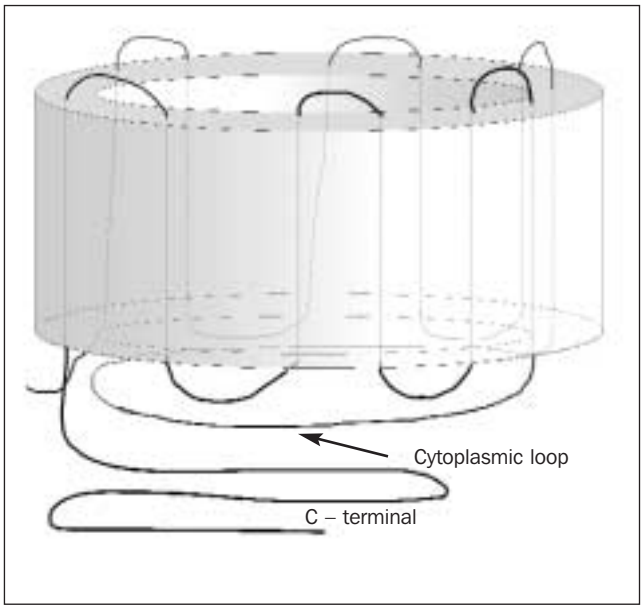

Fig. 6. Schematic to show how the large intracellular loop could play a role in anchoring the transmembrane domains into the expected cylindrical conduit in the cell membrane.

of RFC at the endoplasmic reticulum.[27] This sequence lies in the C-terminal and any mutations affecting or causing loss of the C-terminus will influence the time it takes for the protein to pass along the secretory pathway and embed itself in the cell membrane. This not only hinders correct positioning of the protein but also blocks any further protein synthesis. The C-terminal contains a di-leucine motif at residues 498 and 499 that is also likely to play a role in protein processing and localisation.[27]

The role of chaperones during the synthesis and transport of the protein has yet to be investigated but are likely to prove important – as they are for other proteins – in forming the appropriate tertiary structure. RFC maintains a stable tertiary structure using, among other bonds, charged pair attraction between negatively charged aspartate residues at 88 and 453 and positively charged arginine at 133 (TMD 4).[23]

Liu *et al*.[23] noted a significant drop in activity (V_{max}) when Asp88 was replaced with a neutral valine. However, when substituted with another negatively charged amino acid (glutamic acid), activity only dropped slightly. Similar replacement of the positive Arg133 by a neutral amino acid (leucine) caused loss of activity. It is interesting to note that when both residues 133 and 88 lose their charge the tertiary structure can still be formed and activity is maintained. This latter finding suggests that these residues are involved in stabilisation of the protein structure and are not required for direct recognition of folate or transport.

The size of the large intracellular loop also appears to be crucial to development of the correct RFC conformation.[27,28] This suggests that it could have a role in anchoring the TMDs into the cylindrical conformation expected of a transmembrane carrier (Figure 6).

While the concept is worthy of further study, recent work by Liu *et al*.[28] indicates that it is unlikely. They deleted sections of the loop and then replaced them with similar sections from the loop of the thiamine transporter (ThTr1) to form chimaeric constructs. With the exception of a short sequence of 11 amino acids (K_{204}RPKRSLFFNR_{214}), the majority of residues in the loop were not conserved. However, deletion of the whole or substantial parts of the loop significantly reduced RFC activity. Once passed the first 11 residues of the loop (the importance of which is unknown), the actual sequence of amino acids had minimal effect. These findings support the theory of Sadlish *et al*.[27] that only six TDMs can be inserted into the cell membrane at one time, as the loop keeps them far enough apart for two insertions but maintains the proximity of each.

Glycosylation on residue 58, asparagine, accounts for the significant difference in molecular weight described by Matherly and Angeles.[29] In the presence of tunicamycin to block glycosylation, or following elimination of the *N*-glycosylation site by site-directed mutagenesis, RFC lost

some of its activity.[30] The most likely explanation is that, as it migrates along the endoplasmic reticulum (ER), the usual glycosylation of proteins increases the viscosity of the ER lumen, causing longer retention and ultimately reduces the amount arriving at the cell membrane.[31] However, Wong *et al*.[30] concluded that glycosylation of RFC played no role in its function or its ability to migrate to the cell membrane and establish itself as a transmembrane structure.

RFC-1 gene

Once the protein structure was identified, it was relatively easy to generate complementary DNA (cDNA) for the protein and find a match in the human genome built from contig alignment. A sequence on chromosome 21 at SLC19A1 was identified and confirmed using fluorescence *in situ* hybridisation (FISH). The gene coding for the human RFC protein is located on the long arm of chromosome 21, specifically the 21q22.2-q22.3 region.[21, 32]

The structure of the gene has been studied in mouse, hamster and human cell lines and is referred to as RFC-1. Tolner *et al*.[33] studied cDNA from 16 genomic clones and obtained two overlapping sequences covering the RFC-1 gene. The first strand, labelled λhRFC1-1, is a 19 kb segment containing exons 1, 2, 3 and 4. The second strand, λh-RFC1-2, is slightly shorter at 17 kb and contains exons 5 and 6. The 5′ end of λhRFC1-2 overlaps 1.5 kb of the 3′ end of λhRFC1-1 and thus together they cover 34.5 kb. The full-length cDNA was confirmed as the RFC-1 gene by transfection into a methotrexate-resistant cell line. These cells have become resistant to this folate analogue by producing a mutation in the protein, and no longer take up MTX. When the proposed gene is transfected into such cells and they start to transport MTX across the cell membrane, this is strong evidence that the genetic material introduced codes for RFC.

The exonic sequences have been submitted to both the National Center for Biotechnology Information (NCBI) and European Molecular Biology Laboratory (EMBL) databases, using the accession numbers shown in Table 2. Following rapid amplification of cDNA ends (RACE) and gene walking studies, the intronic regions between the exons have been defined and show several intron/exon/intron splice sites that comform to the GT-AC rule. The boundaries and exonic sequences are mainly conserved across the species, while the introns can vary greatly in size and sequence.

A shorter cDNA sequence was submitted to GenBank (accession number U19870) by Matherly's group,[25] and contained a 1776 bp open reading frame (ORF) flanked by a 98 bp 5′-UTR and 864 bp 3′-UTR. The cDNA ORF from either source predicts a protein of 64,873 Da, which is in the range for the deglycoslyated protein obtained experimentally and demonstrated by Western blot.[30]

As can be seen from Table 2, Tolner *et al*.[33] described three alternative untranslated sequences in exon 1, labelled 1a, 1b and 1c. Each ends with the AG sequence that provides the opportunity for alternative splicing and produces three RFC-1 variants: variant I contains exon 1a plus exons 2-6; variant II contains exon 1b plus exons 2-6; and variant III contains exon 1c plus exon 2 and part of 3.

Variants I and II are active proteins, but the truncated RFC from variant III is inactive. A truncated variant was noted in hamster cell lines in 1994[34] and the suggestion that it was the result of alternative splicing first postulated. The 5′-end untranslated sequence contains promoters that have been studied using inserted downstream indicator genes (e.g., luciferase). Using this technique, it was shown that variant II produces the most efficient transcription.

Table 2. Accession numbers for RFC-1 exons on the NCBI Entrez (http://www.ncbi.nlm.nih.gov/Entrez/) and EMBL (http://www.ebi.ac.uk/embl/index.html) databases

Exon	Accession No.	Size (bp)
1a, 1b and 1c	U92868	3772
2	U92869	250
3	U92870	772
4	U92871	214
5	U92872	151
6	U92873	1451

Using different cell lines, Gong *et al*.[35] showed that the alternative splicing appeared to be tissue-specific and introduced a fourth alternative of exon 1. This new exon, 1d, was only found in fetal liver cells. Exons 1a and 1d are separated from their neighbours by large intronic regions and appear to have independent promoters. With only 21 bases between exons 1b and 1c, they are likely to share the same promotor upstream of exon 1b.

The tissue-specific differences recently highlighted by Whetstine *et al*.[36] also use the alternative splicing theory of 5′-UTRs. The seven possible non-coding exons contain either promoters or promotor enhancers that result in 18 unique splice variants; however, the role of each is yet to be completely understood.

While Tolner *et al*.[33] confirmed that variant III causes a truncated protein, a paper by Zhang *et al*.[18] in the same year proposed more subtle alterations in post-transcriptional processing without altering the coding sequence of the protein. To confuse exon identification further, Zhang *et al*.[18] used a different numbering system for the exons, whereby exon 3 contained the Kozak start sequence (ATG) and corresponds to exon 2 in Tolner's model.[33, 37]

Although there is increased RFC-1 expression in response to very low folate concentration,[14] little is known about the regulation of RFC-1 expression. When 5-formyl folate is reintroduced to 'starved' cells they revert to normal transcription rates, suggesting that a feedback mechanism between intracellular folate level and RFC transcription must exist.[38] Gong *et al*.[35] measured messenger RNA (mRNA) levels throughout the normal cell cycle and noted that transcription is linked to cellular requirements for folate, reaching a peak during the G_1/S phase. In fact, they showed that the variable transcripts show a chronological pattern during the cell cycle, with exon 1c being expressed earlier than 1b and 1d. The significance of this is unknown.

In a series of experiments to determine the promoters for the RFC-1 gene, Zhang *et al*.[18] studied a 342 bp intronic region between exon 2 and 3 (1b and 2 in Tolner's model), which they labelled Pro32, and a 996 bp region upstream of exon 1 (1a in Tolner's model) was labelled Pro43. These regions were later reclassified as hRFC-A and hRFC-B, respectively.[39] The presence of two promoter regions for the

RFC-1 gene indicates the importance of the RFC protein and provides a mechanism to enhance transcription to a higher level when need for the protein is greatest.

Using the luciferase gene as an indicator sequence, these two promoter regions were studied to determine the exact mode of control. Sequential loss of 5′ upstream regions showed that promoters in Pro32 reside between -501 and -455 (where +1 is the translational start site). This 47 bp sequence is GC-rich and contains a CRE/AP-1 element at -485 to -471. The bZip DNA binding proteins (e.g., CREB-1, ATF-1 and c-Jun) bind to CRE/AP-1 elements and cause transcription as part of the cAMP secondary messenger system. An additional AP binding site is present in a recently identified variable nucleotide tandem repeat (VNTR), which increases promoter activity of the 5′-UTR by 63%.[40]

The more efficient Pro43 region that lies between -1088 and -1043 (46 bp) is even more GC-rich and, while there is no TATA box, the high GC content does provide a GC box (CCCGCCC) between -1081 to -1078. Whetstine *et al*.[39] also suggest that there is a second GC box between -1077 and -1071. GC boxes are known to bind Sp1-activating factors and Sp3-antagonistic factors. With opposing actions, Sp1 and Sp3 may be involved in fine control of gene expression and splice variant transcription.

The intronic sequence of the 5′-untranslated region (UTR) may also contain an inhibitory site between -2338 and -1935. This was indicated during the progressive deletion of regions to find the promoters, when increased expression was noted on removal of this particular section.[39]

It is interesting to note that the *p*53 gene may have a role in controlling RFC-1 expression. p53 protein is expressed when a defect in DNA (usually occuring during replication) is detected and inhibits cell replication until either the error is repaired or apoptosis is induced.[41] In this situation, it makes sense to decrease folate uptake and starve the cell of components necessary for replication. This is achieved, to some degree, by p53 suppression of RFC-1 expression.[42] p53 acts on the hRFC-B promoter rather than hRFC-A.[24]

While much effort has been devoted to the 5′-UTR, it appears that exon 6 at the other end may be just as variable. This would explain some of the differences seen between species for the cytoplasmic C-terminal.[43] Brigle *et al*.[44] reported similar C-terminal variation in murine RFC-1 that may play a role in controlling folate uptake. Restriction enzyme analysis of the murine gene indicates that the alleles are heterozygous.[44] The conclusion, therefore, is that if the wild-type allele is present in the cell lines studied, there must be some silencing of the normal allele. The proposed mechanism for this effect is DNA methylation, known to occur in suppression of other genes and also likely to occur in humans.[45]

Acute lymphoblastic leukaemia

Leukaemias are a group of diseases resulting from the uncontrolled proliferation of leucocytes. Broadly, they are classified as either acute or chronic, and subgroups including acute lymphoblastic leukaemia (ALL).[46] ALL originates in the bone marrow and by the time the clinical symptoms are evident it has replaced most of the normal marrow and metastasised.

Presentation of ALL is essentially the same in adults and children at relapse or original diagnosis. Pallor, malaise and weakness due to anaemia are the principal symptoms, but bruising, epistaxis and petechiea indicate associated thrombocytopenia. Both are the result of the normal marrow being replaced by malignant tissue, which also leads to increased susceptibility to infection as normal leucocyte production is disordered.

ALL is diagnosed twice as often in children than adults, but the former show a better response to treatment, with an 80% cure rate possible.[47] This success rate can be improved by initiating a more rigorous treatment regimen from the start,[48] but this requires tighter classification of the malignant cell line, particularly in relation to specific gene mutations.

Although some of the gene mutations leading to ALL can be identified by cytogenetics (e.g., translocations that lead to inactivation of suppressor genes – the Philadelphia chromosome being the most common), there are few specific morphological markers and classification is often based on immunological identification using cluster of differentiation (CD) markers.[49] Reverse transcription polymerase chain reaction (RT-PCR), FISH and other techniques available to the molecular biologist are increasingly important in identifying genes associated with poor prognosis (e.g., multiple drug resistance gene).[50]

There are three phases in the treatment of ALL: induction of remission (returns blood profile to normal), consolidation (keeps blood profile normal) and continuation. The final phase is rarely required for more than three years, but attempts to reduce this sadly correlate with a higher relapse rate.[50]

Relapse is more common in adults and is likely to be due to the higher frequency of genetic abnormalities in this group. Relapse in both children and adults can result from the lower drug concentrations in some body compartments (e.g., cerebrospinal fluid, testes) that are sanctuaries for the malignant cells.[49] Exposure to drug levels below the minimum effective dose gives the cells a chance to evolve and develop drug resistance. Proliferation of these rogue cells leads to relapse and a poor prognosis.

Methotrexate

Methotrexate (4-NH_2,*N*-10 methyl analogue of folic acid) is an effective and widely used cytotoxic agent. Rots *et al*.[47] describe how it has been used with success in the treatment of ALL since the 1940s. Similarity in structure between MTX and the folates (Figure 1) allows it to compete with and inhibit the key enzymes DHFR and TS. This inhibits purine and thymidine synthesis and regeneration of THF, thereby reducing DNA replication and cell proliferation.[51] The ratio of dUMP to dTMP is increased, making misincorporation during replication more likely, leading to fragmentation of the DNA.[47]

T-lineage ALL usually requires higher doses of MTX than do precursor B-ALL malignancies.[47] In high-dose therapy, used in some cases of MTX resistance, extracellular MTX concentrations >20 μmol/L are achieved regularly, and are high enough to facilitate simple diffusion across the cell membrane.[52] When lower routine doses of MTX are used, effective intracellular concentrations can only be achieved by using the RFC transport mechanism.

The anion exchange mechanism recognises the negatively

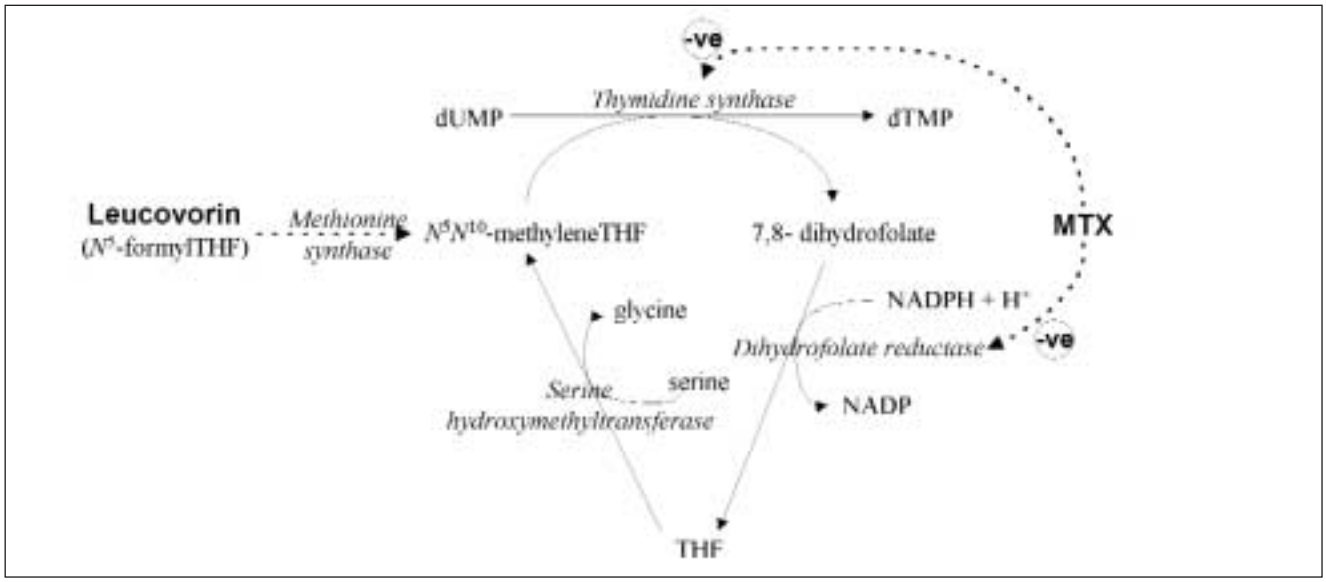

Fig. 7. Schematic of the reactions shown in Figure 4 to indicate where MTX and leucovorin are involved.

charged glutamate residue, which is also used to transport true folates. Once inside the cell, it must be polyglutamylated by folylpolyglutamate synthetase to prevent diffusion back out of the cell or active excretion via the multidrug resistance protein. Retention in the cell is proportional to the number of glutamyl residues.[53] MTX fits into the cleft of DHFR, but because the pteridine ring is inverted, it forms tighter bonds with the active site.[54] This means that the drug binds to the enzyme for longer, making it more effective. Another advantage of MTX polyglutamylation is that it directly inhibits TS.[53]

Mature cells have little demand for THF and are affected only minimally by MTX, but cells undergoing rapid replication (e.g., cells of the normal bone marrow and gastrointestinal mucosa, as well as cancer cells) take up the drug when their demand is highest, effectively poisoning themselves.[55] Cancer cells with hyperploidy for chromosome 21 express extra copies of the RFC-1 gene and have larger amounts of RFC in the cell wall, which makes them more sensitive to MTX.[56]

The most effective MTX regimen is high exposure over a long period of time, but the side effects are too severe. Slightly higher doses of MTX can be tolerated if leucovorin (N^5-formylTHF) is given to support the normal cells. This works because normal cells contain the enzyme methionine synthase, which converts N^5-formylTHF to N^5N^{10}-methyleneTHF, thus by-passing the need for DHFR to generate THF (Figure 7).

MTX's weak negative charge, which hinders its diffusion across cell membranes, also reduces its distribution into the CNS and other lipophilic tissues. Any cells in these sanctuaries will not be exposed to high enough concentrations of MTX to be effective, but they could be sufficiently high for the cells to develop a resistance.[53]

Methotrexate resistance

Natural selection in living things is based on the fact that mutations (which occur all the time) in a cell's genome may give it a survival advantage in a modified environment. Thus, if cells are exposed to MTX levels insufficient to be lethal, they will have a chance to mutate and replicate. If the mutation somehow decreases the intracellular concentration of active polyglutamylated MTX, then they will be able to tolerate higher doses in the future and survive standard treatment regimens (i.e., they will have developed a resistance).

The four possible mechanisms for resistance[8, 47, 53, 57-59] are:

- decreased membrane transport into the cell by down-regulation of the RFC-1 gene, gross modification of the protein structure so that it is cleared from the membrane faster, impaired transport function, and decreased affinity of the RFC protein for MTX;
- altered DHFR response to MTX by decreased affinity for MTX, increasing true folates to overcome competitive inhibition by MTX,[60] and increasing the amount of DHFR;[59]
- MTX rendered less effective by reducing folylpolyglutamate synthetase activity; and,
- increased efflux of MTX from the cell by increasing folylpolyglutamate hydrolase activity,[47] and increasing activity of the multiple drug resistance protein.

While the final three mechanisms provide resistance once the drug is inside the cell, the first option – down-regulation of the RFC-1 gene – aims to prevent or reduce the amount of drug to which the intracellular systems are exposed, and is considered the first line of defence.

Down-regulation of the RFC-1 gene

The simplest and most responsive way to reduce the amount of RFC in the cell membrane is to suppress the gene coding for the protein.[61] MTX^RZR-75-1 and K500E are MTX-resistant cell lines that have mRNA levels lower than those found in the corresponding wild-type cells.[21,62] One way to achieve

this is to simply down-regulate expression of the RFC-1 gene, possibly by a mutation in one of the upstream promoter regions, or a chromosomal translocation that produces transcriptional silencing.[62]

This suppression could be specific to particular spliced mRNA arrangements; for example, exon 1c was found to be the most frequently suppressed in studies by Gong *et al*.[35] The work by Ding *et al*.[41] into the control of RFC-1 expression by the *p*53 gene opens up the possibility that MTX resistance can be achieved indirectly by a mutation in the *p*53 gene.

Gorlick *et al*.[63] report decreased expression in a series of relapsed ALL cases, indicating that this mechanism is used *in vivo* and not just in 'forced' resistant *in vitro* cell lines. Measurement of RFC-1 mRNA is not just a research tool, it could also be used to determine those patients who will not respond to standard-dose MTX-based treatment.

Gross modification of the RFC protein structure

In resistant cell lines where the mRNA is normal, or even increased,[38] the explanation must be a modified protein. These modifications can be small (e.g., one amino acid) or gross changes in the structure as a whole and have tended to cluster into specific regions of the protein.[64]

A 7 bp deletion at the intron/exon 6 splice site (i.e., nucleotides 1152 to 1158) in the murine MtxRIIOua^{R}2-4 cell line causes the loss of two residues and a frame shift.[57] The amino acid sequence resulting from the frame shift has no homology with the original, and a premature stop codon is introduced. The resulting truncated protein with a modified C-terminal is less stable and, while normal RFC in the membrane has a turnover of approximately 50% per day,[65] the defective protein is cleared from the membrane faster than normal RFC.[57, 65]

In humans, similar truncating mutations have been demonstrated in MTX-resistant sublines of MOLT-3[58] and CCRF-CEM.[65] The MOLT-3/MTX$_{10,000}$ cell line contains two truncating mutations.[58] One is the result of a G to A mutation at position 74 that changes the TGG codon (tryptophan) at position 25 to a stop codon, TAG. The second is a C to T transition involving codon 40, CAG (glutamine) to TAG. When both mutations occur together, one in each allele, the resistance is high (i.e., 10,000-fold). The CEM/Mtx-1 cells, studied by Wong *et al*,[65] have a frameshift that also produces an early stop codon. The four-base CATG segment inserted at position 191 causes a heterologous amino acid sequence, (similar to that described in the MtxRIIOua^{R}2-4 cells[57]) and an early stop codon at position 1176. The hydropathy plot of the translated mutant sequence no longer favours stable localisation in the cell membrane, and is therefore not active.

Not all truncated RFCs produce MTX resistance. A 987 base deletion in the CEM-7A leukaemic cell line results in the loss of 160 normally coded amino acids from the C-terminal and the normal stop codon, (Figure 8). The deletion of bases 1389 to 2376 allows the 5′-end sequence from base 2377 to be spliced to 1388, and produces an alternative ORF. This alternative sequence now codes for 58 new C-terminal residues before reaching a new stop codon in the previously untranslated 3′-region. The proposed structure of the resulting RFC is similar to that shown in Figure 5 but, because it lacks the last transmembrane domain, the truncated C-terminal end is left outside the cell membrane.[6]

The truncated protein alone is unable to transport MTX and correctly considered as non-functional by Matherly.[24]

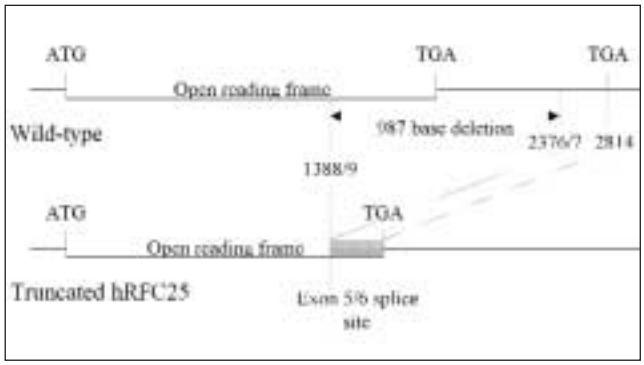

Fig. 8. Alternative splicing of cDNA from human leukaemic CEM-7A cells, producing a truncated RFC.[6]

However, Drori *et al*.,[6] in their discussion, suggest that over-expression of this 'alternatively spliced gene' and subsequent protein synthesis in heterozygotes may have modified the influx/efflux rates that render the cell MTX-sensitive. Brigle *et al*.[44] describe a similar 'alternatively spliced' variant in their studies of mouse cells, which lack several C-terminal end transmembrane domains.

Antibodies against RFC will not detect the truncated proteins described because the epitope sequence is missing in most cases. If immunoassay does detect a full-length translated protein then MTX resistance is likely to be the result of a single but critical residue alteration. These single amino acid alterations can affect the transport function of the protein, or its recognition and affinity for MTX/folates. A negative immunoassay does not exclusively indicate a truncated protein, because it is possible that a single amino acid substitution in the epitope will prevent antigen-antibody recognition.

Proline residues in the α-helical TMD are important features that the RFC should adopt in its tertiary structure. Thus, it is not suprising that the introduction of more proline, or substitution of existing proline, will produce kinks and significantly alter the protein structure. When this is done by site-directed mutagenesis, a loss of transport activity is noted, but affinity is unaffected.[65] This fits with the theory that the transmembrane domains are not involved directly in folate recognition.

Impaired transport function

A predicted conformational change in the RFC to transport folates and MTX requires several of the transmembrane domains to interact with intracellular domains.[66] Previously, the connecting intracellular loop sequences between the TMDs were thought to be just that, connectors, but when the primary structure was modified by the insertion of an immunogenic marker sequence, RFC activity was modified.[26] For example, insertion of this sequence into the loop between the 11th and 12th TMDs caused a total loss of activity. While the result indicates that the insertion of additional amino acids will push the TMDs sufficiently far apart to disrupt RFC function, such large insertions are not likely to occur *in vivo*.

When the charge on residue 45 is changed from negative glutamate to positive lysine, a decrease in the fluidity of the protein is thought to occur, rendering it non-functional. Evidence for this lies in the fact that chloride ions appear to

Table 3. Summary of mutations in the RFC-1 gene affecting the function of the RFC protein (i.e., change in V_{max} but not K_M)

Nucleotide affected	Amino acid residue affected	Species	Domain affected and effect on RFC	Ref
G133A	Glu45Lys	m	1st TMD. Makes the protein rigid	66
G137A	Ser46Asn	m	1st TMD. Allosteric restriction of RFC mobility	68
G175A	Arg27His	H	TMD1. Truncated at 225	64
C264G	Asp88Glu	H	2nd TMD. Slightly $\downarrow V_{max}$ $\rightarrow K_M$	23
G388C	Ala130Pro	m	4th TMD. Proline causes a kink in the protein structure	65
G398A	Arg133His	H	4th TMD. Total loss of activity	23
G398T	Arg133Leu	H	4th TMD. Loss of ion pair with Asp 88 causing total loss of activity	23
CGC397-9GAG	Arg133Glu	H	4th TMD. Total loss of activity	23
C769A	Ser225stop	H	Truncated protein	64
A1033G	Gly345Arg	h	9th TMD. The introduction of the +ve charge of the arginine modifies the 2°/3° structure. Decreased half-life due to poor stability	57
G1118A	Arg373His	m	10th TMD. Loss of positively charged arginine reduces transport as much as 50-fold	67
AAG1210 – 1212CTG	Lys404Leu	m	11th TMD. Loss of positive charged lysine removes the inhibitory effect of Cl- on MTX uptake	69

Footnote
m = mouse, h = hamster, H = human
Nucleotide numbering is taken from the start codon of the open reading frame

Table 4. Summary of mutations in the RFC-1 gene affecting the binding affinity (i.e., V_{max} remains the same but K_M changes)

Nucleotide affected	Amino acid residue affected	Species	Domain affected and effect on RFC	Ref
G131A	Gly44Glu	m	1st TMD	20
G227A	Glu45Lys	H	1st TMD. Modifies sensitivity to Cl-	45
G133A	Glu45Gln	m	1st TMD. $\uparrow K_M$ for MTX, $\downarrow K_M$ for natural folates	70
GAA133 – 135CGT	Glu45Arg	m	1st TMD. $\rightarrow K_M$ for MTX, $\downarrow K_M$ for natural folates, significantly for folic acid	70
GAA133 – 135GAC	Glu45Asp	m	1st TMD. $\uparrow K_M$ for all substrates, including MTX	70
GAA133 – 135CTC	Glu45Leu	m	1st TMD. $\uparrow K_M$ for all substrates, including MTX	70
GAA133 – 135TGG	Glu45Try	m	1st TMD. $\uparrow K_M$ for reduced folates, but $\downarrow K_M$ for folic acid	70
G137A	Ser46Asn	m	1st TMD. $\uparrow K_M$ for MTX, $\downarrow K_M$ for natural folates 68	
A142T	Ile48Phe	m	1st TMD. $\rightarrow K_M$ for MTX, $\downarrow K_M$ for folic acid. $\uparrow K_M$ for $N^{5,10}$ dideazatetrahydafolate	71
T313G	Trp105Gly	m	3rd TMD. $\downarrow K_M$ for folic acid	71
G890A	Ser297Asn	m	External loop between TMD 7-8. $\uparrow K_M$ 72	
C926T	Ser309Phe	m	8th TMD	73

Footnote
m = mouse, H = human
Nucleotide numbering is taken from the start codon of the open reading frame

neutralise the lysine and return function.[66] Larger anions, (e.g., sulphate, ATP) are too large to reach the lysine and have no neutralising capacity. The ability of RFC to function when this amino acid is neutralised indicates that the negatively charged glutamate at position 45 is not important to the binding of folates or to transport activity.

Forced mutations of Arg133 and Asp88[23] provide further evidence that TMDs interact with each other to produce a functional carrier. The two oppositely charged amino acids form an ion-pair. When both are mutated to neutral amino acids they can still form an association and have minimal effect on activity, but if just one of the pair is changed then it prevents the tertiary structure being formed and results in a total loss of activity.

The conclusion was complicated somewhat by the finding that substitution of the negatively charged Asp88 with Glu,

another negatively charged amino acid, produced a drop in activity. This suggests that the additional methylene group of glutamate is sufficiently large to cause a slight structural change, and that the conformation of the protein at this point is not dependent solely on charge interaction.[23]

Similar experiments by Sadlish *et al*.[67] on Arg373 in murine cells indicated the charge of this residue was just as important. The accepted topology of RFC puts this polar amino acid in TMD10, a non-polar region, and therefore suggests that this residue could play a role in temporarily holding the polar folates on their way through the RFC. A summary of these mutations is shown in Table 3.

Modified binding

The most effective way for a cell to obtain sufficient reduced folate to sustain a high replication rate, yet exclude MTX from it, is to make the RFC selective. This can be achieved by substituting amino acid residues responsible for binding recognition or affinity, and is demonstrated experimentally by changes in K_M. A summary of mutations that modify K_M are shown in Table 4.

Amino acids 45-48 appear to form an aqueous pocket in the first TDM that would be important for the binding of folates and MTX. It is not surprising, therefore, that mutations in the first TMD have an effect on affinity, some directly and others indirectly.

Results of targeted mutation studies performed by Zhao *et al*.[70] (Table 5) show that substitution of the negatively charged glutamic acid at position 45 with the negatively charged amino acid aspartic acid significantly decreases affinity for all four compounds. This indicates that it is not the negative charge at position 45 that is essential for RFC function. If substituted by a hydrophobic amino acid (i.e., leucine or tryptophan) then affinity is also decreased, but substitution with hydrophilic glutamine provides decreased affinity for MTX while increasing affinity for folates. The benefit of a hydrophilic amino acid gives support to the aqueous pocket theory.

The Glu45Arg substitution has little effect on the affinity for MTX, and relies on the increased affinity for reduced folates and folic acid to provide the resistance (i.e., by selecting their uptake in preference to MTX). The sensitivity of RFC to chloride ions in the Glu45Lys substitution is thought to result from an allosteric effect caused by small anions on a remote domain of the protein.[66]

Experiments by Sharina *et al*.,[69] who studied the chloride effect on other positively charged residues, showed that the substitution of lysine at residue 404 of murine RFC resulted in chloride losing the competitive inhibitory effect seen in wild-type RFC. The conclusion was that this residue could be a site for control of RFC activity by chloride ions.

Further evidence that the first TMD is involved in the binding function of RFC is provided by Ser46Asp[68] and Ile48Phe[71] mutations, which show similar transport selectivity. Work by both Zhao *et al*.[66] and Tse *et al*.[71] suggests that the third TMD may also play a part in forming this hydrophilic pocket. The increased affinity for folic acid seen in the Trp105Gly substitution provides resistance to another antifolate, $N^{5,10}$dideazatetrahydrofolate (DDATHF). This is achieved by the selective uptake of folic acid to increase the intracellular concentration, which saturates PFGS and prevents DDATHF taking its active polyglutamylated form.[71]

Not all mutations identified so far affect RFC activity. Some will be silent mutations (where the triplet code is changed, but the same amino acid is coded for) or the substitution affects a non-crucial amino acid. Table 6 provides a summary of mutations with no known affect. While these may not be useful to those studying MTX resistance, they may help to elucidate the 3-D structure of RFC.

Table 5. Effect on binding affinity of MTX, N^5– formylTHF, N^5– methylTHF and folic acid when glutamate at residue 45 is substituted (modified from Zhao *et al*.[70])

	K_M (μmol/L)			
Mutation	MTX	N^5–formylTHF	N^5–methylTHF	Folic acid
Wild-type	7.0	5.6	1.5	260
Glu45Gln	15.8	1.6	0.6	42.0
Glu45Arg	8.0	1.2	1.4	27.0
Glu45Asp	25.0	50.0	4.3	400.0
Glu45Leu	13.0	11.4	6.5	117.0
Glu45Try		163.0	15.0	130.0

Table 6. Summary of mutations in the RFC-1 gene with no significant effect on folate or MTX transport

Nucleotide affected	Amino acid residue affected	Species	Domain affected and effect on RFC	Ref
G80A	Arg27His	H	1st TMD 1,	71
AAC171-4CAG	Asn58Gln	H	Extracellular loop connecting TMD 1-2. Removes the *N*-glycosylation site	30
A263T	Asp88Val	H	2nd TMD	23
CGC397-9AAG	Arg133Lys	H	4th TMD	23
C352T	Leu86Leu	H	Intracellular loop connecting TMD 2-3. Silent mutation	45
C696T	Pro232Pro	H	Silent mutation	74
C1242A	Ile414Ile	H	Silent mutation	74

Footnote
H = human
Nucleotide numbering is taken from the start codon of the open reading frame

Summary

Understanding of the biochemistry and molecular biology of cell metabolism means that new drugs can be designed to overcome resistance. RFC is the principal route for the uptake of reduced folates and cytotoxic antifolates into cells and is therefore one site at which resistance can be achieved.

Resistance to antifolate drugs, such as MTX, may be the result of altered RFC affinity, synthesis of a functionally inadequate carrier, or bypass of a transporter altogether by down-regulating RFC-1 expression and translation.

Trimetrexate is one of the new generation of antifolates produced using this information. It lacks the glutamate residue, leaving it non-polar and able to diffuse directly across the cell membrane (i.e., it bypasses any resistant mutation of RFC). Another strategy of therapeutic potential is the restoration of MTX sensitivity using transfection of intact mRFC.[76]

The majority of studies considered here have been performed on specific cell lines grown in the presence of different folates and/or antifolates. While these are 'forced' *in vitro* mutations, they do provide invaluable information on the structure and function of RFC. They are also believed to mimic *in vivo* resistance, and hopefully further studies will characterise clinically significant naturally occurring mutations.

Although we are still a long way from confirming the 3-D structure of the RFC, mutational studies indicate that the 12 TMDs form a cylindrical structure, which would form a channel in the cell membrane through which folates can be passed. The hydrophilic pocket formed by amino acids of TMD 1 and 3 at the extracellular surface of this channel is responsible for binding reduced folates by recognising and binding the pteridine domain. Here it waits for exchange with inorganic phosphate leaving the cell. To ensure it is transferred successfully through the channel, a series of key positively charged amino acids on the TMDs exposed to the channel are probably used.

Variety in the human genome is becoming apparent, with new single nucleotide polymorphisms (SNPs) revealed regularly. Important proteins, such as RFC, have highly conserved genes, which may go some way to explain why so few SNPs have yet to be assigned to this sequence.

As the proteomics age gathers pace, elucidation of protein structure and function will become commonplace. This will prove necessary to provide biomedical scientists, biotechnologists and clinicians with information to better understand disease, develop new tests, design new drugs and, ultimately, provide more effective treatment of disease.

References

1 Chango A, Emery-Fillon N, Potier de Courcy G *et al*. A polymoprhism (80G-A) in the reduced folate carrier gene and its association with folate status and homocysteinema. *Mol Genet Metab* 2000; **70**: 310-5.

2 Salooja N, Catto A, Carter A, Tudnesham EGD, Grant PJ. Methylene tetrahydrofolate reductase C677T genotype and stroke. *Clin Lab Haematol* 1998; **20**: 357-61.

3 Voet D, Voet J, Pratt CW. *Fundamentals of biochemistry* New York: John Wiley & Sons, 1999: 631-2, 693-721.

4 Wang Y, Zhao R, Russell RG, Goldman D. Localization of the murine reduced folate carrier as assessed by immunohistochemical analysis. *Biochim Biophys Acta* 2000; **1513**: 49-54.

5 Zeng H, Liu G, Rea PA, Kruh GD. Transport of amphipathic anions by human multidrug resistance protein 3. *Cancer Res* 2000; **60**: 4779-84.

6 Drori S, Sprecher H, Shemer G, Jansen G, Goldman ID, Assaraf YG. Characterization of a human alternatively spliced truncated reduced folate carrier increasing folate accumulation in parental leukemia cells. *Eur J Biochem* 2000; **267**: 690-702.

7 Trippett T, Schlemmer S, Elisseyeff Y *et al*. Defective transport as a mechanism of acquired resistance to methotrexate in patients with acute lymphocytic leukemia. *Blood* 1992; **80**: 1158-62.

8 Trippett TM, Bertino JR. Therapuetic strategies targeting proteins that regulate folate and reduced folate transport. *J Chemother* 1999; **11**: 3-10.

9 Brigle KE, Spinella MJ, Westin EH, Goldman ID. Increased expression and characterization of two distinct folate binding proteins in murine erythroleukemia cells. *Biochem Pharmacol* 1994; **47**: 337-45.

10 Wang X, Shen F, Freisheim JH, Gentry LE, Ratnam M. Differential stereospecificities and affinities of folate receptor isoforms for folate compounds and antifolates. *Biochem Pharmacol* 1992; **44**: 1898-1901.

11 McKusick VA. Solute carrier family 19, member 1: SLC19A1. *Medelian inheritance in Man*. Johns Hopkins University. <http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=600424>

12 Westerhof GR, Jansen G, van Emmerik N *et al*. Membrane transport of natural folates and antifolate compounds in murine L1210 leukemia cells: role of carrier- and receptor-mediated transport systems. *Cancer Res* 1991; **51**: 5507-13.

13 Dixon KH, Mulligan T, Chung K-N, Elwood PC, Cowan KH. Effects of folate receptor expression following stable transfection into wild type and methotrexate transport-deficient ZR-75-1 human breast cancer cells *J Biol Chem* 1992; **267**: 24140-7.

14 Sierra EE, Goldman ID. Recent advances in the understanding of the mechanism of membrane transport of folates and antifolates. *Semin Oncol* 1999; **26** (Suppl 6): 11-23.

15 Rajgopal A, Sierra EE, Zhao R, Goldman ID. Expression of the reduced folate carrier SLC19A1 in IEC-6 cells result in two distinct transport activities. *Am J Physiol Cell Physiol* 2001; **281**: C1579-C1586.

16 Trippett TM, Garcia S, Manova K *et al*. Localization of a human reduced folate carrier protein in the mitochondrial as well as the cell membrane of leukemia cells. *Cancer Res* 2001; **61**: 1941-7.

17 Horton HR, Moran LA, Ochs RS, Rawn JD, Scrimgeour KG. *Principles of biochemistry* (3rd Ed). Englewood Cliffs, NJ: Neil Patterson Publishers (Prentice-Hall), 1993: 582.

18 Zhang L, Wong SC, Matherly LH. Transcript heterogeneity of the human reduced folate carrier results from the use of multiple promoters and variable splicing of alternative upstream exons. *Biochem J* 1998; **332**: 773-80.

19 Williams FMR, Flintoff WF. Isolation of a human cDNA that complements a mutant hamster cell defective in methotrexate uptake. *J Biol Chem* 1995; **270**: 2987-92.

20 Zhao R, Sharina IG, Goldmann ID. Pattern of mutations that results in loss of reduced folate carrier function under antifolate selective pressure augmented by chemical mutagenesis. *Mol Pharmacol* 1999; **56**: 68-76.

21 Moscow JA, Gong M, He R *et al*. Isolation of a gene encoding a human reduced folate carrier (RFC1) and analysis of its expression in transport-deficient, methotrexate-resistant human breast cancer cells. *Cancer Res* 1995; **55**: 3790-4.

22 Dixon KH, Lampher BC, Chiu J, Kelly K, Cowan KH. A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells. *J Biol Chem* 1994; **269**: 17-20.

23 Liu XY, Matherly LH. Functional interaction between arginine-133 and aspartate-88 in the human reduced folate carrier: evidence for a charge-pair association. *Biochem J* 2001; **358**: 511-6.

24 Matherly LH. Molecular and cellular biology of the human reduced folate carrier. *Prog Nucleic Acid Res Mol Biol* 2001; **67**: 131-62.

25 Wong SC, Proefke SA, Bhushan A, Matherly LH. Isolation of human cDNAs that restore methotrexate sensitivity and reduced folate carrier activity in methotrexate transport-defective chinese hamster ovary cells. *J Biol Chem* 1995; **270**: 17468-75.

26 Ferguson PL, Flintoff WF. Topological and functional analysis of the human reduced folate carrier by hemagglutinin epitope insertion. *J Biol Chem* 1999; **274**: 16269-78.

27 Sadlish H, Williams FM, Flintoff WF. Cytoplasmic domains of the reduced folate carrier are essential for trafficking, but not function. *Biochem J* 2002; **364**: 777-86.

28 Liu XY, Witt TL, Matherly LH. Restoration of high-level transport activity by human reduced folate carrier/ThTr1 chimeric transporters: role of the transmembrane domain 6/7 linker region in reduced folate carrier protein. *Biochem J* (ePublished: BJ20020419).

29 Matherly LH, Angeles SH. Role of N-glycosylation in the structure and fucntion of the methotrexate membrane transporter from CCRF-CEM human lymphoblastic cells. *Biochem Pharmacol* 1994; **47**: 1094-8.

30 Wong SC, Zhang L, Proefke SA, Matherly LH. Effects of the loss of capacity for N-glycoslylation on the transport activity and cellular localization of the human reduced folate carrier. *Biochim Biophys Acta* 1998; **1375**: 6-12.

31 Nehls S, Snapp EL,Cole NB *et al*. Dynamics and retention of misfolded proteins in native ER membranes. *Nat Cell Biol* 2000; **2**: 288-95.

32 Lapenta V, Sossi V, Gosset P *et al*. Construction of a 2.5-Mb integrated physical and gene map of distal 21q22.3. *Genomics* 1998; **49**: 1-13.

33 Tolner B, Roy K, Sirotnak FM. Structural analysis of the human RFC-1 gene encoding a folate transporter reveals multiple promoters and alternatively spliced transcripts with 5' end heterogeneity. *Gene* 1998; **211**: 331-41.

34 Williams FMR, Murray RC, Underhill M, Flintoff WF. Isolation of a hamster cDNA clone coding for a function involved in methotrexate uptake. *J Biol Chem* 1994; **269**: 5810-6.

35 Gong M, Cowan KH, Gudas J, Moscow JA. Isolation and characterization of genomic sequence involved in the regulation of the human reduced folate carrier gene (RFC1). *Gene* 1999; **233**: 21-31.

36 Whetstine JR, Flatley RM, Matherly LH. The human reduced folate carrier gene is ubiquitously and differentially expressed in normal human tissues: identification of seven non-coding exons and characterization of a novel promoter. *Biochem J* 2002; **367**: 629-40.

37 Tolner B, Roy K, Sirotnak F M. Organization, structure and alternate splicing of the murine RFC-1 gene encoding a folate transporter. *Gene* 1997; **189**: 1-7.

38 Jansen G, Westerhoff GR, Jarmuszewski MJA, Kathmann I, Rijksen G, Schornagel JH. Methotrexate transport in variant human CCRF-CEM leukemia cells with elevated levels of the reduced folate carrier. *J Biol Chem* 1990; **265**: 18272-7.

39 Whetstine JR, Matherly LH. The basal promoters for the human reduced folate carrier gene are regulated by a GC-box and a cAMP-response element/AP-1-like element. *J Biol Chem* 2001; **276**: 6350-8.

40 Whetstine JR, Witt TL, Matherly LH. The human reduced folate carrier gene is regulated by the AP2 and Sp1 transcription factor families and a functional 61 base pair polymorphism. *J Biol Chem* 2002 (ePublished: M208296200).

41 Ding BC, Whetstine JR, Witts TLM Schuetz JD, Matherly LH. Repression of human reduced folate carrier gene expression by wild-type p53. *J Biol Chem* 2001; **276**: 8713-9.

42 Lohrum MAE, Vousden KH. Regulation and function of the p53-related proteins: same family different rules. *Trends Cell Biol* 2000; **10**: 197-202.

43 Zhang L, Wong SC, Matherly LH. Structure and organization of the human reduced folate carrier gene. *Biochem Biophys Acta* 1998; **1442**: 389-93.

44 Brigle KE, Spinella MJ, Sierra EE, Goldman ID. Organization of the murine reduced folate carrier gene and identification of variant splice forms. *Biochem Biophys Acta* 1997; **1353**: 191-8.

45 Jansen G, Mauritz R, Drori S *et al*. A structurally altered human reduced folate carrier with increased folic acid transport mediates a novel mechanism of antifolate resistance. *J Biol Chem* 1998; **273**: 30189-98.

46 Hanson CA. Haematology. In: McLactchey KD, ed. *Clinical laboratory medicine*. London: Williams and Wilkins, 1994: 817-63, 939-69.

47 Rots MG, Pieters R, Kaspers GJL, Veerman AJP, Peters GJ, Jansen G. Classification of *ex vivo* methotrexate resistance in acute lymphoblastic and myeloid leukaemia. *Br J Haematol* 2000; **110**: 791-800.

48 Rots MG, Pieters R, Peters GJ *et al*. Methotrexate resistance in relapsed childhood acute lymphoblastic leukaemia. *Br J Haematol* 2000; **109**: 629-34.

49 Mauer AM. Acute lymphocytic leukemia. In: Beutler E, Lichtman M A, Coller BS, Kipps TJ, eds. *William's hematology* (5th Ed). New York: McGraw Hill, 1995: Chapter 105.

50 Pui C-H, Evans WE. Acute lymphoblastic leukaemia. *N Engl J Med* 1998; **339**: 605-15.

51 Belur LR, Boelk-Galvan D, Diers MD, McIvor RS, Zimmerman CL. Methotrexate accumulates to similar levels in animals transplanted with normal versus drug-resistant transgenic marrow. *Cancer Res* 2001; **61**: 1522-6.

52 Warren RD, Nichols AP, Bender RA. Membrane transport of methotrexate in human lymphoblastoid cells. *Cancer Res* 1978; **38**: 668-71.

53 Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. *N Engl J Med* 1983; **309**: 1094-104.

54 Blakley R L, Sorrentino B P. *In vitro* mutations in dihydrofolate reductase that confers resistance to methotrexate: Potential for clinical application. *Hum Mutat* 1998; **11**: 259-63.

55 Hoffbrand AV, Tripp E, Catovsky D, Das KC. Transport of methotrexate into normal haemopoietic cells and into leukaemic cells and its effects on DNA synthesis. *Br J Haematol* 1973; **25**: 497-511.

56 Masson E, Relling MV, Synold TW *et al*. Accumulation of methotrexate polyglutamates in lymphoblasts is a determinant of antileukemic effects *in vivo*, a rationale for high-dose methotrexate. *J Clin Invest* 1996; **97**: 73-80.

57 Sadlish H, Murray RC, Williams MR, Flintoff WF. Mutations in the reduced-folate carrier affect protein localization and stability. *Biochem J* 2000; **346**: 509-18.

58 Gong M, Yess J, Connolly T *et al*. Molecular mechanism of antifolate transporter-deficiency in a methotrexate-resistant MOLT-3 human leukemia cell line. *Blood* 1997; **89**: 2494-9.

59 Ohnuma T, Lo RJ, Scanlon KJ *et al*. Evolution of methotrexate resistance of human acute lymphoblastic leukemia cell *in vitro*. *Cancer Res* 1985; **45**: 1815-22.

60 Peters GJ, Jansen G. Folate homeostasis and antiproliferative activity of folates and antifolates. *Nutrition* 2001; **17**: 737-8.

61 Zhang L, Taub JW, Williamson M *et al*. Reduced folate carrier gene expression in childhood acute lymphoblastic leukemia: relationship to immunophenotype and ploidy. *Clin Cancer Res* 1998; **4**: 2169-77.

62 Ding BC, Witt TL, Hukku B, Heng H, Zang L, Matherly LH. Association of deletions and translocations of the reduced folate carrier gene with profound loss of gene expression in methotrexate-resistant K562 human erythroleukemia. *Biochem Pharmacol* 2001; **61**: 665-75.

63 Gorlick R, Goker E, Trippett T *et al*. Defective transport is a common mechanism of acquired methotrexate resistance in acute lymphocytic leukemia and is associated with decreased reduced folate carrier expression. *Blood* 1997; **89**: 1013-8.

64 Rothem L, Ifergan I, Kaufman Y, Priest DG, Jansen G, Assaraf YG. Resistance to multiple novel antifolates is mediated via defective drug transport resulting from clustered mutations in the reduced folate carrier gene in human leukaemia cell lines. *Biochem J* 2002; **367**: 741-50.

65 Wong SC, Zhang L, Witt TK, Proefke SA, Bhushan A, Matherly LH. Impaired membrane transport in methotrexate-resistant CCRF-CEM cells involves early translation termination and increased turnover of a mutant reduced folate carrier. *J Biol Chem* 1999; **274**: 10388-94.

66 Zhao R, Assaraf YG, Goldmann ID. A mutated murine reduced folate carrier (RFC1) with increased affinity for folic acid, decreased affinity for methotrexate, and an obligatory anion requirement for transport function. *J Biol Chem* 1998; **273**: 19065-71.

67 Sadlish H, Williams FMR, Flintoff WF. Functional role of arginine 373 in substrate translocation by the reduce folate carrier. *J Biol Chem* 2002; **277**: 42105-12.

68 Zhao R, Assaraf YG, Goldmann ID. A reduced folate carrier mutation produces substrate-dependent alterations in carrier mobility in murine leukemia cells and methotrexate resistance with conservation of growth in 5-formyltetrahydrofolate. *J Biol Chem* 1998; **273**: 7873-9.

69 Sharina IG, Zhao R, Wang Y, Babani S, Goldman ID. Mutational analysis of the functional role of conserved arginine and lysine residues in transmembrane domains of the murine reduced folate carrier. *Mol Pharmacol* 2001; **59**: 1022-8.

70 Zhao R, Gao F, Wang PJ, Goldmann ID. Role of the amino acid 45 residue in reduced folate carrier function and ion-dependent transport as characterized by site-directed mutagenesis. *Mol Pharmacol* 2000; **57**: 317-23.

71 Tse A, Brigle K, Taylor SM, Moran RG. Mutations in the reduced folate carrier gene which confer dominant resistance to 5,10-dideazatetrahydrofolate. *J Biol Chem* 1998; **273**: 25953-60.

72 Roy K, Tolner B, Chiao JH, Sirotnak FM. A single amino acid difference within the folate transporter encoded by the murine RFC-1 gene selectively alters its interaction with folate analogues. *J Biol Chem* 1998; **273**: 2526-31.

73 Zhao R, Gao F, Goldmann ID. Descrimination among reduced folates and methotrexate as transport substrate by a phenylalanine substitution for serine within the predicted eighth transmembrane domain of the reduced folate carrier. *Biochem Pharmacol* 1999; **58**: 1615-24.

74 Whetstine JR, Gifford AJ, Witt T *et al*. Single nucleotide polymorphism in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His^{27} and Arg^{27} carriers. *Clin Cancer Res* 2001; **7**: 3416-22.

75 Rots MG, Willey JC, Jansen G *et al*. mRNA expression levels of methotrexate resistance-related proteins in childhood leukemia as determined by a standardized template-based RT-PCR method. *Leukemia* 2000; **14**: 2166-75.

76 Liu S, Song L, Bevins R, Birhiray O, Moscow JA. The murine-reduced folate carrier gene can act as a selectable marker and suicide gene in hematopoietic cells *in vivo*. *Hum Gene Ther* 2002; **13**: 1777-82.