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Folates as adjuvants to anticancer agents: Chemical rationale and mechanism of action

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ABSTRACT

Folates have been used with cytotoxic agents for decades and today they are used in hundreds of thousands of patients annually. Folate metabolism is complex. In the treatment of cancer with 5-fluorouracil, the administration of folates mechanistically leads to the formation of [6R]-5,10-methylene-tetrahydrofolate, and the increased concentration of this molecule leads to stabilization of the ternary complex comprising thymidylate synthase, 2′-deoxy-uridine-5′-monophosphate, and [6R]-5,10-methylene-tetrahydrofolate. The latter is the only natural folate that can bind directly in the ternary complex, with other folates requiring metabolic activation. Modulation of thymidylate synthase activity became central in the study of folate/cytotoxic combinations and, despite wide use, research into the folate component was neglected, leaving important questions unanswered. This article revisits the mechanisms of action of folates and evaluates commercially available folate derivatives in the light of current

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research. Better genomic insight and availability of new analytical techniques and stable folate compounds may open new avenues of research and therapy, ultimately bringing increased clinical benefit to patients.

1. Introduction

Since the 1970s, tetrahydrofolates such as leucovorin have been used in combination with antimetabolites for the treatment of various malignant diseases. The rationale for including tetrahydrofolates, often somewhat incorrectly alluded to as just 'folates', has been to reduce side effects (enhance safety) of folate analogs such as methotrexate, and/or improve the antimalignant effects (enhance efficacy) of antimetabolites such as 5-fluorouracil (5-FU) and its derivatives. The clinical use of tetrahydrofolates has shown no tendency to diminish over time, and they have become an established component of several therapeutic strategies. In some instances, their use preceded understanding of, for example, the different folate derivatives, modes of administration, dose–effect relationships (particularly for multidrug combinations), and necessary target organ metabolite concentrations, as well as the development of precise and direct analytical methods.

The dominating research efforts regarding anticancer combinations have been devoted to the cytotoxic or cytostatic antimetabolites, and less interest has been paid to the folate component as such. Indeed, much of the folate research was performed during the 1970s–1990s, but important contributions are still made, especially regarding the role and importance of subcellular compartmentalization. However, there are still large poorly understood areas, for example differences between physiological situations and pharmacological conditions when high doses of folates are administered. It is possible that the role of compartmentalization is diminished when subcompartmental levels also become influenced by high pharmacological concentrations, which is a focus of this paper. In view of the extensive continuing use of folate derivatives in oncology, the aim here is to review the field in order to stimulate renewed research interest and to point at information gaps, with special relevance for therapeutic applications.

2. Chemical structure, transport, and metabolism of folates

Leafy vegetables are the principal nutritional source of folates, with the term 'natural folate' being used for those folates, and their derivatives, that are found in living organisms. All tetrahydrofolate structures are built from three units: tetrahydropterin, *p*-aminobenzoic acid, and l-glutamic acid (Fig. 1). Naturally occurring tetrahydrofolates contain two asymmetric stereocenters. The chiral Cα-atom, which is part of the l-glutamic acid moiety, has the natural [αS]-configuration. Due to the fixed asymmetry of the l-glutamic acid moiety, there are no enantiomers (and no racemates) among the tetrahydrofolates; only diastereoisomers exist, which have either the [6R]- or [6S]-configuration at the C-6 atom. Therefore, when a natural diastereoisomeric form of a particular tetrahydrofolate is administered, the enzymatic interconversions never change the absolute configuration at the C-6 atom of the tetrahydropterin ring. This chiral form determines and stays unchanged for all naturally occurring folates, and structurally also defines the term 'natural folates'. However, according to the [R,S] system of nomenclature, the same absolute configuration at the C-6 atom may be named differently from one compound to another.

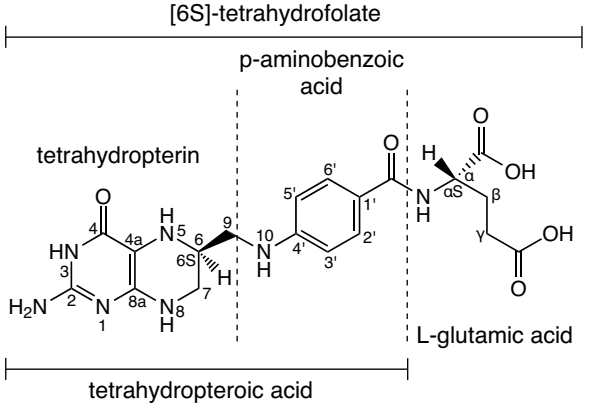

Fig. 1. Tetrahydropterin, p-aminobenzoic acid, and l-glutamic acid are the three units from which all tetrahydrofolate structures are built.

There is only one exception to this rule. Although disputed, a non-enzymatic interconversion has been described for [6R]-5-formyl-tetrahydrofolate (THF) back to 7,8-dihydrofolate (DHF), which is non-asymmetric at the C-6 atom (Baggott and Tamura, 1999; Baggott et al., 2001). This interconversion (Fig. 2) may explain why an intravenous dose of [6R,S]-5-formyl-THF produces slightly more bioactive folates than one-half that of [6S]-5-formyl-THF (DeVito et al., 1993).

Serum folates circulate as monoglutamate derivatives and are imported into cells through the reduced folate carrier (RFC), the proton-coupled folate transporter (PCFT), or via folate receptor (FR)-mediated endocytosis (Qiu et al., 2006; Zhao and Goldman, 2013; Zhao et al., 2009; Chattopadhyay et al., 2007; Desmoulin et al., 2012). RFC has a preference for reduced folates and is a high-capacity, low-affinity transporter, whereas FR is a high-affinity, low-capacity transporter; both RFC and FR have pH optima at physiological levels. PCFT has a low pH optimum (around pH 5.5), can transport both oxidized and reduced folates and antifolates, and plays a major role in folate uptake in the gut and in some tumors.

Once in the cytoplasm, folates are processed to metabolic cofactors by sequential addition of l-glutamic acid residues by the enzyme folyl-polyglutamate synthetase (FPGS) (Turner et al., 2000; Rots et al., 1999; Gonen and Assaraf, 2012; Walling, 2006). The polyglutamate chain comprises up to nine residues linked by gamma peptide bonds, and is linked to tetrahydropteroic acid carrying one-carbon units of various oxidation levels at the N5 and/or N10 positions (Fig. 3). The hydrophilic polyglutamate chain serves both to retain the folate within the cell and to increase the affinity for folate-dependent enzymes (Radparvar et al., 1988; Boarman and Allegra, 1992; Lawrence et al., 2014). Folate polyglutamates regulate the rate of reaction of the key metabolic enzymes in one-carbon metabolism and allow channelling of the substrates between enzymes (Lawrence et al., 2014; Lowe et al., 1993). FPGS is located in both the cytosol and mitochondria; however, mitochondrial FPGS has a higher specific activity than cytosolic FPGS, leading to greater accumulation of folates with a higher chain length in the mitochondria (Lin et al., 1993). The two pools appear to be compartmentalized, meaning that mitochondrial FPGS is required for maintaining mitochondrial pools and cytosolic FPGS is required for maintaining cytosolic pools (Anderson et al., 2012).

With synthetic folates that include the same amounts of both the [6R]- and [6S]-diastereoisomers (e.g. [6R,S]-5-formyl-THF, [6R,S]-

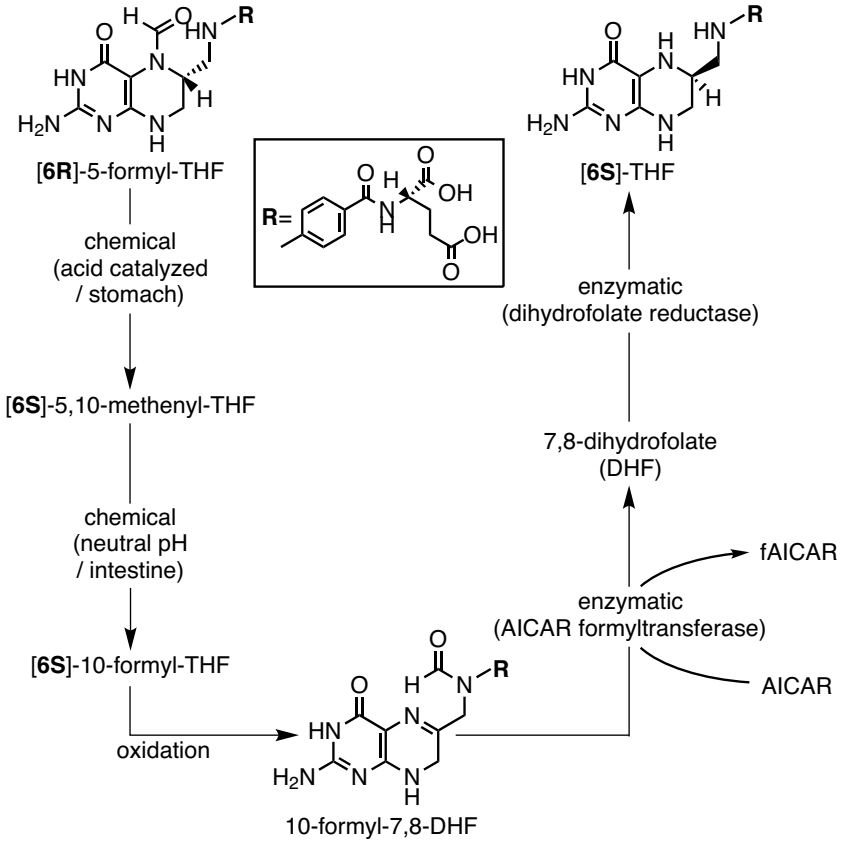

Fig. 2. Non-enzymatic chemical interconversions for the transformation of [6R]-5-formyl-THF to [6S]-THF. The reaction from [6R]-5-formyl-THF involves chemical oxidation to [6S]-5,10-methenyl-THF and then, via two chemical steps, to [6S]-10-formyl-THF and 10-formyl-7,8-DHF. The latter compound is non-asymmetric and can be transformed enzymatically to 7,8-DHF, which may then be reduced to [6S]-THF.
Modified with permission from Baggott JE, Tamura T. Biochim Biophys Acta 1999;1472:323-32 (Baggott and Tamura, 1999).
AICAR: 5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamide; DHF: 7,8-dihydrofolate; fAICAR: 5-formamido-1-(5-phospho-D-ribosyl)imidazole-4-carboxamide; THF: tetrahydrofolate.

5-methyl-THF, and [6R,S]-5,10-methylene-THF), the unnatural diastereoisomer will lack the activity of the natural diastereoisomer and will be treated differently in the organism. Indeed, the unnatural [6R]-isomer of 5-formyl-THF (leucovorin, folinic acid) has a longer plasma half-life and lower clearance, which may lead to systemic accumulation (Straw et al., 1984; Newman et al., 1989; Zittoun, 1993; Zittoun et al., 1993), whereas the unnatural [6S]-isomer of 5,10-methylene-THF has been shown to be a relatively weak competitive inhibitor of the [6R]-isomer at the enzyme thymidylate synthase (TS) (Leary et al., 1974; Cisneros and Dunlap, 1990; Sharma and Kisliuk, 1975). However, [6S]-5,10-methylene-THF is found in plasma only as a result of direct administration of [6R,S]-5,10-methylene-THF, and does not result from administration of other folates. Furthermore, it has been reported that certain unnatural folate forms can affect the formation of the ternary complex (see Section 5) and, as such, influence the efficacy of TS inhibitors (Van der Wilt et al., 1993; Van der Wilt et al., 2002).

3. Tetrahydrofolates and their interconversions

There are six known one-carbon-substituted derivatives of [6S]-THF, each associated with a particular metabolic cycle (Fig. 4) (Jolivet et al., 1996). [6R]-10-formyl-THF is involved in purine synthesis (cycle A), [6R]-5,10-methylene-THF in thymidylate synthesis (cycle B), and [6S]-5-methyl-THF in methionine synthesis (cycle C). Tetrahydrofolate derivatives not directly involved in biosynthetic pathways are [6S]-5-formimino-THF, [6R]-5,10-methenyl-THF, and [6S]-5-formyl-THF (levoleucovorin) – all of which are involved in a 'futile cycle' (cycle D) (Jolivet et al., 1996).

The reversible conversion of [6S]-THF and serine to [6R]-5,10-methylene-THF and glycine, catalyzed by serine hydroxymethyltransferase (SHMT), is the primary entry point of one-carbon units into one-carbon metabolism (Stover and Field, 2011; Locasale, 2013). Notably, however, there is also a non-enzymatic conversion of one-carbon units via a fully reversible chemical reaction: an acetalisation of [6S]-THF with formaldehyde to form an N,N-acetal, which is [6R]-5,10-methylene-THF, a reaction that can be used to synthesize [6S]-5,10-methylene-THF (Moran et al., 1979). This reaction is known to be highly pH-dependent (Baggott and Tamura, 1999; Jackson and Harrap, 1973; Spears et al., 1995).

The equilibrium between [6R]-5,10-methylene-THF and [6S]-THF plus formaldehyde (called 'instability equilibrium') is the reason behind the chemical instability of [6R]−5,10-methylene-THF. Also, the exceptionally high susceptibility of THF to become oxidized can drive the equilibrium towards further decomposition to THF and formaldehyde. Also, other folates (including THF, DHF, [6S]-5-methyl-THF, and [6R]-10-formyl-THF) are chemically labile and susceptible to irreversible oxidative degradation. Thus, *in vitro* in solution, tetrahydrofolate has a half-life of minutes in the absence of reducing agents such as thiols and ascorbate, yet the mean residency time of whole-body folate in humans is much longer, due to polyglutamylation of several intermediates that are formed when an exogenous source such as leucovorin is administered to individuals (Stover and Field, 2011; Allegra and Voeller, 1993; Goldman and Matherly, 1987; Cook et al., 1987). The exact

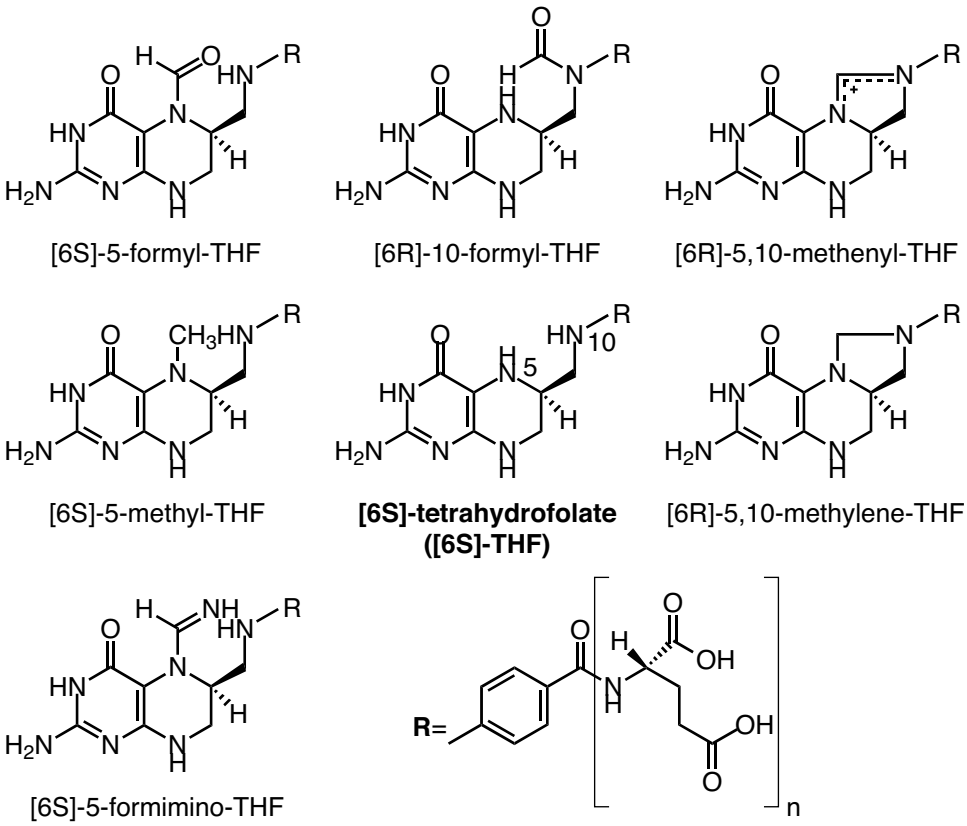

Fig. 3. Chemical structures of natural folates. THF: tetrahydrofolate.

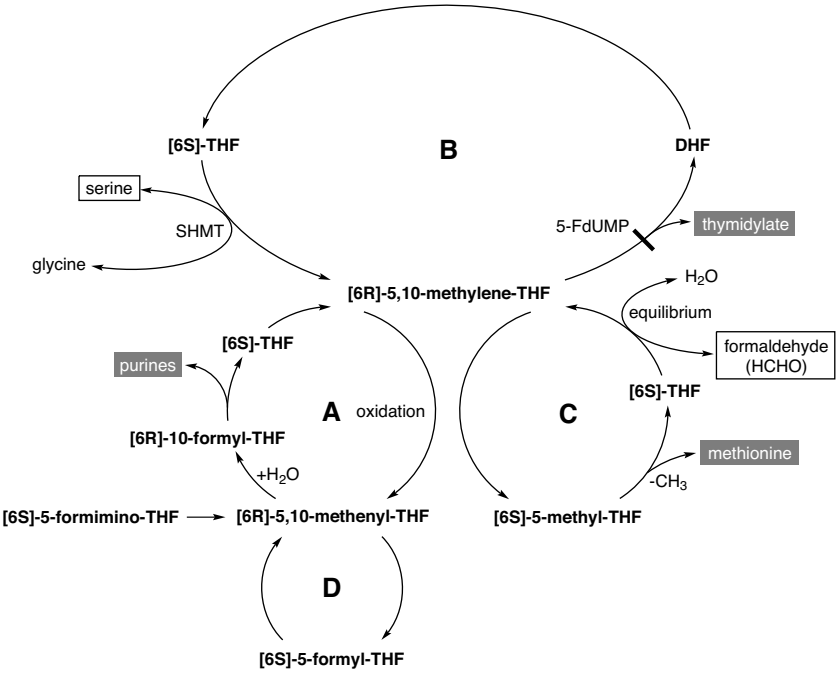

Fig. 4. Metabolic pathways associated with cytosolic one-carbon metabolism. [6R]-10-formyl-THF is involved in purine synthesis (cycle A); [6R]-5,10-methylene-THF is involved in thymidylate synthesis (cycle B); [6S]-5-methyl-THF is involved in methionine synthesis (cycle C); tetrahydrofolate derivatives not directly involved in biosynthetic pathways are [6S]-5-formimino-THF, [6R]-5,10-methenyl-THF, and [6S]-5-formyl-THF (levoleucovorin), which are involved in a 'futile cycle' (cycle D).
Modified with permission from Jolivet J et al. Oncologist 1996;1:248-54 (Jolivet et al., 1996).
DHF: 7,8-dihydrofolate; 5-FdUMP: 5-fluoro-2′-deoxy-uridine-5′-monophosphate; SHMT: serine hydroxymethyltransferase; THF: tetrahydrofolate.

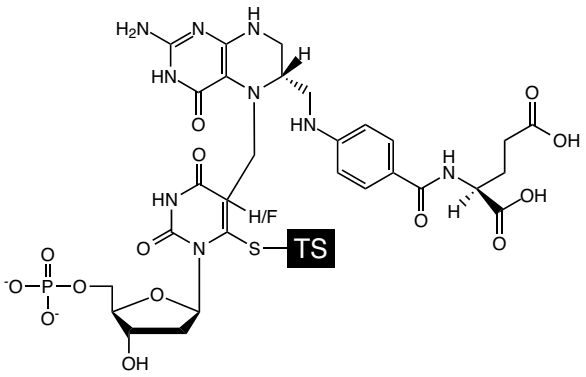

Fig. 5. Ternary complex formed between [6R]-5,10-methylene-THF, thymidylate synthase, and 2′-deoxy-uridine-5′-monophosphate (or 5-fluoro-2′-deoxy-uridine-5′-monophosphate).
THF: tetrahydrofolate; TS: thymidylate synthase.

retention time is not known, but indirect evidence for a long retention of polyglutamylated intermediates has been obtained from studies with folate analogs, such as lometrexol, which was retained for a long period in several organs (e.g. the liver), leading to delayed toxicity several months after administration (Habeck et al., 1998; Laohavinij et al., 1996). Folates are also stabilized and protected from oxidation when bound to cellular proteins, which regulate folate availability in the cell. While proteins bind folate co-factors with dissociation constants in the nanomolar range, folate enzymes are present in the micromolar range in liver cells (Suh et al., 2001; Madhukar et al., 2015). This means that cellular folates are protein bound, with limited opportunity to accumulate in the cellular milieu (Stover and Field, 2011).

4. Key role of [6R]-5,10-methylene-THF

[6R]-5,10-methylene-THF is the key endogenous one-carbon donor and a co-substrate of the TS enzyme for methylation of 2′-deoxy-uridine-5′-monophosphate (dUMP) to 2′-deoxy-thymidine-5′-monophosphate (dTMP). This product is one of the necessary nucleotide substrates for DNA synthesis and repair, and is thus essential for cell division and life. Indeed, the widely used antitumor agent 5-FU is known to exert its cytotoxic activity, to a major extent, through inhibition of the TS enzyme. Importantly, for either *synthesis* of dTMP, via TS catalysis, or *inhibition* of TS, by 5-FU, a ternary complex is formed between TS, [6R]-5,10-methylene-THF, and the respective third component, either dUMP or 5-fluoro-2′-deoxy-uridine-5′-monophosphate (5-FdUMP; Fig. 5).

In vivo, [6R]-5,10-methylene-THF is the only folate that is a direct component of the ternary complex, together with TS and dUMP or 5-FdUMP: of the two diastereoisomers, only [6R]-5,10-methylene-THF is the active form (Leary et al., 1974). Indeed, the work by Galivan et al. became ground-breaking in the area of asymmetry and stereoisomerism requirements in relation to the ternary complex (Galivan et al., 1975). As co-substrate, [6R]-5,10-methylene-THF fulfils all the highly specific chemical characteristic requirements for transfer of the one-carbon unit in this catalytic process. With its N-CH_2-N structural moiety, being a masked formaldehyde, it is the only natural folate that could have the important ability to get its one-carbon unit in equilibrium with a cationic species (=N(+)=CH_2). Such a highly reactive electrophile is probably necessary to obtain the crucial attack from the relatively weakly anionic 'active methylene', being the C-5 atom in dUMP. The C-5 atom 'active methylene' in the uracil moiety obtains a weakly anionic character because of an attack on the C-6 atom in uracil by the sulfhydryl group in TS. All this, together with a specific hydride (H^-) transfer reaction from THF to finalize the methyl group in the thymidine moiety, and a simultaneous formation of DHF, which is reused, makes it a remarkable phenomenon created during evolution. [6R]-5,10-methylene-THF is a 'central' intermediate among the natural folates undergoing the various one-carbon interconversions. Its one-carbon unit has the oxidation state of formaldehyde (HCHO), between formic acid (HCOOH) and methanol (CH_3OH) (Stover and Field, 2011).

5. The 'ternary complex'—a central chemical phenomenon for life

The concept of the ternary complex, formed by the substrate dUMP, the TS enzyme, and the co-substrate [6R]-5,10-methylene-THF, was put forward over 40 years ago (Langenbach et al., 1972; Santi, 1972). The theory has not been altered or contradicted in any significant way since then. The ternary complex comprising 5-FdUMP, instead of dUMP, subsequently became important as a tool for further study; the F-atom, instead of the H-atom in uracil, eliminates a crucial proton abstraction in the reaction from dUMP to dTMP. The biosynthesis of dTMP from dUMP takes place in all cells and is a vital component of DNA synthesis. It proceeds via the ternary complex, the formation of which drives the reductive methylation process. Not unexpectedly, the ternary complex seems to be conserved in nearly all living species. Indeed, TS has been purified, crystallized, and the structure determined in the bacterium *Lactobacillus casei* (Hardy et al., 1987) as well as in humans (Schiffer et al., 1995). Furthermore, TS has also been shown to be coded for in many viruses (Stroud and Finer-Moore, 1993).

5.1. Formation—the chemical mechanism

The formation mechanism can be divided into four events (Fig. 6) (Carreras and Santi, 1995).

1. The C-5 position of dUMP is activated by the TS enzyme attacking the C-6 position with a thiol group. This is a well-known reaction in organic chemistry (i.e. 'Michael Addition' of an alpha, beta-unsaturated ketone). This converts the C-5 position of dUMP to an active anionic enolate form (I).
2. There is simultaneous activation of [6R]-5,10-methylene-THF by opening of the 5-membered imidazolidine ring to give iminium ion formation that includes the CH_2-folate carbon (i.e. +N=CH_2). The CH_2-folate moiety with its two nitrogens is in equilibrium with the (probably transient) 5-iminium moiety being a highly reactive cationic nucleophile acceptor.
3. The C-5 enolate anion (I) in the activated dUMP attacks the 5-iminium CH_2 in the folate, forming a methylene bridge between the dUMP moiety and the folate moiety [i.e. the ternary complex (II)]. The corresponding complex with 5-FdUMP, instead of dUMP, has been isolated, and nuclear magnetic resonance and X-ray studies have confirmed the chemical structure. With 5-FdUMP, however, the further reaction to dTMP is not possible because, unlike the H-atom, the F-atom cannot be abstracted.
4. The [6S]-THF part is eliminated under formation of the intermediate III, followed by reduction of the one-carbon unit by H^- (hydride ion) transfer from [6S]-THF. The latter then provides dTMP as the product and DHF as the by-product.

5.2. Covalent but reversible binding

The ternary complex is built by covalent bonds between the components, and the reactions are fully reversible (Cisneros and Dunlap, 1990; Stroud and Finer-Moore, 1993; Santi et al., 1974; Lockshin and Danenberg, 1981; Sato et al., 1986; Spears et al., 1989; Matthews et al., 1990; Rode, 1993; Jarmuła et al., 2005). Thus, in a human cell preparation study, both 5-FdUMP

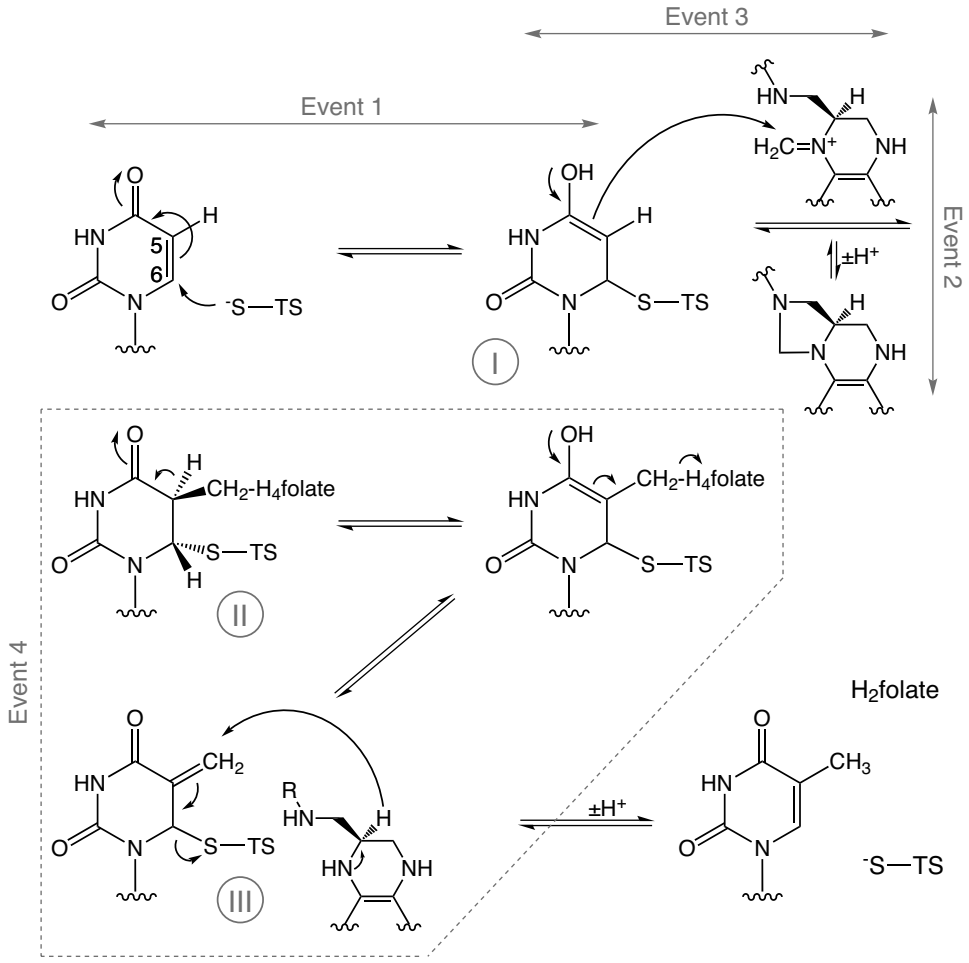

Fig. 6. Formation of the ternary complex. *Event 1:* The C-5 position of dUMP is activated by the TS enzyme attacking the C-6 position with a thiol group. This converts the C-5 position of dUMP to an active anionic enolate form (I). *Event 2:* There is simultaneous activation of [6R]-5,10-methylene-THF by opening of the 5-membered imidazolidine ring to give iminium ion formation that includes the CH_2-folate carbon (i.e. +N=CH_2). *Event 3:* The C-5 enolate anion (I) in the activated dUMP attacks the 5-iminium CH_2 in the folate, forming a methylene bridge between the dUMP moiety and the folate moiety [i.e. the ternary complex (II)]. *Event 4:* The [6S]-THF part is eliminated under formation of the intermediate III, followed by reduction of the one-carbon unit by H^- (hydride ion) transfer from [6S]-THF. The latter then provides dTMP as the product and DHF as the by-product.
DHF: 7,8-dihydrofolate; dTMP: 2′-deoxy-thymidine-5′-monophosphate; dUMP: 2′-deoxy-uridine-5′-monophosphate; THF: tetrahydrofolate; TS: thymidylate synthase.

$$\text{TS} + \text{5-FdUMP} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{TS–5-FdUMP} \underset{k_4}{\overset{k_3}{<=======>}} \text{TS–5-FdUMP–[6R]-5,10-methylene-THF} + \text{[6R]-5,10-methylene-THF}$$

and [6R]-5,10-methylene-THF were shown to be dissociable (Lockshin and Danenberg, 1981), and the TS enzyme from certain human leukemic cells regained the capacity to synthesize dTMP if excess dUMP was present. Importantly, by increasing the concentration of the [6R]-5,10-methylene-THF co-substrate, dissociation from the TS – 5-FdUMP – [6R]-5,10-methylene-THF[6*R*]-5,10-methylene-THF ternary complex was markedly diminished. On the contrary, the concentration of 5-FdUMP had no effect on the dissociation of the co-substrate. These observations are in accordance with a proposed ordered mechanism of ligand release and binding, since the co-substrate has to be dissociated prior to 5-FdUMP release (Danenberg and Danenberg, 1978). These data are in line with the 'principle of microscopic reversibility', whereby 5-FdUMP must bind to the enzyme first, followed by the [6R]-5,10-methylene-THF co-substrate. The reverse reaction must then start with the folate, as shown below (Lockshin and Danenberg, 1981).

Once formed, the ternary complex containing 5-FdUMP is quite stable for long periods, provided that excess [6R]-5,10-methylene-THF is present and the temperature does not exceed 37 °C. Indeed, the ternary complex can be isolated *in vitro* using Sephadex G-25 gel filtration. It is seen that, at equilibrium, the free and bound components undergo numerous association – dissociation events without apparent structural changes. However, degradation occurs when the complex is freed of unbound [6R]-5,10-methylene-THF by gel filtration, possibly due to oxidative degradation of the small amount of [6R]-5,10-methylene-THF present, and its replenishment results in rebinding of [^{3}H]5-FdUMP to the enzyme (Santi et al., 1974).

5.3. Role of intracellular folate pools in the formation and stability of the ternary complex

Spears et al. support the theory of an ordered mechanism, stating that the concentration of [6R]-5,10-methylene-THF must be in excess for maximal formation of the TS – 5-FdUMP – [6R]-5,10-methylene-THF*R* ternary complex and complete inhibition of TS

activity (Spears et al., 1995). In particular, a high concentration of dUMP may lead to poor TS inhibition by 5-FdUMP when the [6R]-5,10-methylene-THF concentration is low. Hence, for anticancer therapy, there is a need for increased concentrations of intratumoral [6R]-5,10-methylene-THF (Spears et al., 1995), which will direct the competitive binding between 5-FdUMP and dUMP in the ternary complex to favor 5-FdUMP (Lockshin and Danenberg, 1981).

The total concentration of [6R]-5,10-methylene-THF plus [6S]-THF in cells is estimated to be 3 μM or less, although several reports give concentrations in terms of pmol/mg protein, which requires a conversion factor that is not always supplied by the authors. As [6R]-5,10-methylene-THF also serves as the substrate for other enzymes (e.g. it is the precursor for [6S]-5-methyl-THF), the overall concentration available for TS may be less than 1 μM (Lockshin and Danenberg, 1981). Indeed, in cancer biopsy tissues, Saif et al. reported [6R]-5,10-methylene-THF levels of 0.1–0.5 μM and lower (Saif et al., 2010), which has also been shown by others (e.g. (Priest et al., 1991; Porcelli et al., 2011)). However, it is proposed that maximum ternary complex formation is generally achieved at [6R]-5,10-methylene-THF concentrations approaching 12 μM (Strohle et al., 2005), and that high intratumoral levels of [6R]-5,10-methylene-THF allow for greater TS inhibition, which is also prolonged. It is important to note that these results do not consider differences between intracellular compartments.

Chéradame et al. measured reduced folate levels in tumor types considered to be sensitive to 5-FU treatment and observed large interpatient variation (Chéradame et al., 1997a). However, [6R]-5,10-methylene-THF levels in the 96 tumor samples (50 head and neck, 16 colon, 30 liver metastases from colorectal cancer) were generally sufficiently high enough to sustain 5-FU-mediated TS inhibition (Chéradame et al., 1997a). Dohden et al. also observed large interpatient variation in the pools of [6R]-5,10-methylene-THF in tumors of patients with colorectal and gastric cancers (Dohden et al., 1993). High folate concentrations were maintained better in patients with high FPGS activity, since FPGS served to form polyglutamates, which became trapped in cells due to their increased polarity.

Administration of leucovorin in combination with a therapeutic dose of 5-FU in patients with colorectal cancer not only increased [6R]-5,10-methylene-THF levels several-fold (Porcelli et al., 2011), but also maintained TS inhibition after 48 h at 75%, compared with 50% inhibition when 5-FU was administered alone (Peters et al., 1994). In addition, in another study in patients with head and neck cancer, Chéradame et al. reported that treatment with leucovorin enhanced the concentrations of [6R]-5,10-methylene-THF (Chéradame et al., 1997b); likewise, Dohden et al. observed a 2 – 3-fold increase in [6R]-5,10-methylene-THF 3 h after the administration of 15 mg leucovorin (Dohden et al., 1993). Trave et al. demonstrated that the increase in tissue [6R]-5,10-methylene-THF levels was schedule-dependent (Trave et al., 1988): a classical 2-h infusion of 500 mg/m^2 leucovorin increased [6R]-5,10-methylene-THF levels 5 – 12-fold in seven patients, whereas a long infusion only increased [6R]-5,10-methylene-THF levels up to twofold. Chéradame et al. also showed that the patients (n = 12) who had a complete response to 5-FU-based therapy had a significantly higher distribution of [6R]-5,10-methylene-THF than those (n = 29) with either a partial response or no response (Chéradame et al., 1997b).

Preclinical data have shown that polyglutamate formation is essential for the maintenance of reduced folate pools (Houghton et al., 1994). This was observed in the study by Chéradame et al. (Chéradame et al., 1997a), and explains the maintenance of high levels of reduced folates in other studies (Strohle et al., 2005). However, recent findings from patients with colorectal cancer have shown large interpatient variability in tissue folate levels after supplementation with leucovorin at standardized dosage (Taflin et al., 2014). High leucovorin doses were needed to exceed baseline [6R]-5,10-methylene-THF values, particularly in patients with rectal cancer. The results indicate that standardized leucovorin doses might be insufficient to attain the full antitumor effect of 5-FU for all patients. Furthermore, it has been suggested that direct administration of [6R]-5,10-methylene-THF allows for greater TS inhibition ((Saif et al., 2010) and references cited therein). In a randomized comparison of equimolar intravenous bolus doses of levoleucovorin or [6R]-5,10-methylene-THF, the mucosal and tumor tissue levels of [6R]-5,10-methylene-THF were several-fold higher after direct administration of [6R]-5,10-methylene-THF than after levoleucovorin administration (Wettergren et al., 2015).

Considering the subcompartmental level, the dissociation reaction is fully reversible, releasing free [6R]-5,10-methylene-THF, which can either re-attack the resulting binary complex in the reversible reaction or stay in solution. Thus, the higher its concentration in solution, the easier [6R]-5,10-methylene-THF can re-attack. For this reason, an increased [6R]-5,10-methylene-THF concentration will counteract dissociation. With high enough concentrations of [6R]-5,10-methylene-THF, the ternary complex can be expected to be fairly stable, which is the ultimate goal for optimizing the cytotoxic effect of 5-FU. If high plasma concentrations of [6R]-5,10 −methylene-THF can be maintained, it is reasonable to believe that cellular concentrations, including tumor concentrations, can also be kept high. However, maintenance of high [6R]-5,10-methylene-THF levels in the tumor are predominantly dependent on the capability of the tumor to polyglutamate reduced folates, as these polyglutamates are too polar to efflux from the cell. This possibly explains high [6R]-5,10-methylene-THF levels in the tumor 48 h after a 2-h infusion of leucovorin (Porcelli et al., 2011).

To summarize, there is extensive, detailed, chemical evidence for the mechanism of formation of the ternary complex, both for 5-FdUMP and dUMP together with TS and [6R]-5,10-methylene-THF, which is the only known natural tetrahydrofolate that has the ability to bind and perform the known reactions. Indeed, [6S]-5-formyl-THF and [6S]-5-methyl-THF do not have the correct oxidation states, [6S]-THF does not have the one-carbon unit, and all other tetrahydrofolates need to be transformed to [6R]-5,10-methylene-THF.

6. Compartmentation of folate biosynthetic pathways

Folate co-factors have been identified in all subcellular organelles, but the highest concentrations are found in the cytoplasm and mitochondria. This metabolic compartmentation requires trafficking of folate metabolites along intercompartment pathways between, for example, the cytoplasm, nucleus, and mitochondria. Much detail regarding the relative function and importance of the different compartments remains to be understood. Mitochondria contain as much as 40% of total cellular folates, and the mitochondrial folate polyglutamates constitute a distinct pool not in equilibrium with folate polyglutamates in the cytoplasm (Stover and Field, 2011). Mitochondrial and cytosolic FPGS maintain the subcellular compartmentation of folates, as no exchange of polyglutamates takes place over the mitochondrial membrane (Lawrence et al., 2014). Monoglutamated [6R]-5,10-methylene-THF is transported across the mitochondrial membrane by the mitochondrial folate transporter, which is a member of the mitochondrial carrier family and is responsible for trafficking of the reduced folates over this membrane (Lawrence et al., 2011). Eukaryotic one-carbon metabolism occurs in two parallel processes, one cytosolic and one mitochondrial (Tibbett and Appling, 2010). The one-carbon units generated in the mitochondria, for example from serine, glycine, and formate, are fed from

the mitochondria into the cytosol (Barlowe and Appling, 1988). Compartmentation of folates specifically in tumor tissue and mitochondria can be explained by both the higher activity of FPGS in tumor tissue compared with normal tissue, and by the higher expression of FPGS in mitochondria compared with the cytoplasm (Lin et al., 1993).

Folate metabolism *per se* has always been considered complex, with many loops, interdependencies, and mechanisms of active regulation. Advanced analytical techniques, for example those based on liquid chromatography and tandem mass spectrometry, have been combined with sophisticated mathematical modelling in efforts to assess numerous factors simultaneously. Metabolic flux studies and metabolomics have begun to illuminate details and suggest different interpretations of known facts (Madhukar et al., 2015; Fan et al., 2014).

The total folate pool is necessarily limited and *de novo* purine biosynthesis, *de novo* dTMP biosynthesis, and homocysteine remethylation compete for the folate co-factors. This metabolic competition is particularly relevant for [6R]-5,10-methylene-THF between *de novo* dTMP biosynthesis and homocysteine remethylation (Stover and Field, 2011). Since [6R]-5,10-methylene-THF is also a key metabolite for the conversion to [6S]-5-methyl-THF, [6R]-5,10-methenyl-THF, and further to [6R]-10-formyl-THF and purines, the [6R]-5,10-methylene-THF co-substrate has a particularly central role in cellular folate interconversions (Fig. 7).

The folate-dependent *de novo* synthesis of dTMP from dUMP, involving the TS enzyme, is also dependent on the SHMT, methylene-tetrahydrofolate dehydrogenase (MTHFD), and dihydrofolate reductase (DHFR) enzymes. However, isotope tracer studies have demonstrated that [6R]-5,10-methylene-THF generated by SHMT is preferentially incorporated into dTMP compared with [6R]-5,10-methylene-THF generated by MTHFD. This preferential incorporation into dTMP is consistent with compartmentation of SHMT with TS, subsequently shown to involve cell-cycle-dependent nuclear localization of SHMT, TS, and DHFR. The concept of nuclear one-carbon metabolism was presented some 40 years ago in 1976 (Shin et al., 1976). More recent studies have demonstrated that mouse liver nuclei can synthesize dTMP from serine, indicating that nuclei contain the entire folate-dependent *de novo* dTMP pathway (Anderson et al., 2012; Anderson and Stover, 2009); SHMT, TS, and DHFR have also been found in nuclei isolated from mouse liver (Stover and Field, 2011). Furthermore, several immunohistochemical studies have consistently shown the presence of TS in the nucleus (Van Triest et al., 2000; Bissoon-Haqqani et al., 2006).

7. Regulation of folate biochemical pathways

Active transport of folates into the nucleus may not be required because the nuclear membrane breaks down and reassembles with each cell division, offering the opportunity for folate co-factors to be exchanged between the cytoplasm and nucleus during each mitotic event; this includes [6S]-5-methyl-THF, which is the predominant form of folate in the cytoplasm. However, only [6S]-THF, DHF, and [6R]-5,10-methylene-THF are involved in the dTMP biosynthesis pathway, whereas [6S]-5-methyl-THF is a potent inhibitor of SHMT (the enzyme bringing the methylene group into THF) (Stover and Field, 2011; Stover and Schirch, 1991). Being both the predominant folate in the cytoplasm and a potent inhibitor of SHMT, [6S]-5-methyl-THF may therefore autoregulate biosynthesis of the central intermediate [6R]-5,10-methylene-THF. This means that by increasing firstly plasma, and secondly cellular, concentrations of [6S]-5-methyl-THF there is the potential to downregulate the cytoplasmic concentration of [6R]-5,10-methylene-THF by inhibiting its biosynthesis. [6S]-5-formyl-THF is also an effective inhibitor of SHMT and may likewise be important in regulating the major entry point of one-carbon units (Stover and Schirch, 1991).

To summarize, the high affinity of both [6S]-5-methyl-THF and [6S]-5-formyl-THF for the SHMT enzyme and their high concentrations in the cell suggest that these co-factors are capable of regulating the activity of SHMT *in vivo* (Stover and Schirch, 1991). Furthermore, [6S]-5-methyl-THF is formed by reduction of [6R]-5,10-methylene-THF by the enzyme methylene-tetrahydrofolate reductase (MTHFR), an essentially irreversible reaction *in vivo*. The enzyme is strongly, but slowly, inhibited by S-adenosylmethionine. Regulation of this enzyme is critical to one-carbon homeostasis, as [6S]-5-methyl-THF is committed towards methionine biosynthesis, whereas unnecessary accumulation of this co-factor results in depletion of the one-carbon folate pool and increased inhibition of SHMT (Stover and Schirch, 1991). Low response to 5-FU plus leucovorin therapy in some patients has also been linked to low expression of identified folate-associated genes (Wettergren et al., 2014).

8. Folates in clinical use/development – important biochemical aspects

Folates in clinical use or in development are shown in Table 1. CoFactor®, leucovorin, and mefolinate are 1:1 mixtures of their respective [6R]- and [6S]-diastereoisomeric forms. Modufolin® (Merck & Cie, Schaffhausen, Switzerland), levoleucovorin (Fusilev®, Spectrum Pharmaceuticals, Inc., Irvine, CA, USA), and Metafolin® (Merck & Cie, Schaffhausen, Switzerland) are single and natural diastereoisomers. Although the natural diastereoisomeric forms of N10-unsubstituted THF (i.e. 5-formyl- and 5-methyl-THF) have the [6S]-configuration and Modufolin® has the [6R]-configuration, their stereochemistry or absolute configuration at the C-6 atom are all the same (nomenclature rules; see Section 2).

8.1. Folic acid

Folic acid (vitamin B9, pteroyl-L-glutamic acid) is a synthetic compound that itself is not active in humans, but is converted in the liver to the biologically active DHF, which has no asymmetry in the pteroyl part (see Section 2). DHF is reduced further in the body by DHFR to exclusively form [6S]-THF (Walling, 2006; Tedeschi et al., 2013; Bertino, 1993). This chiral form then stays unchanged. Folic acid is used in conjunction with cytostatics less so than leucovorin, but one example of folic acid use is co-treatment with pemetrexed (Alimta®, Eli Lilly, Indianapolis, IN, USA) to reduce toxicity. This essential role of folic acid to selectively protect normal cells was initially shown in mice; it was demonstrated that the dose of pemetrexed could be increased safely when folic acid was administered before and during pemetrexed therapy (Worzalla et al., 1998; van der Wilt et al., 2001). Furthermore, a randomized clinical study comparing pemetrexed plus cisplatin with cisplatin alone showed an unpredictably variable and severe toxicity that was reduced when pemetrexed was combined with folic acid, which also increased survival in these patients (Vogelzang et al., 2003).

Although it has been suggested by several authors that folic acid could replace leucovorin in 5-FU modulation, it is unlikely to play such a role (Houghton et al., 1994). Folic acid is an oxidized folate that does not increase the concentration of [6R]-5,10-methylene-THF to that required to enhance FdUMP-mediated inhibition of TS, and it is unable to modulate 5-FU cytotoxicity at physiological concentrations (Houghton et al., 1994). Moreover, in humans, administration of folic acid at doses above 1 mg/kg leads to accumulation of unmetabolized folic acid in plasma, but it is inefficient at increasing plasma THF and 5-methyl-THF levels (Bailey and Ayling, 2009; Obeid et al., 2011). Saturation of DHFR seems to occur at

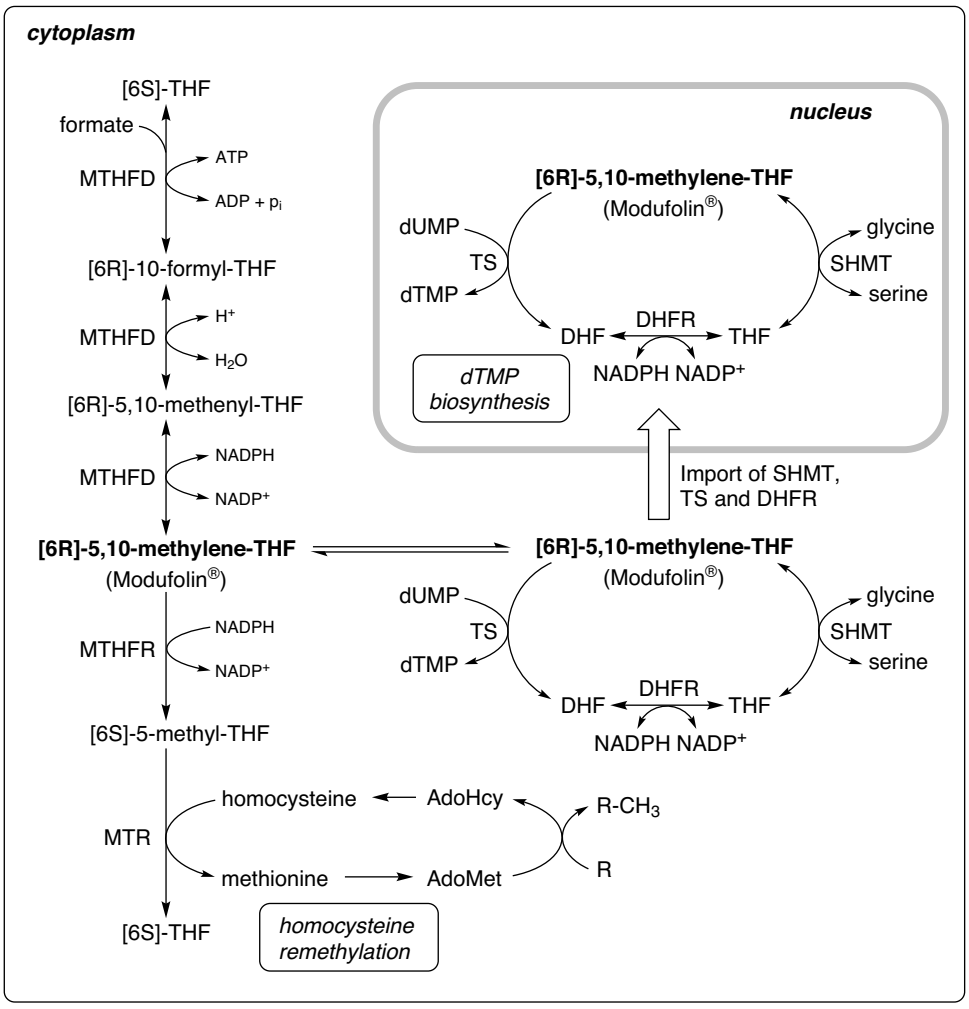

Fig. 7. Compartmentation of biosynthetic pathways. The intermediate metabolic reactions in the cytoplasm and nucleus, respectively.
Modified with permission from Stover PJ, Field MS. Adv Nutr 2011;2:325-31 (Stover and Field, 2011).
AdoHcy: S-adenosylhomocysteine; AdoMet: S-adenosylmethionine; ADP: adenosine diphosphate; ATP: adenosine triphosphate; DHF: 7,8-dihydrofolate; DHFR: 7,8-dihydrofolate reductase; dTMP: 2′-deoxy-thymidine-5′-monophosphate; dUMP: 2′-deoxy-uridine-5′-monophosphate; MTHFD: methylene-tetrahydrofolate dehydrogenase; MTHFR: methylene-tetrahydrofolate reductase; MTR: methionine synthase; NADP: nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate; SHMT: serine hydroxymethyltransferase; THF: tetrahydrofolate; TS: thymidylate synthase.

plasma folic acid concentrations above 5 nmol/l, resulting in a plateau of both THF and 5-methyl-THF. Folic acid cannot therefore be considered an alternative to reduced folates when an increase of 5,10-methylene-THF is desired.

8.2. *[6R,S]-5-formyl-THF (leucovorin) and [6S]-5-formyl-THF (levoleucovorin; Fusilev®)*

Leucovorin ([6R,S]-5-formyl-THF) has been used for many years as rescue treatment to prevent toxicity following high-dose methotrexate therapy, and to modulate the cytotoxicity of 5-FU, and is the most widely used folate in the clinical setting. In both experimental systems and clinical trials, leucovorin has been reported to enhance the antitumor activity of 5-FU, due to expansion of the intracellular reduced folate pool, leading to stabilization of the 5-FdUMP – TS – folate ternary complex. More recently, the pure active (and natural) diastereoisomer levoleucovorin ([6S]-5-formyl-THF) has been developed and used clinically, showing the same efficacy and tolerability profiles as leucovorin (Kovoor et al., 2009). In this review, randomized and non-randomized studies, predominantly in colon and rectal tumors, have been compared, as have schedules utilizing 5-FU alone and 5-FU in combination with other drugs such as irinotecan and oxaliplatin. In all studies, formulations of the [6R,S]-mixture and levoleucovorin showed comparable clinical efficacy.

When 5-FU is given in combination with leucovorin, conversion of leucovorin to the metabolite [6R]-5,10-methylene-THF is responsible for the enhanced antitumor effects of 5-FU (Advanced colorectal cancer meta-analysis project, 1992; Meta-Analysis Group in Cancer et al., 1998). The proposed biochemical basis for this improvement is the metabolic conversion of the parent leucovorin to [6R]-5,10-methylene-THF, which in turn stabilizes the ternary complex with 5-FdUMP and TS, leading to depletion of thymidylate required for DNA synthesis.

Studies of intravenous leucovorin previously used indirect methods for determining [6R]-5,10-methylene-THF (Priest et al., 1991; Straw and Newman, 1988). After oral administration, the [6R]-5,10-methylene-THF plus [6S]-THF concentration is about 100 nM; after intravenous and oral administration, [6R]-10-formyl-THF, [6R]-5,10-methylene-THF, and [6S]-THF exhibit peak levels earlier and are eliminated more rapidly than [6S]-5-methyl-THF. Accumulation of all metabolites after intravenous administra-

Table 1
Natural and unnatural folates in clinical use/development as adjuvant therapy.

Name	Folate content	Folate chemical structure	Key steps in activation cascades					Target	Metabolite
Folic acid	Pteroyl-L-glutamic acid		DHF	⇑			[6S]-THF	[6R]-5,10-CH_2-THF	[6S]-5-CH_3-THF
Leucovorin	[6R,S]-5-formyl-THF	[6R]-5-CHO-THF	Unnatural xenobiotic[a]						
		[6S]-5-CHO-THF	[6S]-5-CHO-THF		[6R]-5,10-CH^+-THF	[6R]-10-CHO-THF	[6S]-THF	[6R]-5,10-CH_2-THF	[6S]-5-CH_3-THF
Levoleucovorin Fusilev®	[6S]-5-formyl-THF	[6S]-5-CHO-THF							
Mefolinate	[6R,S]-5-methyl-THF	[6R]-5-CH_3-THF	Unnatural xenobiotic						
		[6S]-5-CH_3-THF		⇑			[6S]-THF	[6R]-5,10-CH_2-THF	[6S]-5-CH_3-THF
Metafolin®	[6S]-5-methyl-THF	[6S]-5-CH_3-THF							
CoFactor®	[6R,S]-5,10-methylene-THF	[6S]-5,10-CH_2-THF	Unnatural xenobiotic						
		[6R]-5,10-CH_2-THF				⇑		[6R]-5,10-CH_2-THF	[6S]-5-CH_3-THF
Modufolin®	[6R]-5,10-methylene-THF	[6R]-5,10-CH_2-THF							

DHF: 7,8-dihydrofolate;
THF: tetrahydrofolate.
[a] Partial conversion to activated compound; see Section 8.2 for further explanation.

tion is linearly dose-dependent, while oral administration results in saturation. In order to reach plasma levels high enough to enhance the effect of 5-FU, intravenous administration is advocated. More recently, direct biochemical determination of [6R]-5,10-methylene-THF has become possible, and intravenous bolus administration is shown to give higher exposure and tissue concentrations when compared with levoleucovorin (Wettergren et al., 2015).

The reported enhancement of cytotoxicity by leucovorin ([6R,S]-5-formyl-THF) and mefolinate ([6R,S]-5-methyl-THF) has focused attention on these two folates as precursors of [6R]-5,10-methylene-THF in tumor tissue. Host metabolism of leucovorin, yielding [6R]-5,10-methylene-THF in the circulatory system, could also play a role in elevating levels of the active metabolite at the tumor site. Indeed, metabolism of leucovorin takes place at more than one site, rather than in a single organ (Priest et al., 1991). Red blood cells have been reported to metabolize leucovorin *in vitro*, giving rise to the same reduced folates as those observed *in vivo*. For leucovorin metabolism, it has been suggested that [6R]-5,10-methylene-THF, [6S]-THF, and [6R]-10-formyl-THF occur in plasma as intermediates, and are further metabolized to [6S]-5-methyl-THF. A probable sequence of metabolic steps, leading from leucovorin to [6S]-5-methyl-THF, is as follows (Straw et al., 1984; Priest et al., 1991):

[6S]-5-formyl-THF → [6R]-5, 10-methenyl-THF

→ [6R]-10-formyl-THF → [6S]-THF

→ [6R]-5, 10-methylene-THF → [6S]-5-methyl-THF

[6S]-5-methyl-THF is the major, and virtually the only, metabolite found in human plasma and urine after [6S]-5-formyl-THF metabolism (usually over 95% (Baggott and Tamura, 1999)). Alternatively, [6R]-5,10-methenyl-THF can also be directly metabolized to [6R]-5,10-methylene-THF by the MTHFD enzyme (Pawelek and MacKenzie, 1998).

As well as being highly dependent on the mode of administration (with intravenous administration resulting in lower conversion of leucovorin to [6S]-5-methyl-THF than oral administration), the bioconversion process is also saturable (with a lower proportion of leucovorin converted to [6S]-5-methyl-THF at higher intravenous doses compared with lower doses). In addition, the plasma half-lives of both [6S]-5-formyl-THF and [6S]-5-methyl-THF are considerably longer at higher doses (Borsi et al., 1990). Borsi et al. suggested that constant rate infusion of high leucovorin doses could significantly expand the intracellular pool of active folates; however, only a twofold increase of [6R]-5,10-methylene-THF concentration was observed in tumor tissue after continuous infusion, compared with an eightfold increase after a bolus injection of the same dose (500 mg/m^2) (Borsi et al., 1990). In tumor tissues, though, the folate concentration may be increased for a prolonged period (Porcelli et al., 2011). Practical intravenous administration relies on either bolus injection or infusion over various times periods, and the impact of these different administrations on absolute intratissue levels, proportion of patients treated in whom high tissue levels are reached, and the importance of tissue levels for therapeutic response all require further clarification (Wettergren et al., 2015).

8.3. [6S]-5-methyl-THF (Metafolin®) and [6R,S]-5-methyl-THF (mefolinate)

Metafolin® ([6S]-5-methyl-THF), the natural diastereoisomeric form, is the folate normally found in the circulation and is also the predominant folate present in food. It is available commercially

both as the natural form and as mefolinate, the diastereoisomeric 1:1 mixture [6R,S]-5-methyl-THF. The MTHFR enzyme is responsible for the irreversible reduction of [6R]-5,10-methylene-THF to [6S]-5-methyl-THF. This folate is needed for the biosynthesis of methionine from homocysteine via donation of the methyl group as a one-carbon unit. There is a genetic polymorphism associated with human MTHFR enzyme activity, and decreased plasma activity of MTHFR is associated with low plasma [6S]-5-methyl-THF concentration and elevated levels of homocysteine (Prinz-Langenohl et al., 2009).

Studies in humans have shown that administration of Metafolin® is at least as effective as folic acid at increasing both the plasma folate area under the curve and red blood cell folate levels, and at lowering homocysteine levels. This may mean higher relative bioavailability of [6S]-5-methyl-THF when compared with administration of folic acid (Prinz-Langenohl et al., 2009; Lamers et al., 2004; Lamers et al., 2006).

Similar therapeutic benefit has been reported with single intravenous administrations of mefolinate or leucovorin in combination with 5-FU for the treatment of colorectal cancer ((Mader et al., 1995) and references cited therein). Administration of 200 mg/m^2 of either mefolinate or leucovorin resulted in a threefold elevation of [6R]-5,10-methylene-THF for both agents. Similar plasma concentration – time profiles were found, with faster elimination from the blood and higher renal clearance of the [6S]- compared with the [6R]-isomer. Intratumoral folate levels were also shown to be the same.

8.4. [6R,S]-5,10-methylene-THF (CoFactor®)

CoFactor® ([6R,S]-5,10-methylene-THF), a 1:1 mixture of the two diastereoisomers, is very sensitive to oxygen (Odin et al., 1998). As the [6R]-isomer is the directly active co-substrate of TS, it was anticipated that administration of CoFactor®, rather than leucovorin, would be advantageous due to lower inter- and intrapatient variability regarding both clinical safety and efficacy. However, the unnatural [6S]-isomer is a partial competitive inhibitor of the natural [6R]-isomer regarding its effect as co-substrate for TS (Leary et al., 1974), which is likely an undesirable property. The unnatural [6S]-isomer is handled by detoxification mechanisms after administration, and the chemical stability of [6R,S]-5,10-methylene-THF has been shown to be concentration-dependent (Odin et al., 1998).

CoFactor® clinical development programs were initiated, but never brought firm conclusive findings. There may have been various reasons for this, with the route of administration possibly one such factor that was never resolved at the time. Studies using intravenous bolus injection tended to show positive disease impact, but one study relying on intravenous infusion over a more extended period of time did not show the same result (A Vedin, unpublished data: Clinical CoFactor trials meta-analysis, Isofol Medical, January 2015). In this context, the importance of the aforementioned concentration-dependent stability has never been resolved. Indeed, the general field of therapy with reduced folates may have neglected possible important differences between various modes of administration and plasma pharmacokinetics/pharmacodynamics (e.g. tissue levels of different fractions), as well as their relationships to therapeutic impact. With recent advances in analytical techniques, such studies may now be possible (Odin et al., 2013).

8.5. [6R]-5,10-methylene-THF (Modufolin®)

Modufolin® ([6R]-5,10-methylene-THF; Fig. 8) is a biomodulator that can directly enhance the desired cytotoxic antitumor effect of 5-FU. It bypasses the metabolic pathway required by leucovorin. With the exception of CoFactor® ([6R,S]-5,10-methylene-THF), all other folates need to be activated. Furthermore, the unclear influence of equal quantities of unnatural xenobiotics present in the diastereoisomeric pairs (i.e. leucovorin, mefolinate, and CoFactor®) is avoided, which is often seen as an advantage by regulatory authorities.

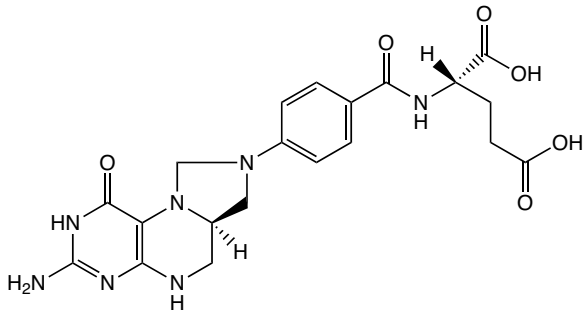

Fig. 8. Chemical structure of Modufolin® ([6R]-5,10-methylene-THF).

In patients with colorectal cancer, a direct comparison of levoleucovorin and Modufolin®, given as equimolar bolus injections, showed that levoleucovorin produced barely measurable plasma levels of [6R]-5,10-methylene-THF and [6S]-THF, whereas Modufolin® resulted in high levels of both within 2 h of injection (Wettergren et al., 2015). There were high 'steady-state' levels of [6S]-5-methyl-THF, for at least 6 h, after administration of either agent. The bioconversion of levoleucovorin to the active [6R]-5,10-methylene-THF requires four consecutive steps. It is possible, however, that following levoleucovorin administration the relatively slowly formed [6R]-5,10-methylene-THF is quickly consumed by an enzymatic reduction to [6S]-5-methyl-THF in the blood, whereas following Modufolin® administration the corresponding reduction to [6S]-5-methyl-THF may be maximized by enzyme saturation due to the very high concentration of [6R]-5,10-methylene-THF (Borsi et al., 1990). From this, it is highly probable that the major effect exerted by levoleucovorin relates to the ultimate re-formation of the active [6R]-5,10-methylene-THF (two steps) from the generated [6S]-5-methyl-THF pool (i.e. seven steps from the original levoleucovorin administration). Furthermore, a several-fold higher tumor concentration of [6R]-5,10-methylene-THF was seen 2 h following Modufolin® administration, compared with levoleucovorin administration, whereas high levels of [6S]-5-methyl-THF were detected more than 2 h after administration of either agent. The high [6S]-THF levels in the initial phase following Modufolin® administration can be interpreted by a shift of the association – dissociation equilibrium of [6R]-5,10-methylene-THF towards [6S]-THF and formaldehyde, by dilution of the injection solution in the blood.

Activation of folates requires the presence of the relevant enzymes, which in turn requires high expression of the genes regulating these enzymes. Patients with colorectal cancer and high levels of expression of these genes have significantly enhanced disease-free survival when treated with 5-FU plus leucovorin, when compared with patients with low levels of expression of these genes (Wettergren et al., 2014). Thus, it may be speculated that patients with low folate-related gene expression levels may respond favorably to direct administration of Modufolin®; controlled trials are needed to confirm this.

9. Conclusion

For many years, folates have been used with cytostatic and cytotoxic agents to enhance both efficacy and safety. The mechanism of action of all folates has been shown to converge on the ternary complex comprising TS, dUMP, and [6R]-5,10-methylene-

THF. Modulation of the concentration of this metabolite has been the central theme in all clinical studies. Application of several of the precursors is complicated because of the chemical and metabolically unstable nature. This can be improved by changing the formulation, while a more effective modulation can perhaps be achieved by administration of either the active metabolite itself or a more direct precursor. Another major question for administration is the timing, i.e. should the metabolite be given before or after the cytotoxic drug? For modulation of 5-FU it is generally agreed that the metabolite should be given before 5-FU, although for capecitabine the metabolite should not be given at the same time because of the likelihood of increased toxicity (Schneiders et al., 2011), and in order to rescue from toxicity leucovorin should be given after methotrexate (Bertino, 1993). In contrast, in order to administer pemetrexed safely, folic acid should be started a few days before pemetrexed is first administered (Chattopadhyay et al., 2007).

Despite the fact that many years have passed since co-administration of folates and cytotoxic agents was first introduced, numerous central questions remain unanswered. Treatment regimes have become empirically established and have evolved over time, often not based on tightly controlled comparisons. This is partly caused by the nature of the disease and the ensuing ethics. Many important factors have been clarified *post hoc* and, had knowledge been available earlier, other regimes might have arisen.

Actual dosages, pharmacokinetics of folates, modes of administration, tissue concentrations, gene profiles in normal and tumor tissues, and the relationships between genes, enzyme levels, tissue folate concentrations, key enzyme inhibition, and clinical outcomes are all examples of potentially fruitful areas for future scientific research. With the availability of new analytical and other techniques, as well as new stable folate compounds, it is hoped that the entire field again will be the center of added research focus, ultimately bringing patients increased clinical benefit.

10. Remark

Product formulations under the registered trademarks Fusilev®, Metafolin®, CoFactor®, and Modufolin® contain different salt forms of the respective folate. For the sake of clarity, only the term folate is used to describe the active ingredient.

Conflict of interest statement

PD, EO, and NP have declared no competing interest. BG is a consultant/advisor for Isofol Medical, is a stockholder of Isofol Medical, and receives funding from Isofol Medical. PJ is a consultant/advisor for Sanofi Aventis, Pfizer, and Chugai Pharmaceuticals, and a stockholder of Fusion Antibodies. PL has received a consultancy fee from Isofol Medical for his work on this manuscript. GJP has received funding from Spectrum. RM is scientific director at Merck & Cie, producing folates including Modufolin®.

References

Allegra, C.J., Voeller, D., 1993. Clinical use of leucovorin: intracellular metabolism. Adv. Exp. Med. Biol. 339, 43–49.

Advanced colorectal cancer meta-analysis project, 1992. Modulation of 5-fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. J. Clin. Oncol. 10: 896–903.

Anderson, D.D., Stover, P.J., 2009. SHMT1 and SHMT2 are functionally redundant in nuclear de novo thymidylate biosynthesis. PLoS One 4, e5839.

Anderson, D.D., Woeller, C.F., Chiang, E.P., Shane, B., Stover, P.J., 2012. Serine hydroxymethyltransferase anchors de novo thymidylate synthesis pathway to nuclear lamina for DNA synthesis. J. Biol. Chem. 287, 7051–7062.

Baggott, J.E., Tamura, T., 1999. Bioactivity of orally administered unnatural isomers, [6R]-5-formyltetrahydrofolate and [6S]-5,10-methenyltetrahydrofolate, in humans. Biochim. Biophys. Acta 1472, 323–332.

Baggott, J.E., Robinson, C.B., Johnston, K.E., 2001. Bioactivity of [6R]-5-formyltetrahydrofolate, an unnatural isomer, in humans and *Enterococcus hirae*, and cytochrome c oxidation of 10-formyltetrahydrofolate to 10-formyldihydrofolate. Biochem. J 354, 115–122.

Bailey, S.W., Ayling, J.E., 2009. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. Proc. Natl. Acad. Sci. U. S. A. 106, 15424–15429.

Barlowe, C.K., Appling, D.R., 1988. In vitro evidence for the involvement of mitochondrial folate metabolism in the supply of cytoplasmic one-carbon units. Biofactors 1, 171–176.

Bertino, J.R., 1993. Karnofsky memorial lecture. Ode to methotrexate. J. Clin. Oncol. 11, 5–14.

Bissoon-Haqqani, S., Moyana, T., Jonker, D., Maroun, J.A., Birnboim, H.C., 2006. Nuclear expression of thymidylate synthase in colorectal cancer cell lines and clinical samples. J. Histochem. Cytochem. 54, 19–29.

Boarman, D.M., Allegra, C.J., 1992. Intracellular metabolism of 5-formyl tetrahydrofolate in human breast and colon cell lines. Cancer Res. 52, 36–44.

Borsi, J.D., Sagen, E., Romslo, I., Moe, P.J., 1990. Rescue after intermediate and high-dose methotrexate: background, rationale, and current practice. Pediatr. Hematol. Oncol. 7, 347–363.

Carreras, C.W., Santi, D.V., 1995. The catalytic mechanism and structure of thymidylate synthase. Annu. Rev. Biochem 64, 721–762.

Chéradame, S., Etienne, M.C., Chazal, M., Guillot, T., Fischel, J.L., Formento, P., et al., 1997a. Relevance of tumoral folylpolyglutamate synthetase and reduced folates for optimal 5-fluorouracil efficacy: experimental data. Eur. J. Cancer 33, 950–959.

Chéradame, S., Etienne, M.C., Formento, P., Schneider, M., Dassonville, O., Demard, F., et al., 1997b. Tumoral-reduced folates and clinical resistance to fluorouracil-based treatment in head and neck cancer patients. J. Clin. Oncol. 15, 2604–2610.

Chattopadhyay, S., Moran, R.G., Goldman, I.D., 2007. Pemetrexed: biochemical and cellular pharmacology, mechanisms, and clinical applications. Mol. Cancer Ther. 6, 404–417.

Cisneros, R.J., Dunlap, R.B., 1990. Characterization of the parameters affecting covalent binding stoichiometry in binary and ternary complexes of thymidylate synthase. Biochim. Biophys. Acta 1039, 149–156.

Cook, J.D., Cichowicz, D.J., George, S., Lawler, A., Shane, B., 1987. Mammalian folylpoly-gamma-glutamate synthetase: 4. In vitro and in vivo metabolism of folates and analogues and regulation of folate homeostasis. Biochemistry 26, 530–539.

Danenberg, P.V., Danenberg, K.D., 1978. Effect of 5,10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2′-deoxyuridylate from thymidylate synthetase: evidence for an ordered mechanism. Biochemistry 17, 4018–4024.

DeVito, J.M., Kozloski, G.D., Tonelli, A.P., Johnson, J.B., 1993. Bioequevalence of oral and injectable levoleucovorin and leucovorin. Clin. Pharmacol. 12, 293–299.

Desmoulin, S.K., Hou, Z., Gangjee, A., Matherly, L.H., 2012. The human proton-coupled folate transporter: biology and therapeutic applications to cancer. Cancer Biol. Ther. 13, 1355–1373.

Dohden, K., Ohmura, K., Watanabe, Y., 1993. Ternary complex formation and reduced folate in surgical specimens of human adenocarcinoma tissues. Cancer 71, 471–480.

Fan, J., Ye, J., Kamphorst, J.J., Shlomi, T., Thompson, C.B., Rabinowitz, J.D., 2014. Quantitative flux analysis reveals folate-dependent NADPH production. Nature 510, 298–302.

Galivan, J.H., Maley, G.F., Maley, F., 1975. The effect of substrate analogs on the circular dichroic spectra of thymidylate synthetase from *Lactobacillus casei*. Biochemistry 14, 3338–3344.

Goldman, I.D., Matherly, L.H., 1987. Biochemical factors in the selectivity of leucovorin rescue: selective inhibition of leucovorin reactivation of dihydrofolate reductase and leucovorin utilization in purine and pyrimidine biosynthesis by methotrexate and dihydrofolate polyglutamates. NCI Monogr. 5, 17–26.

Gonen, N., Assaraf, Y.G., 2012. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. Drug Resist. Update 15, 183–210.

Habeck, L.L., Chay, S.H., Pohland, R.C., Worzalla, J.F., Shih, C., Mendelsohn, L.G., 1998. Whole-body disposition and polyglutamate distribution of the GAR formyltransferase inhibitors LY309887 and lometrexol in mice: effect of low-folate diet. Cancer Chemother. Pharmacol. 41, 201–209.

Hardy, L.W., Finer-Moore, J.S., Montfort, W.R., Jones, M.O., Santi, D.V., Stroud, R.M., 1987. Atomic structure of thymidylate synthase: target for rational drug design. Science 235, 448–455.

Houghton, J.A., Williams, L.G., Adkins, S.A., Loftin, S., Houghton, P.J., 1994. Comparative efficacy of folate species in expanding reduced folate pools and potentiating 5-fluorouracil cytotoxicity in colon adenocarcinoma cells. Cell. Pharmacol. 1, 53–62.

Jackson, R.C., Harrap, K.R., 1973. Studies with a mathematical model of folate metabolism. Arch. Biochem. Biophys. 158, 827–841.

Jarmuła, A., Cieplak, P., Montfort, W.R., 2005. 5,10-Methylene-5,6,7,8-tetrahydrofolate conformational transitions upon binding to thymidylate synthase: molecular mechanics and continuum solvent studies. J. Comput. Aided Mol. Des. 19, 123–136.

Jolivet, J., Dayan, A., Beauchemin, M., Chahla, D., Mamo, A., Bertrand, R., 1996. Biochemical and molecular studies of human methenyltetrahydrofolate synthetase. Oncologist 1, 248–254.
Kovoor, P.A., Karim, S.M., Marshall, J.L., 2009. Is levoleucovorin an alternative to racemic leucovorin: a literature review. Clin. Colorectal Cancer 8, 200–206.
Lamers, Y., Prinz-Langenohl, R., Moser, R., Pietrzik, K., 2004. Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. Am. J. Clin. Nutr. 79, 473–478.
Lamers, Y., Prinz-Langenohl, R., Brämswig, S., Pietrzik, K., 2006. Red blood cell folate concentrations increase more after supplementation with [6S]-5-methyltetrahydrofolate than with folic acid in women of childbearing age. Am. J. Clin. Nutr. 84, 156–161.
Langenbach, R.J., Danenberg, P.V., Heidelberg, C., 1972. Thymidylate synthetase: mechanism of inhibition by 5-fluoro-2′-deoxyuridylate. Biochem. Biophys. Res. Commun. 48, 1565–1571.
Laohavinij, S., Wedge, S.R., Lind, M.J., Bailey, N., Humphreys, A., Proctor, M., et al., 1996. A phase I clinical study of the antipurine antifolate lometrexol (DDATHF) given with oral folic acid. Invest. New Drugs 14, 325–335.
Lawrence, S.A., Hackett, J.C., Moran, R.G., 2011. Tetrahydrofolate recognition by the mitochondrial folate transporter. J. Biol. Chem. 286, 31480–31489.
Lawrence, S.A., Titus, S.A., Ferguson, J., Heineman, A.L., Taylor, S.M., Moran, R.G., 2014. Mammalian mitochondrial and cytosolic folylpolyglutamatesynthetase maintain the subcellular compartmentation of folates. J. Biol. Chem. 289, 29386–29396.
Leary, R.P., Gaumont, Y., Kisliuk, R.L., 1974. Effects of the diastereoisomers of methylenetetrahydrofolate on the reaction catalyzed by thymidylate synthetase. Biochem. Biophys. Res. Commun. 56, 484–488.
Lin, B.F., Huang, R.F., Shane, B., 1993. Regulation of folate and one-carbon metabolism in mammalian cells: III. Role of mitochondrial folylpoly-gamma-glutamate synthetase. J. Biol. Chem. 268, 21674–21679.
Locasale, J.W., 2013. Serine, glycine and one-carbon units: cancer metabolism in full circle. Nat. Rev. Cancer 13, 572–583.
Lockshin, A., Danenberg, P.V., 1981. Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding to human thymidylate synthetase. Biochem. Pharmacol. 30, 247–257.
Lowe, K.E., Osborne, C.B., Lin, B.F., Kim, J.S., Hsu, J.C., Shane, B., 1993. Regulation of folate and one-carbon metabolism in mammalian cells: II. Effect of folylpoly-gamma-glutamate synthetase substrate specificity and level on folate metabolism and folylpoly-gamma-glutamate specificity of metabolic cycles of one-carbon metabolism. J. Biol. Chem. 268, 21665–21673.
Mader, R.M., Steger, G.G., Rizovski, B., Djavanmard, M.P., Scheithauer, W., Jakesz, R., et al., 1995. Stereospecific pharmacokinetics of rac-5-methyltetrahydrofolic acid in patients with advanced colorectal cancer. Br. J. Clin. Pharmacol. 40, 209–215.
Madhukar, N.S., Warmoes, M.O., Locasale, J.W., 2015. Organization of enzyme concentration across the metabolic network in cancer cells. PLoS One 10, e0117131.
Matthews, D.A., Villafranca, J.E., Janson, C.A., Smith, W.W., Welsh, K., Freer, S., 1990. Stereochemical mechanism of action for thymidylate synthase based on the X-ray structure of the covalent inhibitory ternary complex with 5-fluoro-2′-deoxyuridylate and 5,10-methylenetetrahydrofolate. J. Mol. Biol. 214, 937–948.
Meta-Analysis Group in Cancer, Piedbois, P., Rougier, P., Buyse, M., Pignon, J., Ryan, L., et al., 1998. Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. J. Clin. Oncol. 16, 301–308.
Moran, R.G., Spears, C.P., Heidelberger, C., 1979. Biochemical determinants of tumor sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2'-deoxyuridylate, 2'-deoxyuridylate, and thymidylate synthetase. Proc. Natl. Acad. Sci. U. S. A. 76, 1456–1460.
Newman, E.M., Straw, J.A., Doroshow, J.H., 1989. Pharmacokinetics of diastereoisomers of (6R,S)-folinic acid (leucovorin) in humans during constant high-dose intravenous infusion. Cancer Res. 49, 5755–5760.
Obeid, R., Kirsch, S.H., Kasoha, M., Eckert, R., Herrmann, W., 2011. Concentrations of unmetabolized folic acid and primary folate forms in plasma after folic acid treatment in older adults. Metabolism 60, 673–680.
Odin, E., Carlsson, G., Frösing, R., Gustavsson, B., Spears, C.P., Larsson, P.A., 1998. Chemical stability and human plasma pharmacokinetics of reduced folates. Cancer Invest. 16, 447–455.
Odin, E., Wettergren, Y., Carlsson, G., Gustavsson, B., 2013. Determination of reduced folates in tumour and adjacent mucosa of colorectal cancer patients using LC-MS/MS. Biomed. Chromatogr. 27, 487–495.
Pawelek, P.D., MacKenzie, R.E., 1998. Methenyltetrahydrofolate cyclohydrolase is rate limiting for the enzymatic conversion of 10-formyltetrahydrofolate to 5,10-methylenetetrahydrofolate in bifunctional dehydrogenase-cyclohydrolase enzymes. Biochemistry 37, 1109–1115.
Peters, G.J., Van der Wilt, C.L., Van Groeningen, C.J., Meijer, S., Smid, K., Pinedo, H.M., 1994. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: implications for treatment with fluorouracil. J. Clin. Oncol. 12, 2035–2042.
Porcelli, L., Assaraf, Y.G., Azzariti, A., Paradiso, A., Jansen, G., Peters, G.J., 2011. The impact of folate status on the efficacy of colorectal cancer treatment. Curr. Drug Metab. 12, 975–984.
Priest, D.G., Schmitz, J.C., Bunni, M.A., Stuart, R.K., 1991. Pharmacokinetics of leucovorin metabolites in human plasma as a function of dose administered orally and intravenously. J. Natl. Cancer Inst. 83, 1806–1812.
Prinz-Langenohl, R., Brämswig, S., Tobolski, O., Smulders, Y.M., Smith, D.E.C., Finglas, P.M., et al., 2009. [6S]-5-Methyltetrahydrofolate increases plasma folate more effectively than folic acid in women with the homozygous or wild-type 677C → T polymorphism of methylenetetrahydrofolate reductase. Br. J. Pharmacol. 158, 2014–2021.
Qiu, A., Jansen, M., Sakaris, A., Min, S.H., Chattopadhyay, S., Tsai, E., et al., 2006. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. Cell 127, 917–928.
Radparvar, S., Houghton, P.J., Houghton, J.A., 1988. Characteristics of thymidylate synthase purified from a human colon adenocarcinoma. Arch. Biochem. Biophys. 260, 342–350.
Rode, W., 1993. Specificity of thymidylate synthase inactivation by 4,5-bisubstituted dUMP analogues. Acta Biochim. Pol. 40, 363–368.
Rots, M.G., Pieters, R., Peters, G.J., Noordhuis, P., Van Zantwijk, C.H., Kaspers, G.J., et al., 1999. Role of folylpolyglutamate synthetase and folylpolyglutamate hydrolase in methotrexate accumulation and polyglutamylation in childhood leukemia. Blood 93, 1677–1683.
Saif, M.W., Makrilia, N., Syrigos, K., 2010. CoFactor: folate requirement for optimization of 5-fluouracil activity in anticancer chemotherapy. J. Oncol. 2010, 934359.
Santi, D.V., McHenry, C.S., Sommer, H., 1974. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. Biochemistry 13, 471–481.
Santi, D.V., 1972. McHenry CS. 5-fluoro-2′-deoxyuridylate: covalent complex with thymidylate synthetase. Proc. Natl. Acad. Sci. U. S. A. 69, 1855–1857.
Sato, J.K., Newman, E.M., Moran, R.G., 1986. Preparation of (6R)-tetrahydrofolic acid and (6R)-5-formyltetrahydrofolic acid of high stereochemical purity. Anal. Biochem. 154, 516–524.
Schiffer, C.A., Clifton, I.J., Davidsson, V.J., Santi, D.V., Stroud, R.M., 1995. Crystal structure of human thymidylate synthase: a structural mechanism for guiding substrates into the active site. Biochemistry 34, 16279–16287.
Schneiders, F.A., Van den Berg, H.P., Peters, G.J., Verheul, H.M., Van der Vliet, H.J., 2011. Severe toxicity of capecitabine following uncomplicated treatment with 5-fluorouracil/leucovorin. Med. Oncol. 28, 1136–1139.
Sharma, R.K., Kisliuk, R.L., 1975. Quenching of thymidylate synthetase fluorescence by substrate analogs. Biochem. Biophys. Res. Commun. 64, 648–655.
Shin, Y.S., Chan, C., Vidal, A.J., Brody, T., Stokstad, E.L., 1976. Subcellular localization of gamma-glutamyl carboxypeptidase and folates. Biochim. Biophys. Acta 444, 794–801.
Spears, C.P., Hayes, A.A., Shahinian, A.H., Danenberg, P.V., Frösing, R., Gustavsson, B.G., 1989. Deoxyuridylate effects on thymidylate synthase – 5-fluorodeoxyuridylate – folate ternary complex formation. Biochem. Pharmacol. 38, 2985–2993.
Spears, C.P., Carlsson, G., Muggia, F.M., Jaresko, G., Gustavsson, B.G., 1995. Aspects of one-carbon folate cycling related to fluoropyrimidine and antifolate therapy. Cancer Treat. Res. 78, 115–134.
Stover, P.J., Field, M.S., 2011. Trafficking of intracellular folates. Adv Nutr 2, 325–331.
Stover, S., Schirch, V., 1991. 5-Formyltetrahydrofolate polyglutamates are slow tight binding inhibitors of serine hydroxymethyltransferase. J. Biol. Chem. 266, 1543–1550.
Straw, J.A., Newman, E.M., 1988. Pharmacokinetic analysis of [6S]-5-formyltetrahydrofolate (l-CF), [6R]-5-formyltetrahydrofolate (d-CF) and 5-methyltetrahydrofolate (5-CH3-THF) in patients receiving constant i.v. infusion of high-dose [6RS]-5-formyltetrahydrofolate (leucovorin). Adv. Exp. Med. Biol. 244, 53–58.
Straw, J.A., Szapary, D., Wynn, W.T., 1984. Pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res. 44, 3114–3119.
Strohle, A., Wolters, M., Hahn, A., 2005. Folic acid and colorectal cancer prevention: molecular mechanisms and epidemiological evidence (Review). Int. J. Oncol. 26, 1449–1464.
Stroud, R.M., Finer-Moore, J.S., 1993. Stereochemistry of a multistep/bipartite methyl transfer reaction: thymidylate synthase. FASEB J. 7, 671–677.
Suh, J.R., Herbig, A.K., Stover, P.J., 2001. New perspectives on folate catabolism. Annu. Rev. Nutr. 21, 255–282.
Taflin, H., Wettergren, Y., Odin, E., Derwinger, K., 2014. Folate levels measured by LC–MS/MS in patients with colorectal cancer treated with different leucovorin dosages. Cancer Chemother. Pharmacol. 74, 1167–1174.
Tedeschi, P.M., Markert, E.K., Gounder, M., Lin, H., Dvorzhinski, D., Dolfi, S.C., et al., 2013. Contribution of serine, folate and glycine metabolism to the ATP, NADPH and purine requirements of cancer cells. Cell. Death. Dis. 4, e877.
Tibbett, A.S., Appling, D.R., 2010. Compartmentalization of mammalian folate-mediated one-carbon metabolism. Annu. Rev. Nutr. 30, 57–81.
Trave, F., Rustum, Y.M., Petrelli, N.J., Herrera, L., Mittelman, A., Frank, C., et al., 1988. Plasma and tumor tissue pharmacology of high-dose intravenous leucovorin calcium in combination with fluorouracil in patients with advanced colorectal carcinoma. J. Clin. Oncol. 6, 1184–1191.
Turner, F.B., Taylor, S.M., Moran, R.G., 2000. Expression patterns of the multiple transcripts from the folylpolyglutamate synthetase gene in human leukemias and normal differentiated tissues. J. Biol. Chem. 275, 35960–35968.
van der Wilt, C.L., Backus, H.H., Smid, K., Comijn, L., Veerman, G., Wouters, D., et al., 2001. Modulation of both endogenous folates and thymidine enhance the therapeutic efficacy of thymidylate synthase inhibitors. Cancer Res. 61, 3675–3681.

Van Triest, B., Loftus, B.M.K., Pinedo, H.M., Backus, H.H., Schoenmakers, P., Telleman, F., et al., 2000. Thymidylate synthase expression in patients with colorectal carcinoma using a polyclonal thymidylate synthase antibody in comparison to the TS 106 monoclonal antibody. J. Histochem. Cytochem. 48, 755–760.

Van der Wilt, C.L., Pinedo, H.M., De Jong, M., Peters, G.J., 1993. Effect of folate diastereoisomers on the binding of 5-fluoro-2′-deoxyuridine-5′-monophosphate to thymidylate synthase. Biochem. Pharmacol. 45, 1177–1179.

Van der Wilt, C.L., Smid, K., Peters, G.J., 2002. Effects of antifolates on the binding of 5-fluoro-2′-deoxyuridine-5′-monophosphate to thymidylate synthase. Biochem. Pharmacol. 64, 669–675.

Vogelzang, N.J., Rusthoven, J.J., Symanowski, J., Denham, C., Kaukel, E., Ruffie, P., et al., 2003. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J. Clin. Oncol. 21, 2636–2644.

Walling, J., 2006. From methotrexate to pemetrexed and beyond: a review of the pharmacodynamic and clinical properties of antifolates. Invest. New Drugs 24, 37–77.

Wettergren, Y., Odin, E., Sondén, A., Gustavsson, B., Carlsson, G., 2014. Expression of folate pathway genes and risk of recurrent disease in stage III colorectal cancer. J. Clin. Oncol. 32 (May (20) Suppl), e14567.

Wettergren, Y., Taflin, H., Odin, E., Kodeda, K., Derwinger, K., 2015. A pharmacokinetic and pharmacodynamic investigation of Modufolin® compared to Isovorin® after single dose intravenous administration to patients with colon cancer: a randomized study. Cancer Chemother. Pharmacol. 75, 37–47.

Worzalla, J.F., Shih, C., Schultz, R.M., 1998. Role of folic acid in modulating the toxicity and efficacy of the multitargeted antifolate, LY231514. Anticancer Res. 18, 3235–3239.

Zhao, R., Goldman, I.D., 2013. The proton-coupled folate transporter: physiological and pharmacological roles. Curr. Opin. Pharmacol. 13, 875–880.

Zhao, R., Matherly, L.H., Goldman, I.D., 2009. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. Expert Rev. Mol. Med. 11, e4.

Zittoun, J., Tonelli, A.P., Marquet, J., De Gialluly, E., Hancock, C., Yacobi, A., et al., 1993. Pharmacokinetic comparison of leucovorin and levoleucovorin. Eur. J. Clin. Pharmacol. 44, 569–573.

Zittoun, J., 1993. Pharmacokinetics and in vitro studies of l-leucovorin Comparison with the d and d,l-leucovorin. Ann. Oncol. 4 (Suppl. 2), 1–5.

Biographies

Peter Danenberg PhD, is Professor of Biochemistry and Molecular Biology in the Keck School of Medicine, University of Southern California, Los Angeles, California, USA. His research interests have been in the mechanism of action of fluorinated pyrimidines, especially as they interact with thymidylate synthase. More recently, he has been interested in identifying biochemical and genetic biomarkers of response to anticancer drugs. He has over 200 peer-reviewed publications to his name in these areas.

Bengt Gustavsson is Professor of Surgery at the Sahlgrenska University Hospital, Sweden. For many years, his special interest has been the pharmacokinetics and pharmacodynamics of fluorinated pyrimidines in the treatment of gastrointestinal tumors. He was one of the founders of the Nordic gastrointestinal tumor group, particularly important for the introduction of translational research in colorectal cancer. His special focus has also included metabolism of the folates and the interaction between the fluorinated pyrimidines and the target enzyme thymidylate synthase. He has been part of the project SpectaCOLOR, which involves the collaboration of some of the most important centers in Europe in translational research in colorectal cancer. He is the author of more than 200 peer-reviewed manuscripts.

Patrick Johnston is President and Vice-Chancellor of Queen's University Belfast, UK. He received his medical degree with distinction from University College Dublin, Ireland, in 1982, followed by his MD and PhD in Medicine in 1988. His research focus has been on the understanding of mechanisms of drug resistance to fluoropyrimidines in gastrointestinal cancers. Professor Johnston has been awarded many national and international awards, is a Fellow of the Academy of Medical Sciences, and in 2013 he was named winner of the international Bob Pinedo Cancer Care Prize.

Per Lindberg, PhD, is Associate Professor, Organic Chemistry, Chalmers University of Technology, Gothenburg, Sweden (1982). He has 36 years' experience of pharmaceutical research: 6 years with Professor Arvid Carlsson (Nobel Prize Winner, Medicine, 2000) at the University of Gothenburg, and 30 years with Astra, Sweden, as Head of Medicinal Chemistry for the Gastrointestinal Department, Director of the Preclinical Alliances Group, Advisor in the Scientific Patent Support Team, and later as Senior Scientific Advisor engaged in patent strategy. He was deeply involved in the development of the first proton pump inhibitor omeprazole (Losec®/Prilosec®), and now leads his own consultancy firm. He has authored approximately 60 manuscripts.

Rudolf Moser gained his PhD in Biochemistry from the University of Zurich, Switzerland. He has more than 20 years of R&D work experience in the field of synthesis and development of small molecule active pharmaceutical ingredients. Since 2002, he has been Scientific Director at Merck & Cie in Switzerland. His expertise is on the B9 vitamin folate, with a main focus on chemistry, biochemistry, as well as pharmaceutical and nutritional applications.

Elisabeth Odin has, for more than 30 years, focused her research on the biochemistry and enzymology of folate-mediated one-carbon metabolism. She has published more than 20 articles in this field, and developed pioneering ultrasensitive bioanalytical methods for determination of tissue concentrations, with importance for practical therapeutics.

Godefridus (Frits) J Peters is currently at the Laboratory Medical Oncology, VU University Medical Center in Amsterdam, The Netherlands. He obtained a master's degree in 1977 and PhD in 1982, and was appointed Associate Professor in 1992 and full Professor in 2003. His major research interests include pharmacology of anticancer agents, with emphasis on antimetabolites, antifolates, platinum analogs, taxanes, antisignalling and third-generation targeted agents. He is (co)-author of more than 500 peer-reviewed papers in these fields. Professor Peters' group described the predictive value of thymidylate synthase in the antitumor activity of 5-fluorouracil. One major research line focuses on the role of folate homeostasis in the efficacy of anticancer drugs. His current research also involves the role of genetic polymorphisms in drug metabolism and response to anticancer agents. He has been Chairman of the EORTC Pharmacology and Molecular Mechanisms Group for 6 years, editor-in-chief and a member of the editorial board for several journals, and has edited a number of books and proceedings of meetings.

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