

Multitargeted antifolate (Pemetrexed): A comprehensive review of its mechanisms of action, recent results and future prospects

Review Article

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Key Words: Pemetrexed, antifolate, 5-Fluorouracil, malignant mesothelioma

Abbreviations: 5-fluoro-2'-deoxyuridine-5'-monophosphate, (FdUMP); glycinamide ribonucleotide formyltransferase, (GARFT); aminoimidazolecarboxamide ribonucleotide formyltransferase, (AICARFT); dihydrofolate reductase, (DHFR); tetrahydrofolate, (THF); Folylpolylglutamate synthase, (FGPS); thymidylate synthase, (TS).

Received: 24 November 2003; Revised: 22 December 2003

Accepted: 24 December 2003; electronically published: December 2003

Summary

Antifolate drugs belong to the anticancer class of antimetabolites drugs, among which methotrexate and 5-Fluorouracil have been used for years in the treatment of various cancers such as colorectal and breast cancer, as well as lymphomas. 5-Fluorouracil exerts its action through incorporation of its triphosphate form into RNA, and through inhibition (by 5-FdUMP) of thymidylate synthase, the enzyme catalyzing the conversion of deoxyuridine 5' monophosphate into deoxythymidine 5' monophosphate. Conversely, methotrexate inhibits dihydrofolate reductase, an enzyme required for reduction of folates to di- or tetra-hydrofolates. Pemetrexed is a novel antifolate that targets various enzymes of the folate metabolism as well as thymidylate synthase, and is therefore called multitargeted antifolate. The aim of this work is to comprehensively expose pemetrexed's mechanisms of action, and review its advantages over 5-fluorouracil or methotrexate in the treatment of solid tumors. Recent results of pemetrexed chemotherapy in breast, pancreas and colorectal cancer are discussed, as well as promising prospects for malignant mesothelioma.

I. Introduction

Among the armamentarium of anticancer agents, antimetabolites are a class of anticancer drugs widely used in a variety of conditions, including colorectal cancer, breast cancer, and hematologic malignancies such as non-Hodgkin lymphomas. Antimetabolites exert their anticancer action either through direct inhibition of a key enzyme of folate metabolism (for example inhibition of dihydrofolate reductase by methotrexate), or through their action as irreversible enzyme false ligands (deoxyfluorouridine 5' monophosphate and thymidylate synthase). Although synthesized in 1957, 5-Fluorouracil is still a cornerstone in the chemotherapy of digestive malignancies usually co-administered with leucovorin in order to positively modulate thymidylate synthase

inhibition. As a consequence, 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) covalently binds to thymidylate synthase with N₅-N₁₀-methylene-THF (a metabolite of leucovorin), inducing a protein conformational change, trapping the enzyme at the intermediate step. Specific inhibitors to thymidylate synthase such as (Raltitrexed, Tomudex®, Astra-Zeneca) have been designed, and were shown to have similar efficacy when compared to 5-Fluorouracil/leucovorin. Further development in anticancer drug research led to the synthesis of pemetrexed in 1992. Pemetrexed (Alimta®, Eli Lilly) was designed as a multitargeted antifolate, inhibiting thymidylate synthase, dihydrofolate reductase, and two other enzymes: glycinamide ribonucleotide formyltransferase (GARFT) and aminoimidazole

carboxamide ribonucleotide formyltransferase (AICARFT).

II. The metabolism of folates: a key to understanding pemetrexed mechanisms of action on pyrimidine synthesis

The folate coenzymes are found in mitochondria as well as in the cytosol of eukaryotic cells. Their role is to accept one-carbon units from donor molecules and to pass them on via various biosynthetic reactions. Folic acid is the root of the folate family. Reduction of folic acid by dihydrofolate reductase (DHFR) to dihydro- and tetrahydro-folate (THF) is mandatory for its biological activity. However, these unsubstituted forms are chemically unstable, and therefore undergo a further polyglutamation. Folylglutamate synthase (FGPS) (E.C. 6.3.2.17) is required for the addition of glutamate residues to THF. As compared to the native forms, polyglutamated folates are retained in the cell, and are more effective enzymes substrates and regulators of folate-dependent enzymes. Interestingly, loss of FGPS activity through mutations is a major cause of antifolate resistance.

One-carbon substituted THF derivatives are associated with particular metabolic cycles, *e.g.* 10-formyl THF in purine synthesis; N_5-N_{10} -methylene-THF in dTMP synthesis and 5-Methyl THF in methionine synthesis. The interconversion of serine and THF to N_5-N_{10} -methylene-THF is catalyzed by the serine hydroxymethyltransferase (SHMT, E.C. 2.1.2.1). Interestingly, N_5-N_{10} -methylene-THF is also the final metabolite of leucovorin, interacting with both thymidylate synthase (TS) and deoxyuridine 5' monophosphate for the purpose of TS inhibition (Figure 1).

C_1 -tetrahydrofolate synthase is a trifunctional protein catalyzing the sequential reactions specified by the enzymes 10-formyl TRF synthase (E.C. 6.3.4.3), N_5-N_{10} -methenyl-THF cyclohydrolase (E.C. 3.5.4.9) and N_5-N_{10} -methylene-THF dehydrogenase (E.C. 1.5.1.5), which are structurally related to distinct domains (Strong et al, 1990). These three activities supply the activated one-carbon units required for the biosynthesis of thymidylate, purines, methionine, serine, glycine and many other compounds. Thus C_1 THF synthase plays a pivotal role in the regulation of folate coenzymes interconversion, allowing the cell to satisfy its most immediate requirements.

Thymidylate synthase (E.C. 2.1.1.45) is a key enzyme for the synthesis of deoxythymidine 5' monophosphate (dTMP) from deoxyuridine 5' monophosphate, and represents the only way to synthesize thymidylate *de novo*. Inhibition of TS is a major anticancer target, and has been achieved with fluoropyrimidines among which is 5-Fluorouracil, and more recently, with specific TS inhibitors such as raltitrexed. The metabolism of pyrimidines has been extensively investigated in prokaryotes and eukaryotes, to better characterize the enzymes involved in the activation of fluorinated prodrugs, such as 5-Fluorouracil.

III. Folate derivatives: a bridge between the metabolism of pyrimidines and purines

The *de novo* biosynthesis of inosine 5' monophosphate, a precursor of purine nucleotides, is a complex metabolic pathway, which involves ten successive enzymatic steps.

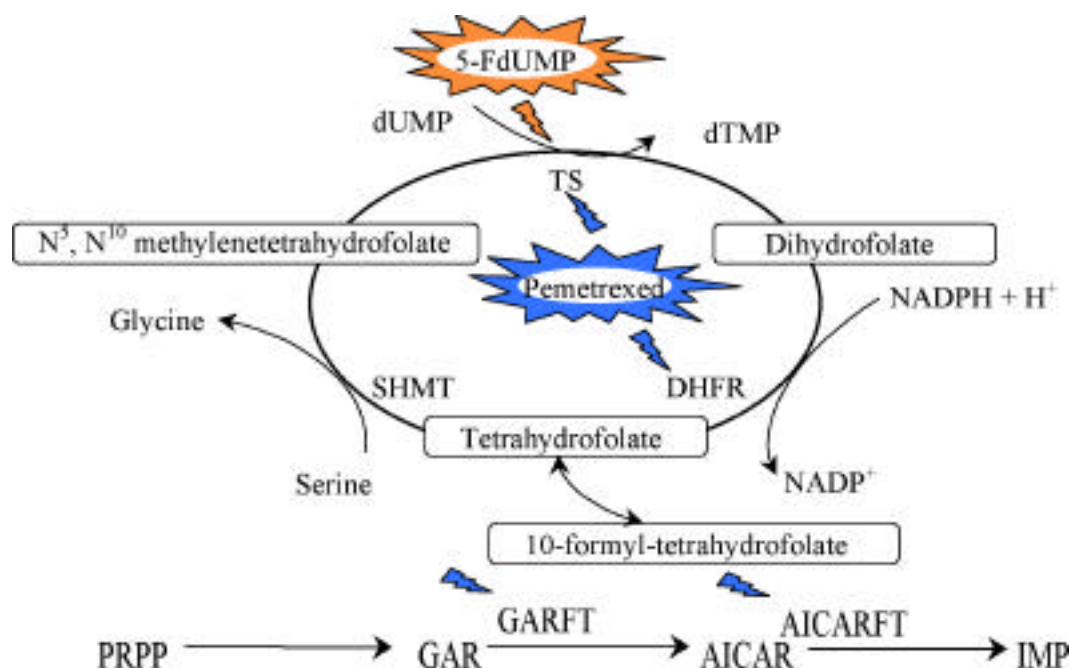


Figure 1. The folate cycle. dUMP: deoxyuridine 5' monophosphate; dTMP: deoxythymidine 5' monophosphate. TS: thymidylate synthase; DHFR: dihydrofolate reductase; SHMT Serine hydroxy methyl transferase. Anticancer agents 5-FdUMP (5-Fluoro deoxyuridine 5' monophosphate) and pemetrexed inhibit TS and TS plus DHFR, respectively.

Among these, two enzymes are folate dependent: glycylamide ribonucleotide formyltransferase (GARFT) and aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFT). Some drugs interacting with the metabolism of folates may also have activity towards folate-dependent enzymes within purine biosynthesis and have significant antiproliferative activity. Specific inhibitors to GARFT such as lometrexol were obtained for this purpose but clinical development was abandoned due to unacceptable myelotoxicity (Boger et al, 2000).

A. Pemetrexed: How does it work ?

Pemetrexed or N-4 (2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl) ethyl) benzoyl-L-glutamic acid is a structural analogue of methotrexate and lometrexol (Lansiaux et al, 1999) (**Figure 2**) that enters the cell through the reduced folate carrier, by a mechanism similar to that of raltitrexed and methotrexate (Zhao et al, 2000). Of note is the fact that a decreased expression of the reduced folate carrier may lead to acquired pemetrexed resistance (Wang et al, 2003). Once in the intracellular compartment, pemetrexed undergoes polyglutamation, catalyzed by folylpolyglutamate synthase, for which it has a very low K_m , as compared to methotrexate (Habeck et al, 1995). Polyglutamation of pemetrexed reduces the drug clearance from the intracellular compartment, and increases its activity towards some of its enzymatic targets. A reduced rate of polyglutamation has been described as a mechanism of resistance to methotrexate, but also for pemetrexed (McCloskey et al, 1991, Mauritz et al, 2002). Similarly, an increased activity of γ -glutamyltransferase may result in pemetrexed resistance (Yao et al, 1995), as polyglutamated derivatives of pemetrexed are substrates for this enzyme (Rhee et al, 1993) (**Table 1**) Resistance to antifolates also involves the modification of cell cycle genes. The loss of pRb has been associated with a higher DHFR level of expression and resistance to methotrexate and 5-fluorodeoxyurine (DFUR) (Banerjee et al 2002). Similarly, it has been shown that high levels of cyclin D1 correlate with high level of DHFR transcription (Hochauer et al, 1996). Whether resistance to pemetrexed is also associated with an altered expression of cell cycle genes remains to be determined.

Pemetrexed was synthesized in an effort to discover structural analogues to lometrexol, for the purpose of finding new GARFT inhibitors. However, in cell culture models, the addition of hypoxanthine did not rescue the

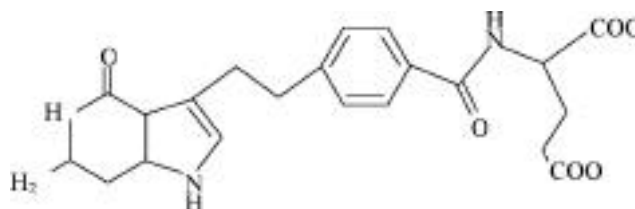


Figure 2. Chemical structure of pemetrexed

antiproliferative effect of pemetrexed, suggesting an alternate/additional mode of action. As a result, adding both thymidine and hypoxanthine rescued cell growth, showing that pemetrexed exerted its activity on both purine and pyrimidine biosynthesis (Shih et al, 1997, Smith et al, 1999). Pemetrexed was found to be an inhibitor of five enzymes involved in folate metabolism and purine / pyrimidine synthesis, *i.e.*: thymidylate synthase, dihydrofolate reductase, GARFT, AICARFT and C1 THF synthase.

B. Thymidylate synthase (TS) and dihydrofolate reductase

Although native pemetrexed is a poor inhibitor of TS, its polyglutamated (glu-3 and glu-5) forms show a 70 to 80-fold lower K_i for the enzyme. Conversely, polyglutamation has no effect on pemetrexed activity against DHFR, as the drug, either native or polyglutamated is a potent inhibitor of DHFR. Inhibition of both TS and DHFR results in a cytotoxic effect, as shown in various cultured cell lines (Chen et al, 2000).

C. GARFT and AICARFT

Unlike its properties on DHFR, polyglutamated pemetrexed is a potent inhibitor of GARFT, mainly in its (glu-5) status, for which the K_i is 144-fold lower than the parent compound, thus strongly inhibiting *de novo* purine biosynthesis. However, in contrast to TS and DHFR inhibition, the pemetrexed-related inhibition of GARFT results in a cytostatic effect (Chen et al, 2000). As shown for GARFT, polyglutamated (glu-5) pemetrexed inhibits AICARFT in a significantly better range (13-fold) than pemetrexed itself (Shih et al, 1997).

D. C1 THF synthase

As compared to TS, DHFR, GARFT and AICARFT, pemetrexed is a less potent inhibitor of C1 THF synthase.

Table 1: Mechanisms of resistance to pemetrexed

Mechanism of resistance to pemetrexed	Consequence	Reference
Mutation of the Reduced Folate Carrier	Decreased accumulation	Wang et al
Decreased activity of folylpolyglutamate synthase	Decreased polyglutamation	Mauritz et al
Increased activity of γ -glutamylhydrolase	Decreased polyglutamation	Rhee et al
Increased activity of thymidylate synthase	Decreased activity of pemetrexed	Sigmond et al

However, the observed intracellular pemetrexed concentration (up to 50µM) suggests that C1 THF synthase might still be *in vivo* a relevant target for pemetrexed.

In summary, these effects induce an imbalance in nucleotide pools, consisting in an important decrease in dTTP, concomitantly with dCTP and dGTP, whereas the pool of dATP increases (Chen et al, 1998). The inhibition of TS results in the decrease of dTMP, and as a consequence, of dTTP. However, dTTP negatively regulates deoxycytidine deaminase, an enzyme that catalyzes the deamination of dCMP into dUMP. This modification of metabolic flux in favour of dUMP reduces the amount of dCMP available for further phosphorylation, and ultimately, the pool of dCTP.

IV. Pemetrexed single-agent therapy: a review of phase I and II trials

In phase I studies, three different schedules of administration have been evaluated for pemetrexed single-agent chemotherapy, including a daily injection for 5 days every 3 weeks; a weekly injection for 4 weeks out of 6, and a single injection every 3 weeks (Rinaldi et al, 1995, 1999; McDonald et al, 1998). Severe myelotoxicity was the dose-limiting side-effect in the first two regimens. However, the weekly regimen was recommended at a weekly dose of 600mg/m². Again, myelotoxicity was the dose-limiting toxicity, but at the recommended dose level, it was acceptable. Hematological and other toxicities from a total of 872 non-supplemented patients who entered phase II studies with pemetrexed (at 500 and 600mg/m² every 3 weeks) appear in **Table 2**.

Once the tolerance/dosing data had been obtained, pemetrexed was assessed in phase II trials in a variety of tumours, including non small cell lung cancer (NSCLC), malignant mesothelioma, breast, colorectal, gastric and pancreatic cancer.

In NSCLC, pemetrexed has been evaluated in first (Rusthoven et al, 1999, Clarke et al, 2002) and second line therapy (Smit et al, 2003). Efficacy and tolerance data from these trials are reported in **Table 3**. From these data, one can assume that the efficacy of pemetrexed is consistent with that of standard therapy (platin doublets), with perhaps a better toxicity profile although grade 3 neutropenia is a concern.

V. Malignant mesothelioma

Among the various cancers in which pemetrexed was evaluated, malignant mesothelioma might represent the most promising for its future use in current practice, in combination chemotherapy rather than as single agent. Pemetrexed monotherapy was investigated in malignant pleural mesothelioma (Scagliotti et al, 2003), and showed an overall response rate of 14% and a median progression-free survival of 4.7 months in 64 patients among whom 50% had stage IV disease. The toxicity profile was

consistent with other reports. These data suggested that pemetrexed was a promising drug for combination chemotherapy trials, especially with platinum in malignant mesothelioma.

VI. Miscellaneous solid tumors

Pemetrexed has been evaluated in breast cancer patients who had failed previous anthracycline/taxane-based therapy (Miles et al, 2001, Martin et al, 2003). These trials concluded that in this setting of pretreated patients, objective response rates ranged from 20 to 30% with a median duration of response of 5.5 to 8 months. Toxicity was acceptable, mainly consisting of myelotoxicity and skin toxicity, the latter being well prevented with dexamethasone administration.

In metastatic colorectal cancer, single-agent pemetrexed led to response rates of around 15% (Cripps et al, 1999, John et al, 2000), and with disappointing median response durations, of 3.3 and 4.4 months, respectively. Similarly, in gastric cancer, a response rate of 23% with a short median duration of response (4.4 months) was reported (Celio et al, 2002). In this study, toxicity was a major concern, with toxic deaths related to myelotoxicity, requiring folic acid/vitamin B12 prophylaxis in subsequent patients (see *infra*).

In advanced pancreatic cancer, Miller et al, (2000) found a response rate of 6%, consistent with the results of single-agent gemcitabine which is currently the standard treatment for this condition, whereas the toxicity profile was acceptable.

Table 2: Grade 3 or 4 hematologic and non hematologic toxicity adapted from (Paz-Ares et al, 2003)

Toxicity	NCI-CTC toxicity grade (% of patients)	
	3	4
ANC	23	27
Hemoglobin	14	3
Platelets	8	7
Nausea	7	<1
Emesis	3	2
Stomatitis	3	<1
Diarrhea	3	2
Alk phos	3	0
ALT	3	0
AST	8	<1
Bilirubin	6	2
Creatinine	<1	0
Cutaneous	5	2
Fatigue	6	<1
Infection	3	2
Pulmonary	2	2

Table 3: Results of pemetrexed single-agent therapy in NSCLC

<i>Trial</i>	<i>Nb of evaluable patients</i>	<i>Dose (mg/m²)</i>	<i>Overall response rate (%)</i>	<i>Median survival (months)</i>	<i>Toxicity (Gr 3& 4)</i>
Rusthoven et al, 1999	30	600 (3 pts) 500 (30 pts)	23.3	9.2	Neutropenia Skin toxicity (rash)
Clarke et al, 2002	42	600	15.8	9.8	Neutropenia Skin toxicity (rash)
Smit et al, 2003	81	600	8.9		Neutropenia Thrombopenia

VI. Pemetrexed-based combinations: is it the future ?

Several phase I trials have investigated the maximal tolerated dose of pemetrexed in combination with platinum compounds. They showed (Todtmann et al, 1999, Hughes et al, 2002, Misset et al, 2002), that pemetrexed 500mg/m² every 3 weeks was the recommended dose in combination with cisplatin, carboplatin or oxaliplatin. Similarly the same schedule of pemetrexed was shown to be well tolerated in combination with gemcitabine 1250mg/m² at day 1 and 8 (Adjei et al, 2000). Conversely, combination with protracted intravenous 5-Fluorouracil induced severe toxicity, although a bolus 5-Fluorouracil regimen showed better tolerance with pemetrexed (Schwartz et al, 1999). Other preliminary reports of pemetrexed combination with taxanes (MacKay et al, 2002) or vinorelbine (Millward et al, 2001) need further confirmation before phase II trials can be started.

In NSCLC, two phase II trials of the pemetrexed-cisplatin combination have been reported. These investigated pemetrexed at 500mg/m² and cisplatin at 75mg/m² every 3 weeks (Manegold et al, 2000, Shepherd et al, 2001). These trials showed similar response rates (39 and 45%) and median duration of responses of 10.9 and 8.9 months respectively. The toxicity profile was acceptable, as moderate myelotoxicity was the main side-effect. Yet in NSCLC, low response rates (16%) for the combination of pemetrexed with gemcitabine (Ettinger et al, 2002) contrasted with better median survival (11.9 months); further trials are required to define the role of pemetrexed-based combinations in this condition.

Other phase II pemetrexed-based combinations have focused on colorectal cancer, where the drug was combined with oxaliplatin, with disappointing preliminary results (Atkins et al, 2003) as compared to usual response rates of 40-50% in patients receiving 5-FU-oxaliplatin or irinotecan. This is in contrast with promising results in advanced pancreatic cancer, as others (Kindler et al, 2002) reported a 15% objective response rate with a 29% 1-year overall survival.

In the light of promising results of single agent pemetrexed in malignant mesothelioma, a randomized (but not double-blinded) phase III trial was recently reported, comparing cisplatin to the combination of pemetrexed and cisplatin (Vogelzang et al, 2003). In this study, the combination of pemetrexed 500mg/m² and cisplatin 75 mg/m² every three weeks demonstrated a significant superiority as compared to single-agent cisplatin. The

response rate was significantly higher in the combination arm (41.3 vs 16.7%, p<0.0001) that also showed a benefit in overall survival (12.1 vs 9.3 months, p=0.02). However, further trials investigating the superiority of the pemetrexed-cisplatin doublet versus other cisplatin-based combinations are required before pemetrexed is registered.

VII. Prevention of pemetrexed toxicity

The spectrum of antifolate-induced adverse events includes myelotoxicity as well as gastro-intestinal side-effects. Although usually moderate, this toxicity may be unacceptable and prompted the clinical development of new antifolates, such as lometrexol. It has been shown that folic acid supplementation (Laohavini et al, 1996) reduced lometrexol toxicity. As shown before, myelotoxicity, especially neutropenia, is the dose limiting toxicity for pemetrexed. Cutaneous side effects are well controlled with steroid prophylaxis (Adjei et al, 2000). Obviously, the rationale for folic acid supplementation in patients receiving pemetrexed is based on the requirement of folic acid for DHF and THF synthesis. Similarly, methionine synthase and its cofactor, vitamin B12, requires N₅-methyl THF as a methyl group donor for the conversion of homocystein to methionine. Data obtained in mice (Worzalla et al, 1998), as well as in humans (Bunn et al, 2001), have shown that vitamin B12 and folic acid supplementation reduced pemetrexed-induced toxicity. The recommended schedule of vitamin supplementation is oral folic acid (350 to 1000 µg/d), starting one week before pemetrexed therapy, combined with intramuscular vitamin B12 (1000 µg) every 9 weeks (Bunn et al, 2001). Other schedules of supplementation are under investigation (Hammond et al, 2003), but further studies are required to determine whether folic acid supplementation might counterbalance pemetrexed activity on DHFR, and eventually decrease its clinical efficacy. In the above mentioned phase III randomized trial in malignant mesothelioma (Vogelzang et al, 2003), the efficacy parameters did not differ between supplemented and non supplemented patients. However, for all patients, overall survival curves strongly diverged (p= 0.020) whereas in supplemented patients, the difference only approached statistical significance (p=0.051). Surprisingly, patients who received cisplatin with supplementation did better than those with cisplatin alone. The authors suggested that supplementation enabled patients to receive more chemotherapy cycles, which could explain this phenomenon in addition, oral vitamin B12 supplementation might be considered as well to avoid

intramuscular injections in the setting of patients that might experience chemotherapy-induced thrombopenia.

VII. Antifolates: a phoenix in anticancer drugs research

The goal of interfering with the metabolism of nucleotides and folates against cancer was reached decades ago. The old drugs 5-Fluorouracil and methotrexate, are widely used in current oncology practice. Targeting pyrimidine metabolism was achieved through thymidylate synthase inhibition by 5-Fluorodeoxyuridine 5' monophosphate and leucovorin, and more recently with specific inhibitors such as raltitrexed. Although these agents are still part of innovative combination chemotherapy schedules, the concept of inhibiting folate metabolism has been revisited with the discovery of the multiple target antifolate pemetrexed. The search for methotrexate analogues that, supposedly, specifically targeted GARFT led to the development of pemetrexed, as its properties of inhibiting multiple enzymes within the same metabolic pathway were relevant for anticancer therapy. Inhibition of thymidylate synthase might represent the main mechanism of action of pemetrexed, although it is likely to differ from both 5-Fluorouracil and raltitrexed from the molecular point of view. In clinical practice, DHFR inhibition has some effect, at least in terms of toxicity, whereas the *in vivo*, impact of GARFT, AICARFT and C₁ tetrahydrofolate synthase inhibition remains unclear. Therefore, pemetrexed differs from both 5-FU and methotrexate by its mechanisms of action, as well as its clinical spectrum of efficacy. So far, there is no data indicating that pemetrexed will replace 5-FU in the treatment of digestive malignancies or breast cancer, as new drugs such as oral fluoropyrimidines have proven efficacy and are widely used in clinical practice (Kurtz et al. 2003). Advances in the knowledge of pyrimidine metabolism, e.g the enzymes of the *de novo* and salvage pathway of pyrimidines, have led to the "re-birth" of antifolate chemotherapy. Among these new antimetabolites, capecitabine takes advantage of the overexpression of thymidine phosphorylase in tumor tissues, and UFT and S-1 combine 5-FU prodrugs to catabolic inhibitors. These new anticancer agents have now become part of current therapy in a variety of malignancies. Comparison of pemetrexed to methotrexate in terms of efficacy is more difficult, as this drug is mostly administered in combination chemotherapy schedules, except the case of high-dose methotrexate with folinic acid rescue. Clinical research with pemetrexed has led to contrasting results. Its activity on colorectal cancer is disappointing, as it is not better than 5-Fluorouracil or oral fluoropyrimidines, with an increased toxicity. In pancreatic cancer, gastric and breast cancer, large phase II or, optimally, phase III studies, will probably define the future role of pemetrexed. In contrast, there is some evidence that patients with malignant pleural mesothelioma can benefit from the combination of pemetrexed with cisplatin, in terms of response, and more importantly, survival. These data need however to be

confirmed by phase III trials, comparing the pemetrexed-cisplatin combination with a different platin-based doublet, in order to definitely recommend this combination as the gold standard therapy in this condition.

Finally, data on antifolate agents such as pemetrexed emphasize the need for developing drugs which interact with the folate metabolism pathways, and should encourage both new drug development and clinical study of these anticancer agents.

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