Abstract: A method of combating toxicity caused by an antifolate compound of Formula I in an individual who has been administered the compound. The method comprises administering an enzyme that has carboxypeptidase G activity to the individual. A method of cleaving a compound comprising a structural fragment of Formula IV, the method comprising contacting the compound comprising the structural fragment of Formula IV with an enzyme that has carboxypeptidase G activity.
For two-letter codes and other abbreviations, refer to the “Guidance Notes on Codes and Abbreviations” appearing at the beginning of each regular issue of the PCT Gazette.
The present invention relates to the use of an enzyme having carboxypeptidase G activity, and in particular to its use in combating toxicity caused by Pemetrexed and related antifolate compounds.

Natural folates are used by cells in the folate pathway to synthesise DNA, RNA and in protein synthesis, and are therefore essential dietary requirements (Jolivet et al., 1983; Pinedo et al., 1976; Goldman 1975).

The three enzymes in the folate pathway most studied as targets for antifolate drugs are dihydrofolate reductase (DHFR), thymidylate synthase (TS) and glycaminamide ribonucleotide formyltransferase (GARFT). DHFR and TS, along with serine hydroxymethyltransferase (SHMT), comprise the three enzymes of the thymidylate cycle. SHMT catalyzes the conversion of serine to glycine with the formation of methylenetetrahydrofolic acid (MTHF). MTHF, under the influence of TS, donates its methylene group to deoxyuridylic acid to form thymidylate, an essential component of DNA. Importantly, in the TS reaction, tetrahydrofolate (THF) supplies reducing equivalents for the conversion of the methylene group of MTHF to the methyl group of thymidylate (dTMP). Thus, for every molecule of dTMP formed, a molecule of THF is converted to dihydrofolate (DHF). DHF must be converted back to THF so that TS cycle should continue producing dTMP. This reaction is catalyzed by DHFR which utilizes NADPH as the reductant. DHFR also catalyzes the conversion of folic acid to DHF.

GARFT catalyzes the third in the series of ten reactions required for de novo purine biosynthesis, the conversion of glycaminamide ribonucleotide to formylglycinamide ribonucleotide utilizing 10-formylTHF as the formyl donor. GARFT occurs in mammals as a trifunctional protein which catalyzes the second and the fifth steps on this pathway in addition to the third. GARFT activity resides in the carboxy-terminal portion of this tri-functional protein. De novo purine biosynthesis leads to the formation of inosine monophosphate, the precursor of...
the formation of inosine monophosphate, the precursor of ATP and GTP necessary for RNA formation and of dATP and dGTP necessary for DNA formation.

Inhibition of DHFR leads to a deficiency of dTMP because DHF cannot be recycled for use in the TS reaction. This in turn leads to deficient DNA synthesis, DNA breakdown and cell death. Direct inhibition of TS likewise leads to a deficiency of dTMP and cell death. Direct inhibition of GARFT leads to depletion of purine nucleotides, which also leads to cell death, but the degree of cell kill is generally less than that produced by an equally growth-inhibitory concentration of a TS inhibitor (Kisliuk et al, 2003).

Methotrexate (MTX), a synthetic folate analogue, has been in clinical use since 1948 (Bleyer 1978) and is an important component of various chemotherapeutic regimens used for the treatment of patients with neoplastic diseases. The cytotoxic effects of both MTX and its active metabolites is through the inhibition of DHFR leading to inhibition of DNA synthesis, repair and cellular replication. Actively proliferating tissue, such as malignant cells, are in general more sensitive to this cellular interference by MTX. In addition, MTX has immunomodulating effects and is used in the treatment of a number of other diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS) and psoriasis. Application of high doses of MTX, usually administered as a prolonged infusion, is nowadays frequently used in patients with Non-Hodgkin's Lymphoma (NHL), acute lymphoblastic leukemia (ALL) or soft tissue tumours such as osteosarcoma.

Although actively proliferating malignant tissues are most sensitive to MTX, MTX can still be toxic to healthy cells in a dose and time dependent manner through two Principle mechanisms. The first is common to all antifolates. This mechanism is -through the inhibition of DNA synthesis and cellular metabolism, which is the jnderlying mechanism that is responsible for MTX’s cytotoxic anti-cancer action. The risk of significant toxicity to healthy cells correlates with increasing doses of VUX and the time of exposure. MTX therapy is associated with a spectrum of toxicities, with myelosuppression, mucositis, acute hepatitis and nephrotoxicity
being the most frequent and serious complications (Bleyer 1978). Additional toxicities seen with high dose therapy are acute desquamative dermatitis, B-lymphocyte dysfunction, and neurological effects. Similar common toxicities are also caused by other antifolate drugs, although they have generally been administered at lower doses than MTX.

The second mechanism is MTX induced renal tubular obstruction and consequent renal dysfunction (MTX nephrotoxicity). MTX is metabolised by liver aldehyde oxidase to 7-hydroxy-MTX. The aqueous solubility of 7-hydroxy-MTX is 3 to 5 times lower than that of the parent compound and, under certain conditions, is known to precipitate in the renal tubules which is thought to be a principal mechanism in the pathogenesis of the MTX nephrotoxicity (Kintzel 2001, Condit 1969). Normal kidney function will accommodate removal of a particular load in a given time, thereafter accumulation and damage will ensue. If a patient receiving MTX develops nephrotoxicity leading to impaired elimination of MTX, a self-perpetuating cycle is initiated of reduced elimination, sustained high plasma MTX levels and subsequent exacerbation of both non-renal toxicity and progression of renal tubular damage, eventually leading to the death of the patient (although mortality can occur even in the absence of total renal failure).

Renal toxicity has been recorded with other antifolate compounds, but this may not be due to an analogous 7-hydroxylation of the compound. 7-OH-MTX toxicity occurs at high MTX doses, while the typical administered doses of the further antifolates described herein would only be considered to be 'intermediate' doses for MTX.

As a result of fatal outcomes from MTX toxicity, protection measures are routinely included in MTX therapeutic regimes:

1. Leucovorin rescue: Leucovorin calcium is the calcium salt of 5-formyl tetrahydrofolic acid (also known as folinic acid/folinate), the DHFR metabolite of folic acid and an essential coenzyme for nucleic acid synthesis and is not inhibited
by MTX (Immunex Corporation 2001). As a result, leucovorin calcium is able to rescue MTX inhibited cells. However, at high MTX concentrations, leucovorin calcium may fail to prevent systemic toxicities (Goldman, 1975; Pinedo et al., 1976). Reversal of MTX by leucovorin calcium is competitive, with relatively higher concentrations required as the MTX concentration increases. When concentrations of MTX reach 100 µM, even ten-fold higher leucovorin calcium concentrations (1,000 µM) are unable to protect bone marrow cells from toxicity (Pinedo et al., 1976).

2. Hydration and alkalinisation is required to enhance the solubility of MTX and thus prevent MTX nephrotoxicity, which can lead to renal impairment.

With these measures, the incidence of Hfe-threatening MTX toxicity may be lowered to around 1.5%. However, despite these precautions, prolonged MTX-clearance due to drug-related renal insufficiency may develop and lead to severe and life-threatening systemic toxicities, such as myelosuppression, mucositis, hepatitis and dermatitis. In the past, several attempts have been made to ameliorate systemic MTX toxicity in such patients. First, haemo- or peritoneal dialysis may enhance MTX clearance but usually result in only small and transient decreases of toxic serum MTX levels. Second, administration of thymidine or an intensified leucovorin rescue may lower systemic MTX toxicity but does not enhance MTX excretion.

MTX remains the most widely used antifolate anticancer agent in clinical use to this date. Because of the relative safety and utility of MTX, considerable effort has been invested in attempting to design more therapeutically selective antifolates or antifolates with a wider tumor spectrum. Initially, the design was based on the burgeoning knowledge of folate-dependent pathways and the determinants of the mechanism of action of MTX. These determinants include transport, the tight-binding inhibition of its target, DHFR, and metabolism of MTX to poly-γ-glutamate (Glu₅) metabolites. These early studies led to the development of other antifolate inhibitors of two types: (1) "classical" analogs that use the same cellular
transport systems as MTX and are also metabolized to Glu₉; and (2) "nonclassical" (ie, lipophilic) analogs that do not require transport systems and that are not metabolized to Gb₂⁺. Although several of these analogs have undergone clinical trials, none has yet proved superior to MTX (McGuire, 2003).

Detailed examination of the mechanisms of cytotoxicity and selectivity of MTX showed that inhibition of both dTMP synthesis and de novo purine synthesis, secondary to DHFR inhibition, led to DNA synthesis inhibition and subsequent cell death; inhibition of other folate-dependent pathways did not appear necessary for cell death. Further studies showed that the contribution of inhibition of dTMP or purine synthesis to cell death varied in different cell types. These data suggested that inhibition of one of these pathways individually might (at least in some cases) be therapeutically superior to the dual inhibition induced by MTX. Thus in rational design and in structure-based design studies, two new classes of antifolate enzyme inhibitors were elaborated: direct inhibitors of TS and direct inhibitors of one or both of the two folate-dependent enzymes of de novo purine synthesis. Members of each class included both classical and nonclassical types. After preclinical evaluation, several of these have moved into clinical trials. To date only two new antifolate compounds have been approved for routine use; Tomudex (raltitrexed, AstraZeneca) is currently approved in Europe for the treatment of colon cancer and Pemetrexed (Alimta®, Eli Lilly) has been approved in the US for malignant pleural mesothelioma and non-small cell lung cancer (NSCLC). This represents a major step forward for antifolates since, for example, MTX is ineffective against colon cancer.

Antifolate development continues. Based on the immense body of knowledge now extant on antifolates, specific aspects of the mechanism of action have been the focus. Newer antifolates have been described that inhibit more than one pathway in folate metabolism, that have improved delivery, or that inhibit other targets in folate metabolism. These new analogs are in various stages of preclinical and clinical development (McGuire, 2003; Kisliuk, 2003; Purcell & Ettinger, 2003; each of which is incorporated by reference in its entirety).
Edatrexate is a classic polyglutamated antifolate that directly inhibits DHFR with greater potency than does MTX. Preclinically, edatrexate was thought to have an improved therapeutic index compared with MTX. However, in comparison with MTX in several phase II and III trials in multiple solid tumours, edatrexate has shown no improvement over MTX, with similar or slightly worse toxicity (primarily mucositis). Promising results from a phase I trial of edatrexate in combination with vinblastine, Adriamycin, and cisplatin (EVAC), along with filgastrim (granulocyte-colony stimulating factor [G-CSF]) in non-small-cell lung cancer (NSCLC) led to a phase II trial of this regimen. Sixteen of 34 evaluable patients (47.1%) responded to therapy. However, significant myelosuppressive toxicity was reported. Fifty-six percent of the patients had grade 3 or higher leukopenia, and three treatment-related deaths were observed. Decreased quality-of-life measurements led investigators to question the feasibility of this regimen in their patient population (reviewed by Purcell & Ettinger, 2003).

Lometrexol, a folate analogue that specifically inhibits GARFT, has been in clinical development for at least 15 years. More than any other antifolate, this agent requires polyglutamation for its effect on GARFT. It is transported into the cell via both the RFC and FR systems. Lometrexol has no effect on DHFR, TS, or AICARFT, and thus its effectiveness is purely related to decreasing purine synthesis. Early phase I trials of lometrexol were confounded by cumulative delayed myelosuppression that prevented repetitive administration. As with Pemetrexed, further preclinical studies suggested that coadministration of folic acid might favourably modulate lometrexol toxicity without eliminating potential antitumor activity. Previous phase I trials of lometrexol combined with leucovorin rescue have been reported. However, the current recommended phase II dosage combinations is lometrexol, 10.4 mg/hr/wk by IV, with folic acid, 3 mg/πr/d orally. Toxicity from this combination is considered to be manageable, with infrequent grade 3 thrombocytopenia and mucositis. Lometrexol is currently being evaluated in phase I combination studies with each of the following agents: temozolomide, doxorubicin, carboplatin, gemcitabine, and paclitaxel as well as in
single-agent phase II studies in soft-tissue sarcoma, melanoma, breast cancer, NSCLC, and head and neck cancer (reviewed by Purcell & Ettinger, 2003).

Perhaps the most exciting new antifolate is Pemetrexed, a potent polyglutamatable classic antifolate TS inhibitor formerly called LY23 154 and now manufactured as Alimta® (Eli Lilly). Pemetrexed has been reviewed by Calvert (2004) and Norman (2001). Phase I/II studies on Pemetrexed have been reviewed by Hanauske et al (2004), and selected phase II and III clinical trials of Pemetrexed are outlined in Table 3 of Purcell & Ettinger (2003). Clinical trials involving Pemetrexed have been summarised by the US Food and Drug Administration (Hazarika et al (2004) and Cohen et al (2005)). The entire disclosure of each of these references in relation to Pemetrexed is incorporated herein by reference.

Pemetrexed is polyglutamated inside the cell and shows high affinity for folylpolyglutamate synthetase (FPGS). The polyglutamate derivatives are also potent inhibitors of DHFR and GARFT and show less potent inhibition of aminomimidazole carboxamide ribonucleotide formyltransferase (AICARFT). Therefore, Pemetrexed has been referred to as a "multitargeted" antifolate. Preclinical studies have shown that Pemetrexed inhibition of GARFT and AICARFT, in addition to DHFR and TS, is important in that cells need both thymidine and hypoxanthine to overcome the antitumour effect. This is true despite the fact that Pemetrexed TS inhibition is 30 to 200 times greater than GARFT or AICARFT inhibition and seven times greater than DHFR inhibition. Toxicities of Pemetrexed in several early trials included significant myelosuppression, mucositis, and diarrhoea. Subsequently, plasma levels of homocysteine and methylmalonic acid were studied as sensitive surrogate markers for folic acid and vitamin Bi₂ status, respectively. Low levels of homocysteine and methylmalonic acid were found to be strongly correlated with the development of serious drug-related toxicities, suggesting that toxicity was related to related to relative folic acid or vitamin Bi₂ deficiency in some cancer patients. Clinical trial patients who subsequently received folic acid and Bi₂ supplementation had far fewer toxicities. Current studies give oral folic acid, 5 mg from day -2 to day +2 of
every cycle of Pemetrexed. Cobalamin, 1000 µg by intramuscular injection, is given every three cycles (reviewed by Purcell & Ettinger, 2003).

Although Pemetrexed is active in many solid tumors, it has been studied most extensively in mesothelioma and NSCLC. Pemetrexed is also under investigation for breast, gastrointestinal, head and neck urothelial, and cervical cancer.

AAGl 13-161 is a recently developed analogue of Pemetrexed and is a dual inhibitor of DHFR and TS. It was considered that replacing the 4-oxo moiety of Pemetrexed with a methyl group to form AAGl 13-161 would enhance DHFR binding by enabling a hydrophobic interaction of the 4-methyl group with Phe31 and Leu22 of human DHFR. X-Ray crystallographic studies of AAGl 13-161 bound to P. carinii DHFR showed it to be bound in the proposed 2,4-diamino mode and that the 4-methyl group of AAGl 13-161 does occupy the hydrophobic environment predicted. In fact, for L. casei DHFR, AAGl 13-161 is 10,000 times more inhibitory than Pemetrexed, and for human DHFR it is 8 time more inhibitory. With regard to TS, AAGl 13-161 is 55 times more inhibitory than Pemetrexed for E. coli TS and 10 times more inhibitory than Pemetrexed for human TS. AAGl 13-161 has IC50 values for CCRF-CEM human leukaemia and FaDu head and neck squamous cell carcinoma cell lines of 12.5 nM and 7.0 nM, respectively. At IC90 concentrations, inhibition of both cell lines was reversed by 40 µM thymidine, consistent with the proposal that AAGl 13-161 is a dual inhibitor of DHFR and TS. In contrast with Pemetrexed, hypoxanthine alone at 50 µM caused no reversal of inhibition with AAGl 13-161. AAGl 13-161 is an excellent substrate for human FPGS. Its K_m value is below the limit of detection of the assay (reviewed by Kisliuk, 2003).

As with MTX, one major drawback to the clinical use of the newer antifolate drugs is an unacceptable level of toxicity. The ability to degrade these antifolate drugs rapidly in vivo would have two major clinical advantages. Firstly, it would minimize toxicity caused by the antifolate drugs. It also allows a higher dose of the antifolate compounds to be administered, potentially leading to a greater clinical
effect. The lower toxicity and higher efficacy may be sufficient to realise the
clinical promise of a number of drugs that have not progressed through clinical
trials. Furthermore, as toxicity associated with antifolate drugs is often duration-
related rather than dose-related, the ability to rapidly remove excess free drug at a
given time point may be therapeutically very useful.

Carboxypeptidase G₂ (CPG₂) is an enzyme from Pseudomonas sp. strain RS-16
(now reclassified as Variovorax paradoxus) and is a zinc-dependent dimeric protein
of 83,000 - 84,000 Dalton (Kalghati & Bertino, 1981; Chabner et al, 1972;
McCullough et al. 1971; Minton et al, 1983; and Sherwood et al, 1985). It has a
relatively restricted specificity and hydrolyses the C-terminal glutamic acid residue
of folic acid, poly-glutamyl derivatives of folic acid, folate analogues, eg
methotrexate, and sub-fragments of folic acid eg, p-aminobenzoyl glutamate
(Minton et al, 1983). To date carboxypeptidase enzymes have only been
characterised in a small number of Pseudomonas sp. and can be separated on the
basis of their substrate affinities for folate and its analogues (Kalghatgi and Bertino,

The International Nonproprietary Name (INN) of recombinant carboxypeptidase
G₂ is glucarpidase, and it is commercially available as Voraxaze™ (Protherics).

Sherwood et al (1985) have previously reported that CPG₂ follows Michaelis-
Menten kinetics with $K_m$ values of 4μM for folate, 8μM for MTX, 34μM for 5-
methyl THF, and 120μM for 5-formyl THF (leucovorin). Glucarpidase cleaves
methotrexate (MTX) into its inactive metabolites, 4-deoxy-4-amino-N 10-
methylpterolic acid (DAMPA) and glutamate, and thus may provide an alternative
route of MTX elimination particularly in patients who develop renal dysfunction
due to MTX nephrotoxicity (Adamson et al, 1991; Mohty et al, 2000; von
Poblozki et al, 2000; Widemann et al, 2000).

Para-aminobenzoyl glutamate is a substrate of glucarpidase, as are a number of
mustard prodrugs based on p-aminobenzoyl glutamate (Springer et al (1995);
Dowell et al (1996)). However, there have been no published reports that have attempted to assess whether any of the new antifolate drugs are substrates for cleavage by glucarpidase. Indeed, as far as we are aware, there are no published reports whether any folate compounds other than folic acid, MTX, 5-methyl THF, and 5-formyl THF were substrates for glucarpidase. It was not known, and could not predicted, whether any of the new generation of antifolate drugs are substrates for glucarpidase cleavage.

We have now shown that Pemetrexed (Alimta®) is a substrate for glucarpidase and has a $K_m$ of 25.4 $\mu$M and a $k_{cat}$ of 1808 s$^{-1}$ as measured by spectrophotometric assay. All of the known substrates for glucarpidase have N (e.g. an amino or substituted amino group) in the para position to the benzene ring marked by $R^4$ in Formula I, see below. Pemetrexed, by contrast, has a carbon at this position which is not an isosteric or functional replacement for the nitrogen. Cleavage of such antifolates by glucarpidase is totally unexpected since para-aminobenzoyl glutamate is a substrate for glucarpidase but benzoyl glutamate is not. Thus Pemetrexed, along with other similar antifolates such as AAGl 13-161, Edatrexate and Lometrexol would not have been predicted to be substrates of glucarpidase.

Antifolate compounds are useful in treating a range of medical conditions, particularly cancers, and being able to combat toxicity associated with these compounds will significantly increase their therapeutic value.

A first aspect of the invention provides a method of combating toxicity caused by an antifolate compound of Formula I$_5$

![Chemical Structure](image)

wherein

$R^1$ represents $NH_2$, OH or $CH_3$;
R² represents NHₐ or C₁₋₄ alkyl;

the group B represents a structural fragment of Formula Ha, lib, Hc, Hd or He,

![Chemical Structures](image)

in which groups the dashed lines indicate the point of ring fusion with the pyrimidinyl ring and the wavy lines indicate the point of attachment of the bicyclic heterocycle to the rest of the molecule;

R⁷a to R⁷c independently represent H or C₁₋₄ alkyl;

A¹ represents C(R⁸a) or N;

A² represents CH or N;

A³ represents C(H)R⁸b, NR⁸c or S;

A⁴ and A⁵ independently represent CH₂, NH, O or S;

the group B¹-B² represents CH-CH or C=C;

R⁸a to R⁸c independently represent H or C₁₋₄ alkyl, or R⁸c represents C(O)R⁸d;

R⁸d represents H or C₁₋₄ alkyl;

R³ represents H, C₁₋₆ alkyl, C₃₋₆ alkenyl or C₃₋₆ alkynyl;

R⁴ represents H or one or two substituents selected from halo, C₁₋₄ alkyl and C₁₋₄ alkoxy, or R⁴, together with R⁵, when R⁴ is attached at a position that is ortho to the position to which the moiety C(O)NR⁵ is attached, represents C₁₋₂ 7₇-alkylene;

R⁵ represents C(O)NR⁵;
R^5 represents H or C\textsubscript{1-4} alkyl, or R^5, together with R^4, when R^4 is attached at a position that is ortho- to the position to which the moiety C(O)NR^5 is attached, represents

Ci\textsubscript{2} w-alkylene;

R^6 represents -CH\textsubscript{2}C(R\textsuperscript{9a})(R\textsuperscript{9b})-D;

R\textsuperscript{9a} and R\textsuperscript{9b} independently represent H or C\textsubscript{1-4} alkyl, or R\textsuperscript{9a} and R\textsuperscript{9b} together represent ^C(H)R\textsuperscript{10};

R\textsuperscript{10} represents H or C\textsubscript{1-4} alkyl;

D represents C(O)OH, tetrazol-5-yl, (CH\textsubscript{2})\textsubscript{o-i}-NHR\textsuperscript{n}, or, when R\textsuperscript{9a} and R\textsuperscript{9b} together represent =C(H)R\textsuperscript{10}, then D may also represent H, or D represents a structural fragment of Formula H\textsubscript{1a} or H\textsubscript{1b},

\[
\begin{align*}
\text{H} &\text{a} \\
\text{H} &\text{b}
\end{align*}
\]

wherein the wavy lines indicate the point of attachment of the structural fragments;

R\textsuperscript{11} represents H or C(O)R\textsuperscript{12};

R\textsuperscript{12} represents H or phenyl substituted by C(O)OH and optionally substituted by one or two further substituents selected from halo, C\textsubscript{i-4} alkyl and C\textsubscript{i-4} alkoxy; and

alkyl, alkenyl and alkynyl groups, as well as the alkyl part of alkoxy groups, may be substituted by one or more halo atoms;

or a pharmaceutically acceptable salt and/or solvate thereof,

in an individual who has been administered said compound, the method comprising administering to the individual an enzyme that has carboxypeptidase G activity.
The term "halo", when used herein, includes fluoro, chloro, bromo and iodo.

Pharmaceutically acceptable salts of compounds of Formula I (as well as of compounds comprising a structural fragment of Formula IV described below) that may be mentioned include both acid addition salts and metal (e.g. alkali metal, such as sodium or potassium) salts. Solvates that may be mentioned include hydrates.

Compounds of Formula I may exhibit tautomerism. In particular, compounds of Formula I in which R\(^1\) represents OH may alternatively be depicted as follows.

All tautomeric forms and mixtures thereof are included within the scope of the invention.

Compounds of Formula I that may be mentioned include the following.

R\(^1\) represents NH\(_2\) or, particularly, CH\(_3\) or OH;
R\(^2\) represents methyl or, particularly, NH\(_2\);
the group B represents a structural fragment of Formula Ha, Hc or lid;
A\(^1\) and A\(^2\) both represent CH or, particularly, both represent N;
R\(^{7a}\), R\(^{7c}\) and R\(^{7d}\) independently represent H or, when A\(^1\) and A\(^2\) both represent CH, R\(^{7a}\) may also represent methyl;
A\(^3\) represents S or, particularly, CH\(_2\);
A\(^4\) represents NH;
A\(^5\) represents O or, particularly, NH;
the group B\(^1\)-B\(^2\) represents C=C;
R\(^3\) represents 3-prop-l-ynyl or, particularly, H, methyl or ethyl;
'R\(^4\) represents methyl or, particularly, H;
R\(^5\) represents H;
R\(^{9a}\) and R\(^{9b}\) both represent H or R\(^{9a}\) and R\(^{9b}\) together represent =CH\(_2\); R\(^{11}\) represents phenyl ortho-substituted by C(O)OH.

Further compounds of Formula I that may be mentioned include those in which:
R' represents NH\(_2\);
the group B represents a structural fragment of Formula Hd;
A\(^d\) represents NH;
R\(^{7d}\) represents H;
R\(^3\) represents H;
D represents C(O)OH;
R\(^{9a}\) and R\(^{9b}\) both represent H.

In preferred embodiments, the compound of Formula I is one of those listed at (1) to (5) below.

(1)

(a) \(R^3 = H\) (10-deazaaminopterin);
(b) \(R^3 = CH_3\) (10-methyl-10-deazaaminopterin);
(c) \(R^3 = CH_2CH_3\) (10-ethyl-1O-deazaaminopterin, Edatrexate); and
(d) \(R^3 = CH_2C\equiv CH\) (10-propargyl-1O-deazaaminopterin).

(2)
4'-Methylene-5,8,1O-trideazaaminopterin (Mobiltrex).

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5,10-dideaza-5,6,7,8-tetrahydrofolic acid (Lometrexol).
In particularly preferred embodiments, the compound of Formula I is Edatrexate, AAGl 13-161 or, most preferably, Pemetrexed.

It is appreciated that Pemetrexed may exhibit tautomerism, in particular with respect to the 0/OH groups at position $R^1$, and Pemetrexed it is often depicted in its alternative tautomeric form, as shown in Formula Ia.

By "an enzyme that has carboxypeptidase G activity" we include the meaning of an enzyme that hydrolyses the C-terminal L-glutamic acid residue from folic acid, folate analogues, and sub-fragments of folic acid eg, p-aminobenzoyl glutamate.

Preferably, the enzyme that has carboxypeptidase G activity is glucarpidase (recombinant carboxypeptidase $G_2$ (CPG$_2$)), EC number 3.4.22.12.
The sequence of the gene encoding glucarpidase and the glucarpidase amino acid sequence can be found in GenBank Accession Nos. M12599 and AAA62842 and in Minton et al. [Gene 31(1-3), 31-38 (1984)], Minton and Clarke (J Mol. Appl. Genet. 3(1), 26-35 (1985)); and Chambers et al. (Appl. Microbiol. Biotechnol. 29, 572-578 (1998)) and the amino acid sequence is listed in Figure 1.

In an embodiment the enzyme that has carboxypeptidase G activity may be a derivative of glucarpidase that has carboxypeptidase G activity. By a "derivative" of glucarpidase we include a fragment, variant, modification or fusion of glucarpidase, or combinations thereof, which has carboxypeptidase G activity.

The derivatives may be made using protein chemistry techniques for example using partial proteolysis (either exolytically or endolytically), or by de novo synthesis. Alternatively, the derivatives may be made by recombinant DNA technology. Suitable techniques for cloning, manipulation, modification and expression of nucleic acids, and purification of expressed proteins, are well known in the art and are described for example in Sambrook et al (2001) "Molecular Cloning, a Laboratory Manual", 3rd edition, Sambrook et al (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

By "fragment" of glucarpidase we mean any portion of the full length enzyme that has carboxypeptidase G activity. Typically, the fragment has at least 30% of the carboxypeptidase G activity of glucarpidase. It is more preferred if the fragment has at least 50%, preferably at least 70% and more preferably at least 90% of the activity of CPG₂. Most preferably, the fragment has 100% or more of the carboxypeptidase G activity of glucarpidase.

The carboxypeptidase G activity of a derivative of glucarpidase can readily be determined by a person of skill in the art using the enzyme assay described on page

A "variant" of glucarpidase refers to glucarpidase that has been altered by an amino acid insertion, deletion and/or substitution, either conservative or non-conservative, at one or more positions. B) "conservative substitutions" is intended combinations such as Gly, Ala; Val, lie, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such modifications may be made using the methods of protein engineering and site-directed mutagenesis, as described in Sambrook et al 2001, supra.

For example, it may be advantageous to modify one or more residues of one or both of the active site of the enzyme. Such variants may beneficially alter the specificity or activity of the enzyme. The crystal structure of glucarpidase was published by Rowsell et al (1997) and identifies the active sites of the enzyme. In other embodiments, it may be advantageous not to modify residues in the active sites. Sequence variants, typically outside the active sites, may protect the enzyme from in vivo metabolism or decrease antigenicity. Additionally, it may be advantageous to add one or more Cys residues to allow disulphide bonds to be formed.

Preferably, the variant of glucarpidase has at least 70% sequence identity with SEQ ID No: 1. It is more preferred if variant glucarpidase has at least 80%, preferably at least 85% and more preferably at least 90% sequence identity with SEQ ID No: 1. Most preferably, the variant glucarpidase has 91 or 92 or 93 or 94 or 95 or 96 or 97 or 98 or 99% or more sequence identity with SEQ ID No: 1.

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequence has been aligned optimally.
The alignment may alternatively be carried out using the Clustal W program (Thompson et al, (1994) Nucleic Acids Res 22, 4673-80). The parameters used may be as follows:

Fast pairwise alignment parameters: K-tuple(word) size; I$_5$ window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

Scoring matrix: BLOSUM.

Preferably, the variant of glucarpidase, or a fragment of the variant, retains at least 30% of the carboxypeptidase G activity of glucarpidase. It is more preferred if variant glucarpidase has at least 50%, preferably at least 70% and more preferably at least 90% of the activity of glucarpidase. Most preferably, the variant of glucarpidase has 100% or more of the carboxypeptidase G activity of glucarpidase.

Variants of glucarpidase with carboxypeptidase G activity have been described in US patent application no. 2004/0014187.

In an embodiment, the variant of glucarpidase has a substitution at one or more of the Asn residues at positions 222, 264 and 272 which are N-glycosylation sites. Preferably, Asn 222 is substituted with Gln; Asn 264 is substituted with Thr or Ser, most preferably Ser; and Asn 272 is substituted with Gln, independently or in combination. The most preferred combination of substitutions has Gln at positions 222 and 272 and Ser at residue 264. This QSQ motif results in a high catalytic activity and a low $K_m$ for MTX (US 2004/0014187).

A "modification" of glucarpidase refers to glucarpidase in which one or more of the amino acid residues has been chemically modified. Such modifications include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, attaching amino acid protecting groups such as N-t-butoxycarbonyl and glycosylation. Such modifications may protect the enzyme from in vivo metabolism or decrease antigenicity. The
glucarpidase may be present as single copies or as multiples, for example tandem repeats.

The invention also includes a fusion of glucarpidase, or a fragment or variant thereof which has carboxypeptidase G activity, to another compound. Preferably, the fusion retains at least 30% of the activity of glucarpidase. It is more preferred if the fusion has at least 50%, preferably at least 70% and more preferably at least 90% of the activity of glucarpidase. Most preferably, the fusion has 100% or more of the carboxypeptidase G activity of glucarpidase.

The invention may be used to alleviate symptoms of toxicity caused by the antifolate compound of Formula I in an individual (ie palliative use), or may be used to reduce the severity of the toxicity in an individual, or may be used to treat toxicity in an individual, or may be used prophylactically to prevent toxicity in an individual. Thus, by "combating toxicity" we include the meaning of treating, reducing or preventing toxicity caused by the antifolate compound or alleviating the symptoms of it.

The enzyme that has carboxypeptidase G activity typically acts to combat toxicity caused by the antifolate compound of Formula I by rapidly lowering plasma levels of the drug, thereby reducing the duration of exposure of normal tissues to the drug and preventing longer-term uptake.

Whether or not a particular patient is one who is expected to benefit from treatment may be determined by the physician.

By preventing toxicity we include the meaning of treating a patient at risk of toxicity, for example due to high levels and/or delayed elimination of the antifolate compound of Formula I. Any patient who has been administered the antifolate compound may be considered to be at risk of toxicity caused by it.

In an embodiment, the individual at risk of toxicity may be one who has been
administered the antifolate compound and who has not been tested for the presence of a clinical marker of toxicity caused by the antifolate compound.

In another embodiment, the individual at risk of toxicity may be one who has been administered the antifolate compound and has one or more clinical markers or indications of toxicity caused by it.

For example, it is known that Pemetrexed is not metabolised in the body to an appreciable extent and it is primarily excreted in the urine (EH Lilly, 2004). In patients with normal renal function, 70-90% of the Pemetrexed is recovered unchanged in the urine in the 24 hours following administration, with an elimination half life of 3.5 hours. However, the level of Pemetrexed clearance decreases, and hence drug exposure increases, in patients with reduced renal function. Creatinine clearance can be used as a marker for renal function, and a creatinine clearance of <45 mL/min, can be considered to be a clinical marker or indication of Pemetrexed toxicity. Indeed one patient with severe renal impairment (creatinine clearance of <19 mL/min) died of drug related toxicity from receiving Aümta® alone (Eli Lilly, 2004). It is thereby appreciated that the ability to degrade Pemetrexed by glucarpidase may allow Pemetrexed to be administered to patients in need thereof who have reduced renal function.

Thus in an embodiment, the method may comprise the prior step of determining whether the individual who has been administered the antifolate compound of Formula I has a clinical marker of toxicity caused by the antifolate compound.

In an embodiment, the clinical marker of toxicity caused by the antifolate compound of Formula I may be a level of the compound, such as a plasma level, greater than a predetermined level at a given time after administration of the compound. The predetermined plasma level of the antifolate compound indicating toxicity may be 0.1 or 0.2 or 0.3 or 0.4 or 0.5 or 0.6 or 0.7 or 0.8 or 0.9 µmole per litre, or 1 or 2 or 3 or 4 or 5 µmole or more per litre at 24 hours after
administration of the antifolate compound, or at 48, or 72 or 96 or 120 hours, or more, after administration of the antifolate compound.

Thus in another embodiment, the method may comprise the prior step of determining the level of the antifolate compound in the individual at a given time after administration of the compound to the individual, such as at 24 or 48, or 72 or 96 or 120 hours, or more, after administration of the antifolate compound.

The invention includes administering an enzyme that has carboxypeptidase G activity to an individual who has been administered an antifolate compound of Formula I as defined above, whether or not the individual has any symptoms of toxicity caused by the compound.

In an embodiment, it may be preferred to administer the enzyme to every individual who has been administered an antifolate compound of Formula I, for example, at a given time after administration of the compound of Formula I.

Thus the invention can be considered to be an *in vivo* method of cleaving an antifolate compound of Formula I as defined above.

In another embodiment, the individual at risk of toxicity may be one who has been administered the antifolate compound and has one or more clinical symptoms of toxicity caused by it.

Thus in an embodiment, the method may comprise the prior step of determining whether the individual who has been administered the antifolate compound has a clinical symptom of toxicity caused by the antifolate compound.

Symptoms of toxicity for various of the antifolate compounds of Formula I as defined above are well known. For example, toxicities for Pemetrexed include, mucositis, myelosuppression, thrombocytopenia, neutropenia, neutropenic sepsis, septicaemia, fatigue, neurotoxicity, anaemia, parasthesia, dyspnea, nausea and
diarrhoea, although somewhat reduced by supplementation with folic acid and vitamin Bn, skin rash, fatigue and stomatitis (Martin et al, 2003; Hanauske et al, 2004); and toxicities for Edatrexate include mucositis, myelosuppression and leukopaenia (Purcell & Ettinger, 2003); and toxicities for Lometrexol include thrombocytopenia and mucositis (Purcell & Ettinger, 2003).

The individual is typically administered the enzyme that has carboxypeptidase G activity between about 24 and 48 hours after being administered the antifolate compound. Alternatively, the individual may be administered the enzyme between about 12 and 24 hours or between about 48 and 72 hours, or between about 72 and 96 hours, or between about 96 and 120 hours, or more, after being administered the antifolate compound.

The individual may be administered the enzyme that has carboxypeptidase G activity about 6 hours, or about 12 hours, or about 18 hours, or about 24 hours, or about 30 hours, or about 36 hours, or about 42 hours, or about 48 hours, or about 54 hours, or about 60 hours, or about 72 hours, or about 84 hours, or about 96 hours, or about 108 hours, or about 120 hours, or more, after being administered the antifolate compound.

It is appreciated that if the antifolate compound has been administered to the individual in error, the enzyme that has carboxypeptidase G activity is preferably administered as soon as possible once the error is noticed in order to combat toxicity caused by it. Similarly, if the individual has a clinical marker of toxicity caused by the antifolate compound, or a clinical symptom of toxicity caused by the antifolate compound, it may also be preferable to administer the enzyme as soon as possible.

A dose of 600 mg/m² of Pemetrexed administered every 3 weeks led to high but manageable levels of toxicity (Martin et al, 2003), and of the clinical studies reviewed by Norman (2001), the highest dose administered was up 600 to 900 mg/m². The maximum tolerated dose of Edatrexate was found to be 3750 mg/m²,
however, because of the occurrence of leukoencephalopathy in one patient, high
dose Edatrexate treatment was not recommended (Pisters et al, 1996). In each of
these cases, the subsequent administration of an enzyme that has carboxypeptidase
G activity followed by degradation of the antifolate compound, can increase the
maximum tolerated dose of the antifolate compound thus increasing the efficacy of
the drug and minimising any side effects.

The invention thus includes administering to an individual in need thereof, as
described herein, a high dose of an antifolate compound of Formula I, such as 2 or
3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or more times the above doses, and
subsequently administering an enzyme that has carboxypeptidase G activity to the
individual.

The invention thus includes administering a dose of Pemetrexed equivalent to about
1000 mg/m² or 1.5 or 2 or 2.5 or 3 or 3.5 or 4 or 5 or 6 or 7 or 8 or 9 or 10 g/m²
or more on a 3-weekly schedule, and subsequently administering an enzyme that
has carboxypeptidase G activity to the individual.

The invention thus includes administering a dose of Edatrexate equivalent to about
5 or 6 or 7 or 8 or 9 or 10 or 15 or 20 or 25 g/m² or more, and subsequently
administering an enzyme that has carboxypeptidase G activity to the individual.

The enzyme that has carboxypeptidase G activity or a formulation thereof may be
administered by any conventional method including parenteral (eg subcutaneous or
intramuscular) injection. The treatment may consist of a single dose or a plurality of
doses over a period of time.

Most preferably, the enzyme that has carboxypeptidase G activity or a formulation
thereof is administered intravenously.

In some circumstances the enzyme that has carboxypeptidase G activity or a
formulation thereof may be administered intrathecally, typically when the antifolate
compound of formula I has been administered intrathecally.'

Studies in rhesus monkeys indicate that the half life of glucarpidase in plasma following intravenous administration is between 52 and 58 minutes. Following intrathecal administration to rhesus monkeys, glucarpidase's half life in cerebrospinal fluid has been estimated at between 3.3 and 3.9 hours.

Whilst it is possible for the enzyme that has carboxypeptidase G activity to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the compound of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

In a preferred embodiment, the enzyme that has carboxypeptidase G activity is stored as a freeze-dried powder ready to be made up as a solution for injection as required. Typically, the contents of a vial of freeze-dried enzyme are reconstituted with sterile normal saline (0.9% w/v), immediately before use.

In a preferred embodiment, the formulation of the enzyme that has carboxypeptidase G activity also contains lactose as an inactive ingredient, except for patients with hypersensitivity to lactose.

Typically, the enzyme that has carboxypeptidase G activity is administered to the individual at a dose of about 50 Units per kg body weight (1 unit corresponds to the enzyme activity that cleaves 1 micromole of MTX per minute at 37°C) intravenously over 5 minutes.

It is appreciated that the enzyme can be administered at lower doses of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40 or 45 Units per kg/body weight. It is also appreciated that
the enzyme can be administered at higher doses of about 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150 or 200 or higher Units per kg/body weight.

The frequency, timing and dosage of administration of the enzyme that has carboxypeptidase G activity may be determined by the physician, using knowledge of the properties of the enzyme, the levels of the antifolate enzyme in the patient, and the degree of any symptoms of toxicity in the patient.

It is appreciated that proteins and peptides may be delivered using an injectable sustained-release drug delivery system. These are designed specifically to reduce the frequency of injections. An example of such a system is Nutropin Depot which encapsulates recombinant human growth hormone (rhGH) in biodegradable microspheres that, once injected, release rhGH slowly over a sustained period.

An alternative method of protein and peptide delivery is the ReGeI injectable system that is thermo-sensitive. Below body temperature, ReGeI is an injectable liquid while at body temperature it immediately forms a gel reservoir that slowly erodes and dissolves into known, safe, biodegradable polymers. The active drug is delivered over time as the biopolymers dissolve.

It is further appreciated that the individual may be administered a polynucleotide that encodes the enzyme that has carboxypeptidase G activity, leading to in vivo expression of the enzyme. Suitable vectors and methods are well known to a person of skill in the art. For example, Schepelmann et al (2005) describe systemic gene-directed enzyme prodrug therapy using a targeted adenovirus to express carboxypeptidase G2.

The individual to be treated may be any individual who would benefit from such treatment. Typically and preferably the individual to be treated is a human. However, the methods of the invention may be used to treat mammals, such as the cows, horses, pigs, sheep, cats and dogs. Thus, the methods have uses in both human and veterinary medicine.
In an embodiment, the method of combating toxicity caused by an antifolate compound of Formula I as defined above further comprises administering a folate pathway rescue agent to the individual.

By "a folate pathway rescue agent" we include the meaning of an agent that can rescue the folate pathway which is blocked by the antifolate compound. The most commonly used folate pathway rescue agent is leucovorin, the calcium salt of 5-formyl tetrahydrofolic acid. Alternative rescue agents may include other salts of 5-formyl tetrahydrofolic acid, thymidine and folic acid. Typically, if the antifolate compound is an inhibitor of DHFR or of GARFT the folate pathway rescue agent is leucovorin, while if the antifolate compound is an inhibitor of TS, the folate pathway rescue agent is thymidine.

For example, Edatrexate is known to be an inhibitor of DHFR, Lometrexol is known to be a GARFT inhibitor, and Pemetrexed is a multitargeted antifolate that is known to inhibit DHFR, GARFT and TS (Table 2, Purcell & Ettinger, 2003). Thus, the appropriate folate pathway rescue agent can readily be determined.

It is appreciated that it is now routine practice to administer folic acid and vitamin B\textsubscript{i2} to a patient in advance of Pemetrexed.

In an embodiment, the individual is administered the enzyme that has carboxypeptidase G activity prior to the folate pathway rescue agent. Alternatively, the individual may be administered the folate pathway rescue agent prior to the enzyme that has carboxypeptidase G activity. In yet another embodiment, the individual may be administered the folate pathway rescue agent and the enzyme that has carboxypeptidase G activity substantially simultaneously.

The antifolate compounds may be useful in treating a range of cancers, and being able to combat toxicity associated with these compounds increases their therapeutic value.
The invention includes a method of treating cancer comprising administering an antifolate compound of Formula I as defined above to the individual, and subsequently administering to the individual an enzyme that has carboxypeptidase G activity.

The subsequent administration of the enzyme that has carboxypeptidase G activity is to combat toxicity caused by the antifolate compound as described above.

Thus the invention includes a method of treating cancer comprising administering an antifolate compound of Formula I as defined above to the individual, and combating toxicity caused by the antifolate compound by administering an enzyme that has carboxypeptidase G activity.

Cancers which can be treated by the antifolate compounds of Formula I, are well known to a person of skill in the art, some of which are discussed by McGuire (2003); Kisliuk (2003); and Purcell & Ettinger (2003), as well as in the specific references mentioned herein.

The invention includes a method of treating cancer, the method comprising administering Pemetrexed to the individual, and combating toxicity caused by Pemetrexed by administering an enzyme that has carboxypeptidase G activity. The cancer to be treated by administration of Pemetrexed may be leukaemia, mesothelioma, NSCLC, lung, breast, colon, pancreas, kidney, bladder, gastrointestinal cancer, head and neck cancer, urothelial cancer or cervical carcinoma (Martin et al (2003); Thodtmarm et al (2003); Ettinger (2002); Calvert (2004)).

The invention includes a method of treating cancer, the method comprising administering AAGl 13-161 to the individual, and combating toxicity caused by AAGl 13-161 by administering an enzyme that has carboxypeptidase G activity. The cancer to be treated by administration of AAGl 13-161 may be leukaemia,
mesothelioma, NSCLC, breast, colon, pancreas, kidney, bladder, gastrointestinal cancer, head and neck cancer, urothelial cancer or cervical carcinoma.

The invention includes a method of treating cancer, the method comprising administering Edatrexate to the individual, and combating toxicity caused by Edatrexate by administering an enzyme that has carboxypeptidase G activity. The cancer to be treated by administration of Edatrexate may be breast, lung, head and neck squamous cell carcinoma, NSCLC, non-Hodgkin's lymphoma, germ cell tumour, pleural mesothelioma or malignant fibrous histiocytoma (Kuriakose et al (2002); Dreicer et al (1997); Pisters et al (1996); and Meyers et al (1998-9)).

The invention includes a method of treating cancer, the method comprising administering Lometrexol to the individual, and combating toxicity caused by Lometrexol by administering an enzyme that has carboxypeptidase G activity. The cancer to be treated by administration of Lometrexol may be soft tissue sarcoma, NSCLC, breast, head and neck cancer or melanoma.

The method of treating cancer may also comprise administering an additional anticancer agent to the individual. Suitable cancer chemotherapeutic agents may include: alkylating agents including nitrogen mustards such as mechlorethamine (HN₂), cyclophosphamide, ifosfamide, melphalan (L-sarcolysin) and chlorambucil; eti/ylenimines and methyknelamines such as hexamethylmelamine, thiotepa; allyĩ sulphonates such as busulphan; nitrosoureas such as carmustine (BCNU), lomustine (CCNU), semustine (methyl-CCNU) and streptozocin (streptozotocin); and triazines such as decarbazine (DTIC; dimethyltriazenoimidazole-carboxamide); Antimetabolites including pyrimidine analogues such as fluorouracil (5-fluorouracil; 5-FU), fioxuridine (fluorodeoxyuridine; FUdR) and cytarabine (cytosine arabinoside); and purine analogues and related inhibitors such as mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG) and pentostatin (2-deoxycoformycin). Natural Products including vinca alkaloids such as vinblastine (VLB) and vincristine; epipodophyllotoxins such as etoposide and teniposide; antibiotics such as dactinomycin (actinomycin D), daunorubicin (daunomycin; rubidomycin), doxorubicin, bleomycin,
plicamycin (mithramycin) and mitomycin (mitomycin C); enzymes such as L-asparaginase; and biological response modifiers such as interferon alphenomes. Miscellaneous agents including platinum coordination complexes such as cisplatin (cis-DDP) and carboplatin; anthracenedione such as mitoxantrone and anthracycline; substituted urea such as hydroxyurea; methyl hydrazine derivative such as procarbazine (N-methylhydrazine, MTH); and adrenocortical suppressant such as mitotane ip,p'-DDD); and aminoglutethimide; taxol and analogues/derivatives; and hormone agonists/antagonists such as flutamide and tamoxifen. However, preferred anticancer agents include cisplatin, carboplatin, oxaliplatin, vinorelbine, doxorubicin, epirubicin, cyclophosphamide, paclitaxel, irinotecan, gemcitabine and docetaxel.

A second aspect of the invention provides the use of an enzyme that has carboxypeptidase G activity in the preparation of a medicament for combating toxicity caused by an antifolate compound of Formula I as defined above in the first aspect of the invention.

Preferences regarding the antifolate compound of Formula I in this and subsequent aspects of the invention are as described above with respect to the first aspect of the invention. Compounds of Formula I that can be mentioned include Pemetrexed, AAAl 13-161, Edatrexate and Lometrexol. Pemetrexed is most preferred.

The invention includes use of an enzyme that has carboxypeptidase G activity for combating toxicity in an individual who has one of more clinical signs, symptoms or markers of toxicity caused by said compound, as described above.

In an embodiment, the invention includes use of the enzyme that has carboxypeptidase G activity in the preparation of a medicament for combating toxicity caused by an antifolate compound of Formula I as defined above in an individual who is administered a folate pathway rescue agent. The individual may be administered the folate pathway rescue agent prior to the medicament, or the individual may be administered the folate pathway rescue agent after the
medicament, or the individual may be administered the folate pathway rescue agent and the medicament substantially simultaneously.

Preferences regarding the folate pathway rescue agent in this and subsequent aspects of the invention are as described above with respect to the first aspect of the invention.

A third aspect of the invention provides the use of a folate pathway rescue agent in the preparation of a medicament for combating toxicity caused by an antifolate compound of Formula I as defined above in an individual who is administered an enzyme that has carboxypeptidase G activity. The individual may be administered the enzyme prior to the medicament, or the individual may be administered the enzyme after the medicament, or the individual may be administered the enzyme and the medicament substantially simultaneously.

A fourth aspect of the invention provides the use of an enzyme that has carboxypeptidase G activity and a folate pathway rescue agent in the preparation of a medicament for combating toxicity caused by an antifolate compound of Formula I as defined above.

The invention includes the use as defined above in the second, third and fourth aspects of the invention for combating toxicity caused by an antifolate compound of Formula I in an individual who is being treated for a cancer by administration of the antifolate compound, as detailed above.

A fifth aspect of the invention provides the use of an antifolate compound of Formula I as defined above in the preparation of a medicament for combating cancer in an individual who is subsequently administered an enzyme that has carboxypeptidase G activity.
The type of cancer that can be combated by any specific antifolate compound of Formula I is known to the person skilled in the art and can be determined by the physician.

In an embodiment, the individual is also administered a folate pathway rescue agent. The enzyme that has carboxypeptidase G activity may be administered before, after, or substantially simultaneously with the folate pathway rescue agent.

In an embodiment, the invention includes the use of Pemetrexed in the preparation of a medicament for treating cancer in an individual who is administered an enzyme that has carboxypeptidase G activity. The invention also includes the use of an enzyme that has carboxypeptidase G activity in the preparation of a medicament for combating toxicity caused by Pemetrexed in an individual who has been administered the Pemetrexed to treat cancer. Cancers that can be treated by administration of Pemetrexed are known in the art and include those listed above.

Similarly, cancers that can be treated by administration of a medicament containing AAGl 13-161, Edatrexate, Lometrexol and other antifolate compounds of Formula I are known in the art, and include those listed above.

The invention thus includes the use of an enzyme that has carboxypeptidase G activity in the preparation of a medicament for complementing the therapy of a cancer that is being treated by administration of an antifolate compound of Formula L.

A sixth aspect of the invention provides an in vitro method of cleaving a terminal L-glutamate moiety from a compound of Formula I as defined above, the method comprising contacting the compound with an enzyme that has carboxypeptidase G activity.
A seventh aspect of the invention provides a method of determining the rate and/or extent of cleavage of a compound of Formula I as defined above by an enzyme that has carboxypeptidase G activity, the method comprising:

- providing a compound of Formula I,
- contacting the compound of Formula I with an enzyme that has carboxypeptidase G activity under conditions such that cleavage of the compound can occur, and
- monitoring the rate and/or extent of cleavage of the compound of Formula I over time.

In an embodiment, the providing step comprises providing a known amount or concentration of the compound of Formula I.

In an embodiment, the monitoring step comprises monitoring the amount and/or concentration of the compound of Formula I over time. Additionally or alternatively, the monitoring step comprises monitoring the amount and/or concentration of one or more break-down products of the compound of Formula I over time.

It is appreciated that the method of determining the rate and/or extent of cleavage can be performed in vitro, or can be performed in vivo.

The method can be used either in vivo or in vitro to monitor the level of the compound of Formula I that remains uncleaved after treatment with the enzyme that has carboxypeptidase G activity. Thus the method can be used to monitor the effectiveness of the enzyme that has carboxypeptidase G activity in combating toxicity associated with the compound of Formula I.

The method may further comprise determining whether an additional dose of the enzyme that has carboxypeptidase G activity is required in order to reduce the amount of the compound of Formula I to a predetermined level, typically a level
which does not cause toxicity. The amount of enzyme to be administered in the additional dose may also be determined.

The method may therefore also comprise contacting the compound of Formula I with an additional dose of the enzyme that has carboxypeptidase G activity under conditions such that cleavage of the compound of Formula I can occur.

An eighth aspect of the invention provides a therapeutic system (or it may be termed a "kit of parts") consisting of or comprising an antifolate compound of Formula I as defined above and an enzyme that has carboxypeptidase G activity. Optionally, the therapeutic system may also contain a folate pathway rescue agent.

Preferably, the therapeutic system contains a preferred compound of Formula I as defined above in the first aspect of the invention, most preferably Pemetrexed. Still preferably, the therapeutic system contains glucarpidase, or a derivative thereof that has carboxypeptidase G activity, as defined above in the first aspect of the invention. Preferred folate pathway rescue agents are also as defined in the first aspect of the invention. The therapeutic system or kit of parts may suitably contain both the compound of Formula I and the enzyme that has carboxypeptidase G activity, and optionally the folate pathway rescue agent, packaged and presented in a suitable formulation either for storage or for use. Thus, for example, the glucarpidase may be a freeze-dried powder ready to be reconstituted as a solution for injection, or may already be reconstituted as a solution for injection. Similarly, Pemetrexed may be a freeze-dried powder ready to be reconstituted as a solution for injection, or may already be reconstituted as a solution for injection. Typically, the compound of Formula I and the enzyme are for separate administration in a particular treatment regime, thus they are packaged or formulated separately. The enzyme and the folate pathway rescue agent may be administered together, and thus may be formulated for co-administration.

A preferred therapeutic system or kit as defined above comprises Pemetrexed and Glucarpidase, and optionally but preferably, folic acid and/or vitamin B12.
A ninth aspect of the invention provides a method of cleaving a compound comprising a structural fragment of Formula IV,

\[ \text{IV} \]

wherein

the wavy line indicates the point of attachment of the structural fragment; and

R\(^3\) to R\(^6\) are as hereinbefore denned;

or a pharmaceutically acceptable salt and/or solvate thereof,

the method comprising contacting the compound comprising the structural fragment of Formula IV with an enzyme that has carboxypeptidase G activity.

Preferences for the groups R\(^3\) to R\(^6\) are the same with respect to the structural fragment of Formula IV as they are with respect to the compounds of Formula I.

In this respect, particular structural fragments of Formula IV that may be mentioned include those in which R\(^4\) and R\(^5\) represents H and R\(^6\) represents \(\text{CH}_2\text{CH}_2\text{C(O)OH}\) or \(\text{CH}_2\text{C(=CH}_2\text{)C(O)OH}\).

Preferences for the enzyme that has carboxypeptidase G activity are the same as for the first aspect of the invention as described above.

In an embodiment, the method may be performed \textit{in vitro}. In an alternative embodiment, the method may be performed \textit{in vivo}.
Typically, the compound comprising the structural fragment of Formula IV is an antifolate compound.

In an embodiment when the antifolate compound comprising a structural fragment of Formula IV is administered to an individual in the course of medical treatment, or otherwise, the invention provides a method of combating toxicity caused by the antifolate compound comprising the structural fragment of Formula IV, the method comprising administering to the individual an enzyme that has carboxypeptidase G activity.

Thus the invention includes the use of an enzyme that has carboxypeptidase G activity in the preparation of a medicament for combating toxicity caused by an antifolate compound comprising a structural fragment of Formula IV.

In the context of medical uses of this ninth aspect of the invention when the compound comprising the structural fragment of Formula IV is an antifolate compound, preferences for the use of folate pathway rescue agents, pharmaceutical formulations, timings and levels of administration, patients and diseases to be treated, and so on are the same as for the first aspect of the invention as described above.

All of the documents referred to herein are incorporated herein, in their entirety, by reference.

The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

The invention will now be described in more detail by reference to the following figures and Examples.

Figure 1 is the amino acid sequence of glucarpidase (SEQ ID No: 1).
Figure 2 shows the chemical structure of five substrates of CPG₂. Pemetrexed, AAGl 13-161, Edatrexate, Lometrexol and Methotrexate (prior art).

Figure 3 is a graph showing spectral differences in Pemetrexed absorbance pre- and post- glucarpidase addition.

Figure 4 is a graph of a Michaelis-Menten plot for MTX (prior art substrate).

Figure 5 is a graph of a Michaelis-Menten plot of Pemetrexed (experiment 1).

Figure 6 is a graph of a Michaelis-Menten plot of Pemetrexed (experiment 2).

**Example 1: Determination of Kinetic Property of Cleavage of Pemetrexed (Alimta®) by Glucarpidase (Voraxaze™)**

**Summary**

The cleavage of Pemetrexed by glucarpidase (Voraxaze™) was evaluated *in vitro* by measurement of change in absorbance spectrum at 250nm. It was found that Pemetrexed was cleaved by glucarpidase with reaction kinetics similar to those of the cleavage of methotrexate by the same enzyme.

This indicates that glucarpidase may have clinical utility as intervention therapy for Pemetrexed toxicity and have a long term role in planned use with Pemetrexed in several malignant conditions.

**Aim of Study**

Determination of the kinetic properties of Pemetrexed and measurement of the affinity ($K_m$) and catalytic rate constant ($k_{cat}$) of Pemetrexed supplied by Eli Lilly using glucarpidase (Voraxaze™).
Glucarpidase Preparation

A 1000 Unit vial of lyophilised glucarpidase (Protherics, batch CAMR 004/1991) was resuspended in ImI of 50mM Tris-HCl pH 7.4 containing 0.2 mM Zn\(^{2+}\), then diluted 1/20 followed by 1/50 to give a working solution 1/1000 of the original concentration, i.e. 1 U/ml. 10 µl of the working solution, 0.01 Units, was used per assay.

Assay Method

The following previously validated kinetic assay of catalyst parameters for Glucarpidase, using methotrexate (MTX) as substrate was used to determine the kinetic properties of Pemetrexed cleavage by Glucarpidase.

Assay overview

This procedure describes the carboxypeptidase G2 activity assay by spectrophotometry at 320 nm, using MTX as substrate (Sherwood et al, 1985).

The carboxypeptidase activity is determined by measuring the MTX hydrolysis speed at 37°C. The reactive mixture (1000 µl) contains Tris/HCl (pH 7.4), MTX and ZnAc\(_2\).2H\(_2\)O. The reaction is initiated by adding the solution containing the enzyme to be assayed and the absorbance at 320 nm is monitored on the Hitachi U-2010 spectrophotometer. The hydrolysis speed is calculated according to the slope of the linear section of the curve A\(_{320}\) = f(time).

In this assay, a unit of carboxypeptidase activity (U) is defined as the quantity of enzyme needed to catalyse the hydrolysis of a micromole of MTX in 1 minute, in 1 ml of the reactive mixture at 37°C.

The carboxypeptidase activity (CA) which is expressed in U.min\(^{-1}\) or in µmoles.min\(^{-1}\).ml\(^{-1}\) is calculated as follows:

\[
CA = \frac{\frac{M \times DiI \times 1000 \times V_{\text{react}}}{A \Sigma_M x M_{\text{mex}} \times V_{\text{sum}}}}{M_{\text{mtx}}}
\]

\textit{Equation 1}

in which,
CA = carboxypeptidase activity;
ΔA = the absorbance variation measured in the period of time selected for measuring the activity;
DiI = the dilution of the sample to be assayed;
Δtime = the duration of the period selected for measuring the activity (min);
V<sub>react</sub> = the volume of the reactive mixture;
V<sub>sam</sub> = the volume of the sample used for measuring; and
ΔΣ<sub>m</sub><sup>320</sup> = the variation of the molar extinction coefficient (in M<sup>-1</sup>.cm<sup>-1</sup>).

The variation of the molar extinction coefficient (ΔΣ<sub>m</sub><sup>320</sup>) of the methotrexate is — 8200 M<sup>-1</sup>.cm<sup>-1</sup>, which means that, for a lcm optical path, the total hydrolysis of a 1M methotrexate solution is accompanied by a decrease in 8200 of the absorbance at 320 am.

\[ V_{react} = 1000 \mu l \]
\[ V_{sam} = 50 \mu l \]
\[ \frac{ΔA}{Δtime} = \text{slope of the line obtained} \]

If these values are replaced in equation 1, the following is obtained:

\[ CA = \frac{-\text{Slope} \times \text{DiI} \times 20}{8.2} = -\text{Slope} \times m \times 2.439 = - \text{Slope} \times \text{DiI} \times K \]

Equation 2

With \( K = 2.439 \)

The specific activity \( SA \) (which is expressed in U.mg<sup>-1</sup>) is calculated as follows:

\[ SA = \frac{CA}{PC} \]

Equation 3

\( SA = \) the specific activity;
\( CA = \) carboxypeptidase activity (in U.ml<sup>-1</sup>);
\( PC = \) protein concentration (mg/ml) determined by UV Spectrophotometry.
**Assay Procedure**

The samples are diluted in the dilution buffer at 40°C just before the assay. The activity (U/ml) of a sample at a given dilution is obtained by the average of two replicates. These two tests are carried out using two independent dilutions. The second dilution of the sample is also prepared just before the assay.

The freeze-dried product activity test is carried out on 10 pools of 2 freeze-dried vials of CPG2 each one resuspended in 1 ml of water to obtain a carboxypeptidase G2 activity of close to 1000 U/ml. Further dilutions in the dilution buffer (+ lactose) of 200Ox, 250Ox and 300Ox can be used for the measurements.

A negative control (without enzyme) is tested. The A$_{320}^2$ is measured and is between 0.80 and 1.00 and does not vary by more than 0.01 units in 1 minute.

The carboxypeptidase activity is measured by adding 50 µl of the diluted sample to be assayed to the sample cell, mixing, and measuring the A$_{320}^2$ once every second for 40 seconds.

- **Slope $\times$ 2.439** ($S =$ slope) is calculated for the first 40 linear seconds of the graph A$_{320}^2$ = f(time), and is between 0.15 and 0.575 U/ml. If the value obtained is not within this range, the assay is repeated with a more appropriate dilution of the sample.

The carboxypeptidase activity is defined as:

$$CA = - \text{Slope} \times \text{DiI} \times K$$

With $K = 2.439$:

The specific activity $SA$ (expressed in U.mg$^{-1}$):
Measurement of Pemetrexed cleavage

In this Example, the rate of the turnover for a fixed, known amount of Glucarpidase was measured over a range of nine different substrate concentrations: 60µM, 40µM, 20µM, 16µM, 12µM, 10µM, 8µM, 6µM, 5µM and 4µM. The rate of cleavage at each different substrate concentration was measured in triplicate. The substrate concentrations were chosen to cover a range that provided data at both saturation and substrate limiting levels in order to allow accurate curve fitting. Rate measurements were made by evaluation of the change in absorbance by Pemetrexed at 250nm using a thermostatted Hitachi U2010 Spectrophotometer s/n 121-01222 with UV solutions v1.2 software.

An example of the changes observed are shown in Figure 3. Subtraction of the pre-spectrum from the post-spectrum shows a maximum change at 250nm. For a 100µM solution, the maximum ΔA250 is 0.487. Therefore the change in molar extinction coefficient for a 1M solution would be 4870.

Pemetrexed was not soluble in aqueous buffer and was therefore dissolved in DMSO at 5.97mg/ml to give a stock solution of 10mM, 1-10µl of which was used to prepare cuvettes containing 10-60µM Pemetrexed; for preparation of cells containing <10µM, a further 1/10 dilution in DMSO was carried out to give a 1mM stock solution. In all cases, the volume of DMSO in the cuvette was kept constant at 1µl by addition of red DMSO as required. Previous studies have demonstrated that the presence of 1% v/v DMSO is not inhibitory to glucarpidase.

The affinity (K_m) and sample rate constant (V_max) of each data set was calculated direct linear using "Enzfitter" software (Biosoft, Cambridge, UK) with the Michaelis-Menten template. The specific catalytic rate constant, kcat, was calculated by division of the calculated V_max value by the known molar enzyme concentration of the Glucarpidase sample measured.
Results
Figure 4 shows the action of methotrexate with glucarpidase over a concentrated range and fitted to demonstrate Michaelis Menten kinetics. Using the Enzfitter programme a Km (affinity constant) for methotrexate can be calculated, and by dividing the calculated Vmax by the known enzyme concentration (starting concentration of glucarpidase was 2.15mg/vial) the kcat was calculated.

Figures 5 and 6 show equivalent data for two studies on Pemetrexed. The results from each experiment are shown below, and are summarised in Table I.

Enzyme Kinetics for Methotrexate

Fitted Parameters
Vmax (limiting maximal rate): 0.067 ± 0.004 (standard error value)
KIs (dissociation constant for ES complex): 10.26 ± 1.81 (standard error value)

kcat Calculation
\[ \Delta E_{max} = 8300 \text{ at } 320\text{nm for MTX}. \] Vmax is therefore \[ \frac{0.067}{8300} = 8.07 \times 10^{-8} \text{ mol/min} \] for the amount of enzyme used. Enzyme used was 1µl of 1.08µg/ml in 1ml reaction mix, =1.08 \times 10^{-6}g = 1.295 \times 10^{-10}M.

Therefore kcat is \[ \frac{1 \times 8.07 \times 10^{-6}}{1.295 \times 10^{-10}} = 62316/\text{min} = 1038.6/\text{sec} \]

Enzyme Kinetics for Pemetrexed (Experiment I)

Fitted Parameters
Vmax: 0.099/min ± 0.008
Ks: 14.44µM ± 2.925

kcat Calculation
Conversion of 1ml containing 1µl of 10mM Pemetrexed gives rise to an OD change of 0.487AU. Therefore the change in molar extinction coefficient is 4870
Vmax is therefore $2.05 \times 10^6 \text{L/min}$ for the amount of enzyme used. Enzyme used was 10µl of 2.15 µg/mL = $2.15 \times 10^{-5} \text{g} = 2.29 \times 10^{10} \text{M}$.

Therefore $kc$ is 

\[
1 \times 2.05 \times 10^6 = \frac{79150}{\text{min}} = 1319/\text{sec} \\
2.59 \times 10^{10}
\]

**Enzyme Kinetics for Pemetrexed (Experiment II)**

**Fitted Parameters**

- **Vmax**: 0.174/min ± 0.028
- **Ks**: 36.286 µM ± 9.522

**kcat Calculation**

Conversion of 1ml containing 10µl of 10mM Pemetrexed gives rise to an OD change of 0.487AU. Therefore the change in molar extinction coefficient is 4870 Vmax is therefore $0.174/4870 = 3.57 \times 10^{-5} \text{L/min}$ for the amount of enzyme used. Enzyme used was 10µl of 2.15µg/ml in 1ml reaction mix, = $2.15 \times 10^{-5} \text{g} = 2.59 \times 10^{10} \text{M}$.

Therefore $k_{cat}$ is 

\[
1 \times 3.57 \times 10^5 = \frac{137840}{\text{min}} = 2297/\text{sec} \\
2.59 \times 10^{10}
\]

**Table 1: Summary of Kinetic Results**

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<th>kcat</th>
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<tr>
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<tr>
<td>Experiment Series II</td>
<td>36.3µM</td>
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<tr>
<td>Mean</td>
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<tr>
<td>Methotrexate Control</td>
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<td>1039s⁻¹</td>
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**Discussion**

The mean of the values obtained for Km and $kcat$ are 25.4µM and 1808s⁻¹ respectively. Both demonstrate that Pemetrexed is a very good substrate for
glucarpidase and suggest that glucarpidase (Voraxaze™) could be used clinically for the control of Pemetrexed levels in the circulation.

Under the same conditions and using the same batch of enzyme, values of 10.3 µM for Km and 1038 s⁻¹ for kcat were determined for methotrexate. Whilst the Km determined for Pemetrexed was higher than that for MTX it is comparable with that of 5-methyl tetrahydrofolate, which has been shown to be depleted by glucarpidase, even in the presence of very high levels of MTX, and the high kcat measured does suggest that glucarpidase should be of clinical value for controlling serum levels of Pemetrexed.

**Example 2: Identification of new substrates for glucarpidase**

**A: AAGU3-161**

AAGl 13-161 is a dual inhibitor of DHFR and TS. AAGl 13-161 is contacted with glucarpidase in vitro and is found to be a substrate of glucarpidase.

Deglutamylation of AAGl 13-161 by glucarpidase is measured spectrophotometrically. A stock solution of 10mM AAGl 13-161 in 100mM Tris-HCl pH 7.3 is prepared and used to prepare a series of dilutions from 0-100 µM in 100mM Tris-HCl pH 7.3. 990 µl of each dilution is placed in a quartz cuvette and pre-warmed to 37°C. 10µl of enzyme solution is added to initiate the reaction and the progress of the reaction is followed by measuring the rate of change in absorbance at a suitable wavelength. The change in extinction coefficient of AAGl 13-161 following complete conversion of a 100µM solution by an excess of glucarpidase is measured in order to calculate the change in extinction coefficient due to the complete conversion of 100nmol of AAGl 13-161. With this data, measured rates are converted to values of µmol/min. Measurement of the rate of reaction at a range of substrate concentrations, from 1-100 µM allows the Kₘ and kₗ for the enzyme to be determined using Enzfitter computer software.
**B: Edatrexate**

Edatrexate is a DHFR inhibitor (Ciba Geigy). Edatrexate is contacted with glucarpidase *in vitro* and is found to be a substrate of glucarpidase.

Deglutamylation of Edatrexate by glucarpidase is measured spectrophotometrically. A stock solution of 10mM Edatrexate in 100mM Tris-HCl pH7.3 is prepared and used to prepare a series of dilutions from 0-100µM in 100mM Tris-HCl pH 7.3. 990µl of each dilution is placed in a quartz cuvette and pre-warmed to 37ºC. 10µl of enzyme solution is added to initiate the reaction and the progress of the reaction is followed by measuring the rate of change in absorbance at a suitable wavelength. The change in extinction coefficient of Edatrexate following complete conversion of a 100µM solution by an excess of glucarpidase is measured in order to calculate the change in extinction coefficient due to the complete conversion of 100nmol of Edatrexate. With this data, measured rates are converted to values of µmol/min.

Measurement of the rate of reaction at a range of substrate concentrations, from 1-100µM allows the $K_m$ and $k_{cat}$ for the enzyme to be determined using Enzfitter computer software.

**E: Lometrexol**

Lometrexol is a GARFT inhibitor (Tularik, originated Lilly). Lometrexol is contacted with glucarpidase *in vitro* and is found to be a substrate of glucarpidase.

Deglutamylation of Lometrexol by glucarpidase is measured spectrophotometrically. A stock solution of 10mM Lometrexol in 100mM Tris-HCl pH7.3 is prepared and used to prepare a series of dilutions from 0-100µM in 100mM Tris-HCl pH 7.3. 990µl of each dilution is placed in a quartz cuvette and pre-warmed to 37ºC. 10µl of enzyme solution is added to initiate the reaction and the progress of the reaction is followed by measuring the rate of change in absorbance at a suitable wavelength. The change in extinction coefficient of Lometrexol following complete conversion of a 100µM solution by an excess of glucarpidase is measured in order to calculate the change in extinction coefficient due to the complete conversion of 100nmol of Lometrexol. With this data,
measured rates are converted to values of µmol/min. Measurement of the rate of reaction at a range of substrate concentrations, from 1-100 µM allows the $K_m$ and $k_{cat}$ for the enzyme to be determined using Enzfitter computer software.

**Example 3: Rescue of Pemetrexed toxicity by administration of gluearpidase**

A patient who has been administered Pemetrexed and has toxic plasma levels of Pemetrexed is administered a dose of 50 Units per kg body weight of gluearpidase by intravenous injection over a period of about 5 minutes. The patient's plasma concentration of Pemetrexed is reduced to non-toxic levels.

**List of References**


1. A method of combating toxicity caused by an antifolate compound of Formula I,

\[
\text{Formula I}
\]

wherein

R\(^1\) represents NH\(_2\), OH or CH\(_3\); 

R\(^2\) represents NH\(_2\) or C\(_{14}\) alkyl;

the group B represents a structural fragment of Formula Ha, lib, Hc, Hd or lie,

\[
\text{Formula IIa, IIb, Hd, lie}
\]

in which groups the dashed lines indicate the point of ring fusion with the pyrimidinyl ring and the wavy lines indicate the point of attachment of the bicyclic heterocycle to the rest of the molecule;

R\(^{7a}\) to R\(^{7e}\) independently represent H or C\(_{14}\) alkyl;

A\(^1\) represents C(R\(^{8b}\)) or N;

A\(^2\) represents CH or N;
A³ represents C(H)R sb, NR sc or S;
A⁴ and A⁵ independently represent CH₂, NH, O or S;
the group B'-B² represents CH-CH or C=C;
R⁸a to R⁸c independently represent H or C₈ alkyl, or R⁸c represents C(0)R sd;
R⁸d represents H or C₁₄ alkyl;

R³ represents H, C₁,₆ alkyl, C₃-₆ alkenyl or C₃-₆ alkynyl;

R⁴ represents H or one or two substituents selected from halo, C₁₄ alkyl and C₁₄ alkoxy, or R⁴, together with R⁵, when R⁴ is attached at a position that is ortho- to the position to which the moiety C(O)NR⁵ is attached, represents Cₙ₋₄/₃-alkylene;

R⁵ represents H or C₁₄ alkyl, or R³, together with R⁴, when R⁴ is attached at a position that is ortho- to the position to which the moiety C(O)NR⁵ is attached, represents C₁₂ 77-alkylene;

R⁶ represents -CH₂C(R⁹a)(R⁹b)-D;
R⁹a and R⁹b independently represent H or C₁₄ alkyl, or R⁹a and R⁹b together represent =C(H)R¹⁰;
R¹⁰ represents H or C₁₄ alkyl;
D represents C(O)OH, tetrazol-5-yl, (CH₂)ₙ-i-NHR π, or, when R⁹a and R⁹b together represent =C(H)R¹⁰, then D may also represent H, or D represent a structural fragment of Formula Ilia or IlIb,

wherein the wavy lines indicate the point of attachment of the structural fragments;
R¹¹ represents H or C(O)R¹²;
R_{12} represents H or phenyl substituted by C(O)OH and optionally substituted by one or two further substituents selected from halo, C_{1-4} alkyl and C_{1-4} alkoxy; and alkyl, alkenyl and alkynyl groups, as well as the alkyl part of alkoxy groups, may be substituted by one or more halo atoms;

or a pharmaceutically acceptable salt and/or solvate thereof;

in an individual who has been administered said compound, the method comprising administering to the individual an enzyme that has carboxypeptidase G activity.

2. A method according to Claim 1 wherein the antifolate compound of Formula I is selected from Pemetrexed, AAGl 13-161, Edatrexate and Lometrexol.

3. A method according to Claim 1 or 2 wherein the individual is administered the enzyme that has carboxypeptidase G activity between about 24 and 48 hours after being administered the antifolate compound.

4. A method according to any of Claims 1 to 3 wherein the individual has one of more clinical markers of toxicity caused by the antifolate compound.

5. A method according to Claim 4 wherein the clinical marker of toxicity caused by the antifolate compound is a plasma level of the compound greater than a predetermined level indicating toxicity at a given time after administration of the compound.

6. A method according to Claim 5 wherein the predetermined blood plasma level of the antifolate compound indicating toxicity is 1µM at 24 hours after administration of the compound.
7. A method according to Claim 5 or 6 further comprising the prior step of determining the plasma level of the antifolate compound in the individual at a given time after administration of the compound.

8. A method according to any of Claims 1 to 7 wherein the individual has one or more clinical symptoms of toxicity caused by the antifolate compound.

9. A method according to Claim 8 wherein the clinical symptom of toxicity caused by the antifolate compound is selected from anaemia, anorexia, asthenia, dehydration, diarrhoea, dyspnea, fatigue, fever, hepatotoxicity, hyperbilirubinemia, leukopaenia, mucositis, myelosuppression, nausea, neurotoxicity, neutropaenia, neutropenic sepsis, parasthesia, rash, reversible transaminitis, septicaemia, stomatitis, thrombocytopaenia and vomiting.

10. A method according to Claim 8 or 9 further comprising the prior step of determining the presence of the one or more clinical symptoms of toxicity caused by the antifolate compound in the individual.

11. A method according to any of Claims 1 to 10 further comprising administering a folate pathway rescue agent to the individual.

12. A method according to Claim 11 wherein the folate pathway rescue agent is selected from leucovorin calcium, thymidine and folic acid.

13. A method according to Claim 11 or 12 wherein the individual is administered the enzyme that has carboxypeptidase G activity prior to the folate pathway rescue agent.

14. A method according to Claim 11 or 12 wherein the individual is administered the folate pathway rescue agent prior to the enzyme that has carboxypeptidase G activity.
15. A method according to Claim 11 or 12 wherein the individual is administered the folate pathway rescue agent and the enzyme that has carboxypeptidase G activity substantially simultaneously.

16. A method according to any of Claims 1 to 15 wherein the individual is administered the enzyme that has carboxypeptidase G activity at a dose of about 50 Units per kg body weight.

17. A method of treating cancer, comprising administering an antifolate compound of Formula I to the individual, and subsequently administering to the individual an enzyme that has carboxypeptidase G activity.

18. A method according to Claim 17 wherein the antifolate compound is Pemetrexed and the cancer to be treated is selected from leukaemia, mesothelioma, NSCLC, lung, breast, colon, pancreas, kidney, bladder, gastrointestinal cancer, head and neck cancer, urothelial cancer and cervical carcinoma.

19. A method according to Claim 17 wherein the antifolate compound is AAG1 13-161 and the cancer to be treated is selected from leukaemia, mesothelioma, NSCLC, breast, colon, pancreas, kidney, bladder, gastrointestinal cancer, head and neck cancer, urothelial cancer and cervical carcinoma.

20. A method according to Claim 17 wherein the antifolate compound is Edatrexate and the cancer to be treated is selected from breast, lung, head and neck squamous cell carcinoma, NSCLC, non-Hodgkin's lymphoma, germ cell tumour, pleural mesothelioma and malignant fibrous histiocytoma.

21. A method according to Claim 17 wherein the antifolate compound is Lometrexol and the cancer to be treated is selected from soft tissue sarcoma, NSCLC, breast, head and neck cancer and melanoma.
22. A method according to any of Claims 17 to 21 further comprising administering an additional anticancer agent to the individual.

23. Use of an enzyme that has carboxypeptidase G activity in the preparation of a medicament for combating toxicity caused by an antifolate compound of Formula I as defined in Claim 1 or Claim 2.

24. Use according to Claim 23 for combating toxicity in an individual who has one or more clinical markers of toxicity caused by the antifolate compound.

25. Use according to Claim 24 wherein the clinical marker of toxicity is a plasma level of the antifolate compound greater than a predetermined level indicating toxicity at a given time after administration of the compound.

26. Use according to Claim 25 wherein the predetermined plasma level of the antifolate compound indicating toxicity is 1µM at 24 hours after administration of the compound.

27. Use according to any of Claims 23 to 26 for combating toxicity in an individual who has one or more clinical symptoms of toxicity caused by the antifolate compound.

28. Use according to Claim 27 wherein the clinical symptom of toxicity caused by the antifolate compound is selected from anaemia, anorexia, asthenia, dehydration, diarrhoea, fatigue, fever, hepatotoxicity, hyperbilirubinaemia, leukopaenia, mucositis, myelosuppression, nausea, neurotoxicity, neutropaenia, neutropenic sepsis, parasthesia, rash, reversible transaminitis, septicaemia, stomatitis, thrombocytopenia and vomiting.

29. Use of an antifolate compound of Formula I as defined in Claim 1 or Claim 2 in the preparation of a medicament for combating cancer in an individual who is subsequently administered an enzyme that has carboxypeptidase G activity.
30. Use according to any of Claims 23 to 29 wherein the individual is one who is administered a folate pathway rescue agent.

31. Use according to Claim 30 wherein the individual is administered the folate pathway rescue agent prior to the enzyme that has carboxypeptidase G activity.

32. Use according to Claim 30 wherein the individual is administered the folate pathway rescue agent after the enzyme that has carboxypeptidase G activity.

33. Use according to Claim 30 wherein the individual is administered the folate pathway rescue agent and the enzyme that has carboxypeptidase G activity substantially simultaneously.

34. Use of a folate pathway rescue agent in the preparation of a medicament for combating toxicity caused by an antifolate compound of Formula I as defined in Claim 1 or Claim 2 in an individual who is administered an enzyme that has carboxypeptidase G activity.

35. Use of an enzyme that has carboxypeptidase G activity and a folate pathway rescue agent in the preparation of a medicament for combating toxicity caused by an antifolate compound of Formula I as defined in Claim 1 or Claim 2.

36. Use according to any of Claims 30 to 35 wherein the folate pathway rescue agent is selected from leucovorin, thymidine and folic acid.

37. Use according to any of Claims 23 to 36 wherein the enzyme that has carboxypeptidase G activity is at a dose of about 50 Units per kg body weight.

38. Use according to any of Claims 23 to 37 for combating toxicity caused by an antifolate compound of Formula I in an individual who is being treated for cancer by administration of the antifolate compound.
39. Use according to any of Claims 23 to 38 wherein the antifolate compound of Formula I is Pemetrexed and the cancer to be treated is selected from leukaemia, mesothelioma, NSCLC, lung, breast, colon, pancreas, kidney, bladder, gastrointestinal cancer, head and neck cancer, urothelial cancer and cervical carcinoma.

40. Use according to any of Claims 23 to 38 wherein the antifolate compound of Formula I is AAGl 13-161 and the cancer to be treated is selected from leukaemia, mesothelioma, NSCLC, breast, colon, pancreas, kidney, bladder, gastrointestinal cancer, head and neck cancer, urothelial cancer and cervical carcinoma.

41. Use according to any of Claims 23 to 38 wherein the antifolate compound of Formula I is Edatrexate and the cancer to be treated is selected from breast, lung, head and neck squamous cell carcinoma, NSCLC, non-Hodgkin's lymphoma, germ cell tumour, pleural mesothelioma and malignant fibrous histiocytoma.

42. Use according to any of Claims 23 to 38 wherein the antifolate compound of Formula I is Lometrexol and the cancer to be treated is selected from soft tissue sarcoma, NSCLC, breast, head and neck cancer and melanoma.

43. A therapeutic system comprising an antifolate compound of Formula I as defined above in Claim 1 or 2, and an enzyme that has carboxypeptidase G activity.

44. A therapeutic system according to Claim 43 further comprising a folate pathway rescue agent.

45. A therapeutic system according to Claim 43 or 44 further comprising an additional anticancer agent.
46. An *ex vivo* method of cleaving a terminal L-glutamate moiety from a compound of Formula I as defined in Claim 1 or Claim 2, the method comprising contacting the compound with an enzyme that has carboxypeptidase G activity.

47. A method of determining the rate and/or extent of cleavage of a compound of Formula I as defined in Claim 1 or Claim 2 by an enzyme that has carboxypeptidase G activity, the method comprising:

   providing the compound of Formula I,

   contacting the compound of Formula I with an enzyme that has carboxypeptidase G activity under conditions such that cleavage of the compound can occur, and

   monitoring the rate and/or extent of cleavage of the compound of Formula I over time.

48. A method according to Claim 47 wherein the monitoring step comprises monitoring the amount and/or concentration of the compound of Formula I.

49. A method according to Claim 47 or 48 wherein the monitoring step comprises monitoring the amount and/or concentration of one or more break-down products of the compound of Formula I.

50. A method according to any of Claims 47 to 49 which is performed *ex vivo*.

51. A method according to any of Claims 47 to 49 which is performed *in vivo*.

52. A method according to Claim 51 further comprising determining whether an additional dose of the enzyme that has carboxypeptidase G activity is required in order reduce the amount of the compound of Formula I to a predetermined level.

53. A method according to Claim 51 or 52 further comprising contacting the compound of Formula I with an additional dose of the enzyme that has
carboxypeptidase G activity under conditions such that cleavage of the compound can occur.

54. A method of cleaving a compound comprising a structural fragment of Formula IV,

\[
\begin{align*}
\text{IV} \\
\text{R}^3 & \quad \text{R}^4 \\
\text{R}^5 & \quad \text{R}^6
\end{align*}
\]

wherein

the wavy line indicates the point of attachment of the structural fragment; and

\( \text{R}^3 \text{ to } \text{R}^6 \) are as defined in Claim I_5

or a pharmaceutically acceptable salt and/or solvate thereof,

the method comprising contacting the compound comprising the structural fragment of Formula IV with an enzyme that has carboxypeptidase G activity.

55. A method according to Claim 54 that is performed \textit{ex vivo}.

56. A method according to Claim 54 that is performed \textit{in vivo}.

57. A method according to Claim 54 wherein the compound comprising the structural fragment of Formula IV is an antifolate compound.

58. A method according to Claim 57 for combating toxicity caused by the antifolate compound in an individual who has \textit{been administered} the said antifolate compound in the course of medical treatment, or otherwise, the method comprising administering to the individual an enzyme that has carboxypeptidase G activity.
59. Use of an enzyme that has carboxypeptidase G activity in the preparation of a medicament for combating toxicity caused by an antifolate compound of Formula IV as defined in Claim 54.

60. A method or a use or a therapeutic system according to any of the preceding claims wherein the enzyme that has carboxypeptidase G activity is glucarpidase, or a derivative thereof which has carboxypeptidase G activity.
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Figure 2 (page 1 of 2)

Pemetrexed

AAG113-161

Lometrexol
Figure 2 (page 2 of 2)

Edatrexate

Methotrexate (prior art)
Figure 3
Figure 6