Therapeutics by Cytotoxic Metabolite Accumulation: Pemetrexed Causes ZMP Accumulation, AMPK Activation, and Mammalian Target of Rapamycin Inhibition

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Abstract

Pemetrexed represents the first antifolate cancer drug to be approved by the Food and Drug Administration in 20 years; it is currently in widespread use for first line therapy of mesothelioma and non–small cell lung cancer. Pemetrexed has more than one site of action; the primary site is thymidylate synthase. We now report that the secondary target is the downstream folate-dependent enzyme in de novo purine synthesis, aminopterinazolecarboxamide ribonucleotide formyltransferase (AICART). The substrate of the AICART reaction, ZMP, accumulated in intact pemetrexed-inhibited tumor cells, identifying AICART as the step in purine synthesis that becomes rate-limiting after drug treatment. The accumulating ZMP causes an activation of AMP-activated protein kinase with subsequent inhibition of the mammalian target of rapamycin (mTOR) and hypophosphorylation of the downstream targets of mTOR that control initiation of protein synthesis and cell growth. We suggest that the activity of pemetrexed against human cancers is a reflection of its direct inhibition of folate-dependent target proteins combined with prolonged inhibition of the mTOR pathway secondary to accumulation of ZMP.

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Introduction

The antifolate pemetrexed is a potent inhibitor of thymidylate synthase and a very efficient substrate for human folypolyglutamate synthetase, creating anabolites that are retained intracellularly after drug exposure (1–4). However, pemetrexed also has at least one other target that becomes apparent from a continued antiproliferative effect in cell cultures exposed to exogenous thymidine, which prevents the cytotoxic effects of thymidylate synthase inhibition (1, 2). A study of the activity of pemetrexed against several recombinant mouse and human enzymes in vitro led to the conclusion that both glycinamide ribonucleotide formyltransferase (GART) and dihydrofolate reductase (DHFR) were potential secondary targets for the polyglutamate forms of pemetrexed (2). The secondary target for pemetrexed is generally taken as GART (5), which was suggested, but not proven, by the in vitro enzyme kinetic data (2). The question of the identity of any secondary targets for pemetrexed has become of substantial interest given the clinical responses to the drug in lung cancers (6), an unusual activity for folate antimetabolites.

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Materials and Methods

Cell culture maintenance and reagents. CCRF-CEM human lymphoblastic leukemia cells were maintained at a density between 106 and 108 cells/ml in RPMI 1640 supplemented with 10% dialyzed fetal bovine serum. Reversal experiments were performed in media supplemented with 20 mmol/L HEPES and 40 mmol/L MOPS. Pemetrexed and (6R)-Ddathf were obtained from Eli Lilly and Co. and were dissolved in PBS. All other culture reagents were from Sigma-Aldrich and were of the highest available quality. 6-[[2-Piperidin-1-yl]-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine (compound C) was also obtained from Sigma-Aldrich. Stock concentrations were determined spectrophotometrically. In studies using modifying agents, 5.6 μmol/L of thymidine, 32 μmol/L hypoxanthine, and 320 μmol/L aminopterinazolecarboxamide (AICA) were used, unless otherwise noted. Cell counts were obtained using a Beckman Coulter Counter. When CEM cells were treated for longer than 48 h, fresh drug-containing media was applied at 48 h.

N-Formyl glycinamide ribonucleotide synthesis from 14C-glycine. Exponentially growing CEM cells were harvested and resuspended in RPMI 1640 without glutamine or serum, and azaserine was added to 10 μmol/L for 30 min at 37°C. Glutamine at 2 μmol/L and 0.25 μCi/ml of 14C-glycine were added, and incubation was continued at 37°C for 1 h. Nucleotides were extracted and radioactivity in the N-Formyl glycinamide ribonucleotide (Fgarn) peak was measured by chromatography on Ag1 resin as previously described (9).

High performance liquid chromatography analysis of ZMP. Following drug exposure, culture densities were determined electronically. Cell pellets were washed with PBS, resuspended in ice-cold 5% trichloroacetic acid at a density of 108 cells per 200 μl, vortexed vigorously, and held on ice for 5 min. Debris was removed by centrifugation at 5,000 × g for 10 min,

In a series of cell culture experiments, we have developed evidence that the target for pemetrexed secondary to thymidylate synthase is the second folate-dependent enzyme in purine synthesis, aminopterinazolecarboxamide ribonucleotide formyltransferase (AICART), not GART, the target for lometrexol (6R-DDATHF). The significance of this is substantial, because the substrate for GART is very labile and is probably inert due to this lability. In contrast, the substrate for AICART is ZMP, a known activator of the AMP-activated protein kinase, AMPK (7), a key controlling element in the mammalian target of rapamycin (mTOR) pathway (8). A therapeutic approach built around reactivation of control on a pathway often dormant in tumor cells due to loss of tumor suppressor gene product function or constitutive activation of c-oncogenes would be very attractive. The accumulation of ZMP offers such an approach, due to the placement of AMPK in the mTOR pathway. Such accumulation of a regulatory intermediate behind a metabolic block had been predicted to be a theoretical avenue for therapeutic drug design, but this case represents a rare instance in which this theory has been reduced to practice. Hence, pemetrexed is indeed a multitargeted inhibitor whose activity seems to extend beyond the traditional targets of antifolates.
and the acid-soluble fractions were neutralized by two extractions with diethyl ether. Samples were passed through a 0.45 μm syringe filter before being injected onto a Phenosphere SAX 250 × 2.0 mm high performance liquid chromatography column (Phenomenex). Absorbance was monitored at 280 nm using a linear gradient of 5 mmol/L NH4H2PO4 (pH 2.8) to 750 mmol/L NH4H2PO4 (pH 3.9) over 25 min at a flow rate of 0.2 mL/min. ZMP concentration was determined by fitting peak absorbance to a standard curve generated from synthetic ZMP. The detection limit of this assay was 25 pmol.

**Immunoblot analysis.** Cells were lysed in buffer containing 62.5 mmol/L Tris (pH 6.5), 5% glycerol, 2% SDS, 5% 2-mercaptoethanol, 50 mmol/L NaF, 0.2 mmol/L Na2VO4, and 1× protease inhibitor complete mixture (Roche Applied Science). Protein concentrations were determined using Bradford assay, against a standard of bovine serum albumin (BSA). Total cellular protein (40 μg) was resolved on 7.5%, 4% to 15%, and 15% SDS-polyacrylamide gels and were transferred to an Immobilon-P polyvinylidene fluoride membrane (Millipore). Membranes were blocked with either 5% milk or Starting Block Buffer (Pierce), washed, and probed with antibodies against AMPKa (1:1000), phospho-AMPKα (T172; 1:250), 4EBP1 (1:1,000), phospho-4EBP1 (T70; 1:1,000), S6 kinase (1:1,000), phospho-S6 kinase (T389; 1:500), acetyl CoA carboxylase (1:1,000), Raptor and phosphorRaptor (S792; 1:1,000), and phosphoacetyl-CoA carboxylase (S79; 1:1,000) at 4°C overnight. All antibodies were purchased from Cell Signaling. Total and phospho-S6 Kinase, -4EBP1, and total and phosphoRaptor antibodies were diluted in Starting Block Buffer (Pierce), all other antibodies were diluted in 5% BSA in TBST. Membranes were incubated with anti-rabbit secondary antibody with horseradish peroxidase conjugate for 1 h at room temperature (Pierce). Chemiluminescence was detected using the SuperSignal West Pico and West Dura Chemiluminescent Substrate kits (Pierce).

**Results**

**Reversal of the growth inhibition of pemetrexed by purines and thymidine.** The growth of human CEM leukemia cells is inhibited by pemetrexed at tens of nanomolar concentrations. This growth inhibition is not reversed by preformed purines, such as hypoxanthine, but inclusion of thymidine in the culture medium does reverse the growth inhibitory effects of pemetrexed, shifting the concentrations of drug needed to affect growth by about 12-fold, as shown for the CEM human leukemia cell line in Fig. I A. Inclusion of both thymidine and hypoxanthine reverses the effects of pemetrexed at even high concentrations (Fig. I A), in agreement with previous literature (1, 2). These observations have previously been interpreted to mean that pemetrexed inhibits thymidylate synthase as its primary target, and has a second site of action within the folate pathways, presumably on purine synthesis, that is only affected at higher drug concentrations (1, 2). To determine whether the first or second folate dependent enzyme of *de novo* purine synthesis, GART or AICART, respectively (Fig. 1C), is affected by drug, we tested the effects of AICA on the inhibition of growth of CEM cells by pemetrexed. AICA is metabolized to the corresponding ribonucleotide, AICAR monophosphate (also known as ZMP) by adenyline phosphoribosyltransferase, and thus, inclusion of AICA in growth medium introduces a purine pathway intermediate into the cell that is downstream of GART but upstream of AICART (Fig. 1C). If the secondary target of pemetrexed were GART, inclusion of thymidine and AICA in the medium should completely reverse growth inhibition (9, 10), whereas if AICART were inhibited, either no effect or a mild exacerbation of growth inhibition would be expected. Others have shown that AICA by itself at 300 μmol/L did not change the potency of pemetrexed against CEM cells (2). However, AICA (320 μmol/L) mildly enhanced the growth inhibition by pemetrexed in the presence of thymidine (Fig. 1A). In contrast, AICA shifted the sensitivity of CEM cells to the GART inhibitor 6R-DDATHF (Fig. 1B), similarly to that seen in mouse cells (9, 10). We concluded that the secondary target of pemetrexed was not GART, and was most likely AICART.

**Effects of pemetrexed on GART and AICART in intact cells.** In the original determination of the site of action of lometrexol within the purine pathway in mouse cells (9), we had used the facts that the enzyme downstream of GART was sensitive to the guanine analogue azaserine, and the enzyme immediately preceding GART in this pathway incorporated glycine into the purine skeleton (Fig. 1C). We previously used these metabolic aspects to show that DDATHF inhibited the accumulation of 14C-glycine into FGAR in azaserine-treated mouse leukemic cells and, hence, had GART as a primary target (9). This is also the case in intact CEM cells for 6R-DDATHF (Fig. 2A) over the concentration range that is growth inhibitory (Fig. 1B). In contrast, pemetrexed gradually inhibited 14C-glycine accumulation (Fig. 2A) to a lower extent that did not reach 50% over the range of concentrations associated with drug action. Hence, any effect of pemetrexed directly or indirectly on GART in intact cells seems to be limited. When the metabolic fate of the intermediates of this pathway were followed further downstream, it was found that the ZMP pool expanded in pemetrexed-treated cells, indicating an effect of pemetrexed or its metabolites predominantly on AICART (Fig. 2B). If inhibition of GART played a role in the secondary effects of pemetrexed, ZMP accumulation would not have been observed (Fig. 1C). To this point, ZMP accumulation did not occur in 6R-DDATHF–treated CEM cells (Fig. 2B). When CEM cells were treated with AICA in the presence of pemetrexed, the accumulation of ZMP was exacerbated, implying that blockage of *de novo* purine synthesis at the AICART step was restricting the flow of intermediates through this pathway (Fig. 1C and see below). The accumulation of ZMP was progressive after drug treatment (Fig. 2C), occurred over the range of concentrations at which pemetrexed was growth-inhibitory, and became more intense at concentrations causing inhibition of a secondary target reversible by hypoxanthine (Figs. 1A and 2A).

The question arises whether the levels of ZMP seen in pemetrexed-treated tumor cells are sufficient to play a role in the inhibitory effects of pemetrexed. One can approach this question by comparing the levels of ZMP that accumulate in pemetrexed-treated cells with those in cells whose growth is inhibited by the ribonucleoside analogue of AICA, AICAR, whose substantial cytotoxicity is generally taken to be solely due to ZMP accumulation (11). At growth-inhibitory concentrations of AICAR, cellular levels of ZMP range from 0.4 to 2 mmol/10^6 cells, the same concentration range seen after pemetrexed treatment. This would correspond to 0.4–2 μmol/L in cell water (Fig. 3). Hence, the ZMP levels seen in pemetrexed-treated cells are sufficient to be at least partially causal of the growth inhibition seen with pemetrexed.

**Effects of accumulating ZMP on AMP-activated protein kinase and the mTOR pathway.** The accumulation of ZMP in cells treated with AICAR and the substantial cytotoxicity of AICAR is thought due to the activation of AMPK by ZMP leading, in turn, to an inhibition of mTOR (11). These effects of AICAR were also seen in CEM cells (Figs. 3 and 4B and C). We studied the activation of AMPK by pemetrexed and thymidine. The phosphorylation of the α-subunit of AMPK enhances the kinase activity of this protein, but the phosphorylation of a direct target of AMPK, acetyl-CoA carboxylase (ACC), is a useful and direct indicator of the activity of AMPK in intact cells (12). A 1-μmol/L concentration of pemetrexed promoted activation of AMPK as shown by both direct

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phosphorylation at T172 and by an enhanced phosphorylation of ACC at residue S79 (Fig. 4B). Activation of AMPK is known to cause inhibition of mTOR by phosphorylation of TSC2, upstream of mTOR, as well as a direct phosphorylation of the Raptor component of the mTORC1 complex (Fig. 4A; refs. 13, 14). Accordingly, a robust hypophosphorylation of two key mTOR targets, 4EBP1 and S6K1, was seen in pemetrexed-treated CEM cells (Fig. 4C), as well as an increase in phosphorylation of S792 of Raptor (Fig. 4B). The phosphorylation of S6K1 by mTOR is confined to a single residue (T389; ref. 15), and this phosphorylation was eliminated by pemetrexed (Fig. 4C), whereas four residues on 4EBP1 are phosphorylated by mTOR (16); the broader migration pattern seen in pemetrexed-treated cells with the pan-4EBP1 antibody (Fig. 3C) suggests that several of these phosphorylation sites are affected by drug. Thymidine was included in these experiments to separate the effects of inhibition of thymidylate synthase from any effect caused by accumulating ZMP. However, phosphorylation of AMPK and hypophosphorylation of 4EBP1 were also observed in cells treated with pemetrexed for 48 hours at doses as low as 0.1 \( \mu \text{mol/L} \) in the absence of thymidine. The effects of pemetrexed on activation of AMPK, as observed by the phosphorylation of the AMPK substrates ACC and Raptor, and the hypophosphorylation of the downstream

Figure 1. Reversal of CEM cell growth inhibition by AICA indicated that the second target of pemetrexed is AICART, not GART. CEM cells were treated with the indicated concentrations of pemetrexed alone (A; No add) or 6R-DDATHF (B; No add) or in the presence of thymidine (dTh; 5.6 \( \mu \text{mol/L} \)), hypoxanthine (Hx; 32 \( \mu \text{mol/L} \)), AICA (320 \( \mu \text{mol/L} \)), or a combination of dTh with either hypoxanthine or AICA. Drug and modifying agents were added simultaneously and drug-containing medium was changed at 48 h. Cell growth was determined after 96 h and cell number is expressed relative to controls without drug. C, the folate-dependent steps of de novo purine synthesis and the site of entry of AICA and AICAR into the pathway. De novo purine synthesis consists of ten sequential enzymatic reactions starting with 1-phosphoribosyl-5-pyrophosphate (PRPP), of which two, GART and AICART, are folate dependent. The figure depicts the substrate of the GART reaction, glycaminide ribonucleotide (GAR), and the product of this reaction FGAR, as well as the salvage of AICA, AICAR, and hypoxanthine catalyzed by adenine phosphoribosyltransferase (APRT), adenosine kinase (AK), and hypoxanthine-guanine phosphoribosyltransferase (HGPRT), respectively.
targetsofmTORwereexacerbatedinthepresenceofAICA(Fig.5B),inconcertwiththeenhancedaccumulationofZMPseenundertheseconditions(Fig.5A).

Reversaloftheeffectsofpemetrexedbyhypoxanthine.

It was clear that a source of preformed purines, such as hypoxanthine, reversed the effects of pemetrexed on its secondary target (Fig. 1A). Initially, we thought this effect indicated a role for a diminution of purine nucleotide pools by pemetrexed inhibition of AICART, but direct measurement of ATP pools by high performance liquid chromatography indicated that ATP levels not only did not decrease but also increased somewhat in pemetrexed-treated cells in the absence of hypoxanthine (data not shown). Rather, the mechanism of this hypoxanthine reversal of the secondary target of pemetrexed seemed to be a prevention of the accumulation of ZMP (Fig. 6A), presumably due to the known feedback effects of an expanded purine pool on early steps in purine synthesis (17, 18). As would be predicted, the inclusion of hypoxanthine in the medium also decreased the phosphorylation of ACC and Raptor as well as the hypophosphorylation of S6K1 and 4EBP1 caused by pemetrexed (Fig. 6B).

Compound C effects support the role of AMPK activation in the pemetrexed-induced mTOR inhibition. The accumulation of targets of mTOR were exacerbated in the presence of AICA (Fig. 5B), in concert with the enhanced accumulation of ZMP seen under these conditions (Fig. 5A).

**Reversal of the effects of pemetrexed by hypoxanthine.** It was clear that a source of preformed purines, such as hypoxanthine, reversed the effects of pemetrexed on its secondary target (Fig. 1A). Initially, we thought this effect indicated a role for a diminution of purine nucleotide pools by pemetrexed inhibition of AICART, but direct measurement of ATP pools by high performance liquid chromatography indicated that ATP levels not only did not decrease but also increased somewhat in pemetrexed-treated cells in the absence of hypoxanthine (data not shown). Rather, the mechanism of this hypoxanthine reversal of the secondary target of pemetrexed seemed to be a prevention of the accumulation of ZMP (Fig. 6A), presumably due to the known feedback effects of an expanded purine pool on early steps in purine synthesis (17, 18). As would be predicted, the inclusion of hypoxanthine in the medium also decreased the phosphorylation of ACC and Raptor as well as the hypophosphorylation of S6K1 and 4EBP1 caused by pemetrexed (Fig. 6B).

**Compound C effects support the role of AMPK activation in the pemetrexed-induced mTOR inhibition.** The accumulation of

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1. Others had previously shown that ATP pools were not diminished by pemetrexed and, in fact, increased by about the factor we observed (37). We would suggest that this effect is due to the block in cell cycle progression with pemetrexed, which prevents the loss of purine nucleotides caused by DNA synthesis.
ZMP in pemetrexed-treated cells and the activating effects of ZMP on AMPK suggest that the inhibition of mTOR by pemetrexed is mediated by a ZMP-dependent activation of AMPK. To determine the intermediacy of activation of AMPK in this mechanism, the effects of an inhibitor of AMPK, compound C (19), on the pemetrexed-induced mTOR inhibition were studied. As expected, compound C inhibited the enhanced AMPK activity in cells treated with pemetrexed and thymidine, as judged by an inhibition of the phosphorylation of ACC (Fig. 6C). The effects of 1 μmol/L compound C on AMPK blocked the inhibition of mTOR by pemetrexed, as judged by the hyperphosphorylation of S6K1 in the presence of pemetrexed and compound C (Fig. 6C), a striking contrast to the marked hypophosphorylation of S6K1 caused by pemetrexed. Interestingly, the phosphorylation of AMPK in the presence of pemetrexed and compound C was greater than that seen with pemetrexed, in spite of the fact that compound C diminished the level of ACC phosphorylation; this suggested that compound C was blocking the activity of AMPK while allowing phosphorylation of the α-subunit of AMPK. Overall, we concluded that the effects of pemetrexed on the mTOR signaling pathway were caused by the activation of AMPK by accumulation of ZMP.

Figure 4. Effects of pemetrexed on activation of AMPK and inhibition of mTOR. A, schematic diagram showing activation of AMPK by either AMP or ZMP that results in inhibition of mTOR and its downstream targets. B and C, Western blot analysis of total and phosphorylated AMPK, ACC, Raptor, S6K1, and 4EBP1. The molecular masses of these bands were 62 kDa (AMPK), 280 kDa (ACC), 150 kDa (Raptor), 70 kDa (S6K1), and 15 to 20 kDa (4EBP1). Equal levels of total protein (40 μg) were loaded in each lane; use of actin as a control showed equal loading between lanes in this and the following figures (data not shown). Vehicle was PBS, pemetrexed was used at 1 μmol/L, AICAR was at 250 μmol/L, and dTh was 5.6 μmol/L; drug exposure was 48 h.

Figure 5. Expansion of the ZMP pool by AICA enhances the effect of pemetrexed on the AMPK-mTOR pathway. Exposure to AICA for 48 h increased cellular levels of ZMP in a dose-related manner in pemetrexed-treated but not untreated CEM cells (A). B, Western blot analysis and drug exposures were as in Fig. 4B; AICA was used at 320 μmol/L.
transformed cells that were not shared with normal stem cells required for host survival. Such steps have rarely been identified, although the Gleevec-sensitive BCR-Abl and Iressa-selective mutations in the epidermal growth factor receptor protein are two well-studied cases (20, 21). One of the most promising approaches seems to be the design of therapeutic agents that affect pathways dependent on tumor suppressor genes whose function is often eliminated or dramatically altered during transformation. The mTOR pathway responsible for balance of energy metabolism, protein and lipid synthesis, and growth involves a series of upstream controlling proteins recognized as tumor suppressor proteins, including LKB1, PTEN, TSC1 and 2, and others recognized as cellular oncogenes, such as AKT and PI3 kinase (8). Perhaps the central element in this pathway that controls the influence of the upstream tumor suppressor gene products/oncogenes on the downstream direct activator of protein synthesis, mTORC1, is AMPK, a protein kinase that phosphorylates the TSC1/TSC2 complex (13) and also directly inhibits mTORC1 by phosphorylation of the Raptor component of this complex (14). The AMPK-phosphorylation of the TSC1/TSC2 complex inactivates the Rheb GTP component needed for mTOR signaling. AMPK is composed of three subunits, and activation of the catalytic α-subunits is facilitated by binding of AMP to the γ-subunit; ZMP is thought to mimic the effect of AMP on the γ-subunit (7, 22).

In this study, we make the case that the accumulation of ZMP behind a block of AICART will activate AMPK and inhibit the downstream activity of mTORC1. The concept that the accumulation of a toxic substrate behind a metabolic block could be used for therapeutics dates back to the classic treatise by Webb on enzyme inhibition in vivo (23). It is difficult to find examples in the chemotherapeutic literature, but the accumulation of ZMP behind an AICART block that we describe here makes a case for the utility and effectiveness of metabolite accumulation as a mechanism of anticancer agents. An inhibitor of any linear metabolic pathway would be expected to cause the accumulation of substrate behind the block, at least transiently, and such accumulation could result in a limitation of the effects of the inhibitor, e.g., the excessive accumulation of dUMP in cells treated with precursors of FdUMP (24), or enhancement of an enzyme inhibitor, e.g., raltitrexed (25).

Why has inhibition of AICART by pemetrexed or its metabolites not been found during prior surveys of folate enzymes for sensitivity to this drug? Earlier in vitro analysis of the kinetics of inhibition of several folate-dependent enzymes by pemetrexed and its polyglutamates led to the conclusion that inhibition of AICART was unlikely to contribute to the biological effects of this drug. Thus, the Ki measured for recombinant human AICART for pemetrexed itself was 3 μmol/L, and for the more potent long-chain polyglutamate metabolites of pemetrexed was 0.26 μmol/L when measured at a fixed concentration of ZMP (50 μmol/L), whereas the activity of these compounds against GART was more favorable but still not very high (Ki, 65 nmol/L). However, we herein show that any effects of pemetrexed on de novo purine synthesis in intact tumor cells are apparently due to effects on AICART, not GART. The solution to this apparent dilemma may be in the binding mechanisms of these two enzymes: GART can bind its two substrates, glycaminide ribonucleotide and 10-formyltetrahydrofolate polyglutamates in a random manner (26), with either substrate binding first, whereas AICART obeys an ordered sequential binding mechanism, with 10-formyltetrahydrofolate polyglutamates binding first before ZMP can bind (27).
For AICART, when ZMP accumulates behind an initial blockade of the pathway, the expanded ZMP pool would promote the reformation of inhibited ternary complex whenever it dissociates to binary AICART-pemetrexed complex. Hence, the likelihood that the bound pemetrexed or its polyglutamates can leave the enzyme is greatly diminished, an example of metabolic trapping of an inhibitor on the enzyme active site by an expanded pool of second substrate. This mechanism is formally identical to the metabolic trapping of FdUMP on the surface of thymidylate synthase responsible for the stabilization of TS ternary complexes by the expanded pool of 5,10-methenyltetrahydrofolate polyglutamates induced by high doses of folic acid (5-formyltetrahydrofolate; refs. 28, 29). In theory, the secondary effect of pemetrexed reversible by a combination of thymidine and purine (Fig. 1) might be due to an inhibition of DHFR causing a cellular deficiency of 10-formyltetrahydrofolate. There are four pieces of evidence that argue against this interpretation: (a) The potency of pemetrexed and its metabolites against DHFR in vitro is quite low (72 nmol/L) compared with the activity of pemetrexed polyglutamates against thymidylate synthase (1.3 nmol/L) (ref. 4). (b) The substantial excess of DHFR over thymidylate synthase in leukemic cells implies that DHFR must be almost completely inhibited before any inhibition of cell growth can occur (30). (c) The primary inhibition of thymidylate synthase by pemetrexed polyglutamates would eliminate the importance of any inhibition of DHFR, given evidence that DHFR becomes irrelevant to cell survival or growth in the absence of thymidylate synthase activity (31, 32). That is, substantial levels of a DHFR inhibitor as potent as methotrexate are without effect on the growth of leukemic cells exposed to a complete pharmacologic or genetic block of thymidylate synthase. And (d) accumulation of ZMP has been sought in methotrexate (MTX)-treated tumor cells and does not occur, although MTX treatment does enhance the ZMP accumulation in cells treated with AICAR (33). This enhanced accumulation of ZMP made from AICAR in methotrexate-treated cells is consistent with reports that methotrexate results in inhibition of AICAR both by depletion of 10-formyltetrahydrofolate and by a direct inhibition of AICAR by methotrexate polyglutamates (34).

It was appreciated that pemetrexed targeted more than one site since early in the development of this compound, but the multiple targets were thought to all involve other enzymes on the folate pathways (1, 2). We now present evidence that inhibition of AICART by pemetrexed causes a mechanistically important but indirect effect on the AMP-dependent kinase-mTOR pathway. We note that this should be the case for any drug targeted against AICART, a concept that suggests the development of drugs specifically against AICART but without activity against thymidylate synthase. Pharmacologic inhibition of mTOR with direct inhibitors is currently of substantial interest for cancer therapeutics, and current mTOR inhibitors have shown activity against human lung cancers (35, 36). Perhaps this spillover of the effects of pemetrexed to the mTOR pathway explains the activity of pemetrexed in lung cancers, an unusual pattern for classic antifolates.

Disclosure of Potential Conflicts of Interest

The authors declared no potential conflicts of interest.

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