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Pemetrexed indirectly activates the metabolic kinase AMPK in human carcinomas

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Abstract

The chemotherapeutic drug pemetrexed, an inhibitor of thymidylate synthase, has an important secondary target in human leukemic cells, AICART, the second folate-dependent enzyme of purine biosynthesis. The purine intermediate aminoimidazolecarboxamide ribonucleotide (ZMP), which accumulates behind this block, transmits an inhibitory signal to the mTORC1 complex via activation of the cellular energy sensor AMPK. Given that the PI3K-AKT-mTOR pathway is frequently deregulated during carcinogenesis, we asked whether the indirect activation of AMPK by pemetrexed offers an effective therapeutic strategy for carcinomas with defects in this pathway. Activation of AMPK by ZMP in pemetrexed-treated colon and lung carcinoma cells and the downstream consequences of this activation were strikingly more robust than previously seen in leukemic cells. Genetic experiments demonstrated the intermediacy of AICART inhibition and the centrality of AMPK activation in these effects. While AMPK activation resulted in marked inhibition of mTORC1, other targets of AMPK were phosphorylated that were not mTORC1-dependent. Whereas AMPK activation is thought to require AMPK α T172 phosphorylation, pemetrexed also activated AMPK in carcinoma cells null for LKB1, the predominant AMPK α T172 kinase whose deficiency is common in lung adenocarcinomas. Like rapamycin analogs, pemetrexed relieved feedback suppression of PI3K and AKT, but the prolonged accumulation of unphosphorylated 4E-BP1, a tight-binding inhibitor of cap-dependent translation, was seen following AMPK activation. Our findings indicate that AMPK activation by pemetrexed inhibits mTORC1- dependent and -independent processes that control translation and lipid metabolism, identifying pemetrexed as a targeted therapeutic agent for this pathway that differs significantly from rapamycin analogs.

Keywords

pemetrexed; Non-small cell lung cancer; AMP-activated protein kinase; mTOR; Aminoimidazolecarboxamide ribonucleotide formyltransferase

Introduction

Pemetrexed has shown substantial activity against lung carcinomas not usually considered to be sensitive to classical antifolates (1). The US Food and Drug Administration approved pemetrexed for mesothelioma in 2004, for non-small cell lung cancer (NSCLC) in 2008, and, notably, for maintenance therapy of NSCLC in 2009, the first drug ever approved for

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this purpose (2-4). Pemetrexed and its metabolites were originally shown to be potent inhibitors of thymidylate synthase (TS) (5), but it was clear from the start that there was another target for pemetrexed that led to inhibition of purine synthesis (5,6). We recently discovered that this elusive target was the second folate-dependent enzyme of *de novo* purine synthesis, aminoimidazolecarboxamide ribonucleotide formyltransferase (AICART) in human leukemic cells (7). Others have shown that intracellular pemetrexed is rapidly converted to polyglutamate derivatives by folylpolyglutamate synthetase (8); hence, we presume that inhibition of AICART was brought about by the polyglutamate derivatives of pemetrexed. Inhibition of AICART results in the accumulation of ZMP, an intermediate along the purine *de novo* biosynthesis pathway. ZMP is a mimetic of AMP that also accumulates in cells following exposure to aminoimidazolecarboxamide ribonucleoside (AICAR), with profound consequences (9,10). We recently demonstrated that the accumulation of ZMP in leukemic cells treated with pemetrexed activates AMP-activated protein kinase (AMPK), a protein that senses cellular energy charge and links proliferative capacity to the availability of sufficient ATP (7).

AMPK is composed of three subunits (α , β , and γ). The AMPK β subunit is a scaffold for the assembly of the trimeric protein, with the α subunit responsible for the kinase activity and the γ subunit modulating the catalytic activity of the α subunit (11). The γ subunit responds to energy charge due to the cooperative binding of AMP at two sites competitive with ATP. Enhanced binding of AMP to the γ subunit is seen as concentration rises through the μ M range, even in the presence of physiologic ATP concentrations (12). Activation of AMPK is thought to require phosphorylation of AMPK α T172 (13-15). The tumor suppressor gene product LKB1 has been shown to be the predominant AMPK kinase in most cell types (16-18). T172 can also be phosphorylated by CAMKK β in the presence of Ca⁺ or a calcium ionophore, but absence of AMP (19-21).

AMPK phosphorylates several controlling elements of lipid and energy metabolism, and also phosphorylates two key proteins in the PI3Kinase-AKT-mTOR pathway (22,23). Both phosphorylation events lead to inhibition of the kinase activity of the mTORC1 complex, causing suppression of the phosphorylation of two downstream targets, S6K1 and 4E-BP1, each of which play a critical role in cap-dependent translation (24). Phosphorylation of S6K1 activates its kinase activity, which converts eukaryotic translational initiation factor 4B (eIF4B) to its phosphorylated form, a necessary step in its recruitment to the cap-dependent translational pre-initiation complex. In contrast, unphosphorylated 4E-BP1 tightly binds 5'-cap-bound eIF4E, preventing the binding of eIF4G necessary for assembly of the translational initiation complex. mTORC1-phosphorylated 4E-BP1 does not bind to cap-eIF4E complexes. The control of mTOR-dependent processes is frequently deregulated during carcinogenesis due to mutation of K-Ras, loss of PTEN, hyperactivity of PI3K, or loss of control on AKT (25).

With the far-reaching effects of AMPK activation on processes essential for tumor cell proliferation, it was apparent that the secondary effect of pemetrexed might be instrumental in the activity of this antifolate against lung cancers. In this manuscript, we demonstrate the endogenous accumulation of ZMP to very high levels following the inhibition of *de novo* purine synthesis by pemetrexed and the activation of AMPK by this metabolite in a spectrum of human carcinoma cells, even in cells with an LKB1-null phenotype. Importantly, the site in purine synthesis targeted by pemetrexed was verified by siRNA experiments and by the availability of human fibroblasts that are null for AICART. Overall, in human carcinomas, these studies define pemetrexed as an AMPK activating agent, as well as a TS inhibitor, with effects that include, but extend beyond, inhibition of mTORC1.

Materials and Methods

Cell Culture and Reagents

Cell lines were purchased from ATCC and maintained in RPMI 1640 supplemented with 10% dialyzed fetal bovine serum (dFBS). The phenotype of LKB-1 null H460 and HeLa cells was confirmed by Western blotting. AICART (-/-) skin fibroblasts were a generous gift from Marie-Francois Vincent (Universite Catholique de Louvain, Brussels, Belgium) and were maintained in DMEM supplemented with 15% FBS; experiments were performed in dialyzed serum. Experiments were performed on the AICART(-/-) fibroblasts within two passages after receipt. Pemetrexed disodium was obtained from Eli Lilly and Co. Unless otherwise noted, cells were treated with final concentrations of 1 μ M pemetrexed and 5.6 μ M thymidine. Cell proliferation experiments were evaluated by Coulter counting after 72 hours of continuous drug exposure. GSK690693 was purchased from SYN l thesis Med Chem and was dissolved in DMSO. All other culture reagents were obtained from Sigma Aldrich.

ZMP Measurements

Cell pellets were extracted with 5% trichloroacetic acid and ZMP levels were measured after neutralization by high performance liquid chromatography using $\text{NH}_4\text{H}_2\text{PO}_4$ gradient elution of a SAX 250 $\times$ 2.0 mm column (Phenomenex) (7).

Immunoblotting

Total cellular protein was harvested and quantified as previously described (7). Typically, 20 μ g protein was resolved on 5%, 7.5%, and 12.5% SDS-polyacrylamide gels and transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore). AICART primary antibody was from BD Pharmingen. β -actin primary antibody was from Abcam. All other primary antibodies were from Cell Signaling Technology. Chemiluminescence was detected using Super Signal Substrate kits (Pierce).

AMPK Activity Assay

AMPK activity was measured in AMPK α 1 immunoprecipitates (IPs) using the SAMS peptide (Tocris Bioscience) as substrate (26). Cells were broken in cold lysis buffer (26) and the protein content of the supernatant was determined by Bradford assay. For each sample, a 10 μ l bed volume of protein G sepharose (Amersham) was first washed 3 $\times$ with IP buffer (26). Anti-AMPK α 1 antibody (10 μ g) (Upstate) was bound to equilibrated protein G beads at 4°C. Antibody-bound beads were washed 5 $\times$ with cold IP buffer and incubated overnight with 100 μ g lysate at 4°C. IPs were washed with cold IP buffer containing 1 M NaCl followed by additional washes as described (26). IP was resuspended in 60 μ l assay buffer (50 mM HEPES (pH 7.4), 1 mM DTT, and 0.02% (v/v) Brij-35). Typical reactions were performed in 25 μ l assay buffer containing 0.2 mM SAMS peptide, 0.2 mM AMP, 0.2 mM [γ - 32 P]-ATP (final specific activity $\sim$ 500 cpm/pmol), 5 mM MgCl_2 , and immunoprecipitated AMPK α 1. Reactions were incubated at 37° for 10 min and quenched by spotting onto Whatman P81 disks. Disks were washed 2 $\times$ in 1% (v/v) phosphoric acid, 1 $\times$ in water, and 1 $\times$ in acetone before being counted in scintillation fluid.

RNA Interference

DharmaFECT transfection reagent #2, siGENOME SMARTpool siRNAs targeting human AICART, AMPK α 1, and non-targeting siRNA pool #1 were purchased from Dharmacon. Cells were transfected with 50 nM siRNA with 0.1% DharmaFECT according to the manufacturer's instructions for 24 hr. Mock indicates incubation with transfection reagent alone. Protein was harvested 72 hr after transfection unless noted.

m⁷GTP capture of 4E-BP1-eIF4E complexes

Following the indicated drug treatments, cells were lysed on ice for 30 min in IP buffer (25 mM HEPES pH 7.5, 1% NP40, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM PMSF, 0.1% 2-mercaptoethanol, and 1× Roche Complete Protease Inhibitor Tablet). An aliquot (500 µg) of cleared lysate was incubated with 40 µl of a 50% slurry of m⁷GTP-Sepharose (GE Lifesciences) for 2 hr at 4°C with rotation. m⁷GTP captured complexes were washed with IP buffer four times, resuspended in Laemmli Sample Buffer, and boiled for 5 min before being resolved on a 12.5% SDS-PAGE gel and immunoblotted as described above.

Clonogenic Survival

Cells were plated from a single cell suspension at 100/60-mm and allowed to attach overnight. Cells were then treated continuously with media supplemented with 5.6 µM thymidine containing 0-100 µM combinations of GSK690963 and pemetrexed for 72 hours. Plates were washed with PBS, and fresh media supplemented with thymidine was added every 2-3 days and colonies were fixed with methanol, stained with 5% Wright-Giemsa reagent and counted on day 8.

Results

AMPK is activated following ZMP accumulation in pemetrexed-treated carcinoma cells

A striking inhibition of AICART was seen in a series of human carcinoma cell types treated with pemetrexed, as evidenced by the accumulation of the substrate for the AICART reaction, ZMP (Table 1). Whereas cellular ZMP is normally held below 1 µM, it accumulated to levels of 1-12 mM in different carcinoma cell types due to the continuing operation of the pathway for purine biosynthesis in the face of an AICART block. These experiments were performed in the presence of 5.6 µM thymidine, a salvageable source of thymidylate that prevented the effects of thymidylate synthase inhibition by pemetrexed. In contrast, treatment of HCT116 cells with 1 µM methotrexate in the presence of thymidine for 24 hours did not result in the accumulation of measurable ZMP, indicating that this accumulation was not an unspecific antifolate effect. The substantial levels of ZMP accumulating in pemetrexed-treated HCT116 cells initiated a robust phosphorylation of T172 on the α-subunit of AMPK (Fig. 1A). Activation of the kinase activity of AMPK, assayed *in vitro* using the AMPK-specific SAMS peptide as a substrate, was also detected in immunoprecipitates from HCT116 cells using antibody against the α1 subunit of AMPK, implying that a post-translational event, presumably T172 phosphorylation, was sufficient for AMPK activation in the presence of AMP or ZMP (Fig. 1A). AMPK activity was not measurable in a similar immunoprecipitation using an antibody specific for AMPKα2 (data not shown).

The growth of HCT116 cells is potently inhibited by pemetrexed at low nM concentrations (Fig. 1B), an effect due in part to inhibition of TS (5). Exposure to drug in the presence of thymidine, which prevents the effects of TS inhibition, shifts the growth inhibitory effect of pemetrexed approximately 3-fold (Fig. 1B). Hence, although the most sensitive folate-dependent step in intact carcinoma cells is TS, the secondary target in purine synthesis was affected even at the minimal inhibitory concentrations of this drug. The concentrations of pemetrexed that cause this accumulation of ZMP *in vitro* (0.03-1 µM) (Fig. 1B) are maintained in serum of patients treated with this drug for more than 48 hours (27). Accumulation of ZMP in HCT116 cells occurs over the same concentration range that causes a thymidine-insensitive growth inhibition (Fig 1B), and phosphorylation of AMPKα T172, likewise, incrementally increases over the same concentration range of pemetrexed (data not shown).

When HCT116 cells were transfected with pools of small interfering RNAs directed against AICART, the effects of inhibition of the secondary target of pemetrexed were recapitulated (Fig. 1C, D), confirming the intermediacy of AICART inhibition in the accumulation of ZMP and the activation of AMPK by pemetrexed. Surprisingly, the effect of ~95% knockdown of AICART on ZMP accumulation and AMPK α T-172 phosphorylation was less pronounced than that seen following treatment with pemetrexed (Fig. 1A, C and D). The accumulation of ZMP in AICART knockdown cells exposed to a low concentration of AICAR (100 μ M) indicated that the flow of ZMP through AICART becomes restricted by siRNA depletion of AICART (Fig. 1D), but ZMP accumulation even under conditions of AICART knockdown plus AICAR challenge did not match that seen after pemetrexed treatment of HCT116 cells. Hence, it appears that AICART activity is in excess and not rate-limiting to the flux through the purine synthesis pathway in uninhibited cells and that inhibition of AICART by pemetrexed is quite extensive in intact cells.

A patient was reported who had inactivating mutations in both alleles of the AICART gene and, hence, complete deficiency of this enzyme (28). In skin fibroblasts from this patient (AICART $-/-$ HSF), a > 1000 fold increase in ZMP was detected compared to levels in WI-38 human lung fibroblasts (Table 2), phenocopying the effect of pemetrexed.

Inhibition of mTORC1 by pemetrexed is dependent on the activity of AMPK

AMPK inhibits mTORC1 both by a mechanism involving direct phosphorylation of Raptor and indirectly through phosphorylation of the TSC2 component of the tuberous sclerosis complex (22,23). The phosphorylation of AMPK in response to pemetrexed resulted in a striking hyperphosphorylation of Raptor at S792 and hypophosphorylation of T389 of the downstream target of mTORC1, S6K1 (Fig. 2A). The competitive inhibitor of the AMPK α ATP-binding site, compound C (29), blocked the hypophosphorylation of S6K1 at T389 induced by pemetrexed (Fig. 2B). Likewise, siRNA against the α 1 subunit of AMPK blocked the hypophosphorylation of S6K1 and hyperphosphorylation of Raptor in spite of the continued accumulation of ZMP in HCT116 cells (Fig. 2C and Table 3). This AMPK α 1-specific siRNA pool depleted all AMPK α detectable on immunoblots probed with an antibody that detected both AMPK α 1 and α 2, indicating that AMPK α 2 was not expressed in HCT116 cells. We concluded that the pemetrexed-induced accumulation of ZMP behind an AICART block activated AMPK, signaling downstream to inhibit the activity of the mTORC1 complex.

The secondary effects of pemetrexed are not identical to those of rapamycin

Growth inhibition from an AICART block by pemetrexed was determined across a panel of carcinoma cell lines, many of which had mutations in key controlling elements along the PI3Kinase-AKT-mTOR signaling axis (Fig. 3A). All ten cell lines tested were sensitive to the secondary effect of pemetrexed, albeit to varying degrees. The sensitivity did not correlate with the level of ZMP measured in these cells (Table 1 and Fig. 3A), suggesting that other factors, either biochemical or genetic, contribute to this variability. Notably, the sensitivity of these cell lines to pemetrexed and to a concentration of rapamycin that would cause complete and prolonged inhibition of mTORC1 (25 nM) were not identical nor even correlated, with most cell lines demonstrating higher sensitivity to the AMPK activation by pemetrexed than to direct inhibition of mTOR (Fig. 3A). We note that the cell lines most sensitive to pemetrexed in our limited screen, HCT116 and H460, harbor activating mutations in both PI3Kinase and K-Ras. Interestingly, the HCT116 cell was among the least sensitive to growth inhibition by rapamycin but the most sensitive to the secondary effect of pemetrexed. We concluded that the secondary effects of pemetrexed causing growth inhibition were due to the downstream signaling from activated AMPK rather than solely to inhibition of mTORC1.

LKB1 is not required for AMPK activation in response to pemetrexed

Loss-of-function mutations in the tumor suppressor gene product LKB1, the major upstream kinase of AMPK, is a common occurrence in primary non-small cell lung carcinomas (30). We therefore sought to determine whether the effects of pemetrexed on AMPK signaling remained intact in carcinoma cells lacking LKB1. Human H460 NSCLC and Hela cervical carcinoma cell lines do not express functional LKB1 (Fig 3B); H460 has a premature stop at codon 37 (31) and there is biallelic deletion of LKB1 in Hela (32). In spite of these defects in LKB1 expression, the growth of H460 and Hela is inhibited by pemetrexed in the presence of thymidine, albeit somewhat less potently than HCT116 (Fig 3B). Inhibition of mTORC1 signaling in response to pemetrexed was also seen in both H460 and Hela cells, as indicated by S6K1 T389 hypophosphorylation (Fig 3C). Compound C blocked the initial hypophosphorylation of S6K1 at T389 in H460 cells (Fig 3D), again suggesting that mTORC1 inhibition induced by pemetrexed was mediated by increased AMPK activity; interestingly, compound C by itself also diminished S6K1 phosphorylation.

In LKB1-null cells, enhanced phosphorylation of AMPK α at T172 was not always a clear indicator of AMPK activity. Thus, in H460 cells, pemetrexed stimulation of AMPK α T172 phosphorylation was not detected, primarily because this residue was already hyperphosphorylated in untreated cells (Fig. 3E). However, several observations support the concept that AMPK was activated in H460 cells by pemetrexed: 1) The direct AMPK target acetyl CoA carboxylase (ACC) was hyperphosphorylated at S79 in pemetrexed-treated H460 cells. 2) Eukaryotic elongation factor 2 (eEF2), the substrate for the direct AMPK target eEF2 kinase, was also hyperphosphorylated at T56 after pemetrexed treatment, (Fig 3E,F), and 3) S6K1 was hypophosphorylated at T389 (Fig 3C). The inactivity of AMPK that was extensively phosphorylated at T172 in untreated H460 cells led to the conclusion that T172 phosphorylation was necessary but not sufficient for AMPK activation in these cells, and that such activation only occurred following the accumulation of ZMP either after pemetrexed or AICAR treatment (Fig 3E,F). Interestingly, a marked hyperphosphorylation of both ACC at S79 and eEF2 at T56 was seen in untreated HCT116 cells that had hypophosphorylated AMPK.

Pemetrexed-induced inhibition of mTORC1 results in relief of feedback suppression of AKT

A time course of ZMP accumulation following pemetrexed exposure showed that, after an initial delay of a few hours, ZMP accumulated at a rate of 0.2 mM/hour up to 15 hours, and then remained elevated for at least 48 hours in HCT116 cells (Fig. 4A). AMPK α became hyperphosphorylated at T172 by 15 hours and this phosphorylation persisted for an interval coincident with expansion of the ZMP pool. It appeared that the presence of the expanded ZMP pool was sufficient to trigger AMPK α phosphorylation (Fig 4A).

The time-course of pemetrexed effects on 4E-BP1 and S6K1 phosphorylation (Fig 4B and 4C, respectively) was followed. Inhibition of 4E-BP1 phosphorylation peaked by 15 hours, but recovered slowly, as indicated by western blots probed with phospho-specific antibody and, particularly, by a pan 4E-BP1 antibody (Fig 4B). While only a very low percentage of this protein ran at the position of p-4E-BP1 (pan 4E-BP1 blot, Fig 4B), there was a substantial expansion of the cellular content of unphosphorylated 4E-BP1 after pemetrexed treatment in HCT116 cells. Because unphosphorylated 4E-BP1 is known to tightly bind to eIF4E-capped mRNA complexes, preventing the binding of eIF4G and the initiation of cap-dependent translation, we measured the binding of 4E-BP1 to eIF4E-cap complexes in extracts of pemetrexed-treated cells. The binding of 4E-BP1 to eIF4E-bound 7-methylGTP-beads was enhanced by pemetrexed and was maintained for at least 24 hours after treatment (Fig 4B), indicating a prolonged presence of active inhibitor of cap-dependent translation. In

contrast, the binding of 4E-BP1 to cap complexes promoted by rapamycin was less than seen with pemetrexed and diminished at longer times (12 hr) after treatment (Fig 4B), as previously shown by others (33).

S6K1 phosphorylation was initially inhibited by 15 hours after pemetrexed treatment, but hypophosphorylation of S6K1 was transient and partially recovered by 48 hours (Fig 4C). The recovery of S6K1 phosphorylation occurred in spite of continued phosphorylation of AMPK α T172 and expansion of the ZMP pool (Fig 4A), suggesting that an event that was dominant over continued AMPK activity was being activated. As in HCT116 cells, hypophosphorylation of S6K1 was transient in H460, peaking at 15-24 hours and partially recovering by 48 hours, suggesting that the recovery of mTORC1 signaling also occurred in cells which lack LKB1 (Fig. 4C).

We sought to determine whether the slow recovery of S6K1 phosphorylation following pemetrexed was caused by relief of feedback suppression of PI3K and AKT via stabilization of insulin receptor substrate 1 (IRS-1) and stimulation of mTORC2 with subsequent AKT S473 phosphorylation, as has been seen with rapamycin analogs (34,35). The phosphorylation and stability of IRS-1 was determined with an antibody directed against total IRS-1 protein. An increased level of IRS-1 was measured (Fig 4D), presumably signifying expression and stabilization of this protein (36,37). An enhanced phosphorylation of AKT on S473 was also observed coincident with recovery of pemetrexed-mediated inhibition of S6K1 in HCT116 cells (Figs 4C and D). Therefore, it appeared that the partial recovery of S6K1 T389 phosphorylation (Fig 4C) was a result of enhanced AKT activity, at least partially reactivating mTORC1. In support of this mechanism, treatment of HCT116 cells with the pan-AKT competitive inhibitor GSK690693 prolonged the inhibitory effect of pemetrexed on S6K1 T389 phosphorylation (Fig 4E). Colony formation experiments with this combination indicated that the cytotoxicity of inhibition of AICART by pemetrexed was synergistically enhanced by exposure to the AKT inhibitor (Fig. 4F), with non-cytotoxic concentrations of pemetrexed plus thymidine increasing the cell kill seen with 10 or 32 μ M GSK690693 (Fig 4F).

Discussion

Cellular requirements for AMPK activation

ZMP or AMP binding to the AMPK γ subunit is thought to allosterically stimulate the kinase activity of the α subunit both directly through conformational restraint of an autoinhibitory peptide on the α subunit and by protecting T172 in the α subunit from dephosphorylation (13-15). Following pemetrexed-mediated ZMP accumulation in HCT116 cells, T172 is maintained in a phosphorylated state and the kinase is activated towards some targets, e.g., Raptor. Of note, AMPK activity was easily measurable in immunoprecipitates of untreated HCT116 cells (Fig. 1A), and the mTORC1-independent targets of AMPK, ACC and eEF2, were hyperphosphorylated in the absence of detectable T172 phosphorylation or an expanded ZMP pool in HCT116 (Fig 3E,F). The phosphorylation of ACC in cells with hypophosphorylated AMPK has also been recently observed by others (38). The mechanism of the differential phosphorylation of mTORC1-independent targets ACC and eEF2 and of mTORC1-related AMPK targets in HCT116 cells remains to be elucidated.

One of the more interesting aspects of our results is that the accumulation of ZMP induced by pemetrexed stimulates AMPK activity in cells that have lost LKB1 function, in spite of the fact that the experimental support for LKB1 as the principal kinase for AMPK α T172 phosphorylation is strong (16,17,39). In H460 cells, the AMPK α subunit is hyperphosphorylated at T172 under basal conditions by a kinase other than LKB1. However, AMPK is not catalytically active under these conditions and becomes active only

after accumulation of ZMP. These data support previous conclusions that T172 phosphorylation alone is insufficient to fully activate the kinase activity of AMPK α (13-15), at least towards the substrates Raptor and TSC2.

Opposing influences of AMPK and AKT on mTORC1

Whereas AMPK inhibits mTORC1 through phosphorylation of TSC2 and the Raptor subunit of mTORC1, AKT opposes each of these effects. AKT indirectly inhibits the GTPase activity of the mTORC1 effector Rheb (40-42), likely through phosphorylation of multiple residues including S939 and T1462 on the human TSC2 subunit (43,44), while AMPK phosphorylation of TSC2 S1387 and T1271 are requisite for mTORC1 inhibition in response to energy stress (23). AKT also activates mTORC1 by phosphorylating the PRAS40 subunit of mTORC1 at T246, promoting the dissociation of inhibitory PRAS40 from mTORC1 (45,46), whereas the phosphorylation of Raptor S792 by AMPK results in inhibition of the kinase activity of mTORC1 (22). A question of some significance is whether the effect of AMPK or that of AKT would dominate at each of these two nodes of signal integration. We show that, in spite of continued activation of AMPK in response to pemetrexed, the inhibition of S6K1 phosphorylation is partially relieved, apparently by activation of the opposing influences of AKT on TSC2 and mTORC1. The recovery of 4E-BP1 phosphorylation was less marked, and was dwarfed by the marked accumulation of unphosphorylated 4E-BP1, the tight-binding inhibitor of an early step in initiation of cap-dependent translation, recruitment of eIF4G to the cap-bound eIF4E complex. In the presence of this expanded pool of unphosphorylated 4E-BP1, binding of 4E-BP1 to cap-eIF4E complexes is maintained in pemetrexed-treated cells even after rebound activation of AKT. Others have reported that the stimulation of AKT activity following rapamycin did not prevent suppression of S6K1 phosphorylation nor did it interfere with growth suppression by everolimus (35,47). We note that the relief of feedback induced stimulation of AKT after pemetrexed treatment also did not prevent complete growth suppression of HCT116 cells, presumably due to either the continued inhibition of translational initiation by the expanded pools of unphosphorylated 4E-BP1 (Fig 4B) or to non-mTORC1-dependent effects of AMPK activation, e.g., inhibition of translational elongation by phosphorylation of eEF2 (Fig 3F).

AMPK activators and direct inhibitors of mTOR

A direct inhibitor of mTOR such as rapamycin, binds to a site adjacent to the kinase domain of mTORC1 as a complex with FKBP12 (48). In contrast, activators of AMPK inhibit mTORC1 by phosphorylation events on Raptor and on the TSC2 subunit of the tuberous sclerosis complex (22,23). Pemetrexed-induced accumulation of ZMP does not appear to inhibit the mTORC2 complex, as judged by the enhanced phosphorylation of AKT seen in Figure 4D. While most agents identified to activate AMPK seem to promote AMPK activation through their ATP-depleting effects (49), it is important to note that pemetrexed does not cause ATP depletion (7,50).

A number of other targets are controlled by AMPK that are not mediated by mTORC1 (11). Indeed, we show that ACC phosphorylation is induced by AMPK activation in H460 cells, as is phosphorylation of eEF2 (Figs. 3E-F), both of which are direct targets for AMPK whose phosphorylation is not mediated by mTORC1 (51). It would be expected that pemetrexed-induced activation of AMPK would also affect other direct targets of this kinase, and that the overall effects of AICART inhibition would have components additional to the effects seen with rapamycin analogs. Likewise, activation of AMPK by pemetrexed and inhibition of mTORC1 by rapamycin were not equivalent against a spectrum of cell lines (Table 1). Whether AMPK activation offers a therapeutic advantage over direct mTOR inhibitors remains an open question.

In this manuscript, we present the argument that activation of AMPK by ZMP accumulation following inhibition of AICART poses a viable therapeutic intervention to prevent aberrant proliferation in tumors with genetic defects in the PI3Kinase-AKT-mTOR pathway. Although downstream effects on mTORC1 are involved in pemetrexed action, the spectrum of effects is not confined to mTORC1-dependent processes, and AICART inhibitors represent a unique class of drugs different from the rapamycin analogs.

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The abbreviations used were

AICART	aminoimidazolecarboxamide ribonucleotide formyltransferase
ZMP	aminoimidazolecarboxamide ribonucleotide
AMPK	AMP-activated kinase 1
mTOR	mammalian target of rapamycin
TS	thymidylate synthase
NSCLC	non-small cell lung cancer
IRS-1	insulin receptor substrate 1

Figure 1. AMPK activation by ZMP accumulating in pemetrexed-treated HCT116 cells
 (A) Pemetrexed induces AMPK phosphorylation and activity. HCT116 cells were treated with 1 μM pemetrexed in the presence of 5.6 μM thymidine to offset the effects of TS inhibition. The phosphorylation of T172 on AMPKα was detected by western blot (upper panel), and the kinase activity of immunoprecipitated AMPKα1 was measured using SAMS peptide as a substrate (lower panel). (B) The secondary growth-inhibitory mechanism of pemetrexed correlates with ZMP accumulation. HCT116 cells were treated with pemetrexed for 72 hours in the absence (circles) or presence (open triangles) of thymidine. ZMP (closed triangles) was measured by HPLC following 15 hr exposure to pemetrexed and thymidine, and measured values in nmol/10⁶ cells were converted to mM concentrations in cell water. (C) siRNA knockdown (KD) of AICART mimics the effects of pemetrexed on AMPKα phosphorylation in HCT116 cells. (D) Accumulation of ZMP in HCT116 cells transfected with a pool of siRNAs against AICART. HCT116 cells were transfected with Dharmacon smart pools of AICART or scrambled siRNAs for 24 hours. Cells were lysed after 72 hours and ZMP assayed by HPLC. Where indicated, cells were treated with 100 μM AICAR for 4 hr immediately before harvest. The asterisks indicate values that were below the limits of the assay (< 0.001 mM). For details, see Materials and Methods.

Figure 2. mTORC1 inhibition by pemetrexed is mediated through AMPK activation

(A) Pemetrexed-induced activation of AMPK inhibits mTORC1 signaling. Immunoblots on HCT116 cells treated with pemetrexed for 15 hr were probed for Raptor p-S792 and for p-T389 of S6K1. (B) The AMPK α inhibitor, compound C (1 μ M), blocks the effects of pemetrexed on mTORC1 signaling following 15 hr exposure to both agents in HCT116 cells. All treatments contained 0.1% DMSO and thymidine. (C) Depletion of AMPK α 1 by siRNA knockdown (KD) in HCT116 cells blocks the effects of pemetrexed on mTORC1 signaling. siRNA pools were added to HCT116 cells for 24 hr and protein was harvested after 72 hr. Pemetrexed and thymidine were present during the last 15 hr.

Figure 3. Signaling events dependent on AMPK activity are maintained in LKB1-null carcinoma cells exposed to pemetrexed

(A) Sensitivity of carcinoma cells to pemetrexed-induced AMPK activation and to rapamycin. Cells were exposed to pemetrexed and thymidine or to rapamycin (25 nM) for 72 hr. The genotypes (Sanger Institute Cancer Cell Line Project <http://www.sanger.ac.uk/genetics/CGP/CellLines/>) is listed as mutant (M) for LKB1, PI3K, and KRAS. (B) Sensitivity of LKB1-null cell lines to growth inhibition after 72 hr exposure to pemetrexed in the presence of thymidine. LKB1 expression was assessed by immunoblot. (C) Signaling downstream of mTORC1 is inhibited in LKB1-null carcinoma cells following pemetrexed. (D) AMPK activation mediates mTORC1 inhibition in LKB1-null cells. Inhibition of S6K1 phosphorylation by pemetrexed is prevented by 1 μ M compound C in H460 cells. (E-F) AMPK activation and ACC and eEF2 phosphorylation in cells of wild-type (HCT116) and -null (H460) LKB1 status following 1 μ M pemetrexed plus thymidine or 250 μ M AICAR for 15 hr.

Figure 4. Reactivation of mTORC1-dependent processes in response to relief of feedback inhibition of AKT activity following pemetrexed

(A) Time course of ZMP accumulation and AMPK α T172 phosphorylation in response to pemetrexed and thymidine in HCT116 cells. (B) mTORC1-dependent phosphorylation of 4E-BP1 is inhibited in pemetrexed-treated HCT116 cells, partially recovering by 48 hr (top panel). This is accompanied by substantial accumulation of hypophosphorylated 4E-BP1, which abundantly binds to a m⁷GTP sepharose bead (bottom panel). In the bottom panel, TdR and DMSO controls were for 24 hr. (C) Repression of S6K1 phosphorylation partially recovers following pemetrexed and thymidine treatment in HCT116 and LKB1-null H460 cells. (D) Recovery of mTORC1 signaling is concurrent with release of feedback inhibition of PI3K and AKT in HCT116 cells, as judged by phosphorylation of S6K1 and 4E-BP1. (E) AKT reactivation mediates recovery of mTORC1 signaling after pemetrexed. HCT116 cells were treated with pemetrexed and GSK690693 (100 nM) in the presence of thymidine for the denoted times. (F) The cytotoxicity of pemetrexed and GSK690693 in combination for

72 hr. Representative clonogenic survival plates are shown in the top panel and enumeration of colonies in the bottom panel.

Table 1
ZMP accumulates in human carcinoma cell lines following exposure to pemetrexed and thymidine *

Cell Line	Origin	ZMP (nmol / 10 ⁶ cells) $\approx$ mM	
		TdR	TdR + pemetrexed
HCT116	Colon	< 0.001	4.0 $\pm$ 0.31
H460	NSCLC	< 0.001	1.9 $\pm$ 0.18
MCF7	Breast	< 0.001	3.7 $\pm$ 0.04
WIDR	Colon	< 0.001	2.4 $\pm$ 0.29
TE85	Osteosarcoma	< 0.001	12.4 $\pm$ 1.74
A549	Lung	< 0.001	1.2 $\pm$ 0.05
OVCAR-8	Ovarian	< 0.001	1.8 $\pm$ 0.02
HELA	Cervical	< 0.001	1.3 $\pm$ 0.13
HT1080	Fibrosarcoma	< 0.001	3.8 $\pm$ 0.91
H526	SCLC	< 0.001	1.1 $\pm$ 0.19

* Exponentially growing cells were treated with pemetrexed (1 μ M) and thymidine (5.6 μ M) for 24 hours. ZMP was measured by HPLC from trichloroacetic acid extracts. 1mM ZMP is equivalent to 1 nmol ZMP in 1 μ l cell water. Results are presented as an average of 3 measurements.

Table 2
ZMP accumulates in human skin fibroblasts devoid of AICART activity

Cell Line	ZMP (mM)
AICART -/- HSF	1.16 ± 0.34
WI-38	< 0.001

AICART (-/-) fibroblasts or WI38 fibroblasts were cultured in medium containing dialyzed fetal bovine serum and harvested for ZMP measurements in mid-log growth.

Table 3
ZMP accumulates following pemetrexed and thymidine treatment in HCT116 cells transfected with AMPK α 1 siRNA

Transfection	ZMP (mM)
mock	3.05 ± 0.14
scrambled siRNA	3.67 ± 0.16
AMPK α 1 siRNA	1.91 ± 0.24

48 hours after transfection, cells were treated with pemetrexed (1 μ M) and thymidine (5.6 μ M) for 24 hours. ZMP was measured by HPLC from trichloroacetic acid extracts.