Pemetrexed Indirectly Activates the Metabolic Kinase AMPK in Human Carcinomas

Scott B. Rothbart, Alexandra C. Racanelli, and Richard G. Moran

Abstract

The chemotherapeutic drug pemetrexed, an inhibitor of thymidylate synthase, has an important secondary target in human leukemic cells, aminoimidazolecarboxamide ribonucleotide formyltransferase (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 AMP-activated kinase (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. Whereas 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 AMPKα T172 phosphorylation, pemetrexed also activated AMPK in carcinoma cells null for LKB1, the predominant AMPKα 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. Cancer Res; 70(24): 10299–309. ©2010 AACR.

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 this purpose (2–4). Pemetrexed and its metabo-

Authors’ Affiliation: Department of Pharmacology & Toxicology and the Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia

Current address for S.B. Rothbart: Department of Biochemistry and Biophysics and the Lineberger Cancer Center, University of North Carolina, Chapel Hill, NC 27599.

Corresponding Author: Richard G. Moran, Department of Pharmacology & Toxicology and the Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298.

©2010 American Association for Cancer Research.
Enhanced binding of AMP to the \( \gamma \)-subunit is seen as concentration rises through the \( \mu \)mol/L range, even in the presence of physiologic ATP concentrations (12). Activation of AMPK is thought to require phosphorylation of AMPK\( \alpha \) 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\( \beta \) in the presence of \( \mathrm{Ca}^{2+} \) or a calcium ionophore, but absence of AMP (19–21).

AMPK phosphorylates several controlling elements of lipid and energy metabolism, and also phosphorylates 2 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 2 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 2b (eIF2b) to its phosphorylated form, a necessary step in its recruitment to the cap-dependent translational preinitiation 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 article, 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, and 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 LKB1-null H460 and HeLa cells was confirmed by Western blotting. AICART(-/-) skin fibroblasts were a generous gift from Marie-Francoise Vincent (Universite Catholique de Louvain) and were maintained in Dulbecco's modified Eagle's medium supplemented with 15% FBS; experiments were performed in dialyzed serum. Experiments were performed on the AICART(-/-) fibroblasts within 2 passages after receipt. Pemetrexed disodium was obtained from Eli Lilly and Co. Unless otherwise noted, cells were treated with final concentrations of 1 \( \mu \)mol/L pemetrexed and 5.6 \( \mu \)mol/L thymidine. Cell proliferation experiments were evaluated by Coulter counting after 72 hours of continuous drug exposure. GSK690693 was purchased from SYNthesis Med Chem and was dissolved in dimethyl sulfoxide (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 (HPLC) using \( \mathrm{NH}_{4} \mathrm{H}_{2} \mathrm{PO}_{4} \) gradient elution on a SAX 250 \( \times \) 2.0 mm column (Phenomenex; ref. 7).

**Immunoblotting**

Total cellular protein was harvested and quantified as previously described (7). Typically, 20 \( \mu \)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. \( \beta \)-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\( \alpha \)I immunoprecipitates (IP) 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-\( \mu \)l bed volume of protein-G sepharose (Amersham) was first washed 3 times with IP buffer (26). Anti-AMPK\( \alpha \)I antibody (10 \( \mu \)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 \( \mu \)g lystate at 4°C. IPs were washed with cold IP buffer containing 1 mol/L NaCl followed by additional washes as described (26). IP was resuspended in 60 \( \mu \)l assay buffer [50 mmol/L HEPES (pH 7.4), 1 mmol/L dithiothreitol and 0.02% (v/v) Brij-35]. Typical reactions were performed in 25 \( \mu \)l assay buffer containing 0.2 mmol/L SAMS peptide, 0.2 mmol/L AMP, 0.2 mmol/L \( \gamma^{32P} \)-ATP (final specific activity \( \sim \)500 cpm/pmol), 5 mmol/L MgCl\( \_2 \), and immunoprecipitated AMPK\( \alpha \)I. Reactions were incubated at 37°C for 10 minutes and quenched by spotting onto Whatman P81 disks. Disks were washed 2 times in 1% (v/v) phosphoric acid, 1 time in water, and 1 time in acetone before being counted in scintillation fluid.

**RNA interference**

DharmaFECT transfection reagent no. 2, siGENOME SMARTpool siRNAs targeting human AICART, AMPK\( \alpha \)I, and nontargeting siRNA pool no. 1 were purchased from Dharmacon. Cells were transfected with 50 mmol/L siRNA with 0.1% DharmaFECT according to the manufacturer's instructions for 24 hours. Mock indicates incubation with
transfection reagent alone. Protein was harvested 72 hours after transfection unless noted.

m7GTP capture of 4E-BP1-eIF4E complexes
Following the indicated drug treatments, cells were lysed on ice for 30 minutes in IP buffer [25 mmol/L of HEPES (pH 7.5), 1% NP40, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonylfluoride, 0.1% 2-mercaptoethanol, and 1/2 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 hours at 4°C with rotation. m7GTP captured complexes were washed with IP buffer 4 times, resuspended in Laemmli sample buffer, and boiled for 5 minutes 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 per 60-mm dish and allowed to attach overnight. Cells were then treated continuously with media supplemented with 5.6 mmol/L thymidine containing 0 to 100 mmol/L combinations of GSK690963 and pemetrexed for 72 hours. Plates were washed with PBS, and fresh media supplemented with thymidine was added every 2 to 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 μmol/L, it accumulated to levels of 1 to 12 mmol/L in different carcinoma cell types because of 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 μmol/L thymidine, a salvageable source of thymidylate that prevented the effects of TS inhibition by pemetrexed. In contrast, treatment of HCT116 cells with 1 μmol/L 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 IPs from HCT116 cells using antibody against the α1-subunit of AMPK, implying that a posttranslational 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 nmol/L 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

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>ZMP (nmol/10⁶ cells) ≈ mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TdR</td>
</tr>
<tr>
<td>HCT116</td>
<td>Colon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>H460</td>
<td>NSCLC</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WIDR</td>
<td>Colon</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TE85</td>
<td>Osteosarcoma</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A549</td>
<td>Lung</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OVCAR-8</td>
<td>Ovarian</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HELA</td>
<td>Cervical</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HT1080</td>
<td>Fibrosarcoma</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HS26</td>
<td>SCLC</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Exponentially growing cells were treated with pemetrexed (1 μmol/L) and thymidine (5.6 μmol/L) for 24 hours. ZMP was measured by HPLC from trichloroacetic acid extracts. 1 mmol/L ZMP is equivalent to 1 nmol ZMP in 1 μL cell water. Results are presented as an average of 3 measurements.
(0.03–1 μmol/L; 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 AMPKαT172, likewise, incrementally increases over the same concentration range of pemetrexed (data not shown).

When HCT116 cells were transfected with pools of siRNAs directed against AICART, the effects of inhibition of the secondary target of pemetrexed were recapitulated (Fig. IC and D), confirming the intermediacy of AICART inhibition in the accumulation of ZMP and the activation of AMPK by pemetrexed. Surprisingly, the effect of approximately 95% knockdown of AICART on ZMP accumulation and AMPKαT172 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 μmol/L) indicated that the flow of ZMP through AICART becomes restricted by siRNA depletion of AICART (Fig. 1D), but ZMP accumulation even

---

**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 μmol/L pemetrexed in the presence of 5.6 μmol/L thymidine to offset the effects of TS inhibition. The phosphorylation of T172 on AMPKα was detected by Western blot (top), and the kinase activity of immunoprecipitated AMPKα1 was measured using SAMS peptide as a substrate (bottom). 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-hour exposure to pemetrexed and thymidine, and measured values in nmol/10⁶ cells were converted to mmol/L 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 μmol/L AICAR for 4 hours immediately before harvest. *, values that were below the limits of the assay (<0.001 mmol/L). For details, see Materials and Methods.
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(−/−) human skin fibroblasts], a more than 1,000-fold increase in ZMP was detected compared with 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 AMPKα ATP-binding site, compound C (29), blocked the hypophosphorylation of S6K1 at T389 induced by pemetrexed (Fig. 2B). Likewise, siRNA against the α1-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; Table 3). This AMPKα1-specific siRNA pool depleted all AMPKα detectable on immunoblots probed with an antibody that detected both AMPKα1 and α2, indicating that AMPKα2 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 10 cell lines tested were sensitive to the secondary effect of pemetrexed.

---

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ZMP (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICART(−/−) HSF</td>
<td>1.16 ± 0.34</td>
</tr>
<tr>
<td>WI-38</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AICART(−/−) fibroblasts or WI-38 fibroblasts were cultured in medium containing dialyzed fetal bovine serum and harvested for ZMP measurements in mid-log growth. Abbreviation: HSF, human skin fibroblasts.
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 AMPKα at T172 was not always a clear indicator of AMPK activity. Thus, in H460 cells, pemetrexed stimulation of AMPKα 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 and 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 and 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 mmol/L/hour up to 15 hours, and then remained elevated for at least 48 hours in HCT116 cells (Fig. 4A). AMPKα 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 AMPKα phosphorylation (Fig. 4A).

The time course of pemetrexed effects on 4E-BP1 and S6K1 phosphorylation (Fig. 4B and C, 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). Although 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-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 hours) 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

Table 3. ZMP accumulates following pemetrexed and thymidine treatment in HCT116 cells transfected with AMPKα1 siRNA

<table>
<thead>
<tr>
<th>Transfection</th>
<th>ZMP (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>3.05 ± 0.14</td>
</tr>
<tr>
<td>Scrambled siRNA</td>
<td>3.67 ± 0.16</td>
</tr>
<tr>
<td>AMPKα1 siRNA</td>
<td>1.91 ± 0.24</td>
</tr>
</tbody>
</table>

Forty-eight hours after transfection, cells were treated with pemetrexed (1 μmol/L) and thymidine (5.6 μmol/L) for 24 hours. ZMP was measured by HPLC from trichloroacetic acid extracts.

albeit to varying degrees. The sensitivity did not correlate with the level of ZMP measured in these cells (Table 1; 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 nmol/L) 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 because of the downstream signaling from activated AMPK rather than solely to inhibition of mTORC1.
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 nmol/L) for 72 hours. The genotypes (Sanger Institute Cancer Cell Line Project; http://www.sanger.ac.uk/genetics/CGP/CellLines/) are listed as mutant (M) for LKB1, PI3K, and KRAS. B, sensitivity of LKB1-null cell lines to growth inhibition after 72-hour 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 μmol/L compound C in H460 cells. E and F, AMPK activation and ACC and eEF2 phosphorylation in cells of wild-type (HCT116) and null (H460) LKB1 status following 1 μmol/L pemetrexed plus thymidine or 250 μmol/L AICAR for 15 hours.
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 AMPKα 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 hours (top). This is accompanied by substantial accumulation of hypophosphorylated 4E-BP1, which abundantly binds to an m7GTP sepharose bead (bottom). On the bottom, TdR and DMSO controls were for 24 hours. 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 nmol/L) in the presence of thymidine for the denoted times. F, the cytotoxicity of pemetrexed and GSK690693 in combination for 72 hours. Representative clonogenic survival plates are shown on the top and enumeration of colonies on the bottom.
was transient and partially recovered by 48 hours (Fig. 4C). The recovery of S6K1 phosphorylation occurred in spite of continued phosphorylation of AMPKα 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 to 24 hours and partially recovering by 48 hours, suggesting that the recovery of mTORC1 signaling also occurred in cells that 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 (Fig. 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 ALICRT by pemetrexed was synergistically enhanced by exposure to the AKT inhibitor (Fig. 4F), with nontoxic concentrations of pemetrexed plus thiuridine increasing the cell kill seen with 10 or 32 μmol/L GSK690693 (Fig. 4F).

**Discussion**

**Cellular requirements for AMPK activation**

ZMP or AMP binding to the AMPKα 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 toward some targets, for example, Raptor. Of note, AMPK activity was easily measurable in IPs 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 and 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 AMPKα T172 phosphorylation is strong (16, 17, 39). In H460 cells, the AMPKα 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 AMPKα (13–15), at least toward 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), whereas 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 2 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 in response to energy stress following rapamycin (34, 47). We note that the relief of feedback induced stimulation of AKT 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, for example, 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. Although 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 (Fig. 3E and 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 AICAR 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 (Fig. 3A). Whether AMPK activation offers a therapeutic advantage over direct mTOR inhibitors remains an open question.

In this article, we present the argument that activation of AMPK by ZMP accumulation following inhibition of AICAR 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 AICAR inhibitors represent a unique class of drugs different from the rapamycin analogs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Marie-Francoise Vincent (Universite Catholique de Louvain, Brussels, Belgium) for generously providing AICAR (—/—) fibroblasts, and Sandarwan Garg (Eli Lilly Research Laboratories) and Barton Kamen (Robert Wood Johnson Medical School, New Jersey) for helpful suggestions.

Grant Support

This study was supported in part by Grant R01-CA140416 (R.G. Moran) and Fellowship F30-IL094068 (A.C. Racanelli) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 05/27/2010; revised 08/17/2010; accepted 09/08/2010; published Online 12/15/2010.

References

AMPK Activation by Pemetrexed in Carcinoma Cells

Pemetrexed Indirectly Activates the Metabolic Kinase AMPK in Human Carcinomas

Scott B. Rothbart, Alexandra C. Racanelli and Richard G. Moran

Cancer Res 2010;70:10299-10309.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/70/24/10299

Cited articles
This article cites 51 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/24/10299.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/24/10299.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.