AMP-activated Protein Kinase (AMPK) Control of mTORC1 Is p53- and TSC2-independent in Pemetrexed-treated Carcinoma Cells

Background: The antifolate pemetrexed (PTX) and the nucleoside AICAR both activate AMPK via ZMP accumulation. AICAR-activated AMPK signals to both TSC2 and Raptor arms of mTORC1 control, whereas PTX-activated AMPK uses only the Raptor arm because p53-mediated transcription is not activated. The effects of the loss of the upstream tumor suppressor gene PTEN and mutation or amplification of oncogenic K-Ras, PI3K, and/or EGFR converge on mTORC1 through the activation of AKT (2–4). mTORC1 is a multicomponent complex that includes the catalytic subunit mTOR, an inhibitory subunit, PRAS40, and the Raptor subunit, which acts as a scaffold for the recruitment of the substrates for mTORC1, 4EBP1 and p70-S6K1, two proteins that are rate-limiting to cap-dependent protein synthesis initiation (5–7). Therapeutic agents targeting the catalytic site of mTOR kinase or mTORC1 separately, PI3K, AKT, or EGF receptor, or hybrid molecules targeting more than one of these steps have a central role in current molecularly targeted cancer chemotherapy (8–10). The pathways leading to and downstream of mTORC1 are subject to feedback control loops that are important to the refractoriness of some cancers to inhibitors of mTOR and related nodes of this pathway (11–13). The analysis of the effects of the drugs affecting these targets is pivotal to the understanding of these pathways and how they are perturbed in human carcinomas (13, 14).

Upstream oncogenic mutations activate mTORC1 kinase by at least two mechanisms: down-regulation/ inactivation of TSC2 and phosphorylation of the mTORC1 inhibitor PRAS40 by AKT (15–17). The TSC2 protein integrates signals from three pathways (AKT, ERK/RSK, and LKB1/AMPK); each of these pathways can lead to phosphorylation of several serines and threonines on TSC2. TSC2 in turn acts as a GTPase-activating protein for the small Ras-like protein, Rheb (15, 18–20). Whereas Rheb bound to GDP is thought to be inactive, Rheb-GTP is an essential activator for mTORC1 kinase (19). Phosphorylation of PRAS40 by AKT weakens the binding of PRAS40 for mTORC1 and stimulates mTORC1 kinase (16).

AMP-activated protein kinase (AMPK) regulates cap-dependent translation initiation and glucose and lipid metabolism in response to intracellular energy levels (21, 22). Under energy limitation conditions or decreases in the ATP/AMP ratio, AMP binds to the AMPK γ subunit leading to the sustained phosphorylation of the α-subunit at Thr-172, activating the kinase activity toward several substrates (21–24). Two phos-
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Phosphorylation events catalyzed by AMPK oppose the activating effects of AKT on mTORC1 kinase: TSC2 Ser-1387 phosphorylation, thought to decrease the Rheb-GTP-dependent activation of mTORC1 (15, 17, 19, 20, 25), and Raptor Ser-792 phosphorylation, which inhibits mTORC1 kinase activity directly (26). The stimulation of the GTPase activity of Rheb by AMPK-phosphorylated TSC2 is incompletely understood but appears to involve the dissociation of TSC2 from the lysosomal compartment (29). Likewise, sestrins limit mTORC1 is mediated by the dissociation of TSC2 from the lysosomal membranes (28). The recruitment of protein(s) (27). mTORC1 is present as a large complex under these conditions of limited TSC2 function, and hyperactivated mTORC1 due to robust p53-independent phosphorylation of Raptor.

Experimental Procedures

Cell Culture and Reagents—HCT116 cell lines, a gift from Dr. Bert Vogelstein, were used within eight passages from frozen stocks. Cells were grown in RPMI 1640 with 10% diazylated FBS within eight passages after thawing. Immortalized p53-/- TSC2-/- MEFs and Sestrin2-/- MEFs, generous gifts from Dr. Andrei Budanov, were grown in DMEM supplemented with 10% diazylated FBS. Experiments were performed in diazylated FBS. Unless otherwise noted, cells were treated with 1 μM PTX (Eli Lilly and Co.) and 5.6 μM thymidine. Cell proliferation was evaluated by Coulter counting after 72 h of continuous drug exposure. Etoposide (VP16), G66976, SCH900776, PV1019, and Ku60019 were dissolved in dimethyl sulfoxide. AICAR was purchased from Toronto Research Biochemicals, PV1019 from EMD Millipore, and SCH900776, Ku60019, and G66976 from Selleckchem. All other reagents were from Sigma-Aldrich.

Immunoblotting—Total cellular protein was extracted and immunoblotted, typically using 20 μg of protein as described (38, 39). Chemiluminescence was detected using SuperSignal substrate kits (Pierce). p53 and p21 antibodies were from Calbiochem. HDFM2, BAX, and Sestrin2 antibodies were from Santa Cruz Biotechnology. β-Actin primary antibody was from Abcam. All other primary antibodies were from Cell Signaling Technology.

RNA Interference—DharmaFECT 2 transfection reagent was purchased from Dharmacon. The siRNA pool against TSC2 was made by mixing Qiagen S10001718, S10327339, S100011697, and S100011711 in equal proportions. Cells were transfected with 50 nM siRNA in 0.1% DharmaFECT for 36 h (TSC2). The transfected cells were treated with drugs for an additional 24 h and then harvested.

qPCR—Total RNA was isolated using TRIzol (Invitrogen). DNase treatment, cDNA synthesis followed the manufacturer’s protocol and real-time PCR were as described (29, 36). Values were normalized to the expression of GAPDH. Experiments were performed in triplicate.

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taining a ratio of 1:3 of DNA:Polyjet. After 24 h, each well was split into duplicate wells for thymidine (TdR) and PTX treatments. After 12 h, cells were treated with TdR or PTX/H11001TdR, and lysed for immunoblot analysis 24 h after treatment. Vectors used for transfection pBABE-myc Raptor (ID-18116) and pBABE-myc Raptor Ser722Ala/Ser792Ala (ID-18117) were originally made in the laboratory of Dr. Reuben Shaw (26) and were purchased from Addgene, as was the pBABE Hygro-empty vector (ID-1765).

Clonogenic Survival Assay—Cells plated in triplicate from a single cell suspension at 100/60 mm dish were allowed to attach overnight and then treated continuously with media supplemented with drugs for 72 h. Plates were washed with PBS, and fresh medium was added every 2–3 days until control colonies were easily counted (typically 7–10 days). When rescue agents (hypoxanthine, 32 μM, thymidine, 5.6 μM) were included, they remained in the media throughout the duration of the experiment. Plates were then washed with PBS, fixed with methanol, and stained with Wright-Giemsa reagent. All visible colonies were counted manually.

Results

Differences in AMPK Signaling Activated by PTX and by AICAR—AICAR is converted to the AMPK-activating nucleotide ZMP in several human cancer cell lines (38, 42, 43). Likewise, we have shown previously (38, 39) that interruption of de novo purine synthesis by the antifolate PTX causes a substantial accumulation of ZMP with subsequent activation of AMPK signaling in lung and colon cancer cell lines. In previous studies from this laboratory (38, 39), the sustained and substantial inhibition of mTORC1 by PTX has been demonstrated to be due to activation of AMPK using siRNA against AMPK/1 and also using the AMPK inhibitor, Compound C. In those studies, we used TdR in the culture medium to circumvent the primary effect of PTX on thymidylate synthase and thus to isolate PTX effects on AMPK activation. At concentrations of AICAR (250 μM) or PTX (in the presence of TdR) (1 μM), which are equi-inhibitory to HCT116 cell growth, the activation of AMPK is equivalent as judged by the phosphorylation of AMPKα-Thr172 (Fig. 1A). Activated AMPK has been shown to inhibit
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mTORC1 signaling by two mechanisms: phosphorylation of TSC2 at Ser-1387, which diminishes contact of mTORC1 with an obligatory activator Rheb-GTP (19), and the inhibitory phosphorylation of the mTORC1 subunit Raptor at Ser-792 (26). AMPK activation in AICAR- or PTX-treated WT (p53+/+) HCT116 cells results in robust phosphorylation of Raptor (Fig. 1B). Activation of AMPK by AICAR also results in both phosphorylation of TSC2 at Ser-1387 and an increase in cellular TSC2 levels in HCT116 cells (Fig. 1B). Surprisingly, the activation of AMPK by PTX did not stimulate phosphorylation of TSC2 Ser-1387, nor did it result in higher cellular TSC2 levels in WT HCT116 cells (Fig. 1B). The phosphorylation of TSC2 was not seen after any of the concentrations of PTX (+TdR) studied (Fig. 1D), nor was it observed over a time course as long as 48 h (Fig. 1E). When these signaling events initiated by AICAR and PTX were compared in a single experiment, PTX-activated AMPK signaling involved a higher level of Raptor phosphorylation than seen with AICAR at equi-inhibitory concentrations (Fig. 1, B, D, and E), and the effectiveness of AICAR reflected phosphorylation events at both Raptor and TSC2. Whereas TdR prevented the effects of PTX on thymidylate synthase, it was not causative of the defect in signaling from PTX-activated AMPK to TSC2, as evidenced by the fact that the inclusion of TdR with AICAR did not alter the phosphorylation of TSC2, the accumulation of TSC2, or the phosphorylation of Raptor (Fig. 1C). Hence, we concluded that signaling downstream of AMPK activated by AICAR differed from that initiated by PTX in that PTX did not cause AMPK signaling to TSC2.

Dependence of Signaling by AMPK on p53—Given that both PTX and AICAR activate AMPK by causing the accumulation of ZMP (36, 38, 39), the differences in downstream signaling from AMPK activated by the two drugs were perplexing. The solution to this dilemma was suggested by the observation that phosphorylation of TSC2 was not seen after either AICAR or PTX in HCT116 cells null for p53 (Fig. 1B). That is, AMPK phosphorylation of TSC2 was p53-dependent, apparently subsequent to the much lower levels of TSC2 present in the absence of p53. In contrast, both the expression of Raptor and the phosphorylation of Raptor Ser-792 by AMPK were independent of p53 (Fig. 1B). Because the activation of AMPK by either PTX or AICAR was not affected by the loss of p53 (Fig. 1A), it was clear that the differences in TSC2 activation seen in Fig. 1B were not due to AMPK.

Role of p53 Transcription in the Differences in Signaling from AMPK Activated by PTX and by AICAR—The deficit of TSC2 in p53 null cells (Fig. 1B) agrees with prior studies demonstrating that TSC2 is a transcriptional target of p53 (31, 32). This, in turn, suggests that the increment in TSC2 seen in AICAR-treated HCT116 cells (Fig. 1B) reflects stabilization of p53 followed by increased transcriptional activation by the stabilized p53. Indeed, p53 levels were increased in cells treated with both AMPK-activating agents, AICAR and PTX (+TdR), as well as in cells treated with the strong DNA-damaging agent VP16 (Fig. 2A). The levels of the p53 target proteins p21, Sestrin2, HDM2, and BAX were also enhanced by AICAR in a pattern nearly identical to that seen after treatment with VP16 (Fig. 2A), and AICAR also induced

![FIGURE 2. PTX does not activate TSC2, because it causes a deficiency in p53 transactivation.](image-url)

In this experiment, the level of p53 in each treatment was first estimated by immunoblotting, and then a second gel was loaded with different volumes of lysate to allow equal loading with p53. This approach allowed estimation of stoichiometry of modifications by inspection.
AMPK Activation and Phosphorylation of Chk1 and Chk2—
The N-terminal posttranslational phosphorylations of p53 following DNA damage have been associated with the action of several kinases, ATR and ATM kinases and their downstream effector kinases Chk1 and Chk2, which are thought to be central to the p53 response to DNA damage (46, 47). The phosphorylation of Chk1 did not occur after either AMPK activator but was robust after VP16-induced DNA damage (Fig. 3A). However, phosphorylation of Chk2 was observed in both treatments associated with p53 transcriptional activation, DNA damage by VP16 and AMPK activation by AICAR, but not following treatment with PTX (with TdR) (Fig. 3A). Hence, p53-dependent transcription following activation of AMPK by AICAR is associated with phosphorylation of Chk2 and phosphorylation of p53 Ser-15, whereas PTX activates neither phosphorylation of Chk2 and p53 Ser-15 nor p53-dependent transcription. The phosphorylation of Chk2 after VP16 or AICAR treatment appeared to indicate the activation of ATM in these cells. This concept was strengthened by the fact that Ku60019, an inhibitor of ATM (48), prevented the phosphorylation of Chk2 and Ser-15 on p53 after VP16 or AICAR treatment (see below).

It was of interest to determine whether the activation of AMPK would trigger events downstream of ATM activation in the DNA damage response. Phosphorylation of histone H2A.X was followed after AMPK activation and compared with the time course of DNA damage with VP16. H2A.X phosphorylation was robustly induced by as little as 3 h of exposure to VP16 and was maintained for at least 24 h (Fig. 3B). This time course coincided with the occurrence of robust Chk2 phosphorylation and equally robust and sustained phosphorylation of p53 Ser-15, whereas Chk1 phosphorylation was more delayed in onset, as was the accumulation of p21. Interestingly, after AICAR treatment, H2A.X phosphorylation was also detected on a time scale coincident with Chk2 and p53 Ser-15 phosphorylation and p21 transcription (Fig. 3B). Notably, the phosphorylations of H2A.X, p53 Ser-15, and Chk2 were less pronounced after AICAR than after VP16. The time course of these events in cells treated with PTX + TdR indicated that three phosphorylation events apparently caused by ATM, namely H2A.X, Chk2, and p53 Ser-15 phosphorylation, did not occur nor did the p53-dependent transcription of the p21 gene at any time point studied (Fig. 3B). We concluded that the difference between AICAR and PTX activation of AMPK reflected an ATM activation and Chk2 phosphorylation induced by AICAR as a late event, whereas this response did not occur with PTX when TdR was present. In addition, we concluded that AMPK activation per se, as exemplified by PTX treatment in the presence of TdR, stimulated the stabilization of p53 but did not activate the signaling needed to activate p53 transcription. Notably, the activation of AMPK by PTX did not result in phosphorylation of p53 Ser-15, in contrast to the results of prior in vitro kinase assays that suggested p53 Ser-15 as a target residue for AMPK (49). We concluded that, under PTX (+TdR) treatment, transactivation of p53 was absent due to the lack of an ATM response, as evidenced by downstream signaling to Chk2 and p53 Ser-15.

Modification of the Cellular Response to PTX by Thymidine—
The inclusion of TdR in the culture medium allows both the activation of AMPK and the inhibition of thymidylate synthase but prevents the sequela of thymidylate synthase inhibition due to misincorporation of dUMP into DNA with subsequent excision from DNA by uracil-DNA glycosylase (50). As a control to determine whether TdR alters the effects of AMPK activation by PTX, we compared the effects of AICAR with and without TdR. The addition of TdR did not change the stabilization of p53, the accumulation of pH2A.X, nor the transactivation of p21 caused by AICAR (Fig. 3C). When TdR was not added, the full effect of PTX germane to clinical use of this drug was seen. Under these conditions, the phosphorylation of H2A.X was observed in PTX-treated cells, p53 was stabilized, and Ser-15 of p53 was heavily phosphorylated (Fig. 3D). Chk1, but not Chk2, phosphorylation was seen, suggesting that ATR was activated but ATM was not. Remarkably, p21 transcription again was not observed, a critical clue to the mechanism of these effects (Fig. 3, D and
The lack of transcription of p21 was confirmed by RT-qPCR analysis, which also demonstrated that p53 transcription after PTX without TdR was almost completely quiescent for other p53 targets genes, namely, MDM2 and PUMA, and was minimal for PIG3 and BAX (Fig. 3, D and E). We concluded that the PTX-induced inhibition of DNA synthesis caused the activation of ATR and the downstream Chk1 but not Chk2 phosphorylation and that, as a result, p53 transcription was not activated. This interpretation is consistent with the fact that PTX-treated cells entered S-phase but experienced substantially prolonged and unsuccessful S-phase traverse, whereas the cells treated with PTX in the presence of thymidine were arrested in G1 and never entered S-phase (51).

PTX Blocks AICAR-mediated Transactivation of p53—We sought to understand whether PTX would interfere with p53 transactivation initiated by AICAR. HCT116 cells were treated with PTX (∼Tdr) for 20 h, a period sufficient to bring on the effects of this drug, and then challenged with AICAR for 15 h. Whereas cells treated with AICAR alone showed increased levels of H2A.X, Chk2, and p53 Ser-15 phosphorylation followed by induction of p21, hMDM2, PUMA, PIG3, and BAX mRNA (∼S.D., where n = 3 independent experiments; **, p < 0.001; and *, p < 0.045, relative to controls) (right panel), or RT-qPCR analysis of steady state mRNA levels was performed for the indicated genes (∼S.D., n = 3; **, p < 0.002 for the indicated comparisons) (right panel).
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**FIGURE 4.** PTX-mediated mTORC1 inhibition is independent of TSC2 or Sestrin2. A, p53^{+/+} and p53^{-/-} HCT116 cells were treated with either TdR (5.6 μM) or PTX (1 μM) + TdR for 24 h. Cells were harvested followed by immunoblotting to assess phosphorylation of AMPK and S6K1. B, cell extracts were also used in m^7^-GTP-Sepharose bead immunoabsorption assays to analyze the binding of 4EBP1 after PTX treatment. The lysates were subjected to immunoblot analysis for pThr389 of S6K1 we detected a higher level of pThr70 4EBP1 and 4EBP1 as indices of mTORC1 activity. WT and p53 null HCT116 cells transfected with TSC2 siRNA for 36 h (Fig. 4, A and B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B). By probing the lysates for pThr389 of S6K1 we detected a higher level of mTORC1 activity in untreated p53 null HCT116 cells compared with p53 WT isogenic HCT116 (Fig. 4A, compare lanes 1 and 3), as expected from the loss of TSC2 in p53 WT null HCT116 cells (Fig. 4B).

**Effects of PTX on mTORC1 Activity in p53 Null Cells**—We questioned whether the loss of p53 would compromise the downstream effect of PTX-activated AMPK on mTORC1 activity. Treatment of HCT116 cells indicated that the mTORC1 phosphorylation of S6K1 and 4EBP1 was inhibited by PTX-activated AMPK whether or not the cells had p53 function (Fig. 4, A and B). Thus, probing immunoblots of HCT116 cell lysates for pThr70 4EBP1 indicated an equivalent inhibition of mTORC1 kinase activity toward this substrate in p53 WT (+/+) or null (p53^{-/-}) HCT116 cells (Fig. 4B). Hence, although PTX activation of AMPK inhibited mTORC1 whether or not p53 was expressed, complete suppression of mTORC1 by AMPK appears to be more difficult in the absence of p53.

**Role of TSC2 and Sestrin2 in the Response to PTX-activated AMPK**—With the loss or mutation of p53, the transcription of several p53 targets was decreased (Fig. 1B and Ref. 32), at least two of which, TSC2 and Sestrin2, have been implicated in AMPK control of mTORC1 (17, 27). The effectiveness of PTX-activated AMPK against mTORC1 was determined in cell systems that allowed the separation of the roles of TSC2, p53, and Sestrin2. Sestrin2 is known to mediate the interaction of AMPK with mTORC1 (27) and to suppress the localization of mTOR to lysosomal membranes, the site of mTORC1 activity (28, 54). It was clearly noted that the level of phosphorylation of S6K1 was higher and the binding of 4EBP1 to m^7^-GTP-Sepharose beads was lower in PTX-treated HCT116 cells null for p53 than were these indicators of mTORC1 activity in untreated p53 WT HCT116 (Fig. 4, A and B). Hence, treatment of HCT116 cells null for p53 with PTX suppressed the phosphorylation of S6K1 down to equivalent levels in Sestrin2 null WT MEFs (Fig. 4C, right panel). MEF cells from mice that were genetically null for TSC2 or Sestrin2 were also studied; the deletion of the TSC2 gene can only be made in a p53^{-/-} background because TSC2 loss is embryonic lethal in p53 WT mice. These TSC2 null MEFs represent a more complete depletion of TSC2 function than in

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3 The mechanism whereby carcinoma cells that are null for p53 or that have DNA binding domain p53 mutations have higher mTORC1 activity has been shown to involve a lower TSC2 and higher Rheb distribution to lysosomal membranes and higher mTOR in mTORC1 complexes (32).
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HCT116 cells null for p53. PTX suppressed the phosphorylation of S6K1 in these TSC2−/−, p53−/− MEFs as well as in control WT TSC2 p53−/− MEFs, again despite the fact that mTORC1 activity was higher in the TSC2 null MEFs (Fig. 4C, left panel). Nevertheless, we concluded that TSC2 deficiency did not change the effectiveness of PTX-activated AMPK on mTORC1.

Because these experiments involved both a p53 null genotype cell and null (Fig. 4C) or deficient expression of TSC2 (Fig. 1B), p53 WT HCT116 cells were subjected to siRNA knockdown of TSC2 to separate the effects of a TSC2 deficiency from those resulting from the loss of p53. TSC2 was substantially diminished in these knockdown experiments, and concomitantly, mTORC1 kinase activity was enhanced, as judged by the phosphorylation of both S6K1 and of 4EBP1 (Fig. 4D). However, PTX activation of AMPK was still effective at suppressing mTORC1 kinase activity toward both substrates (Fig. 3D). Hence, it appeared that with a deficiency or complete absence of TSC2 or Sestrin2, or with a p53 loss-induced deficiency of TSC2 and Sestrin2, the activation of AMPK by PTX was still quite effective at suppressing mTORC1.

Phosphorylation of Raptor by PTX-activated AMPK Is Sufficient to Suppress mTORC1 Kinase—We questioned whether the robust p53-independent phosphorylation of Raptor seen in PTX-treated cells (Fig. 1, C–E) was sufficient for suppression of mTORC1 in the absence of TSC2 function or p53 function. p53−/− TSC2−/− MEFs were transfected with an expression vector encoding wild type human Raptor or a construct for Raptor in which the two amino acids phosphorylated by AMPK (serines 722 and 792) were mutated to alanines (AA Raptor (26)). The vectors carrying WT or AA Raptor resulted in the expression of recombinant Raptor in substantial excess over endogenous Raptor levels (Fig. 5A). Raptor was phosphorylated at Ser-792 in empty vector-transfected cells after treatment with PTX and even more so in PTX-treated cells transfected with WT Raptor, but cells transfected with AA Raptor and treated with PTX showed strong suppression of Raptor pSer792 (Fig. 5A). We concluded that the Raptor expressed from transfected Raptor constructs competed successfully with endogenous Raptor for binding to mTORC1 complexes. Treatment of MEFs transfected with empty vector or WT Raptor with PTX resulted in the suppression of mTORC1 kinase activity, as seen by lower levels of S6K1 pSer389, and 4EBP1 pThr70. However, neither of these phosphorylation events was suppressed in AA Raptor-transfected cells after treatment with PTX (Fig. 5A).

Hence, we concluded that the suppression of mTORC1 following PTX treatment was mediated by AMPK phosphorylation of Raptor and that, in cells in which endogenous Raptor in mTORC1 complexes was replaced by a mutant Raptor incapable of AMPK-mediated phosphorylation, mTORC1 suppression after PTX treatment did not occur. It appeared that AMPK phosphorylation of Raptor was necessary and sufficient to suppress mTORC1 in the absence of TSC2.

This same experiment was performed in HCT116 cells WT or null for p53 to determine whether mTORC1 suppression by PTX-stimulated phosphoRaptor was modified by TSC2 or p53 (Fig. 5B). Again, transfection of a WT Raptor or an AA Raptor construct into HCT116 cells appeared to allow the replacement of transfected Raptor for the endogenous Raptor in mTORC1 complexes. PTX treatment of cells transfected with WT Raptor suppressed mTORC1-mediated S6K1 phosphorylation as well as 4EBP1 phosphorylation, but cells transfected with the AA Raptor construct did not show suppression of either phosphorylation event. Notably, the same effects were seen in p53 null HCT116 cells, despite the much higher levels of mTORC1 activity in these cells (Fig. 5B). We concluded that Raptor phos-

FIGURE 5. Phosphorylation of Raptor is necessary and sufficient for inhibition of mTORC1 by PTX-activated AMPK. p53−/− TSC2−/− MEFs (A) or WT and p53 null HCT116s (B) were transfected with pBABE-Hygro or with this vector containing WT or AA Raptor for 36 h and then subsequently treated with TdR or PTX (+TdR) for 24 h. Immunoblots for pThr389 S6K1 and pThr70 4EBP1 were used as an index of mTORC1 activity. C, p53−/− TSC2−/−, and p53−/− TSC2−/− MEFs were treated with TdR (5.6 μM), PTX (1 μM) + TdR, or AICAR (250 μM) for 24 h followed by immunoblotting to analyze pThr389 S6K1 levels as an indicator of mTORC1 activity after these treatments.
phorylation by PTX-activated AMPK had suppressed mTORC1 and that the loss of neither p53 nor TSC2 changed this outcome.

**TSC2 Phosphorylation by AMPK Plays a Role in the Suppression of mTORC1 by AICAR**—Given the suppression of mTORC1 caused by PTX-activated AMPK in the absence of TSC2 function (Figs. 4D and 5A), it was of interest to determine whether the TSC2 control pathway played a role in mTORC1 suppression by AICAR or whether the Raptor arm of this control system was again sufficient with this compound. To test this concept, MEFs null for TSC2 were treated with AICAR, and mTORC1 phosphorylation of S6K1 was used to judge the effectiveness of the Raptor arm of this control circuit. Under these conditions, as expected, PTX-treated cells showed an equivalent suppression of mTORC1 that was independent of TSC2 (Fig. 5C). AICAR treatment, however, suppressed the phosphorylation of S6K1 in MEFs that had TSC2 function but not in TSC2 null cells. Hence, at the dose of AICAR used in these experiments, the suppression of mTORC1 in MEFs was dependent on both TSC2 phosphorylation and Raptor, in contrast to PTX.

**p53 Independence of Growth Inhibition by Pemetrexed**—The phosphorylation of both 4EBP1 and S6K1 was inhibited by PTX-activated AMPK in p53 WT or null HCT116 cells (Fig. 4, A and B). However, the higher activity of mTORC1 clearly made it more difficult to suppress S6K1 phosphorylation in p53 null cells, so that the level of PTX-inhibited pS6K1 in p53 null HCT116 cells was, in fact, substantially higher than the pS6K1 in untreated HCT116 cells with WT p53 (Fig. 4A). Hence, it was unclear whether p53 status would affect the therapeutic effects of PTX. When we compared growth inhibition by PTX in HCT116 cells with p53 null for p53, the growth inhibition in the presence of TdR (thought to be due to AMPK activation) was identical between the two cell lines; growth inhibition in the absence of TdR, which would more accurately reflect clinical circumstances and which also involved the sequelae of inhibition of thymidylate synthase, was also identical in HCT116 cells and their isogenic p53−/− equivalents (Fig. 6A). Interestingly, HCT116 cells with p53 function were more sensitive to growth inhibition by AICAR than were HCT116 p53 null cells (Fig. 6B). The same patterns were also seen in MEFs WT and null for p53 (Fig. 6, C and D), and MEFs that were null for TSC2 and/or p53 were equally as sensitive to PTX plus TdR as the WT MEFs (Fig. 6C). However, both p53−/− TSC2−/− and p53−/− TSC2−/− MEFs were less sensitive to AICAR than were the WT MEFs (Fig. 6D). We concluded that growth inhibition of cells with PTX was p53-
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independent but cells that were WT for p53 were more sensitive to AICAR because the p53-dependent activation of the TSC2 control mechanism between AMPK and mTORC1 played a role in AICAR effects but not PTX effects.

PTX Activation of AMPK Is Cytostatic, but PTX Activation of AMPK with Thymidylate Synthase Inhibition Is Cytotoxic—Very interestingly, colony formation assays indicated that the AMPK activation by PTX was not cytotoxic, but the combination of AMPK activation with thymidylate synthase inhibition was highly cytotoxic (Fig. 6E). Thus, 1 μM PTX suppressed colony formation by >95%, and 10 μM completely prevented the appearance of colonies. The addition of TdR to the PTX-treated cells decreased the size of the colonies, but the number of colonies was identical to control even at 10 μM PTX. The addition of both hypoxanthine (which reinstates feedback control on purine synthesis and prevents ZMP accumulation (38)) and TdR to even 10 μM drug prevented growth inhibition by PTX, and colony size and number were unchanged from controls.

Model for the Biochemical Differences Downstream of AMPK Activation by AICAR and PTX—The effects of AICAR reflect the activation of AMPK by ZMP (Fig. 7D). ZMP accumulation also occurs in PTX-treated cells, again activating AMPK; this effect is most easily studied by circumventing the effects of PTX on thymidylate synthase, although it occurs with or without TdR. Both AMPK activators inhibit mTORC1, AICAR by canonical effects (phosphorylation of both TSC2 and of Raptor), whereas PTX only phosphorylates Raptor, because PTX prevents the p53-driven transcription of TSC2 and Sestrin2, as shown in the model (Fig. 7D, top).

Probing the Mechanism of Suppression of p53 Transcription by PTX—We sought to determine whether the block of p21 transcription in PTX-treated cells was caused by the lack of Chk2 phosphorylation or of p53 Ser-15 phosphorylation. Firstly, it was not clear whether p53 Ser-15 phosphorylation in AICAR-treated cells was mediated by Chk2 or ATM. Co-treatment with the specific Chk2 inhibitor PV1019 (55) and AICAR indicated partial, but incomplete, suppression of p53 Ser-15 phosphorylation (Fig. 7A), but complete suppression was observed with doses of Ku60019, which suppressed both ATM and ATR (Fig. 7B). In these experiments, inhibition of Chk2 was followed by the suppression of the autophosphorylation of Chk2 at Ser-516. Hence, we concluded that both ATM and Chk2 had caused p53 Ser-15 phosphorylation. PV1019 also interfered with the induction of p21 (Fig. 7A), suggesting that Chk2 phosphorylation was essential for p53 transcription after AICAR treatment.

Secondly, the role of Chk1 activation in phosphorylation of p53 Ser-15 and suppression of p21 phosphorylation by PTX (without TdR) was probed with either of two Chk1 inhibitors, Gö6976 or SCH900776. Co-treatment with either of these Chk1 inhibitors did not block p53 Ser-15 phosphorylation in PTX (Fig. 7C), ruling out a direct phosphorylation by Chk1 under these conditions. Ku60019 did block p53 Ser-15 phosphorylation following PTX (Fig. 7B). Hence, it was most likely that the phosphorylation of p53 Ser-15 was being catalyzed by upstream kinases, probably ATM, ATR, or DNA-PK. Interestingly, we noted that the use of Chk1 inhibitors in PTX-treated cells allowed the activation of Chk2 (Fig. 7C), probably explaining the enhanced phosphorylation of p53 Ser-15 seen in cells treated with Gö6976 or SCH900776 and PTX.4

Thirdly, inhibition of Chk1 by either Gö6976 or SCH900776 resulted in p21 transcription in the presence of PTX (Fig. 7C), apparently relieving the block on p53-dependent p21 transcription. This pivotal observation appears to be the same as that reported by Prives and colleagues (57) in which Gö6976 treatment prevents a hydroxyurea block of p53-dependent p21 transcription. The fact that Chk1 inhibitors allowed p21 transcription coincided with the appearance of activated Chk2 (Fig. 7C) strengthened the correlation between Chk2 activity and p21 transcription. Hence, we concluded that the activity of Chk1 interfered with p53-dependent transcription of the p21 gene, and that circumventing the Chk1-induced S-phase block with inhibitors allowed p53-dependent p21 transcription coincident with the activation of Chk2.

In summary, we note that the p53-dependent transcription of p21 coincided with the occurrence of Chk2 activation in the several circumstances we encountered in this study. After treatment with VP16 or AICAR, both Chk2 phosphorylation and p21 transcription occurred; after PTX without TdR, neither Chk2 phosphorylation nor p21 transcription was observed. When Chk1 activation was suppressed with inhibitors in PTX, Chk2 activation reappeared and so did p21 transcription. We propose that activated Chk2 is required for p53-dependent transcription (Fig. 7D, bottom).

Discussion

Pemetrexed—PTX has been a clinically valuable and biochemically interesting drug since early in its development. It was first synthesized as an analog of lometrexol, a specific inhibitor of glycinamide ribonucleotide formyltransferase (40, 58), but the modifications in structure of PTX compared with lometrexol changed the primary cellular target away from glycinamide ribonucleotide formyltransferase to thymidylate synthase (40, 41). Yet, it clearly had another target or targets that initially appeared to involve several folate-dependent enzymes (41) (hence, the trade name Alimta, derived from multiply targeted antifolate) but was later clarified as AICART (38), the second folate-dependent enzyme in the purine synthesis pathway. The nucleotide substrate for AICART, ZMP, does not appear to show appreciable feedback in the early steps of this pathway, so that when AICART is inhibited by PTX, purine synthesis continues and ZMP levels rise from submicromolar to a sustained level as high as 15 mM in some cell lines (39). Such ZMP levels caused robust activation of AMPK, which would help to explain why PTX has found a central role in the therapeutics of lung cancers, given that several of the mutations/losses that are causative of lung carcinogenesis are upstream of AKT and result in hyperactive mTORC1. Activated AMPK can be viewed as a naturally occurring control system that limits, among other things, the activity of mTORC1 (21).

4 Alternatively, it might reflect decreased activity of the Chk1-activated protein phosphatase 2A (PP2A) (56).
The most frequent genetic change in lung cancers is the loss and/or mutation of p53 (33). We have recently reported that p53 null carcinoma cells, or those expressing DNA-binding domain mutations in p53, have a substantially enhanced mTORC1 kinase due to suppression of transcription of TSC2.
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and resultant changes in lysosomal TSC2 and Rheb (32). The hyperactivity of mTORC1 in p53 null cells is clearly seen in the untreated controls in Fig. 4, A and B. The effect of PTX on AMPK signaling is related to the changes we observed in this pathway (32) with the loss of p53, in that PTX-activated AMPK did not activate the TSC2 control arm connecting AMPK and mTORC1 because of a drug-induced defect in p53-dependent transcription. Despite the fact that PTX renders tumor cells functionally null with respect to p53 transactivation, the p53-independent phosphorylation of Raptor by PTX-activated AMPK was sufficient to suppress mTORC1 activity and inhibit cell growth. When the effects of inhibition of thymidylate synthase by PTX are allowed to progress in addition to the activation of AMPK, the potency of PTX to suppress proliferation is enhanced, and the agent becomes highly cytotoxic. Hence, PTX, as a single agent, has elements of a molecularly targeted drug, suppressing the downstream activation of mTORC1 caused by the mutations common in lung cancers, while also stimulating the apoptotic response typical of cytotoxic drugs. However, the potency of PTX is not affected by loss of p53, and one would predict equivalent therapeutic activity in p53–competent and –deficient tumor cells.

Activation of p53 by AMPK Activators—The difference in signaling to p53 after the two AMPK activators, AICAR and PTX, would appear to be due to the activation of ATM elicited by AICAR, manifest by Chk2, H2A.X, and p53 Ser-15 phosphorylation. The lack of an effective p53 transcriptional response in cells in which PTX activates AMPK suggests that AMPK activation per se does not initiate p53-dependent transactivation. The mechanism of stabilization of p53 by events initiated by PTX remains to be determined.

The shift in the effects of PTX upon inclusion of TdR in the culture media is quite dramatic. When PTX (in the absence of TdR) caused both AMPK activation and thymidylate synthase inhibition, a robust phosphorylation of histone H2A.X is induced, a response most often associated with DNA damage, but which is also a response to replication delay (59), and PTX becomes cytotoxic. PTX without TdR causes cells to undergo a markedly slower S-phase (51, 60), explaining the phosphorylation of H2A.X apparently catalyzed by ATR. In the presence of TdR, the effects of thymidylate synthase on DNA synthesis and thymidylate nucleotide pools are prevented, cells are blocked from entering S-phase in a G1 block (51), and PTX is only cytostatic, albeit potently so. This is reminiscent of the effects of the rapamycin analogs, which can be cytostatic and are not cytotoxic to most tumor cell lines in vitro (12, 61–63).

Inactivation of p53 Transactivation by Pemetrexed—The reason that PTX-stabilized p53 does not stimulate transcription from p53 requires explanation. The activation of Chk1, an index of ATR kinase activity (47), with subsequent phosphorylation of p53 Ser-15 and histone H2A.X, was observed following treatment with PTX without TdR, even though p21 transcription was not observed. Notably, Chk2 phosphorylation did not occur with PTX, even when the suppression of DNA synthesis was allowed by exclusion of TdR. The Chk2 activation by AICAR (and VP16) most likely reflects the activation of ATM (47). Chk2 phosphorylation, and therefore presumably ATM activation, was the biochemical event most directly correlated with the transcription activity of p53 in these experiments. Indeed, the inhibition of Chk2 with PV1019 suggested that Chk2 phosphorylation was essential for p21 transactivation. This explanation is supported by the fact that PTX pretreatment (with or without TdR) prevents AICAR-mediated phosphorylation of Chk2, coincidental with a block of AICAR-mediated induction of p21. On the other hand, PTX in the absence of TdR not only prevents AICAR-mediated Chk2 phosphorylation but also causes Chk1 phosphorylation. Activated Chk1 has been shown to inhibit the elongation of mRNA from the p21 locus in S-phase-arrested cells (57). In concurrence with this idea, treatment of HCT116 cells with PTX was found not to interfere with the loading of p53 onto the p21 promoter (data not shown), at the same time also preventing the appearance of mRNA for p21 (Fig. 2B). However, the inhibition of Chk1 relieved the PTX-induced block of p53-dependent transcription, and p21 transcription was observed coincident with the activation of Chk2. The inhibition of Chk1, was previously shown to relieve a block on p21 transcription in hydroxyurea-treated cells, and was traced to a Chk1-induced inhibition of the elongation of p21 transcripts (57). Presumably, the inhibition of Chk1 allowed cells blocked in S-phase to escape into G2 and a premature M-phase. This suggests that the combination of PTX with a Chk1 inhibitor would have a strong cytotoxic effect on carcinoma cells. Three drugs have been reported to cause the accumulation of a transcriptionally limited p53: PTX (Fig. 2A), lometrexol (64), and hydroxyurea (57, 65); all three cause a substantial prolongation of transit through S-phase (51, 57, 64, 65).

Overall, we concluded that, because of a PTX-induced block in p53-dependent transcription, the inhibitory effects of PTX
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on mTORC1 are due to robust AMPK-mediated phosphorylation of Raptor, independent of TSC2 and p53.

Author Contributions—S. A. performed most of the immunoblotting experiments shown in Figs. 1–5 and contributed to the analysis and writing of the manuscript. C. M. B. performed the experiments of Fig. 7 and contributed to the interpretation of all of the data as well as manuscript preparation. S. B. R. contributed the data of Fig 6E and corrected the manuscript. R. G. M. contributed some of the data for Fig. 6, supervised the work, and wrote the early drafts of the manuscript.

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