Inhibition of AMP-activated Protein Kinase Sensitizes Cancer Cells to Cisplatin-induced Apoptosis via Hyper-induction of p53*

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This article has been withdrawn by the authors. The α-actinin immunoblot from Fig. 1A from cells treated with 1-h cisplatin was duplicated in Fig. 3A.

In Fig. 2A, lanes 2-4 and lanes 7-9 of the ACC immunoblot were duplicated. The PARP immunoblot from Fig. 2F was duplicated in Fig. 5B. The last two lanes of the α-actinin immunoblot from Fig. 6B are duplicates. Lanes 1, 5, and 6 of the left ERK immunoblot were duplicated in Fig. 7A. Additionally, lanes 4 and 9 of the same immunoblot were also duplicated. In Fig. 9A, lanes 3 and 5 of the pAMPK immunoblot were duplicated. The Journal also raised questions regarding the ACC immunoblot in Fig. 5A, the ERK immunoblot in Fig. 7B, and the actinin immunoblot in Fig. 9A, which the withdrawing authors were not able to address. Because the original data are no longer available, the authors state that they repeated the above-mentioned experiments and obtained essentially identical results. The authors state that they have full confidence in the conclusions of this paper. The authors apologize to the readers.

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apotosis, both in vitro and in vivo. AMPK is a heterotrimer that consists of a catalytic subunit (α) and two regulatory sub-units (β and γ), and operates as an intracellular energy sensor via the monitoring of the cellular AMP/ATP ratio (11). It is activated sensitively in response to a broad spectrum of physiological and pathological ATP-depleting conditions, including exercise, hypoxia, nutrition deprivation, oxidative stress, and exposure to carcinogenic metals. Moreover, recent studies have shown that the AMPK pathway performs a central function in the regulation of glucose and lipid homeostasis, body weight, food intake, insulin signaling, and mitochondria biogenesis (12).

By way of contrast, studies regarding the role of AMPK in cancer are in an embryonic stage at present. Moreover, the effects of genotoxic stress, including the effects of anti-cancer drugs on AMPK or its role in genotoxic stress-induced apoptosis, remain almost completely unknown. In this work, we examined the role of AMPK in cisplatin-induced apoptosis in AGS (human gastric carcinoma), HCT116 (human colon carcinoma), and HeLa (human cervix adenocarcinoma) cells as well as in a human carcinoma xenograft. We also assessed a relevant signaling pathway involving ATM, ATR, and p53. On the basis of the observations given herein, we propose that AMPK is one of the cellular factors determining the cellular sensitivity to cisplatin, further implying that a strategy in which cisplatin and AMPK inhibition were coupled could be developed for a novel chemotherapeutic approach.

EXPERIMENTAL PROCEDURES

Materials—RPMI medium 1640 was obtained from Invitrogen, G418 was from TOCRIS. Compound C was from Calbiochem. Antibodies recognizing a phosphospecific ERK (Thr202/Tyr204) (9101), p53-Ser15 (9284), and ACC-Ser79 (3732) were from Cell Signaling Technology. Antibodies for p53 (sc126), p21 (3661) were from Santa Cruz Biotechnology. Antibodies for ATGL/CE3 (12691), p53, p21, and p16 (3661) were from Sigma. PD98059 was from TOCRIS. Compound C was from Calbiochem. Antibodies recognizing a phosphospecific ERK (Thr202/Tyr204) (9101), p53-Ser15 (9284), and ACC-Ser79 (3661) were from Cell Signaling Technology. Antibodies for p53 (sc126), p21 (3661), poly(ADP-ribose) polymerase 1 (sc7150), and β-actin (sc5141), and α-actinin (sc17829) were from Santa Cruz Biotechnology (Santa Cruz, CA). AMPKα2 antibody (07181) was from Upstate Biotechnology, Inc. (Lake Placid, NY). GFP-LKB1 was from Dr. J. Yuan (Dept. of Cell Biology, Harvard Medical School, Boston).

Cell Culture and Treatment—AGS (human gastric carcinoma), HCT116 (human colon carcinoma), HeLa S3 (human cervix adenocarcinoma), and GM05849 cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37 °C with 95% air and 5% CO2. Cells were exposed to cisplatin in culture media containing 10% fetal bovine serum. The pretreatment of cells with compound C, caffeine, and PD98059 was conducted for 20 min prior to the addition of cisplatin. HCT11653+/- and HCT11653-/- cells were provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute and The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins Medical Institutions, Baltimore, MD). GM05849 (ATM-/-) cells were purchased from the Coriell Institute (Camden, NJ).

Cell Viability Assay—The Vi-CELL™XR Cell viability analyzer (Beckman Coulter) cell counter that performs an automated trypan blue exclusion assay was used to measure cell viability. The assay is based on uptake of trypan blue dye by dead cells due to loss of their membrane integrity. One milliliter of aliquot of the cell suspension in plastic cuvette was aspirated and mixed with trypan blue and then pumped into the flow cell for imaging. The instrument collected 100 images of cells to compute viability. The dead cells appear darker than the viable cells allowing the contrast between live and dead cells to be used in determining cell viability.

Assessment of Cell Apoptosis—Cell apoptosis was assessed via fluorescence-activated cell sorting (FACS) analysis, Hoechst 33342 staining and internucleosomal DNA fragmentation analysis. Total cells were harvested by trypsinization, collected by centrifugation, and washed with PBS. After fixing with 70% ethanol, cells were resuspended in PBS containing 10 µg/ml propidium iodide. After sorting out the viable cells, fluorescence intensity was measured by FACSCalibur flow cytometry and CellQuest software (BD Biosciences) using excitation and emission wavelengths of 488 and 575 nm, respectively. Internucleosomal DNA fragmentation measured by this assay was measured as previously described (12). Cisplatin exposure was conducted with Hoechst 33342 as previously described (12). The appearance of condensed chromatin and blebbing of membrane indicated apoptotic.

Cell Viability Assay—Cells were seeded into 96-well plates (10,000 cells per well). After stimulation, the addition of lipofectamine 2000 (Invitrogen), and drug treatment, the cells were harvested by trypsinization and mixed with trypan blue and then pumped into the flow cell for imaging. The instrument collected 100 images of cells to compute viability. The dead cells appear darker than the viable cells allowing the contrast between live and dead cells to be used in determining cell viability.
with the cell lysates (5 µl). Luminescence was analyzed by VICTOR\textsuperscript{3TM} luminometer (PerkinElmer Life Sciences) and normalized using the cellular proteins.

**Animals and in Vivo Anti-tumor Assay**—Athymic BALB nu/nu mice were utilized in this study. All animal experiments were approved by the Ethics Committee for Animal Experimentation of the Korea Institute of Radiological & Medical Sciences. Human colon carcinoma cells (HCT116) were injected into the flanks of 5- to 6-week-old nude mice. Five mice were assigned to each of the experimental groups. Intraperitoneal injections of all drugs at 4-day intervals was initiated after the tumor achieved a minimal weight of 200 mg. Tumor weights were evaluated in accordance with the formula \((L \times W^2)/2\) via the measurement of tumor length \((L)\) and width \((W)\) with a set of calipers.

**Statistical Analysis**—Results are expressed as the means ± S.E. We used the Student \(t\) test. Differences were considered significant at a \(p\) value of <0.05.

**RESULTS**

**Cisplatin Rapidly Induces AMPK Activation in AGS Cells via Generation of Reactive Oxygen Species**—Because gastric carcinoma is one of the most intractable cancers and is known for resistance to cisplatin (16), we used AGS gastric cancer cells in the present study as a working model to investigate a possible mechanism determining the cisplatin sensitivity. To examine the effect of cisplatin on AMPK activity, AGS cells in culture media containing 10% serum were exposed to cisplatin in a time- and dose-dependent manner (Fig. 1A). Indeed, cisplatin activated AMPK in a rapid manner, with the phosphorylation level of AMPK being increased at 1 h. This cisplatin-induced AMPK activation was accompanied by the phosphorylation level of ACC-Ser79 (Fig. 2A), indicating that ROS is a mediator for AMPK activation by cisplatin.

**Inhibition of AMPK Activity Sensitizes AGS Cells to Cisplatin-Induced Apoptosis**—To ascertain whether AMPK activity is essential for cell viability after treatment with cisplatin, we have taken a pharmacological and molecular approach to inhibit the AMPK activity, and then we examined the subsequent effect on cell viability via using several different approaches. Compound C is a potent and selective AMPK inhibitor (19), and it has been widely used to test a role of AMPK. Pretreatment of AGS cells with 20 µM compound C for 30 min almost completely prevented the cisplatin-induced AMPK activation, as evidenced by the phosphorylation level of ACC-Ser\textsuperscript{79} (Fig. 2A). We also attempted to confirm our results by using recombinant adenovirus expressing the c-myc-tagged wild-type (Ad-AMPK-WT), dominant negative (Ad-AMPK-DN), or constitutively active (Ad-AMPK-CA) form of AMPK (15). In accordance with the previous report showing that formation of AMPK trimeric complex (\(\alpha, \beta,\) and \(\gamma\) subunits) is required for its activity (20, 21), overexpression of only the wild-type \(\alpha\) subunit did not activate endogenous AMPK activity, as evidenced by the phosphorylation level of ACC-Ser\textsuperscript{79}. However, overexpression of AMPK-DN, which replaces the endogenous \(\alpha\) subunit in the trimeric complex (20), effectively blocked the cisplatin-induced phosphorylation level at ACC-Ser\textsuperscript{79}, whereas AMPK-CA increased its level (Fig. 2A). We further examined the phosphorylation level of AMPK\(\alpha\) Thr\textsuperscript{172}, but there was no difference in its level between cells overexpressing AMPK\(\alpha\) WT and DN (Fig. 2A). Because AMPK-DN contains a mutation in ATP binding site, Thr\textsuperscript{172} residue is still available for phosphorylation by the upstream kinase (20).

AGS cells infected by Ad-AMPK-WT, -DN, or -CA were exposed to cisplatin for 24 h, cell apoptosis was assessed via fluorescence-activated cell scanning analysis (sub-G1 fraction), and the results showed that cell apoptosis increased \(\sim 10\%\) when cisplatin was applied in AGS cells infected with Ad-AMPK-WT. However, the infection with Ad-AMPK-DN in AGS cells did, indeed, result in a augmentation of cisplatin-mediated apoptosis (Fig. 2B), suggesting that AMPK plays a protective role in cisplatin-induced cytotoxicity. The increase
in cisplatin-induced apoptosis was also verified by Hoechst 33342 staining; distinctive nuclear condensation was observed when AMPK activity was inhibited by Ad-AMPK-DN (Fig. 2C). This result was further supported by DNA fragmentation analysis; a ladder pattern of internucleosomal fragmentation of DNA was apparent when AGS cells infected with Ad-AMPK-DN were treated with cisplatin for 24 h (Fig. 2D). Collectively, these results indicate that AMPK activation generates a survival signal after cisplatin treatment in AGS cells.

We next determined the levels of intracellular ATP in AGS cells that were exposed to cisplatin, compound C, or both for different durations (Fig. 2E). Twenty-four hours of cisplatin treatment (60 μM) or compound C (20 μM) resulted in a ~20% reduction in ATP levels. However, combination treatment with cisplatin and compound C on cellular ATP levels decreased dramatically. In accordance with the kinetics inherent to ATP depletion, the cleavage of poly(ADP-ribose) polymerase (PARP), which implies the initiation of apoptosis, was accelerated dramatically as the result of combined treatment with cisplatin and compound C (Fig. 2F). These data indicate, again, that cisplatin-induced AMPK activation exerts a protective effect against both ATP depletion and apoptosis.

Inhibition of Cisplatin-induced AMPK Activity Increases p53 Protein Accumulation, Phosphorylation at Ser15, and the Expression of p53 Target Genes—The p53 tumor suppressor protein is a central mediator of a variety of DNA damage responses, and it has been fairly well established that the p53 protein is stabilized and its level thereby increases in response to cisplatin-induced DNA damage (9). p53, in turn, triggers cell cycle arrest and apoptosis via the up-regulation of its transcriptional target genes, such as p21cip1/waf1 and Bax, respectively (22). Therefore, we attempted to determine whether AMPK is involved in the regulation of p53 in AGS cells (Fig. 3). As had been expected, p53 was detected 3–6 h after cisplatin exposure and increased in a time-dependent manner (Fig. 3A). Treatment of cells with a combination of compound C and cisplatin caused a significant induction of p53, and the time course of its induction was also accelerated (Fig. 3A). In accordance with this time course, the phosphorylation level of p53-Ser15, which is essential for the stabilization of the p53 protein, as well as p21cip1/waf1 expression, exhibited similar kinetic patterns (Fig. 3A). Conversely, the pretreatment of cells with the AMPK activator, 5-aminoimidazole-4-carboxamide-1-B-d-ribofuranoside (AICAR) (11), reduced the cisplatin-induced protein level of p53 and the phosphorylation of p53-Ser15 (Fig. 3B). The effect of the modulation of AMPK activity on p53 was further verified via molecular approaches (Fig. 3C). Infection with Ad-AMPK-DN significantly induced the cisplatin-mediated stabilization of p53, the phosphorylation of p53-Ser15, and p21cip1/waf1 expression in AGS cells. The opposite results were observed when endogenous AMPK activity was stimulated by Ad-AMPK-CA.
Considering the finding that the inhibition of cisplatin-induced AMPK activation resulted in an increase in the levels of the p53 protein, we then assessed the transcriptional activity of p53 and the level of p53 target gene expression. The AGS cells were infected with Ad-AMPK-WT or Ad-AMPK-DN, followed by transfection with pG-13-luc, which harbors the firefly luciferase gene under the control of 13 p53-responsive elements. These cells were then exposed for 12 h to cisplatin. Under these conditions, cisplatin induced p53-dependent luciferase activity by a factor of ~4-fold, but infection with Ad-AMPK-DN resulted in a further elevation of cisplatin-induced luciferase activity, resulting in a ~14-fold induction (Fig. 4A). Cisplatin unambiguously increased the mRNA level of p53 target genes such as p21\(^{cip1/waf1}\) and Bax as shown by reverse transcription-PCR, and such inductions were increased significantly by Ad-AMPK-DN (Fig. 4B). Under these conditions, levels of p53 and \(\beta\)-actin mRNA remained constant. Consequently, these findings suggest that the inhibition of cisplatin-induced AMPK activity had no effect on the levels of p53 mRNA, but rather resulted in an increase in the level of p53-Ser\(^{15}\) phosphorylation, which in turn leads to an increase in protein stabilization, transcriptional activity, and target gene expressions.

**The Effect of AMPK Inhibition on Cisplatin-induced Apoptosis Is Achieved Primarily in a P53-dependent Manner**—Thus far, our data have indicated that inhibition of cisplatin-induced AMPK by compound C and p53-wild type (WT) may be dependent on p53. This idea was further verified when we also observed that the modulation of AMPK activation after cisplatin treatment may be dependent on p53. This idea was further verified when cell viability was assessed under identical conditions (Fig. 5C). Treatment with compound C and cisplatin resulted in a dramatic reduction in the viability of HCT116\(^{p53-/-}\), but HCT116\(^{p53-/-}\)/caspase-3 cells proved highly resistant against cisplatin as well as the combined treatment. However, HCT116\(^{p53-/-}\) became susceptible to cisplatin, as well as the compound C/cisplatin combination modality, after transfection with an expression vector encoding for wild-type p53. This result clearly indicates that the effects of the inhibition of cisplatin-induced AMPK activation are mediated primarily by p53. Furthermore, we also observed that the modulation of AMPK activity exerted identical effects on the phosphorylation level of exogenously introduced p53 in HCT116\(^{p53-/-}\) cells (Fig. 5D).

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**FIGURE 3.** p53 response to cisplatin (60 µM, 6 h) was inhibited by inhibition of AMPK. A, AGS cells were pretreated with AICAR (0.2 mm) or compound C (20 µM) for 30 min, then exposed to cisplatin (60 µM) for the indicated times. B, AGS cells were preincubated with AICAR (0.2 mm) or compound C (20 µM) for 30 min, then exposed for 6 h to cisplatin. C, AGS cells were infected with Ad-AMPK-WT, Ad-AMPK-DN, or Ad-AMPK-CA, and then exposed to cisplatin (60 µM) for 6 h. Under these conditions, Western blot analysis was conducted using anti-p53 (p33), anti-phosphospecific p53-Ser\(^{15}\) (p53-Ser\(^{15}\)/p21), anti-phosphospecific ACC (P-ACC), anti-p21\(^{cip1/waf1}\) (p21), and anti-\(\alpha\)-actinin (\(\alpha\)-actinin) antibody.

**FIGURE 4.** The effect of AMPK inhibition on p53 transcriptional activity and the expression of p53 target genes. A, AGS cells were infected with Ad-AMPK-WT or Ad-AMPK-DN followed by transfection with pG-13-luc. After 12 h of exposure to cisplatin (60 µM), luciferase activity was measured. The data are expressed as the means S.E. for five determinations (*, \(p < 0.05\); compared with control). B, under the identical conditions, the mRNA levels of p21\(^{cip1/waf1}\), Bax, p53, and \(\beta\)-actin were determined via reverse transcription-PCR.
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The inhibition of AMPK by caffeine is associated with an increase in cisplatin-induced p53-Ser15 phosphorylation in GM05849 cells (Fig. 6), suggesting that AMPK signaling may not be directly involved with that of ATM and ATR. Serine 15 of p53 is phosphorylated by both ATM and ATR, but it is generally thought that genotoxic drugs, including cisplatin, preferentially activate ATR (24, 25), whereas ATM responds principally to ionizing radiation-induced DNA damage. This notion was also bolstered by our observation that p53-Ser15 phosphorylation in the ATM-deficient human fibroblast line, GM05849, was still induced strongly by cisplatin, and the inhibition of cisplatin-induced AMPK activation also caused a significant elevation of p53-Ser15 phosphorylation in GM05849 cells (Fig. 6B).

Induction of p53 Phosphorylation at Ser15 via ERK Activation—As is shown in Fig. 6A, caffeine did not completely block the cisplatin-induced phosphorylation of p53-Ser15, which suggests that some signaling pathway other than ATR and ATM may also be involved in phosphorylation of p53-Ser15. In fact, the involvement of mitogen-activated protein kinases (MAPK) in the regulation of the cisplatin sensitivity of cancer cells remains a subject of substantial interest. MAPKs have been classified into three major subfamilies: extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal protein kinase, and p38 kinase. Among them, ERK has become the focus of our attention due to the following reasons. First, cisplatin activates ERK in many types of cancer cells, and ERK activation has been shown to be critical for cisplatin-induced apoptosis (26). Second, cisplatin-induced ERK activation contributes to an elevated level of p53 phosphorylation at serine 15 and to p53 accumulation (27). Third, we previously showed that AICAR treatment results in the inhibition of
of insulin-like growth factor-A-induced ERK activity (28), although another study showed that AICAR can activate ERK under a certain condition (29). As a result, the interrelationship between AMPK and ERK remains elusive so far. Thus, we attempted to elucidate the AMPK signaling pathway by characterizing the cross-talk with ERK signaling. Cisplatin did, indeed, induce rapid ERK activation in AGS cells (Fig. 7A, left panel). The inhibition of AMPK activation after cisplatin treatment via either compound C (Fig. 7A) or Ad-AMPK-DN (Fig. 7B) resulted in the hyper-activation of ERK, thereby indicating that AMPK activation in response to cisplatin may result in a suppression of ERK activity. Because the ERK inhibitor, PD98059, almost completely blocked the hyper-activation of ERK induced by the combined cisplatin and AMPK inhibition treatment, the enhanced level of p53-Ser\(^{15}\) phosphorylation under this condition was simultaneously reduced (Fig. 7, A (right panel) and B). Consequently, these results indicate the presence of a novel signal cascade involving AMPK, ERK, and p53. Our data appear to initially suggest that ERK activation, in addition to ATM and ATR, is required for the cisplatin-induced p53-Ser\(^{15}\) phosphorylation. Second, AMPK activation in response to cisplatin seems to transmit a survival signal to the cells via the suppression of the apoptotic property of p53, which occurs due to a suppression of ERK activity. Caffeine treatment did not significantly affect cisplatin-induced AMPK (Fig. 6A) or ERK activation (Fig. 7C), thereby suggesting that this pathway operates independently of ATR or ATM. These signaling pathways are summarized in the diagram shown below in Fig. 13.

Inhibition of AMPK Activation Increases Cellular Response to Cisplatin in Various Cancer Cells, but Not in HeLa Cells—Next, we determined the sensitivity of a variety of human cancer cells (AGS gastric cancer, HCT116 colon cancer, HepG2 liver cancer, MCF7 breast cancer, and HeLa cervical cancer) to cisplatin. We first examined the level of AMPK activation in these five different cancer cells (Fig. 8A). The results revealed that cisplatin activated AMPK in AGS, HCT116, MCF7, and HepG2 cells, but not in HeLa cells. To ascertain whether AMPK activity is essential for cell viability after treatment with cisplatin, these cells were treated for 24 h with the indicated concentrations of cisplatin in the presence or absence of the AMPK-specific inhibitor, compound C (Fig. 8B). The inhibition of AMPK activation after cisplatin treatment resulted in a dramatic increase of cytotoxicity in AGS, HCT116, MCF7, and HepG2 cells (Fig. 8B). In the case of the HeLa cells, although another study showed that AICAR can activate ERK and induce ROS generation in HeLa cells (Fig. 9A). Moreover, cisplatin did not induce ROS generation in HeLa cells (Fig. 9B), and anti-oxidants such as NAC or catalase showed no effect on AMPK activity (Fig. 9C).

To further demonstrate whether AMPK activation is indeed associated with the sensitivity of cellular response to cisplatin, we attempted to promote AMPK activation in HeLa cells via two different approaches: overexpression of a constitutively active form of AMPK (AMPK CA) or LKB1 which was identified as one of the upstream activating AMPK kinases (30–32). LKB1 is a tumor suppressor kinase, gene mutations of which induce a dominantly inherited cancer, referred to as Peutz-Jeghers syndrome (33). Moreover, a recent study showed that LKB1 is essential for protection of cells against the apoptosis induced by nutritional deprivation (30). It was also reported that HeLa cells do not express LKB1 (34), which led us to suspect that overexpression of LKB1 would promote AMPK acti-
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FIGURE 7. ERK was involved in the hyper-induction of p53 via the inhibition of AMPK. AGS (a human gastric carcinoma), HCT116 (a human colon carcinoma), HepG2 (a human hepatoma), MCF-7 (a human breast cancer cell), and HeLa (a human cervix adenocarcinoma) cells were exposed to cisplatin (60 μM) for 24 h in the presence or absence of 50 μM PD98059, or in the presence or absence of 20 μM compound C (20 μM) for 6 h in the presence or absence of 50 μM PD98059. Cells were then measured via trypan blue exclusion assay. The data are expressed as the means ± S.E. over four determinations. (*, p < 0.05; compared with the control).

FIGURE 8. Effects of cisplatin on AMPK activity and viability in cancer cells. A, AGS (a human gastric carcinoma), HCT116 (a human colon carcinoma), HepG2 (a human hepatoma), MCF-7 (a human breast cancer cell), and HeLa (a human cervix adenocarcinoma) cells were exposed to cisplatin (60 μM) for 1 h in the presence or absence of compound C (20 μM). The total cell extracts were prepared and subjected to Western blot assays using anti-phosphospecific ACC-Ser79 (P-ACC), anti-ACC (ACC) antibody. B, these cells were exposed to the indicated concentration of cisplatin for 24 h in the presence or absence of compound C (20 μM). Cell viability was then measured via trypan blue exclusion assay. The data are expressed as the means ± S.E. over four determinations. (*, p < 0.05; compared with the control).

viation in HeLa cells. Indeed, no LKB1 was expressed in the HeLa cells, as demonstrated by reverse transcription-PCR, whereas it was clearly expressed in the AGS cells (Fig. 10A). Transfection of HeLa cells with green fluorescent protein (GFP)-tagged LKB1 expression vector resulted in a significant elevation of AMPK activity, as evidenced by the phosphorylation level of ACC (Fig. 10B, upper panel), and cell viability after cisplatin treatment (60 μM, 24 h) was also increased by a significant degree (Fig. 10C). Similar results were also observed when HeLa cells were transfected with c-myc-tagged AMPK CA (Fig. 10B, lower panel and C). Consequently, our results thus far appear to suggest that AMPK performs a pivotal function for protection against the cytotoxic effects of cisplatin, thereby implying that AMPK is one of the cellular factors determining the sensitivity of cancer cells to the chemotherapeutic agent cisplatin.

We next attempted to examine whether blunt responsiveness of AMPK in HeLa cells is stimuli-specific or not. To this end, HeLa, AGS, and HCT116 cells were exposed to other apoptotic stimuli such as hypoxia (0.1% O2), H2O2 (0.3 mM), as well as cisplatin (60 μM) (Fig. 11).
LKB1 may stimulate AMPK in HeLa cells in response to hypoxia and H$_2$O$_2$. These three cells were also exposed to hypoxia, H$_2$O$_2$, and cisplatin for 24 h in the presence or absence of the AMPK-specific inhibitor, compound C, and the cell viability was compared (Fig. 11B). Compound C potentiated cell death induced by these stimuli in all tested cell lines except the case of cisplatin-induced HeLa cell death, where AMPK activation did not occur. These results again support that AMPK plays a critical role for protection against the cytotoxic effects of cisplatin.

The Anti-tumor Efficacy of AMPK Inhibitor in Combination with Cisplatin in HCT116 Human Cancer Xenograft Model—Next, to determine whether the in vitro chemosensitizing effects associated with AMPK inhibition can be reproduced in vivo, we evaluated the anti-tumor effects of cisplatin, compound C, and the combination treatment in nude mice harboring HCT116p53$^{−/−}$ tumor xenografts. A total of 2 $\times$ 10$^6$ HCT116p53$^{−/−}$ cells was implanted on a flank subcutaneously in each of five nude mice in each group. When the tumor masses grew to $\sim$2–3 mm in diameter, drugs were intraperitoneally administered once every 4 days (compound C, 2 mg/kg; cisplatin 2 mg/kg; combination of two). Under our experimental conditions, cisplatin alone dened no detectable anti-tumor activity (Figs. 2, 5, and 8). In contrast compound C alone resulted in a moderate slowing of tumor growth as compared with the control. When these two agents were administered in combination, the anti-tumor effects on xenografts were potentiated. Our data show that AMPK inhibition sensitizes cancer cells to cisplatin, mechanisms have been implicated in the development of cisplatin resistance, including the reduced intracellular accumulation of the drug, the inactivation of cisplatin by sulfur-containing molecules, an increase in DNA damage repair, increased levels of anti-apoptotic genes, and alterations in the signal transduction pathways associated with apoptosis (35). Therefore, a great deal of effort has been made to augment the therapeutic indices of cisplatin via the modulation of the aforementioned targets. Apart from these mechanisms, we investigated whether inhibition of AMPK, which performs a pivotal function in energy homeostasis via the monitoring of intracellular energy status, could represent a novel approach to the augmentation of tumor cell cisplatin sensitivity (Fig. 13).

Herein, we showed that cisplatin rapidly but transiently activated AMPK in various cancer cells (Figs. 1, 3, and 8), and the inhibition of AMPK activity resulted in a marked augmentation of cisplatin-induced apoptosis (Figs. 2, 5, and 8). Moreover, identical effects were detected in a xenograft model (Fig. 12). In accordance with the previous report (26), the HeLa cells appeared to be relatively cisplatin-sensitive, and moreover addition of AMPK inhib-

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**FIGURE 9.** AMPK in HeLa cells exposed to cisplatin in an identical manner as in Fig. 1. A, Western blot analysis was performed using anti-phosphospecific AMPK (P-AMPK), anti-AMPK (AMPK-α) and anti-α-actinin antibody. B, after 1-h exposure to cisplatin, the phosphorylation levels of AMPK in HeLa cells were determined by 2′,7′-dichlorofluoroscein diacetate (H$_2$DCFDA) assay. C, after exposure of AGS cells to cisplatin (60 μM, 1 h) in the presence of catalase (1000 units/ml) or 5 mM NAC, the phosphorylation level of AMPK was measured.

**FIGURE 10.** Overexpression of LKB1 or constitutive active form of AMPK in HeLa cell promotes AMPK activation and cell viability in response to cisplatin. A, the mRNA levels of LKB1 and β-actin of the HeLa and AGS cells were compared via reverse transcription-PCR. B, HeLa cells were transfected with a eukaryotic expression vector encoding for GFP-tagged LKB1 (GFP-LKB1) or c-myc-tagged AMPK CA (AMPK CA). The expression level of each form was confirmed via LKB1 or c-myc antibody. The phosphorylation level of ACC-Ser$^{373}$ (P-ACC) was compared with that of the vector- or GFP-transfected HeLa cells after 1-h exposure to cisplatin. C, the above cells were exposed for 24 h to cisplatin (60 μM), and trypan blue exclusion assays were conducted. The data are expressed as the means ± S.E. for four determinations in triplicate (*, $p < 0.05$; compared with the indicated group).

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A

|            | AGS | HCT116 | HeLa |
|------------|-----|--------|------|
| None       | 100 | 100    | 100  |
| Cisplatin  |     |        |      |
| Hypoxia    |     |        |      |
| H₂O₂       |     |        |      |

P-AMPK
P-ACC
α-actinin

B

![Graph showing cell viability](image)

FIGURE 11. A, AGS, HCT116, and HeLa cells were exposed to cisplatin (60 μM), hypoxia (0.1% O₂), or H₂O₂ (0.3 mM) for 1 h, and then the total cell extracts were prepared and subjected to Western blot assays using anti-phosphospecific AMPKα-Thr172 (P-AMPK), anti-phosphospecific ACC-Ser79 (P-ACC), anti-α-actinin (α-actinin) antibody. B, AGS, HCT116, and HeLa cells were exposed to cisplatin (60 μM, Cis), hypoxia (0.1% O₂, Hy), or H₂O₂ (0.3 mM) for 24 h. Cell viability was then measured via trypan blue exclusion assay.

FIGURE 12. In vivo anti-tumor efficacy of AMPK inhibitor alone and in combination with cisplatin. BALB/c nude mice were inoculated subcutaneously into one flank with 2 × 10⁶ HCT116 cells in Matrigel. Five mice were assigned to each of the groups. When the tumor masses reached ~2–3 mm in diameter, drugs were intraperitoneally administered once every 5 days (compound C, 2 mg/kg; cisplatin 2 mg/kg; combination of two). Tumor growth was measured every 4 days, as described under “Experimental Procedures” (*, p < 0.01; compared with control).

It seems rational to view AMPK as a survival factor for cancer cells, on the basis of our knowledge of the probable role of AMPK in the augmentation of energy production via the activation of AMPK may be employed as a component of an anti-cancer therapy (40, 41). The logic of this approach is predicated on recent observations that AMPK also strongly suppresses cell proliferation. This effect is mediated, in part, by several tumor suppressor proteins associated with the AMPK signaling network, including LKB1 (33) and the tuberous sclerosis complex (TSC2) (42). Moreover, Jones et al. recently reported that the activation of AMPK induces p53-Ser¹⁵ phosphorylation in response to glucose deprivation, resulting in replicative senescence (43). The ability of AMPK to promote senescence or to inhibit cell proliferation in response to energy starvation has been interpreted as a check point that couples glucose availability to the progression of the cell cycle; it was implied that the activation of AMPK might promote the conservation of the remaining energy to support the survival and physiological functions of the cell during cell cycle arrest.

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WITHDRAWN
suppressor proteins involved in the AMPK signal network, including LKB1, TSC2, and p53, not only contribute to cell cycle arrest but also protect cells against energy deprivation-mediated apoptosis (30, 42, 43). As a result, active AMPK may facilitate the provision of energy for tumorigenesis, but may also concomitantly diminish the selective advantages of the cancer cells. Therefore, a key question persists as to whether AMPK activation effectively induces apoptosis in malignant cancer cells. Moreover, advanced malignant cancers often retain defective tumor suppressor proteins, including p53, LKB1, and TSC2, and therefore the tumor suppression effect associated with AMPK activation may not be anticipated in the case of certain malignant cancers. One of the most fundamental metabolic alterations occurring during the malignant transformation is the up-regulation of glycolysis, a phenomenon referred to as the Warburg effect, and malignant tumors exploit this pathway for the generation of ATP (44). AMPK also performs a key function in hypoxia-induced glycolysis (45). Therefore, rather than the simple induction of cancer cell arrest, which cannot completely stop the re-emergence of cancer, inhibition of AMPK, which plays a central role in energy homeostasis, is likely to be a more effective approach to cancer chemotherapy, because this approach does, in fact, induce apoptosis in cancer cells. Within a similar conceptual framework, the inhibition of glycolysis can also be considered as a component of an alternative approach to anticancer treatment (46, 47).

In this study, we have also shown that AMPK-sensitized cells to cisplatin via hyper-induction of p53 by AMPK. This was corroborated by the observation that AMPK inhibition significantly affects the cisplatin sensitivity of H11002 cells lacking p53. The introduction of a wild-type p53 into HCT116p53−/− rendered these cells relatively sensitive to the combined AMPK inhibition and cisplatin treatment. Our results are consistent with the idea that AMPK activation may help in excess of a certain threshold to result in apoptosis or in enhanced drug sensitivity (48). Furthermore, the signaling network that connects AMPK and p53 appears to be a highly complex one, because the particular patterns of biochemical regulation and the ultimate physiological outcome appear to depend substantially upon the types of cells and the stresses induced. Jones et al. demonstrated that AMPK induces p53-Ser15 phosphorylation in mouse embryonic fibroblasts, and such metabolic activations of p53 were associated with cell survival, whereas the activation of p53 induced by γ-radiation resulted in a reduction in cell viability (43). However, our results indicate that the inhibition of cisplatin-induced AMPK activation resulted in an upshift in the phosphorylation of p53-Ser15 in cancer cells, which is associated with reduced cell viability. Consistent with our results, the findings of a recent study indicated that treatment with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, a well known AMPK activator, caused a reduction in p53 levels in renal cell carcinoma cells (47). Consequently, to elucidate clearly the AMPK-p53 network, more elaborate and comprehensive studies appear to be required, which take into account the genetic profiles of the cells, the types of stress, its duration, and the degree of persistence of the AMPK activation.

REFERENCES

1. Rosenberg, B. (1977) *Adv. Exp. Med. Biol.* 91, 129–150
2. Prestayko, A. W., D’Aoust, J. C., Issell, B. F., and Crooke, S. T. (1979) *Cancer Treat. Rev.* 6, 17–39
3. Siddik, Z. H. (2003) *Oncogene* 22, 7265–7279
4. Harrison, J. C., and Haber, J. E. (2006) *Annu. Rev. Genet.* 40, 209–235
5. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) *Science* 281, 1674–1677
6. Canman, C. E., Lim, D. S., Cinprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Silicicano, J. D. (1998) *Science* 281, 1677–1679
7. Tibbets, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999) *Genes Dev.* 13, 152–157
8. Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997) *Cell* 91, 325–334
9. El-Dweir, W. S. (2003) *Oncogene* 22, 7486–7495
10. Boulay, J. L., Perruchoud, A. P., Reuter, J., Bolliger, C., Herrmann, R., and Rochlitz, C. (2000) *Cancer Gene Ther.* 7, 1215–1219
11. Hardie, D. G., Carling, D., and Carlson, M. (1998) *Annu. Rev. Biochem.* 67, 821–855
12. Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005) *Cell Metab.* 1, 15–25
13. Zhou, N., Xiao, X., Xiao, Y., and Liu, L. F. (2003) *J. Biol. Chem.* 278, 40216–40223
14. Banin, S., Moyal, L., Shieh, S., Taya, Y., Prives, C., Lee, H. W., Lee, S.-K., Ha, J., and Ziv, Y. (2003) *Cell Death Differ.* 10, 181–191
15. Burkle, A. (2005) *Anticancer Drugs* 7, 137–149
16. Wang, X., Martindale, J. L., and Holbrook, N. J. (2000) *J. Biol. Chem.* 269, 28756–28762
17. Park, O. J. (2006) *J. Biol. Chem.* 281, 19102–19110
18. Choi, S. L., Kim, S. J., Lee, K. T., Kim, J., Mu, J., Birnbaum, M. I., Soo Kim, K., and Carling, D. (2005) *Biochem. Biophys. Res. Commun.* 327, 92–97
19. Kanzawa, K., Kato, T., Fushimi, Y., Kajiyama, G., Fujii, K., Kobayashi, T., Kamada, N., Kameyama, N., and Moller, D. E. (2001) *J. Clin. Invest.* 108, 1167–1174
20. Woods, A., Azzout-Marniche, D., Foretz, M., Stein, S. C., Lemarchand, P., Ferre, P., Foufelle, F., and Carling, D. (2000) *Mol. Cell. Biol.* 20, 6704–6711
21. Stein, S. C., Woods, A., Jones, N. A., Davison, M. D., and Carling, D. (2000) *Biochem. J.* 345, 437–443
22. Yu, J., and Zhang, L. (2005) *Biochem. Biophys. Res. Commun.* 331, 851–858
23. Burkle, A. (2005) *FEBS J.* 272, 4576–4589
24. Damia, G., Filiberti, L., Vikhanskaya, F., Carrassa, L., Taya, Y., D’Incalci, M., and Broggi, M. (2001) *Neoplasia* 3, 10–16
25. Zhao, H., and Piwnica-Worms, H. (2001) *Mol. Cell. Biol.* 21, 4129–4139
26. Wang, X., Martindale, J. L., and Holbrook, N. J. (2000) *J. Biol. Chem.* 275, 39435–39443
27. Persson, D. L., Yazlovskaya, E. M., and Pelling, J. C. (2000) *J. Biol. Chem.* 275, 35778–35785
28. Kim, J., Yoon, M. Y., Choi, S. L., Kang, I., Kim, S. S., Kim, Y. S., Choi, Y. K., and Ha, J. (2001) *J. Biol. Chem.* 276, 19102–19110
29. Chen, H. C., Bandypadhyay, G., Sajan, M. P., Kanoh, Y., Standaert, M., Farese, R. V., and Farese, R. V. (2002) *J. Biol. Chem.* 277, 23554–23562
30. Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Farese, R. V., Jr. (2002) *Annu. Rev. Biochem.* 71, 1215–1219
31. DePinho, R. A., and Cantley, L. C. (2004) *Cancer Gene Ther.* 11, 209–235
32. Alessi, D. R., Sakamoto, K., and Bayascas, J. R. (2006) *Annu. Rev. Biochem.* 75, 137–163
33. Alessi, D. R., Sakamoto, K., and Bayascas, J. R. (2006) *Annu. Rev. Biochem.* 75, 137–163
34. Tiainen, M., Ylikorkala, A., and Makela, T. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 9248–9251

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35. Fojo, T., and Bates, S. (2003) Oncogene 22, 7512–7523
36. Yun, H., Lee, M., Kim, S. S., and Ha, J. (2005) J. Biol. Chem. 280, 9963–9972
37. Kato, K., Ogura, T., Kishimoto, A., Minegishi, Y., Nakajima, N., Miyazaki, M., and Esumi, H. (2002) Oncogene 21, 6082–6090
38. Esumi, H., Izuishi, K., Kato, K., Hashimoto, K., Kurashima, Y., Kishimoto, A., Ogura, T., and Ozawa, T. (2002) J. Biol. Chem. 277, 32791–32798
39. Dyck, J. R., and Lopaschuk, G. D. (2006) J. Physiol. 574, 95–112
40. Motoshima, H., Goldstein, B. J., Igata, M., and Araki, E. (2006) J. Physiol. 574, 63–71
41. Luo, Z., Saha, A. K., Xiang, X., and Ruderman, N. B. (2005) Trends Pharmacol. Sci. 26, 69–76
42. Inoki, K., Zhu, T., and Guan, K. L. (2003) Cell 115, 577–590
43. Jones, R. G., Plas, D. R., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M. J., and Thompson, C. B. (2005) Mol. Cell 18, 283–293
44. Warburg, O. (1956) Science 123, 309–314
45. Marsin, A. S., Bouzin, C., Bertrand, L., and Hue, L. (2002) J. Biol. Chem. 277, 30778–30783
46. Pelicano, H., Martin, D. S., Xu, R. H., and Huang, P. (2006) Oncogene 25, 4633–4646
47. Galban, S., Martindale, J. L., Mazan-Mamczarz, K., Lopez de Silanes, I., Fan, J., Wang, W., Decker, J., and Gorospe, M. (2003) Mol. Cell Biol. 23, 7083–7095