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Overcoming Hypoxia-Induced Apoptotic Resistance through Combinatorial Inhibition of GSK-3β and CDK1

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Abstract

Tumor hypoxia is an inherent impediment to cancer treatment that is both clinically significant and problematic. In this study, we conducted a cell-based screen to identify small molecules that could reverse the apoptotic resistance of hypoxic cancer cells. Among the compounds, we identified were a structurally related group that sensitized hypoxic cancer cells to apoptosis by inhibiting the kinases GSK-3β and cyclin-dependent kinase (CDK) 1. Combinatorial inhibition of these proteins in hypoxic cancer cells and tumors increased levels of c-Myc and decreased expression of c-IAP2 and the central hypoxia response regulator hypoxia-inducible factor (HIF) 1α. In mice, these compounds augmented the hypoxic tumor cell death induced by cytotoxic chemotherapy, blocking angiogenesis and tumor growth. Taken together, our findings suggest that combinatorial inhibition of GSK-3β and CDK1 augment the apoptotic sensitivity of hypoxic tumors, and they offer preclinical validation of a novel and readily translatable strategy to improve cancer therapy. *Cancer Res; 71(15); 5265–75. ©2011 AACR.*

Introduction

A key feature of malignant tumors is the ability to adapt and survive under low oxygen (hypoxic) conditions (1). Prolonged or recurrent tumor hypoxia selects for cancer cells with increased survival signaling and loss of apoptotic potential (2, 3). Tumor hypoxia and expression of hypoxic biomarkers has been associated with resistance to radiotherapy, chemotherapies, and a number of molecularly targeted therapies including TNF-related apoptosis-inducing ligand (TRAIL; refs. 4, 5).

Multiple agents have been developed that target hypoxic tumor cells through inhibition of hypoxia-inducible factor (HIF) 1 (6–8), a master transcriptional regulator. Various strategies for pharmacologic inhibition of HIF-1 activity have been proposed, including inhibition of HIF-1 transcriptional activity, suppression of HIF-1α translation through inhibition of mTOR signaling, as well as increasing HIF-1α proteasomal degradation (8). Other strategies aim to exploit the hypoxic phenotype of tumors to increase the achievable therapeutic dose of cytotoxic chemotherapies by targeting them specifically to hypoxic cells as prodrugs (9, 10).

Here, we have conducted a small molecule screen in cancer cells cultured under low oxygen conditions to identify compounds which sensitize hypoxic cancer cells to apoptosis. We identified compounds which sensitize hypoxic tumor cells to TRAIL or chemotherapy-induced apoptosis through the combined inhibition of GSK-3β and cyclin-dependent kinase (CDK) 1. We show that inhibition of GSK-3β in hypoxic tumor cells promotes tumor cell death by elevating c-Myc expression and reducing c-IAP2 levels in a p53- and HIF-1α–independent manner. Inhibition of CDK1 results in decreased HIF-1α expression and transcriptional activity which leads to apoptotic sensitization of cancer cells and decreased tumor angiogenesis.

Materials and Methods

Caspase-3/7–specific assay and bioluminescent library screening

Apoptosis was imaged using the Caspase-Glo 3/7 Assay (Promega) as described previously (11). Hypoxic cell culture

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P.A. Mayes and N.G. Dolloff contributed equally to this work.

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experiments were carried out at 0.2% to 0.5% O_2 using the INVIVO2 Hypoxia Workstation (TOUCAN Technologies). Human, recombinant, His-tagged TRAIL was produced in DH5α *E. coli* and subsequently purified using Ni-NTA Superflow beads (Qiagen).

Flow cytometry, sub-G_1, and Apostain analysis

Cells were fixed with ethanol (sub-G_1) or methanol (Apostain) overnight at 4°C. Cell membranes were permeabilized using either phosphate citric acid buffer or formamide. For Apostain, cells were incubated with mouse primary antibody to ssDNA (F7-26). Cells were incubated with propidium iodide for 30 minutes at room temperature and then analyzed by flow cytometry.

Clonogenic survival assay

Cells were fixed (10% methanol, 10% acetic acid) and stained with crystal violet (0.4% crystal violet in 20% ethanol). Quantification of colonies was conducted by solubilizing the crystal violet in 33% acetic acid and measuring the absorbance at 540 nm in triplicate for each plate.

Tumor xenograft studies

HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells lines were a gift from the laboratory of Dr. Bert Vogelstein. SW620 and HT29 cells were purchased from the American Type Culture Collection. One million HCT116 $p53^{-/-}$ or SW620 cells were suspended in 50% Matrigel and injected subcutaneously into the flanks of Nu/J mice (Jackson Laboratory). Mice were housed and maintained in accordance with Institutional Animal Care and Use Committee and state and federal guidelines for the humane treatment and care of laboratory animals. *In vivo* vascular imaging was conducted as described previously (12).

Immunohistochemical and immunofluorescent analysis

Resected tumors were weighed then fixed in 4% paraformaldehyde. CD34 (MEC 14.7; Abcam) primary antibody was used at a 1:50 dilution. Blood vessel number and area were quantified per 10× field of view using IP Lab Software. Pimonidazole (60 mg/kg; Hypoxyprobe) was administered 90 minutes prior to sacrifice of the mice. For quantification of hypoxic tumor cell apoptosis via immunofluorescence, tumors were snap frozen in optimum cutting temperature (OCT) and cryosectioned. Cryosectioned slides were postfixed for 5 minutes with 10% neutral-buffered formalin. Fixed frozen sections were sequentially incubated with ApopTag TdT enzyme (Millipore) for 1 hour for the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay and Hypoxyprobe-1 mouse monoclonal antibody (1:10; Hypoxyprobe) at 4°C overnight. Sections were then coincubated with Cy3-antidigoxygenin (1:500) and Cy5-goat anti-mouse (1:200) for 30 minutes at 37°C.

Statistical analysis

We used the Student's *t* test assuming unequal variance (heteroscedastic *t* test), for calculation of significance. All values of $P < 0.05$ were considered significant.

Western blot analysis and siRNA-mediated knockdown

Primary antibodies for caspase-8, caspase-9, caspase-3, PARP, phospho-c-Myc (T58/S62), GSK-3β, phospho-β-catenin (Ser33/37/Thr41), β-catenin, c-IAP2, survivin (Cell Signaling) were used at a 1:1,000 dilution. HIF1-α (1:500) and RAN (1:5,000) were from BD Transduction Labs. c-Myc (9E10; 1:200), Mcl-1 (S19; 1:200), CDK1/cdc2/p34 (sc-54; 1:500), and p-survivin (Thr34; 1:250) were from Santa Cruz Biotechnology. c-FLIP (NF6; 1:500) was from ALEXIS Biochemicals and β-actin (Sigma) was used at a 1:5,000 dilution. For siRNA-mediated gene knockdown, HCT116 p53-null cells were transfected with gene-specific (10 μmol/L) or control siRNA (10 μmol/L; Santa Cruz Biotechnology) using Lipofectamine RNAiMAX Reagent (Invitrogen).

Results

Hypoxia induces apoptotic resistance in human colon carcinoma cells lacking p53

We tested the sensitivity of HCT116 colon carcinoma cells to TRAIL, 5-fluorouracil (5-FU), and CPT-11. At ambient oxygen cell culture conditions (normoxia), the absence of p53 was associated with relative apoptosis resistance in response to 5-FU and to a lesser extent CPT-11 and TRAIL (Fig. 1A). This was in accordance with our previous reports (13, 14). Hypoxic cell culture conditions imparted resistance following treatment with CPT-11 and 5-FU, regardless of p53 status, while making only the p53-null cells significantly resistant to TRAIL (Fig. 1A and Supplementary Fig. S1A). TRAIL treatment under hypoxia led to less caspase-8 cleavage and almost complete inhibition of caspase-9, caspase-3, and PARP cleavage as compared with normoxia (Fig. 1B). Flow cytometric analysis confirmed the decreased sensitivity of hypoxic HCT116 $p53^{-/-}$ cells to TRAIL, CPT-11, and 5-FU (Fig. 1C). Other human colon carcinoma cell lines with mutant or null p53 status also showed hypoxia-induced TRAIL resistance (Supplementary Fig. S1).

We next tested whether TRAIL and chemotherapy combinations sensitize resistant HCT116 $p53^{-/-}$ cells to apoptosis under hypoxia. Combinations of chemotherapy and TRAIL potently induced apoptosis in wild-type and p53-null HCT116 cells under normoxia (Fig. 1D). However, under hypoxic conditions, the combinations of TRAIL and chemotherapy induced apoptosis in p53 wild-type cells but failed to induce significant apoptosis in HCT116 p53-null cells (Fig. 1D). In addition, the hypoxia-activated prodrug tirapazamine (TPZ) failed to sensitize p53-deficient cells to TRAIL under hypoxic conditions (Supplementary Fig. S1D). This prompted us to conduct a small molecule screen to identify compounds capable of resensitizing p53-deficient hypoxic colon cancer cells to apoptosis.

A chemical library screen identifies compounds that reverse apoptotic resistance in hypoxic cancer cells

The National Cancer Institute chemical diversity library of 1,990 small molecules was screened against hypoxic HCT116 $p53^{-/-}$ colon carcinoma cells in the presence of TRAIL (Fig. 2A). Follow-up analysis of 36 small molecules identified

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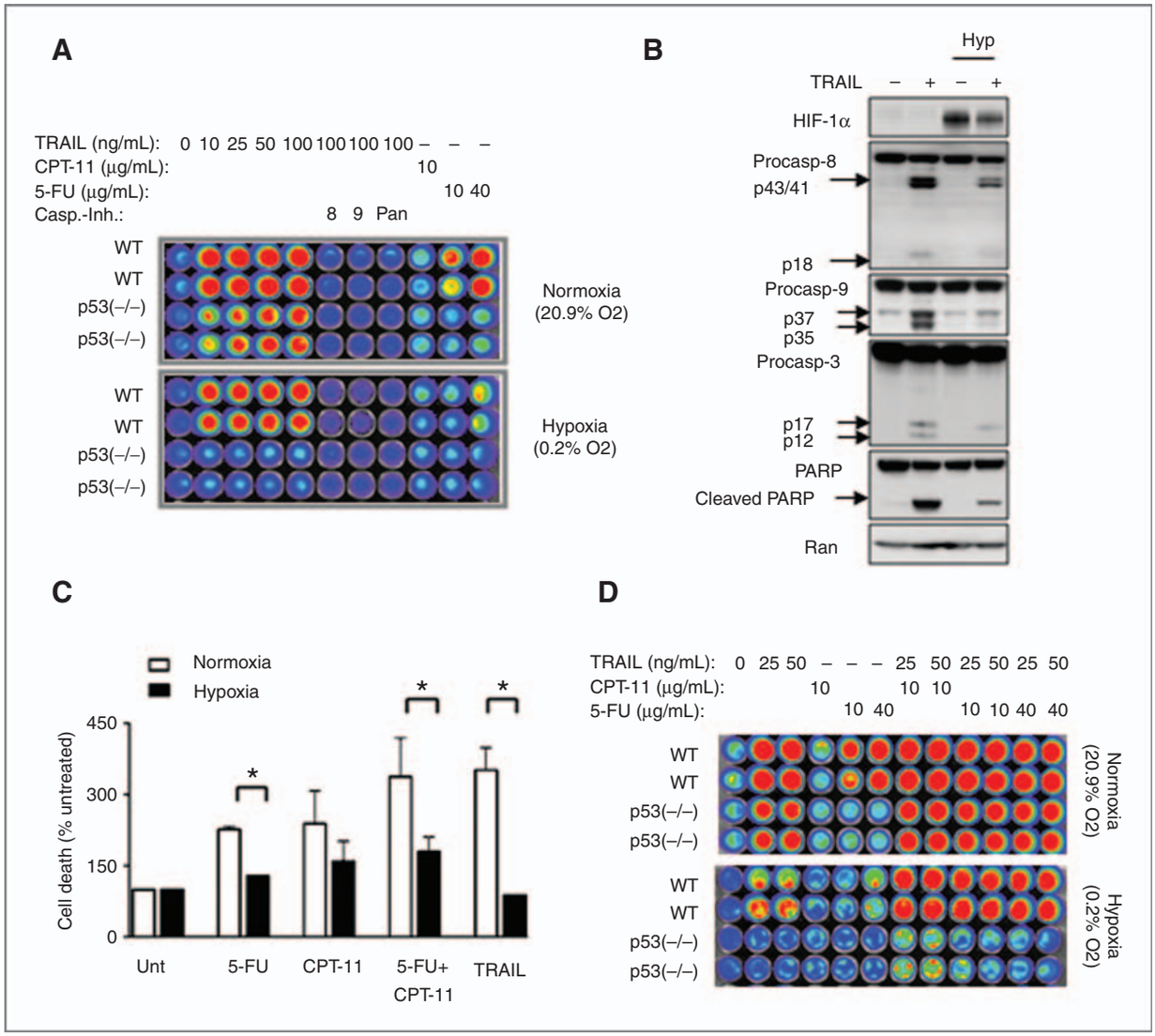

Figure 1. Hypoxic HCT116 *p53*$^{-/-}$ cells are resistant to therapy-induced apoptosis. A, HCT116 *p53*$^{+/+}$ and *p53*$^{-/-}$ cells were cultured under normal or low oxygen conditions for 24 hours in the presence of CPT-11, 5-FU, or TRAIL (final 6 hours). A bioluminescence-based assay was employed to measure caspase-3 activity. B, cleavage of caspase-8, caspase-9, caspase-3, and PARP was assessed by Western blot analysis in TRAIL-treated (3 hours) HCT116 *p53*$^{-/-}$ cells under normoxia or hypoxia (hyp). C, HCT116 *p53*$^{-/-}$ cells were cultured under normoxic or hypoxic conditions for 48 hours in the presence or absence of 5-FU (30 μmol/L), CPT-11 (50 μmol/L), 5-FU and CPT-11 (30/50 μmol/L), or TRAIL (50 ng/mL, 6 hours). Bars represent mean ± SEM (*, $P < 0.05$). D, caspase-3/7 activity was measured in normoxic and hypoxic HCT116 *p53*$^{+/+}$ and *p53*$^{-/-}$ cells exposed to combinations of TRAIL, CPT-11, and 5-FU. See also Supplementary Figure S1.

8 that sensitized hypoxic HCT116 *p53*$^{-/-}$ cells to TRAIL-induced apoptosis at a concentration of 5 μmol/L or less (Supplementary Fig. S2A and Table S1). Four of the 8 small molecules had structural homology to sangivamycin; hereafter, we refer to them as sangivamycin-like molecules (SLM; Fig. 2B and Supplementary Table S1). At 10 μmol/L, SLM2 and SLM3 were more potent apoptosis sensitizers than SLM1 or SLM4 (Supplementary Fig. S2B). At this dose, SLM2 and SLM3 caused significant G_2 cell-cycle arrest, whereas SLM1 and SLM4 did not (Supplementary Fig. S2B). In response to TRAIL treatment, SLM2 and SLM3 significantly increased caspase-8, caspase-9, and caspase-3 activation and increased PARP cleavage in hypoxic HCT116 *p53*$^{-/-}$ cells (Supplementary Fig. S2C). Therefore, SLM2 and SLM3 were selected for further analysis.

Submicromolar concentrations of SLM3 increased TRAIL-induced caspase-3 activation (Fig. 2C) and apoptosis (Fig. 2D) and decreased clonogenic survival (Fig. 2E) compared with TRAIL alone. Compared with tirapazamine or CPT-11, SLM3 caused significantly greater apoptotic induction under hypoxia (Fig. 2F). SLM3 also sensitized hypoxic HCT116 *p53*$^{-/-}$ cells to apoptosis induced by 5-FU and CPT-11

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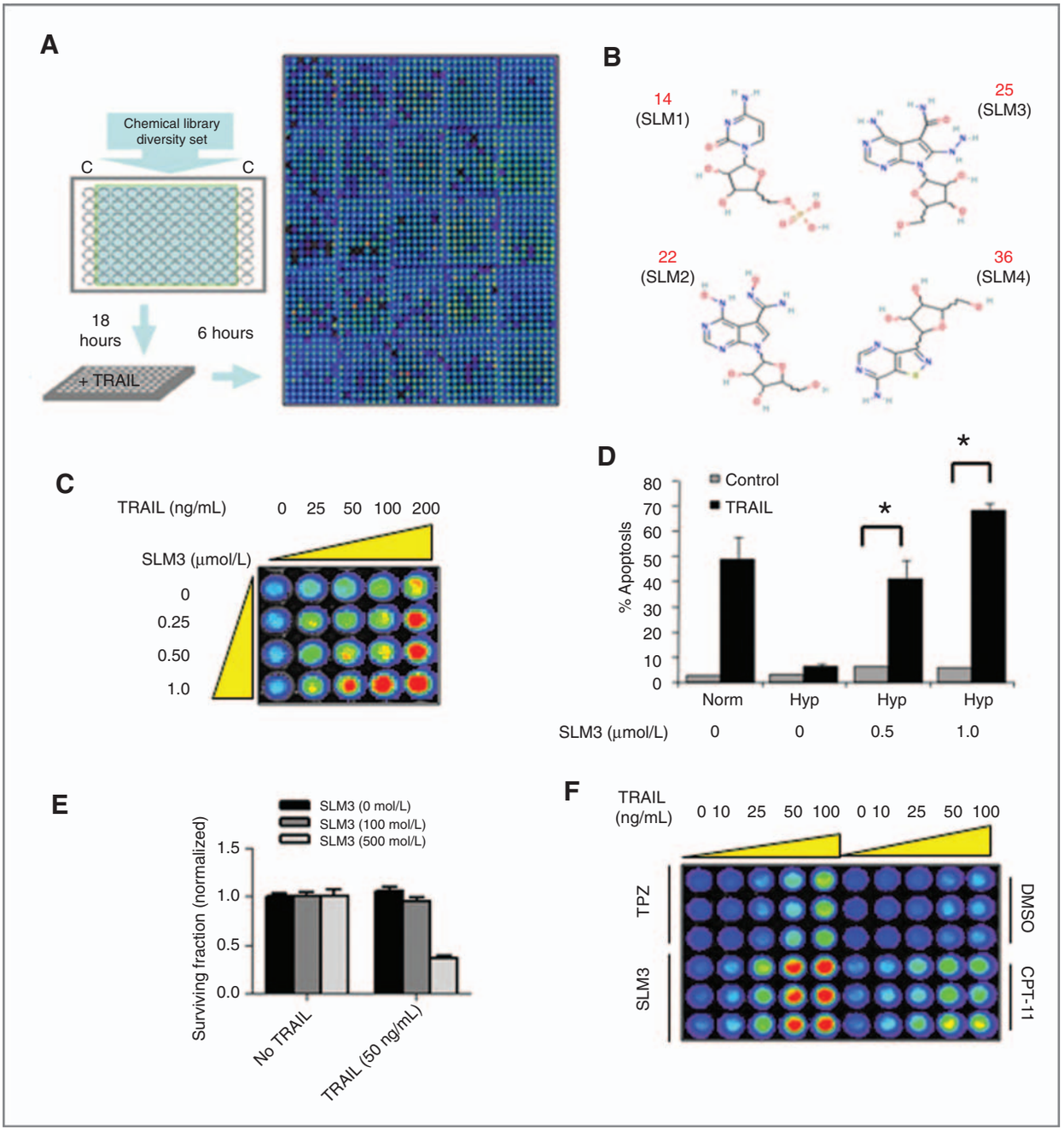

Figure 2. A chemical library screen identifies compounds that overcome hypoxia-induced apoptosis resistance. A, scheme representing the screen for small molecules (50 μmol/L) that sensitize hypoxic HCT116 $p53^{-/-}$ cells to TRAIL. B, structure of compounds 14, 22, 25, and 36, renamed SLM 1–4, respectively. C, caspase-3 activity was measured in hypoxic HCT116 $p53^{-/-}$ cells treated with increasing doses of SLM3 (24 hours) and TRAIL (final 6 hours). D, HCT116 $p53^{-/-}$ cells were treated with increasing doses of SLM3 (24 hours) combined with TRAIL (final 6 hours). Bars represent mean ± SEM (*, $P < 0.05$). E, clonogenic survival was assayed in HCT116 $p53^{-/-}$ cells treated with varying doses of SLM3 plus TRAIL. (Mean ± SEM; *, $P < 0.05$). F, bioluminescent caspase-3 activity was measured in HCT116 $p53^{-/-}$ cells treated with increasing concentrations of TRAIL (final 6 hours) in the presence or absence of SLM3 (1 μmol/L; 24 hours), CPT-11 (1 μmol/L), or tirapazamine (1 μmol/L). See also Supplementary Figure S2. DMSO, dimethyl sulfoxide.

(Supplementary Fig. S2D). SLM3 caused TRAIL sensitization in a panel of other cancer cell lines while having much less activity in 2 normal cell lines tested (Supplementary Fig. S2). We also found that other SLM-related structures possessed potent apoptotic sensitization activity (Supplementary Fig. S2). These results show that SLMs, particularly SLM3, effectively sensitize cancer cells, including multidrug resistant hypoxic cells, to TRAIL and chemotherapy-induced apoptosis.

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SLM3 increases c-Myc expression causing apoptotic sensitization in hypoxic cells

To determine the mechanism of SLM3-mediated apoptotic sensitization, we probed for changes in the expression of proteins that are known to mediate TRAIL-induced apoptosis. We have previously shown that expression of the c-Myc positively correlates with TRAIL sensitivity (15) and that hypoxia downregulates c-Myc expression (16). Upon examination of c-Myc protein levels, we found that SLM3 induced a dose-dependent increase in c-Myc expression in p53-deficient colon cancer cell lines under hypoxia (Fig. 3A and Supplementary Fig. S3). SLM2 also caused a significant increase in c-Myc protein expression in hypoxic cancer cells (Supplementary Fig. S3). In HCT116 cells, p53 status did not affect hypoxia-induced downregulation of c-Myc or induction of Mxi-1, an antagonist of c-Myc transcriptional activity (Supplementary Fig. S3C and D). To directly test the significance of SLM3-induced c-Myc in the apoptotic sensitization of hypoxic cancer cells, we inhibited c-Myc with siRNA then treated cells with SLM3 and TRAIL. RNA interference–mediated knockdown of c-Myc conferred TRAIL resistance in normoxic cells and inhibited SLM3-mediated TRAIL sensitization in hypoxic cells (Fig. 3B). In addition, we overexpressed c-Myc in hypoxic HCT116 $p53^{-/-}$ cells which resulted in significant resensitization to TRAIL-induced apoptosis (Fig. 3C). In hypoxic cancer cells, we observed an increase in c-IAP2 that corresponded with decreased c-Myc expression (Fig. 3A), and treatment with SLM3 resulted in a dose-dependent decrease in c-IAP2 protein expression, and to a lesser extent, c-FLIP and Mcl-1 (Fig. 3A and Supplementary Fig. S3E). Therefore, we hypothesized that c-IAP2 was a mediator of hypoxia-induced apoptotic resistance in hypoxic cancer cells. To test this hypothesis, we inhibited c-IAP2 with siRNA, which resulted in a significant increase in TRAIL-induced apoptosis in hypoxic HCT116

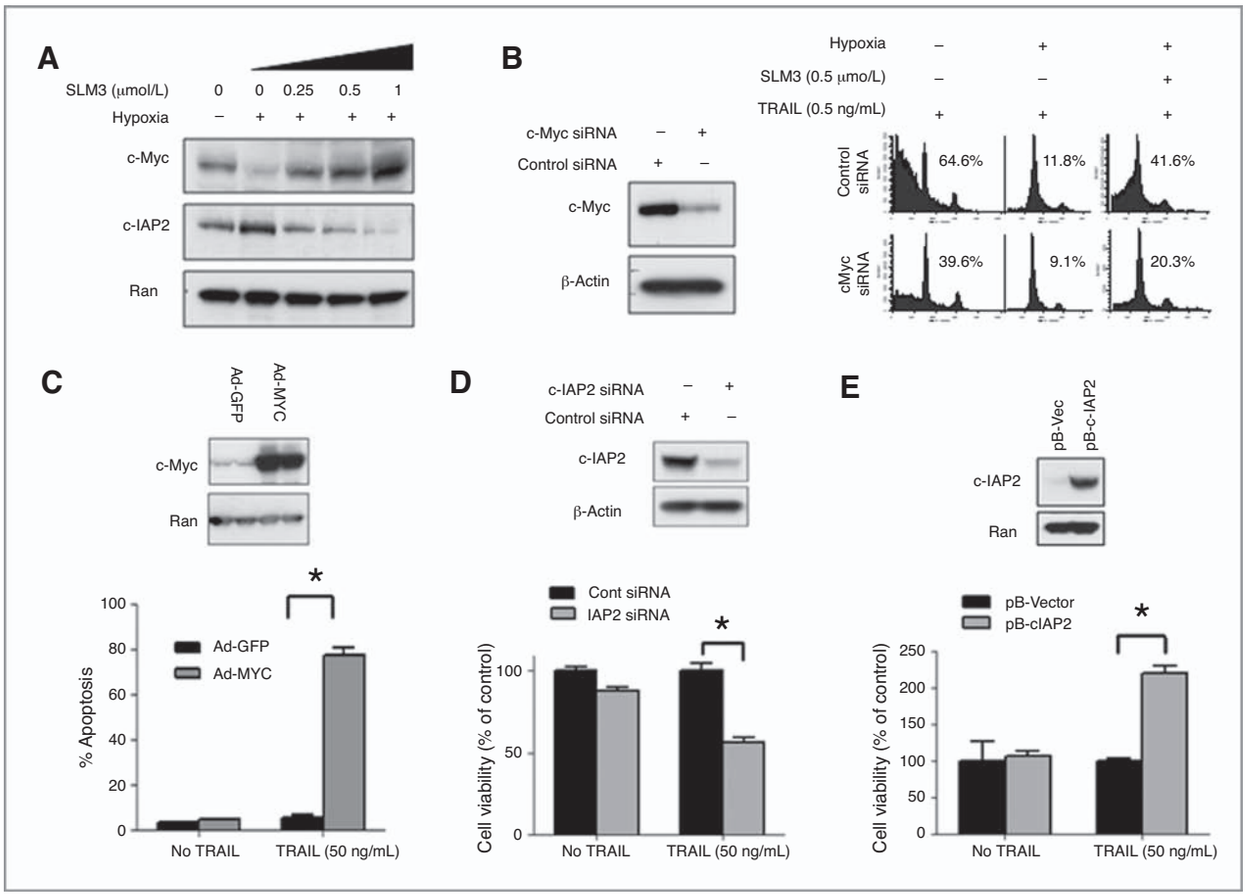

Figure 3. SLM3-mediated induction of c-Myc protein expression is critical for apoptotic sensitization. A, c-Myc expression was assessed by Western blotting in hypoxic HT29 cells treated with increasing concentrations of SLM3. B, HCT116 $p53^{-/-}$ cells were treated with c-Myc–targeted siRNA and cell death induced by $SLM3^{+/-}$ TRAIL was measured by sub-G_1 fluorescence-activated cell-sorting (FACS) analysis. Immunoblot showing the efficiency of c-Myc knockdown is shown (left). C, c-Myc or green fluorescent protein (GFP; control) were overexpressed in HCT116 $p53^{-/-}$ cells using adenoviral infection. Apoptosis was assessed by active caspase-3 FACS analysis in hypoxic cells following TRAIL treatment. Bars represent mean ± SEM (*, $P < 0.05$). D, c-IAP2 was inhibited with siRNA in HCT116 $p53^{-/-}$ cells, which were then treated with TRAIL (6 hours) under hypoxia (16 hours). c-IAP2 knockdown efficiency was confirmed by Western blotting (left). Bars represent mean ± SEM (*, $P < 0.05$). E, cell viability was measured in c-IAP2–overexpressing HCT116 $p53^{-/-}$ cells after treatment with a high dose of TRAIL (100 ng/mL) under hypoxic conditions. Bars represent mean ± SEM. (*, $P < 0.05$). See also Supplementary Figure S3.

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p53$^{-/-}$ cells (Fig. 3D). We also overexpressed c-IAP2 in hypoxic HCT116 *p53*$^{-/-}$ cells, which provided further protection from TRAIL-induced apoptosis (Fig. 3E). These data support the role of c-Myc and c-IAP2 in mediating TRAIL resistance and suggest that induction of c-Myc and repression of c-IAP2 by SLM contributes to TRAIL resensitization under low oxygen conditions.

SLM3 inhibits GSK-3β signaling leading to c-Myc protein stabilization and apoptotic sensitization

We investigated the effects of SLM3 on c-Myc phosphorylation and protein stability. We found that treatment with SLM3 inhibited hypoxia-induced c-Myc phosphorylation in a dose-dependent manner (Fig. 4A). Treatment with SLM3 also resulted in reduced c-Myc phosphorylation in HCT116

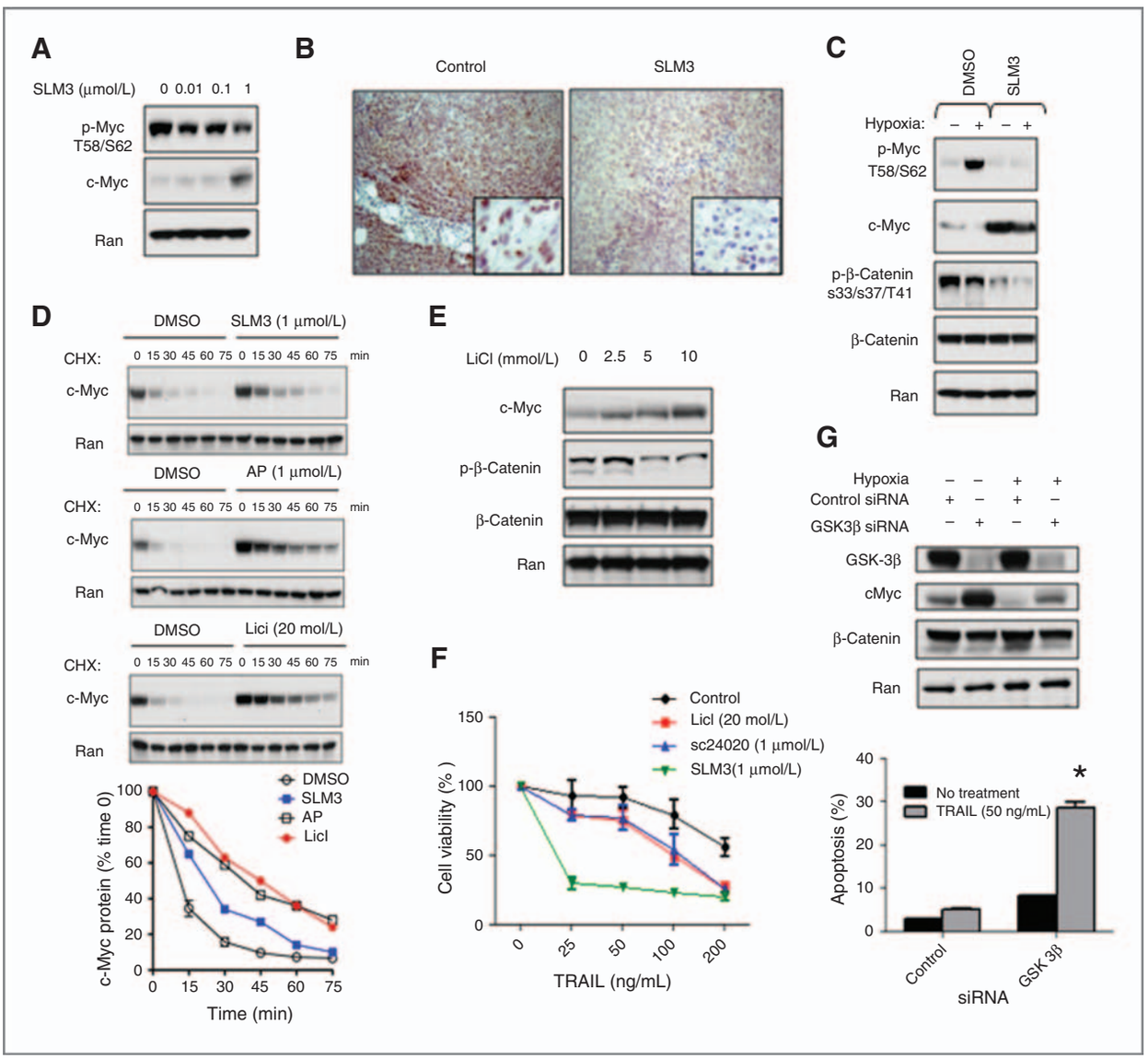

Figure 4. SLM3 inhibits GSK-3β–induced c-Myc phosphorylation, leading to stabilization of c-Myc protein. A, HCT116 *p53*$^{-/-}$ cells were treated with increasing concentrations of SLM3 for 12 hours under hypoxia. Western blots are shown. B, tumors from mice treated with SLM3 (25 mg/kg) were analyzed by immunohistochemistry for phospho-c-Myc (T58/S62) staining. Images are 100× with 400× inserts. C, HCT116 *p53*$^{-/-}$ cells were cultured under normoxic or hypoxic conditions in the presence or absence of SLM3 (1 μmol/L). Western blots are shown. D, HCT116 *p53*$^{-/-}$ cells were treated for 4 hours under hypoxic conditions in the presence of SLM3, AP, or LiCl. Cells were then exposed to cyclohexamide (CHX; 12.5 μg/mL) for the indicated times. Western blots and quantification of c-Myc band densitometry are shown. E, hypoxic HCT116 *p53*$^{-/-}$ cells were treated with increasing concentrations of LiCl. Immunoblots are shown. F, hypoxic HCT116 *p53*$^{-/-}$ cells were treated with increasing concentrations of TRAIL in the presence or absence of the GSK-3β inhibitors LiCl, sc-24020, and SLM3. Markers represent mean viability ± SEM (*, $P < 0.05$ for control compared with LiCl, sc-24020, and SLM3). G, HCT116 *p53*$^{-/-}$ cells were treated with GSK-3β–targeted siRNA under normoxic and hypoxic conditions (24 hours). Western blots are shown (top). HCT116 *p53*$^{-/-}$ cells were treated with GSK-3β–targeted siRNA, then TRAIL under hypoxia. Bars represent mean sub-G_1% ± SEM (*, $P < 0.05$). See also Supplementary Figure S4.

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p53$^{-/-}$ xenograft tumors (Fig. 4B). We next tested the effect of SLM3 on phosphorylation of β-catenin, another GSK-3β substrate. Consistent with the activity of a GSK-3β inhibitor, SLM3 inhibited phosphorylation of β-catenin (Fig. 4C). We conducted a rabbit reticulocyte lysate GSK-3β kinase assay using c-Myc as protein substrate. SLM3 partially inhibited GSK-3β kinase activity toward c-Myc in this cell-free system (Supplementary Fig. S4). To test whether the GSK-3β inhibitory activity of SLM3 is direct, we conducted *in vitro* kinase assays using purified recombinant GSK-3β. In this cell-free system, SLM3 did not inhibit GSK-3β kinase activity (data not shown), suggesting an indirect mechanism of GSK-3β inhibition by SLM3.

Phosphorylation of c-Myc by GSK-3β at threonine 58 is known to target the protein for proteasomal degradation (17, 18). Therefore, we conducted cyclohexamide chase experiments to investigate the effects of SLM3 on c-Myc protein stability. c-Myc protein is highly unstable under hypoxic conditions, with a half-life of approximately 15 minutes (Fig. 4D). However, in the presence of SLM3, LiCl, and alsterpaullone (AP), c-Myc protein stability was significantly increased (Fig. 4D). We also found that SLM3 moderately induced c-Myc mRNA expression in hypoxic cells through a β-catenin–independent mechanism (Supplementary Fig. S4). Treatment with other pharmacologic GSK-3β inhibitors also resulted in increased c-Myc protein expression (Fig. 4E) as well as increased TRAIL sensitivity (Fig. 4F) in hypoxic cells, albeit to a lesser extent than SLM3. In addition, siRNA knockdown of GSK-3β resulted in increased c-Myc protein expression and an increase in TRAIL-induced apoptosis under hypoxia (Fig. 4G).

Inhibition of CDK1 contributes to the apoptotic sensitization of hypoxic cancer cells

Upon examination of cell-cycle profiles, we found that the effects of SLM3 more closely resembled those of the dual GSK-3β/CDK inhibitor AP, than LiCl, a selective GSK-3β inhibitor (Fig. 5A). SLM3 and AP, but not LiCl, caused G_2–M phase cell-cycle arrest, an effect that is consistent with CDK1 inhibition. Tumors from mice treated with SLM3 also had a significantly higher percentage of cells in G_2–M phase (Fig. 5B). SLM3

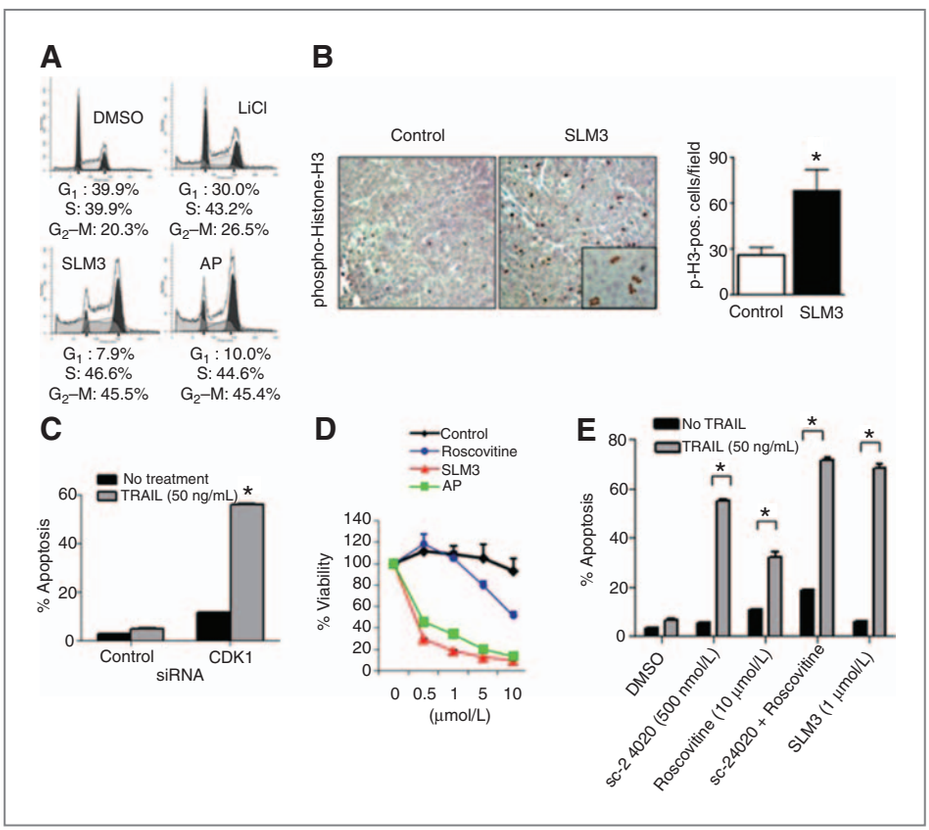

Figure 5. Inhibition of CDK1 contributes to SLM3-induced apoptotic sensitization in hypoxic cancer cells. A, HCT116 *p53*$^{-/-}$ cells were treated with LiCl (20 mmol/L), SLM3 (1 μmol/L), and AP (1 μmol/L) for 30 hours under hypoxia. Cell-cycle analysis was conducted by FACS analysis of propidium iodide–stained cells. B, tumors from mice treated with SLM3 (25 mg/kg) were analyzed by immunohistochemistry for phospho-histone-H3–positive staining. Images are 100× with 400× inserts. Bars represent mean ± SEM (*, $P < 0.05$). C, HCT116 *p53*$^{-/-}$ cells were treated with CDK1-specific siRNA followed by TRAIL treatment under hypoxia. Bars represent mean sub-G_1% ± SEM (*, $P < 0.05$). D, hypoxic HCT116 *p53*$^{-/-}$ cells were treated with TRAIL (50 ng/mL) in the presence of increasing concentrations of roscovitine, SLM3, or AP. Markers represent mean% viability ± SEM (*, $P < 0.05$ for control compared with roscovitine, AP, and SLM3). E, HCT116 *p53*$^{-/-}$ cells were treated with roscovitine, sc-24020, sc-24020/roscovitine combination, or SLM3 followed by TRAIL treatment under hypoxia. Bars represent mean sub-G_1% ± SEM (*, $P < 0.05$). See also Supplementary Figure S5.

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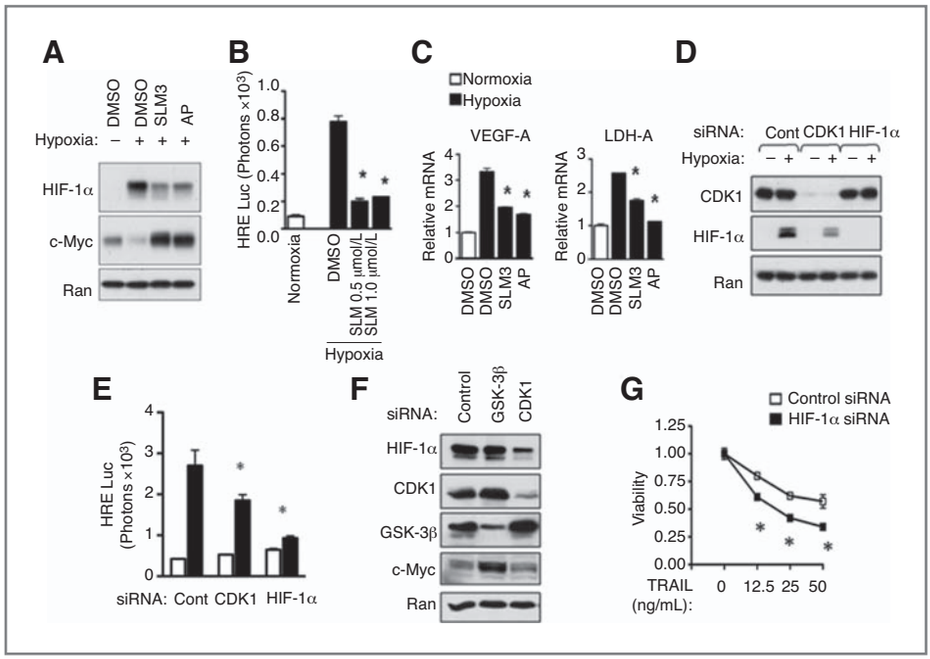

Figure 6. Inhibition of CDK1 causes repression of HIF-1α expression and activity under hypoxia. A, HCT116 $p53^{-/-}$ cells were treated with SLM3 (1 μmol/L) or AP (1 μmol/L) for 16 hours. Western blots are shown. B, *HRE* luciferase activity was measured in HCT116 $p53^{-/-}$ cells that were treated with increasing concentrations of SLM3. Bars represent mean ± SEM (*, $P < 0.05$). C, HCT116 $p53^{-/-}$ cells were treated as in (A). Quantitative real-time PCR was carried out for the HIF-1α target genes, *VEGF-A* and *LDH-A*. Bars represent mean ± SEM (*, $P < 0.05$). D, CDK1 and HIF-1α were knocked down using siRNA in HCT116 $p53^{-/-}$ cells. Lysates from normoxic and hypoxic cells were analyzed by Western blotting. E, *HRE* luciferase activity was assessed in cells treated as in (D). Bars represent mean ± SEM (*, $P < 0.05$). F, CDK1 and GSK-3β expression were inhibited in HCT116 $p53^{-/-}$ cells using siRNA. Cells were cultured under hypoxic conditions for 16 hours. Western blots are shown. G, HIF-1α was inhibited with siRNA in HCT116 $p53^{-/-}$ cells, which were treated with increases concentrations of TRAIL for 24 hours. Cell viability was measured using a bioluminescence-based assay. Markers represent mean ± SEM (*, $P < 0.05$). See also Supplementary Figure S6.

inhibited purified recombinant CDK1/cyclin B in a dose-dependent manner (Supplementary Fig. S5), and treatment with SLM3 resulted in the decreased phosphorylation of the CDK1 substrate survivin, an affect not observed with GSK-3β–specific inhibitors LiCl and sc-24020 (Supplementary Fig. S5). To validate the functional significance of CDK1 inhibition, we used siRNA to directly inhibit CDK1 in hypoxic *HCT116* $p53^{-/-}$ cells, which resulted in significant sensitization to TRAIL-induced apoptosis (Fig. 5C). Furthermore, the dual-GSK-3β/CDK1 kinase inhibitor AP increased TRAIL-induced apoptosis to a greater extent than roscovitine, a CDK inhibitor with little activity toward GSK-3β, and exhibited TRAIL sensitization under hypoxia with efficacy that was comparable to SLM3 (Fig. 5D and Supplementary Fig. S5). When we combined GSK-3β (sc-24020) and CDK inhibitors (roscovitine) in hypoxic cells, we found that the combination was a more potent TRAIL sensitizer than either agent alone (Fig. 5E and Supplementary Fig. S5E). Therefore, combined inhibition of GSK-3β and CDK1 is an effective strategy for overcoming resistance to TRAIL under hypoxia.

CDK1 inhibitors increase the apoptotic sensitivity of hypoxic cells through inhibition of HIF-1α

We discovered that treatment of hypoxic cancer cells with SLM3 or AP decreased HIF-1α expression (Fig. 6A) and transcriptional activity (Fig. 6B). Treatment with SLM3 or AP also resulted in a corresponding decrease in the expression of the HIF-1 transcriptional target genes *VEGF-A* and *LDH-A* (Fig. 6C). SLM3 and AP inhibited HIF-1α through the targeting of CDK1, as inhibition of CDK1 with siRNA also decreased hypoxia-induced HIF-1α expression (Fig. 6D) and transcriptional activity (Fig. 6E). This action is specific to the CDK1 inhibitory activity of SLM3 and AP, as siRNA knockdown of GSK-3β had no effect on HIF-1α expression (Fig. 6F). Reduction of HIF-1α expression was also not an effect of increased c-Myc expression (Supplementary Fig. S6). Likewise, HIF-1α induction under hypoxia was not responsible for the decreased expression of c-Myc protein (Supplementary Fig. S6B).

Treatment with SLM3 had no effect on the half-life of HIF-1α protein under hypoxia (Supplementary Fig. S6). In addition, treatment of hypoxic cells with the proteasome inhibitor bortezomib failed to rescue SLM3-mediated repression of HIF-1α (Supplementary Fig. S6). However, treatment with SLM3, AP, and CDK1 siRNA resulted in decreased HIF-1α mRNA expression (Supplementary Fig. S6F), suggesting that inhibition of CDK1 may reduce transcription of HIF-1α. To directly test the functional significance of HIF-1α inhibition in hypoxic cancer cells, we inhibited HIF-1α with siRNA, which resulted in a modest but significant increase in

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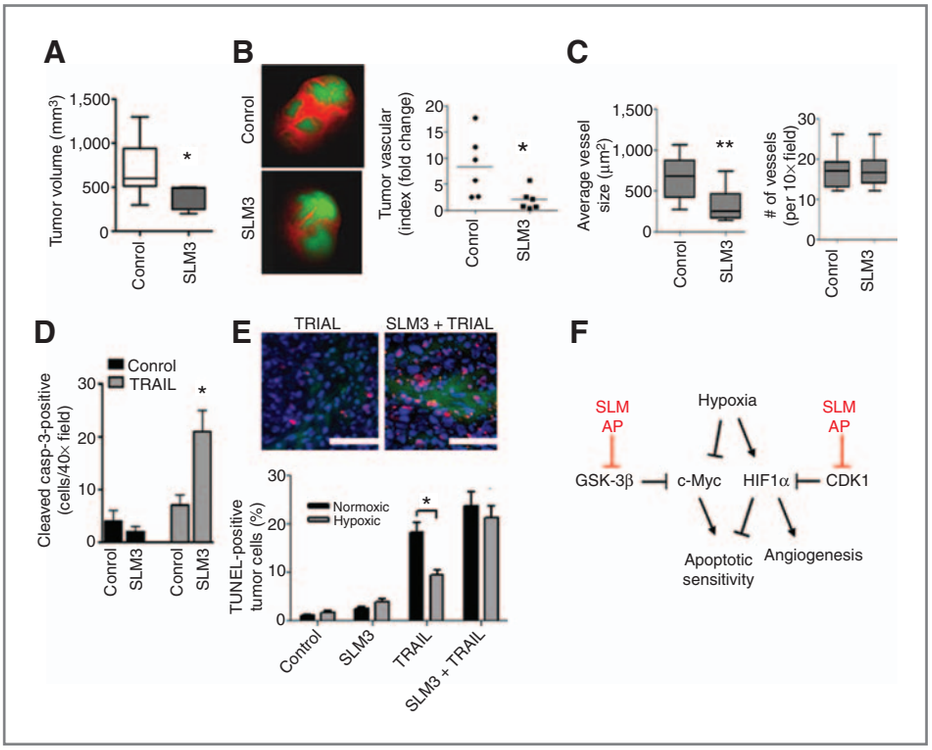

Figure 7. SLM3 inhibits tumor growth and sensitizes tumor cells to TRAIL-induced apoptosis. A, mice with HCT116 $p53^{-/-}$ tumors were treated with SLM3 (25 mg/kg). Mean ± SEM (*, $P < 0.05$). B, representative images of HCT116 $p53^{-/-}$ xenograft tumors imaged using noninvasive vascular imaging (#, average vascular indices of tumors; *, $P < 0.05$). C, quantification of tumor vessels by immunostaining of CD34 (Mean ± SEM; *, $P < 0.05$). D, immunostaining of cleaved caspse-3 in SW620 tumors treated with SLM3 (12.5mg/kg), TRAIL (100 μg), or SLM3 and TRAIL combination. E, hypoxia and apoptosis were measured in tumors by dual fluorescence imaging of Hypoxyprobe-1 (green) and ApopTag (red), respectively. White scale bars represent 50 μm. Bars represent mean% TUNEL–positive cells per 100× field of view ± SEM (*, $P < 0.05$). F, mechanistic model of SLM3 activity in tumor cells. See also Supplementary Figure S7.

TRAIL-induced death in hypoxic HCT116 $p53^{-/-}$ cells (Fig. 6G). SLM3 and AP also repressed hypoxia-induced HIF-2α protein expression (Supplementary Fig. S6). However, unlike HIF-1α, the knockdown of HIF-2α by siRNA failed to increase the apoptotic sensitivity of hypoxic cells (Supplementary Fig. S6).

SLM3 exhibits *in vivo* antitumor and antiangiogenic activity and enhances the activity of TRAIL and 5-FU

We next evaluated the *in vivo* activity of SLM3. Treatment with SLM3 significantly inhibited tumor growth and increased peripheral tumor necrosis relative to controls (Fig. 7A and Supplementary Fig. S7). SLM3 also showed a robust antiangiogenic effect, as tumors from SLM3-treated mice were significantly less vascular (Fig. 7B and C). In short-term combination studies, we found that 2 consecutive days of low-dose SLM3 (12.5 mg/kg) treatment prior to TRAIL treatment significantly increased cleaved (active) caspase-3 staining in SW620 tumor xenografts (Fig. 7D). SLM3 treatment significantly increased TRAIL-induced apoptosis in both normoxic and hypoxic tumor tissue (Fig. 7E). We also detected significant reductions in tumor growth when SLM3 was combined with 5-FU in studies using both wild-type and p53-deficient HCT116 tumor xenografts (Supplementary Fig. S7). Taken together, we propose a model whereby the combined inhibition of GSK-3β and CDK1 under hypoxia results in the stabilization of c-Myc and inhibition of HIF-1α expression (Fig. 7F).

Discussion

We conducted a chemical screen to identify small molecules that overcome apoptotic resistance in hypoxic tumors, which uniquely pinpointed compounds that are dual inhibitors of GSK-3β and CDK1. Small molecule kinase inhibitors with dual specificity for GSK-3β and CDK1 have previously been described, and this cross-reactivity is attributed to the similarity in ATP-binding pocket structure of these kinases (19). The chemical structures of the SLMs identified here are closely related to sangivamycin, which is a nucleoside analogue that was found nearly 40 years ago to have antitumor activity. Later studies identified sangivamycin as a potent inhibitor of protein kinases including protein kinase C (PKC; ref. 20). More recently some of the SLMs that we describe here, including SLM3 (NSC188491), were identified as having potent cancer growth inhibitory and antiangiogenic activity (21). Like sangivamycin, SLM3 has also been shown to possess activity as a PKC inhibitor (22). We did not investigate the role of PKC in regulating the apoptotic sensitivity of hypoxic cancer cells. However, we believe that PKC represents a candidate upstream regulator of GSK-3β in hypoxic cancer cells. Future studies should also address the potential inhibitory activity of SLMs toward other cellular kinases.

GSK-3β is a serine/threonine protein kinase that regulates diverse cellular and physiologic processes. In cancer, GSK-3β is commonly recognized as a putative tumor suppressor due to its function as a repressor of β-catenin signaling (23), and

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the phosphorylation-dependent downregulation of cell-cycle regulators cyclin D1 (24), cdc25 (25), and c-Myc (17, 18). On the other hand, GSK-3β can promote cell survival and oppose apoptosis (26–28). Therefore, GSK-3β functions as a mediator of cancer cell survival in some contexts, particularly through the regulation of c-Myc. We have previously reported that overexpression of c-Myc confers increased sensitivity to TRAIL, through repression of NF-κB–induced antiapoptotic factors c-FLIP, Mcl-1, and c-IAP2 (15, 29). Elevated expression of c-Myc has also been associated with increased sensitivity and survival of colon carcinoma patients that received 5-FU treatment (30, 31). Under hypoxic conditions, c-Myc protein expression and transactivation are repressed. Repression of c-Myc activity was shown to occur through HIF1-α–dependent induction of Mxi-1, a repressor of c-Myc transcriptional activity (16). In addition, phosphorylation of c-Myc protein targets it for proteasomal degradation under hypoxic conditions (16, 32). Induction of c-IAP2 in hypoxic cells via an undescribed, HIF-1–independent mechanism has also been previously reported (33).

GSK-3β kinase inhibitors are in clinical trials, primarily for the treatment of type II diabetes and neurodegenerative disorders. Our data suggest that these inhibitors may also be good candidates for anticancer drug development. Epidemiologic data show that long-term use of LiCl in patients with bipolar disorder is not associated with increased cancer prevalence (34), and LiCl does not increase the number of tumors in a mutant APC mouse model (35). A recent report has also shown that pharmacologic inhibition of GSK-3 causes growth inhibition in leukemias with oncogenic MLL mutations (36).

CDK1/cdc2 is a cyclin-dependent protein kinase, which regulates the mitotic G_2–M cell-cycle checkpoint. CDK1 promotes cell survival through the regulation of the antiapoptotic factors survivin (37, 38) and Mcl-1 (39, 40). The potential of CDK inhibitors including CDK1 has been proposed for the treatment of cancer (13), and small molecule CDK inhibitors are in clinical trials for a number of cancer types. Inhibition of CDK1 sensitizes cancer cells to TRAIL-induced apoptosis (14, 41) and also has a synthetic lethal effect in MEFs overexpressing c-Myc (42). We show that inhibition of CDK1 leads to apoptotic sensitization in hypoxic cancer cells in part due to decreased HIF-1α expression and transcriptional activity and also through decreased survivin phosphorylation. To our knowledge, this is the first report of CDK1-dependent regulation of HIF-1α expression and activity. Flavopiridol, a pleiotropic kinase inhibitor whose targets include CDKs, was previously reported to inhibit HIF-1α expression (43). Future studies should address the specific mechanism of CDK1-dependent HIF-1α regulation.

In summary, we have shown the potential for targeting hypoxic regions of human tumors with novel therapeutic combinations. Future studies should test dual GSK-3β/CDK1 inhibitors, such as AP, in other tumor types where tumor hypoxia is thought to play a role in therapeutic resistance.

Disclosure of Potential Conflicts of Interest

W.S. El-Deiry is an American Cancer Society Research Professor. No potential conflicts of interest were disclosed.

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