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 performed 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 CDK1. 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 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.
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 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (4, 5).

Multiple agents have been developed that target hypoxic tumor cells through inhibition of hypoxia-inducible factor 1 (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 pro-drugs (9, 10).

Here we have performed a small molecule screen in cancer cells cultured under low oxygen conditions in order 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 CDK1. 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 experiments were carried out at 0.2-0.5% O₂ 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-G1 and APOSTAIN analysis.

Cells were fixed with ethanol (sub-G1) 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 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 performed 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 lab of Dr. Bert Vogelstein. SW620 and HT29 cells were purchased from the ATCC. One million HCT116 p53-/- or SW620 cells were suspended in 50% Matrigel and injected subcutaneously into the flanks of Nu/J mice (Jackson Labs). 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 performed as described previously (12).

Immunohistochemical (IHC) and immunofluorescent (IF) 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 was quantified per 10x field of view using IP Lab Software. Pimonidazole (60 mg/kg) (Hypoxprobe) was administered 90 min prior to sacrifice of the mice. For quantification of hypoxic tumor cell apoptosis via immunofluorescence, tumors were snap frozen in OCT and cryosectioned. Cryosectioned slides were post-fixed for 5 minutes with 10% neutral-buffered formalin. Fixed frozen sections were sequentially incubated with ApopTag TdT enzyme (Millipore) for 1 hr for the TUNEL assay and Hypoxprobe-1 mouse monoclonal antibody (1:10) (Hypoxprobe) at 4°C overnight. Sections were then co-incubated with Cy3-antidigoxygenin (1:500) and Cy5-goat anti-mouse (1:200) for 30 min at 37°C.

Statistical analysis
We used the Student’s \textit{t}-test assuming unequal variance (heteroscedastic \textit{t}-test), for calculation of significance. All P values below 0.05 were considered significant.

**Western blot analysis and siRNA mediated knockdown**

Primary antibodies for Caspase-8, Caspase-9, Caspase-3, PARP, phospho-cMyc (Thr58/Ser62), GSK3\(\beta\), phospho-\(\beta\)-catenin (Ser33/37/Thr41), \(\beta\)-catenin, cIAP2, Survivin, (Cell Signaling) were used at a (1:1000) dilution. HIF1-\(\alpha\) (1:500), and RAN (1:5000) 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 \(\beta\)-actin (Sigma) was used at a (1:5000) dilution. For siRNA-mediated gene knockdown HCT116 p53-null cells were transfected with gene-specific (10 \(\mu\)M) or Control siRNA (10 \(\mu\)M) (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-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 (Figure 1A). This was in accordance with our previous reports (39, 40). 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 (Figures 1A and 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 to normoxia (Figure 1B). Flow cytometric analysis confirmed the decreased sensitivity of hypoxic HCT116 p53-/- cells to TRAIL, CPT-11 and 5-FU (Figure 1C). Other human colon carcinoma cell lines with mutant or null p53 status also demonstrated hypoxia-induced TRAIL resistance (Figure 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 (Figure 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 (Figure 1D). In addition, the hypoxia-activated pro-drug, tirapazamine (TPZ), failed to sensitize p53-deficient cells to TRAIL under hypoxic conditions (Figure S1D). This prompted us to perform a small molecule screen to identify compounds capable of re-sensitizing p53-deficient hypoxic colon cancer cells to apoptosis.

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

The NCI chemical diversity library of 1,990 small molecules was screened against hypoxic HCT116 p53-/- colon carcinoma cells in the presence of TRAIL (Figure 2A). Follow-up analysis of 36 small molecules identified eight that sensitized hypoxic HCT116 p53-/- cells to TRAIL-induced apoptosis at a concentration ≤ 5 µM (Figure S2A and Table S1). Four of the eight small molecules had structural homology to sangivamycin; hereafter,
we refer to them as sangivamycin-like molecules (SLMs) (Figure 2B and Table S1). At 10 µM, SLM2 and SLM3 were more potent apoptosis sensitizers than SLM1 or SLM4 (Figure S2B). At this dose, SLM2 and SLM3 caused significant G2 cell-cycle arrest whereas SLM1 and SLM4 did not (Figure S2B). In response to TRAIL treatment, SLM2 and SLM3 significantly increased caspase-8, -9 and -3 activation and increased PARP cleavage in hypoxic HCT116 p53-/− cells (Figure S2C). Therefore, SLM2 and SLM3 were selected for further analysis.

Sub-micromolar concentrations of SLM3 increased TRAIL-induced caspase-3 activation (Figure 2C), apoptosis (Figure 2D) and decreased clonogenic survival (Figure 2E) compared to TRAIL alone. Compared with tirapazamine or CPT-11, SLM3 caused significantly greater apoptotic induction under hypoxia (Figure 2F). SLM3 also sensitized hypoxic HCT116 p53-/− cells to apoptosis induced by 5-FU and CPT-11 (Figure S2D). SLM3 caused TRAIL sensitization in a panel of other cancer cell lines while having much less activity in two normal cell lines tested (Figure S2). We also found that other SLM-related structures possessed potent apoptotic sensitization activity (Figure S2). These results demonstrate that sangivamycin-like molecules (SLMs), particularly SLM3, effectively sensitize cancer cells, including multi-drug resistant hypoxic cells, to TRAIL and chemotherapy-induced apoptosis.

**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 (25), and that hypoxia down-regulates c-Myc expression (29). 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 (Figures 3A and S3). SLM2 also caused a significant increase in c-Myc protein expression in hypoxic cancer cells (Figure S3). In HCT116 cells, p53 status did not affect hypoxia-induced down-regulation of c-Myc or induction of Mxi-1, an antagonist of c-Myc transcriptional activity (Figure S3C, 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. RNAi-mediated knockdown of c-Myc conferred TRAIL-resistance in normoxic cells and inhibited SLM3-mediated TRAIL-sensitization in hypoxic cells (Figure 3B). In addition, we over-expressed c-Myc in hypoxic HCT116 p53-/− cells which resulted in significant re-sensitization to TRAIL-induced apoptosis (Figure 3C). In hypoxic cancer cells we observed an increase in c-IAP2 that corresponded with decreased c-Myc expression (Figure 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 (Figure 3A and Figure 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 p53-/− cells (Figure 3D). We also over-expressed c-IAP2 in hypoxic HCT116 p53-/− cells, which provided further protection from TRAIL-induced apoptosis (Figure 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 re-sensitization 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 (Figure 4A). Treatment with SLM3 also resulted in reduced c-Myc phosphorylation in HCT116 p53−/− xenograft tumors (Figure 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 (Figure 4C). We performed 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 (Figures S4). To test if the GSK-3β inhibitory activity of SLM3 is direct we performed 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 (13,14). Therefore, we performed 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 (Figure 4D). However, in the presence of SLM3, LiCl, and AP, c-Myc protein stability was significantly increased (Figure 4D). We also found that SLM3 moderately induced c-Myc mRNA expression in hypoxic cells through a β-catenin-independent
mechanism (Figure S4). Treatment with other pharmacologic GSK-3β inhibitors also resulted in increased c-Myc protein expression (Figure 4E) as well as increased TRAIL sensitivity (Figure 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 (Figure 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 alsterpaullone (AP), than LiCl, a selective GSK-3β inhibitor (Figure 5A). SLM3 and AP, but not LiCl, caused G2/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 G2/M phase (Figure 5B). SLM3 inhibited purified recombinant CDK1/cyclinB in a dose-dependent manner (Figure 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 (Figure 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 (Figure 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 towards GSK-3β, and exhibited TRAIL sensitization under hypoxia with efficacy that was comparable to SLM3 (Figure 5D, 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 (Figure 5E and 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 (Figure 6A) and transcriptional activity (Figure 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 (Figure 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 (Figure 6D) and transcriptional activity (Figure 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 (Figure 6F). Reduction of Hif-1α expression was also not an effect of increased c-Myc expression (Figure S6). Likewise, Hif-1α induction under hypoxia was not responsible for the decreased expression of c-Myc protein (Figure S6B).

Treatment with SLM3 had no effect on the half-life of Hif-1α protein under hypoxia (Figure S6). In addition, treatment of hypoxic cells with the proteasome inhibitor bortezomib failed to rescue SLM3-mediated repression of Hif-1α (Figure S6). However, treatment with SLM3, AP and CDK1 siRNA resulted in decreased Hif-1α mRNA expression (Figure 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 TRAIL-induced death in hypoxic HCT116 p53-/- cells (Figure 6G). SLM3 and AP also repressed hypoxia-induced Hif-2α protein expression (Figure S6). However, unlike Hif-1α, the knockdown of Hif-2α by siRNA failed to increase the apoptotic sensitivity of hypoxic cells (Figure S6).

**SLM3 exhibits in vivo anti-tumor and anti-angiogenic 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 S7). SLM3 also demonstrated a robust anti-angiogenic effect, as tumors from SLM3-treated mice were significantly less vascular (Fig. 7B,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-fluorouracil (5-FU) in studies using both wild-type and p53-deficient HCT116 tumor xenografts (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 (Figure 7F).
Discussion:

We performed 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 (15). The chemical structures of the SLMs identified here are closely related to sangivamycin, which is a nucleoside analog that was found nearly 40 years ago to have anti-tumor activity. Later studies identified sangivamycin as a potent inhibitor of protein kinases including protein kinase C (PKC) (16). More recently some of the SLMs that we describe here, including SLM3 (NSC188491), were identified as having potent cancer growth inhibitory and anti-angiogenic activity (17). Like sangivamycin, SLM3 has also been shown to possess activity as a PKC inhibitor (18). We did not investigate the role of PKC in regulating the apoptotic sensitivity of hypoxic cancer cells. However, we believe PKC represents a candidate upstream regulator of GSK-3β in hypoxic cancer cells. Future studies should also address the potential inhibitory activity of SLMs towards other cellular kinases.

GSK-3β is a serine/threonine protein kinase that regulates diverse cellular and physiological processes. In cancer, GSK-3β is commonly recognized as a putative tumor suppressor due to its function as a repressor of β-catenin signaling (19), and the phosphorylation-dependent downregulation of cell cycle regulators cyclin D1 (20), cdc25 (21) and c-Myc (13, 14). On the other hand, GSK-3β can promote cell survival and oppose apoptosis (22-24). 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 over-expression of c-Myc confers increased sensitivity to TRAIL, through repression of NF-κB-induced anti-apoptotic factors c-FLIP, Mcl-1 and c-IAP2 (25, 26). Elevated expression of c-Myc has also been associated with increased sensitivity and survival of colon carcinoma patients that received 5-FU treatment (27, 28). 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 (29). In addition, phosphorylation of c-Myc protein targets it for proteasomal degradation under hypoxic conditions (29, 30). Induction of c-IAP2 in hypoxic cells via an undescribed, HIF-1 independent, mechanism has also been previously reported (31).

GSK-3β kinase inhibitors are in clinical trials, primarily for the treatment of type II diabetes and neurodegenerative disorders. Our data suggests that these inhibitors may also be good candidates for anti-cancer drug development. Epidemiological data shows that long-term use of LiCl in patients with bipolar disorder is not associated with increased cancer prevalence (32), and LiCl does not increase the number of tumors in a mutant APC mouse model (33). A recent report has also shown that pharmacologic inhibition of GSK-3 causes growth inhibition in leukemias with oncogenic MLL mutations (34).

CDK1/cdc2 is a cyclin-dependent protein kinase, which regulates the mitotic G2/M cell-cycle checkpoint. CDK1 promotes cell-survival through the regulation of the anti-apoptotic factors survivin (35, 36) and Mcl-1 (37, 38). The potential of cyclin-dependent kinase (CDK) inhibitors including CDK1 has been proposed for the treatment of cancer (39), 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 (40, 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 demonstrated the potential for targeting hypoxic regions of human tumors with novel therapeutic combinations. Future studies should test dual GSK-3β/CDK1 inhibitors, such as alsterpaullone, in other tumor types where tumor hypoxia is thought to play a role in therapeutic resistance.

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References:


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Figure Legends:

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 hr in the presence of CPT-11, 5-FU or TRAIL (final 6 hr). A bioluminescence-based assay was employed to measure caspase-3 activity (B) Cleavage of caspase-8, -9, -3 and Parp was assessed by western blot analysis in TRAIL-treated (3 hr) HCT116 p53-/- cells under normoxia or hypoxia (C) HCT116 p53-/- cells were cultured under normoxic or hypoxic conditions for 48 hr in the presence or absence of 5-FU (30 μM), CPT-11 (50 μM), 5-FU and CPT-11 (30/50 μM), or TRAIL (50 ng/mL, 6 hr). Bars represent (mean ± S.E.M., *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 Figure S1.

Figure 2. A chemical library screen identifies compounds that overcome hypoxia-induced apoptosis resistance (A) Scheme representing the screen for small molecules (50 μM) 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 hr) and TRAIL (final 6 hr) (D) HCT116 p53-/- cells were treated with increasing doses of SLM3 (24 hr) combined with TRAIL (final 6 hr). Bars represent (mean ± S.E.M., *P<0.05) (E) Clonogenic survival was assayed in HCT116 p53-/- cells treated with varying doses of SLM3 plus TRAIL. (mean ± S.E.M., *P<0.05) (F) Bioluminescent caspase-3 activity was measured in HCT116 p53-/- cells treated with increasing concentrations of TRAIL (final 6
hr) in the presence or absence of SLM3 (1 μM; 24 hr), CPT-11 (1μM) or tirapazamine (1 μM; TPZ). See also Figure S2.

Figure 3. SLM3-mediated induction of c-Myc protein expression is critical for apoptotic sensitization (A) c-Myc expression was assessed by western blotting analysis in hypoxic HT29 cells 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-G1 FACS analysis. Immunoblot showing the efficiency of c-Myc knockdown is shown (left). (C) c-Myc or 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 ± S.E.M., *P<0.05). (D) c-IAP2 was inhibited with siRNA in HCT116 p53-/- cells, which were then treated with TRAIL (6 hr) under hypoxia (16 hr). c-IAP2 knockdown efficiency was confirmed by western blotting (left). Bars represent (mean ± S.E.M., *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 ± S.E.M., *P<0.05). See also Figure S3.

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 hr 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 100x with 400x inserts. (C) HCT116 p53-/- cells were cultured under normoxic or hypoxic conditions in the presence or absence of SLM3 (1 μM). Western blots are shown. (D) HCT116 p53-/- cells were treated for 4 hours under hypoxic conditions in the presence of SLM3, Alsterpaullone, or lithium chloride. 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, sc2420 and SLM3. Markers represent (mean viability ± S.E.M., *P<0.05 for control compared to LiCl, sc24020 and SLM3). (G) HCT116 p53-/- cells were treated with GSK-3β-targeted siRNA under normoxic and hypoxic conditions (24 hr). Western blots are shown (top). HCT116 p53-/- cells were treated with GSK-3β-targeted siRNA, then TRAIL under hypoxia. Bars represent (mean Sub-G1 % ± S.E.M., *P<0.05). See also Figure S4.

**Figure 5. Inhibition of CDK1 contributes to SLM3-induced apoptotic sensitization in hypoxic cancer cells** (A) HCT116 p53-/- cells were treated with LiCl (20 mM), SLM3 (1 μM) and Alsterpaullone (1 μM) for 30 hours under hypoxia. Cell cycle analysis was performed 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 100x with 400x inserts. Bars represent (mean ± S.E.M., *P<0.05). (C) HCT116 p53-/- cells were treated with CDK1-specific siRNA followed by TRAIL treatment under hypoxia. Bars represent (mean sub-G1% ± S.E.M.,
*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 Alsterpaullone. Markers represent (mean % viability ± S.E.M., *P<0.05 for control compared to Roscovitine, Alsterpaullone and SLM3). (E) HCT116 p53-/ cells were treated with roscovitine, sc24020, sc24020/ros covitine combination or SLM3 followed by TRAIL treatment under hypoxia. Bars represent (mean sub-G1% ± S.E.M., *P<0.05). See also Figure S5.

**Figure 6. Inhibition of CDK1 causes repression of Hif-1α expression and activity under hypoxia** (A) HCT116 p53-/ cells were treated with SLM3 (1 μM) or Alsterpaullone (1 μM) for 16 hr. 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 ± S.E.M.,*P<0.05) (C) HCT116 p53-/ cells were treated as in (A). Quantitative real-time PCR was performed for the HIF-1α target genes, VEGFa and LDHa). Bars represent (mean ± S.E.M., *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 ± S.E.M., *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 hr. Cell viability was measured using a bioluminescence-based assay. Markers represent (mean ± S.E.M., *P<0.05) See also Figure S6.
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 ± S.E.M., *P<0.05) (B) Representative images of HCT116 p53-/- xenograft tumors imaged using non-invasive vascular imaging (hashes indicate average vascular indices of tumors (*P<0.05). (C) Quantification of tumor vessels by immunostaining of CD34 (mean ± S.E.M., *P<0.05). (D) Immunostaining of cleaved caspse-3 in SW620 tumors treated with SLM3 (12.5mg/kg), TRAIL (100ug) 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 100x field of view ± S.E.M., (*P<0.05). (F) Mechanistic model of SLM3 activity in tumor cells. See also Figure S7.
Figure 3
Figure 5

A

B

Control

SLM3

Phospho-Histone-H3

p-H3 pos. cells/field

0
30
60
90
Control
SLM3

C

D

E

Apoptosis (%)

% Viability

0
20
40
60
80
Control
CDK1

No Treatment
TRAIL (50ng/mL)

Control
Rosco
SLM3
Alsterpaullone

0
0.5
1
5
10
0.5M

DMSO

sc2020 (500nM)

Roscovitine (10μM)

sc2020+Roscovitine

SLM3 (1μM)
Overcoming hypoxia-induced apoptotic resistance through combinatorial inhibition of GSK-3β and CDK1

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