Inhibiting p53 Acetylation Reduces Cancer Chemotoxicity

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Abstract

Chemotoxicity due to unwanted p53 activation in the bone marrow remains an unmet clinical challenge. Doxorubicin, a first-line chemotherapy drug, often causes myelosuppression in patients, thus limiting its effectiveness. In this study, we discovered that C646, a reversible p300 inhibitor, downregulates p53 transcription and selectively protects noncancerous cells from p53-dependent apoptosis. C646 treatment blocked acetylation of specific lysine residues that regulate p53 activity. Exploitation of differential p53 genetic backgrounds between human hematopoietic and colorectal cancer cells improved the therapeutic index of doxorubicin with C646 cotreatment. C646 administration in mice afflicted with p53-mutant tumors protected them from doxorubicin-induced neutropenia and anemia while retaining antitumor efficacy. We deduce that temporary and reversible inhibition of p53 acetylation in cancer subjects, especially those with p53-mutant tumors, may protect them from severe chemotoxicity while allowing treatment regimens to effectively proceed.

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Introduction

Chemotoxicity is an unsolved clinical problem that affects the quality of life in many cancer patients. Doxorubicin, an anthracycline antibiotic, is commonly used to treat a wide range of leukemias, lymphomas, carcinomas, and sarcomas (1). Although effective against many cancers, doxorubicin is limited by negative effects such as cardiotoxicity and myelosuppression (2). Adverse reactions, such as heart failure, stroke, infections, or internal bleeding, may also interrupt the chemotherapy regimen, thereby increasing the likelihood of ineffective tumor clearance or cancer recurrence.

Doxorubicin works by DNA intercalation and formation of a topoisomerase II inhibitory complex (3), which prevents the ligation of double-strand breaks (4). The DNA damage triggers activation of p53-dependent (5) or p53-independent signaling pathways (6), leading to cell-cycle arrest or apoptosis (7). As this process interrupts DNA replication, it affects actively proliferating cancer cells more so than the relatively quiescent normal cells. Nonetheless in the bone marrow, where blood cells are produced constantly, the multipotent hematopoietic stem cells and their progenitors actively proliferate, differentiating into erythrocytes, neutrophils, leukocytes, and other blood types in the circulatory and lymphatic systems. Disturbingly, this means that doxorubicin also results in collateral damage to bone marrow cells (8), alongside actively dividing stem cells in the hair follicles, intestinal crypts, and other parts of the body.

Cancers with p53-inactivating mutations are found in over half of the cancer population (9) and the patients carrying them have a much poorer prognosis compared to those with p53 wild-type cancers (10). When p53-positive mice were treated with doxorubicin, upregulation of p53 was exhibited in the rapidly dividing cells of the bone marrow, spleen, thymus, and small intestine, associating with massive apoptosis in these tissues. In p53-null mice, this toxicity was largely avoided (11), hinting that p53 inhibition may offer cancer patients some degree of protection from the toxicity of p53-activating chemotherapeutics (12).

Doxorubicin treatment results in posttranslational modifications such as p53 phosphorylation, acetylation, or ubiquitination (13). These play important roles in p53 regulation, influencing protein stability, promoter binding, transcriptional activity, or biomolecular interactions (14). The complete loss of p53 acetylation has been shown to abolish doxorubicin-induced cell death. Of the eight lysine residues reported to be critical for p53 activity, seven are catalyzed by the catalytic domain of p300/CBP (15). Prior to this study, it was unclear whether C646 could prevent p300-dependent acetylation of p53. In this study, we establish C646 as a de facto p53 inhibitor that works by preventing the acetylation of specific lysine residues.

C646 was also shown to protect normal bone marrow cells from doxorubicin-induced toxicity, while allowing doxorubicin to keep its cancer-killing efficacy through E2F1-dependent mechanisms. The findings presented in this study serve as a reference for future development of small molecule p300 inhibitors in chemoprotective strategy.

Materials and Methods

Human bone marrow cells

Patient studies were conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving...
Human Subjects (CIOMS). Cells from human donors were legally procured from StemCell Technologies Inc. and comply with approved ethical guidelines. The cells were collected in accordance with Local, State and Federal U.S. requirements and were obtained from normal volunteers participating in a donor program that was approved by an Institutional Review Board or Human Subject Committee. A signed and witnessed consent form was obtained from each donor prior to starting the protocol. All of the volunteers have either donated their cells or were reasonably compensated for their commitment and such compensation was legally approved by an Institutional Review Board or Human Subject Committee. All of the volunteers were aware that the cells may be used for any research applications and waive any rights generated from the research applications. Each normal adult volunteer was screened for general health, HIV, hepatitis B, and hepatitis C, by FDA-approved methods. All products were handled at Biosafety Level 2 (BSL-2) or higher and universal handling precautions for biological samples were used. Strict controls on personal identifiers of volunteers were in place to preserve anonymity and protect individual privacy. The extracted CD34+ human bone marrow cells (StemCell, #70002) were maintained with StemSpan CD34+ Expansion Supplement (StemCell, #02691).

Animal models

All animal experimentation was approved by the A*STAR Institutional Animal Care and use Committee (IACUC protocol no. 130885) and performed in compliance with IACUC regulations. Mice were reared in cages (up to a maximum of five animals per cage) and fed with sterile food and water. Xenograft experiments were performed using female BALB/c nude mice between 8 and 2 weeks of age. p53W and p53KO mice were males between 8 and 10 weeks of age. Tp53 knockout mouse strain was generated by crossing Trp53 conditional knockout mice with mice expressing Cre under the control of the β-actin promoter. The mice were backcrossed with C57Bl/6 albino mice for at least 10 generations to generate near congenic (>99%) mice. Genotyping was performed by PCR analysis of DNA from ear clips obtained at the time of weaning.

Mice subjects were randomly assigned an arbitrary number known only to the primary investigator, and the experimenters were blind to the group assignment and outcome assessment. The number of mice used per group was based on the minimum required for statistical analysis. Tumor images were acquired using IVIS SpectrumCT in vivo imaging system (PerkinElmer, 128201). Complete blood counts were obtained by submandibular extraction. Hematopoietic cells were obtained by flushing the murine tibia and femurs with RPMI, then disaggregating the cells through gentle pipetting. The cells were passed through a 30 μmol/L nylon mesh to exclude cell clumps and washed by centrifugation at 2,000 rpm at 4°C for 10 minutes. Details are available in the mouse lineage cell depletion kit protocol (Miltenyi Biotec, #130-090-858).

Cell lines

U2OS, MC27, H1299, HCT116, and HFF1 cells were procured from the ATCC from 2015 to 2017. All cell lines were authenticated by short tandem repeat (STR) profiling by ATCC at the time of purchase. All cultured cells were tested biweekly for contamination using the MycoAlert Plus Mycoplasma Detection Kit (Lonza) according to the manufacturer's protocol. The number of cell passages from the day of collection or thawing to experimental use was limited to 30.

Chemicals

C646, 5-fluorouracil, cisplatin, and camptothecin were purchased from Tocris. Doxoruubicin, etoposide, and actinomycin D were from Sigma.

Fluorescence microscopy

Hematopoietic cells were cytospun from suspension onto slides using a Shandon cytocentrifuge and were fixed with 100% methanol for 5 minutes. Next, they were incubated with 1% BSA, 10% normal goat serum, 0.3 M glycine, and 0.1% PBS-Tween for 1 hour to permeabilize the cell membranes and block nonspecific protein–protein interactions. The treated cells were incubated with rabbit anti-K382 (Abcam) and mouse anti-p53 (Abcam) primary antibodies, washed, and incubated with secondary antibody coupled to Alexa Fluor 647 and Alexa Fluor 488, respectively. After washing, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and embedded in Prolong Gold Antifade (Invitrogen). Images were acquired with a LSM 510 laser scanning microscope (Zeiss) using a 40× plan apo objective using appropriate filter sets. The display lookup table (LUT) was linear and covered the full range of the data. Processing software was Carl Zeiss ZEN 2.1 (black).

p53 reporter assay

The p53 reporter cell line T22 comprised p53 wild-type murine fibroblasts stably integrated with a p53-responsive RGCAlacZ gene. An Envision 2104 Multilabel reader was used to detect the fluorescent intensities. The excitation and detection wavelengths were 390 and 460 nmol/L, respectively (Supplementary Methods).

Plasmids

Mutations were introduced to wild type human p53 gene by site-directed mutagenesis (SDM) using pCI-neoHp53 plasmid as starting material. Three rounds of SDM were performed using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies), resulting in a total of seven nucleotide changes at sites K164, K370/372/373 cluster, and 381/382/386 cluster. All constructs were sequenced after each round of mutation to ensure that the correct clone was obtained before proceeding with further rounds of SDM. The 7mut-pCI-neoHp53 plasmid obtained at the end of the three rounds of cloning was verified by sequencing with vector primers. The plasmids pcDNA3.1-p300 (Addgene, #23252) and pcDNA3.1-p300(HAT-)(Addgene, #2354) were gifts from Warner Greene's lab (Supplementary Methods).

siRNA

DharmaFECT siRNA transfection reagent (GE Dharmacon) and Opti-MEM I Reduced Serum Media (Gibco) were used for siRNA transfection. On-TargetPlus siRNA against human TP53 and P300, including nontargeting control, were purchased from GE Dharmacon.

CellTiter-Glo and caspase-3/7-Glo assays

Cells were seeded in 96- or 384-well plates and then treated with the indicated compounds. Plates were sealed with BreathEasy sealing membrane (Sigma-Aldrich, Z380059) during incubation to reduce evaporation. CellTiter-Glo luminescence cell
viability reagent (Promega, C7570) or Caspase-Glo 3/7 substrate (Promega, G8090) was added according to manufacturer’s protocol. An Envision 2104 Multilabel reader was used for detection of luminescent signals.

Immunoblotting

Apafl (sc-65891), E2F1 (sc-251), Mdm2 (sc-965), Noxa (sc-515840), p21(CIP1) (sc-6246), p53-HP (sc-126), p300 (sc-585), Puma-α (sc-377015), and ubiquitin (sc-8017) antibodies were purchased from Santa Cruz. p53-AcK120 (AB78316), p73 (AB26123), and cleaved PARP (AB32064) antibodies were obtained from AbCam. The rest, actin-HRP (Sigma-Aldrich, A8385), p35-AcK320 (Merck Millipore, 06-1283), and p53-AcK382 (Cell Signaling Technology, 25255S) were from their respective suppliers. Anti-rabbit p300 (P0217) and anti-mouse (P0161) secondary antibodies were purchased from Dako (see Supplementary Methods).

Reverse transcription quantitative PCR

Total RNA was isolated from cells with the AxyPrep total RNA Miniprep Kit (Axygen). For each sample, 1 μg of isolated RNA was reverse transcribed to cDNA using iScript reverse transcription supermix for RT-qPCR (Bio-Rad) and qPCR reactions were performed by adding SoAdvanced Universal SYBR Green Supermix (Bio-Rad), 500 nmol/L primers and cDNA template (see Supplementary Methods).

Ubiquitination assay

MCF7 cells were transfected with His-ubiquitin plasmid using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. At 20 hours posttreatment, the cells were treated with control or 20 μmol/L of MG132 and further incubated for 4 hours. The corresponding cell lysates were then subjected to standard immunoblotting and immunoprecipitation protocols.

Cycloheximide assay

H1299 cells were transfected with p53 plasmid and treated with control or C646, followed by control or doxorubicin for 48 hours. One hundred μg/mL cycloheximide (Sigma, #C7698) was added and the cells were harvested at the indicated time points. Immunoblotting was performed to visualize relative protein levels. The corresponding band intensities were quantified using ImageJ software (NIH) and plotted to determine their decay constants. The protein half-lives were calculated from the natural log of 2/x, where x is the decay constant.

Colony formation

A total of 1,000 cells per well were seeded in six-well plates and the cell confluences were monitored daily over 7 to 10 days to determine the optimal experimental end points. During the harvesting process, the culture media was aspirated gently to avoid physical damage to the cells. The cells, still adhered to the wells, were briefly rinsed with cold PBS and promptly stained with fresh Crystal violet dye. Within 2 minutes, the dye was removed and the plates were rinsed with copious amount of autoclaved deionized water. The plates were then air dried and their images were captured using an Epson scanner. Quantification of cell density was performed using ImageJ software.

Therapeutic index

The therapeutic index of doxorubicin in this study is defined as the ratio of the concentration that kills half the population of normal cells (IC50 normal) to the concentration that kills half the population of cancer cells (IC50 cancer).

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2013. Normally distributed variables were compared using unpaired two-tailed t tests. Null hypotheses were rejected for P-values below 0.05.

Results

Identifying C646 as an inhibitor of p53 activity

C646 is a pyrazoline-containing small molecule identified from a virtual ligand screening for novel p300 acetyltransferase inhibitors (Fig. 1A). In the pilot study, C646 was characterized as a reversible linear competitive inhibitor with high selectivity to p300 compared to other acetyltransferases (16). Because p53 is a known substrate of p300 (17), we set out to investigate whether the p300-mediated acetylation of p53 can be inhibited by C646. Using the T22 murine fibroblast cell line, which contains a fluorescent reporter construct controlled by a p53-dependent promoter (18, 19), we performed reporter assays after exposure to two p53-activating agents of different nature (Fig. 1B). Doxorubicin is a genotoxic agent that upregulates p53 activity through the DNA-damage repair pathway, triggering p53 lysine acetylation in the process. In contrast, nutlin-3 stabilizes p53 protein in a non-genotoxic fashion by disrupting the interaction between p53 and its negative regulator Mdm2 (20). When cells were pretreated with C646, they showed resistance to doxorubicin-induced p53 activation. However, these cells were unable to counter nutlin-3-induced p53 activation. These findings not only indicate that C646 is an inhibitor of p53 activity, but also suggest that it is able to impede a regulator in the DNA-damage repair pathway.

To test whether the reporter assays results reflected real changes in gene transcription and protein expression, we performed RT-qPCR and immunoblotting experiments on similarly treated MCF7 cells. Because p53 does not bind or activate its own gene promoter, the treatment did not cause significant changes to p53 mRNA levels (Fig. 1C). Chromatin immunoprecipitation (ChIP) results also suggest that the binding between p53 and its target gene promoters was not disrupted by C646 (Supplementary Fig. S1A–S1C). Analysis of the p53 target genes revealed a contrasting picture, as the mRNA and protein levels of all three genes behaved in tandem to the p53 expression changes not only indicate that C646 is an inhibitor of p53 activity, but also suggest that it is able to impede a regulator in the DNA-damage repair pathway.

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deactivated doxorubicin through chemical reaction, we repeated the experiment using a broad range of DNA damaging agents and found that C646-treated cells were more resistant to toxicity induced by cisplatin, actinomycin D, and doxorubicin (Supplementary Fig. S1E). As C646 only showed protective effects against agents that resulted in p53 lysine acetylation (Supplementary Fig. S1F), we deduced that the mechanism of action of C646 is inclined more towards inhibition of acetyltransferase activity rather than chemical sequestration.

To assess how these molecular changes impacted growth and viability, we performed colony assays and found that C646-treated cells possessed some resistance against doxorubicin toxicity compared to control-treated cells (Fig. 1E and F). Flow cytometry results also showed that C646 reduced both doxorubicin-triggered apoptosis and necrosis (Fig. 1G and H). These findings reinforced the hypothesis that C646 protected cells from doxorubicin toxicity.

**Elucidating C646’s mechanism of action**

To confirm the on-target effects of C646 on the p300 histone acetyltransferase (HAT) activity, we first depleted endogenous p300 using siRNA that targeted the 3′-untranslated region of
the p300 mRNA, then transfected ectopic p300WT or p300HAT– plasmid contained six-point mutations, which inactivated the acetyltransferase catalytic activity (24). Immunoblot results confirmed that p300 HAT activity was essential for p53 acetylation and transcription (Fig. 2B and C). Colony assays (Fig. 2D) and flow cytometry (Fig. 2E) also reflected the importance of p300 HAT in doxorubicin-induced toxicity. Notably, the outcomes of C646 treatment in p300WT–expressing cells closely matched those of control-treated p300HAT–expressing cells. This was consistent with our prediction that C646 acted as an inhibitor of p300-catalyzed acetylation of p53.

Figure 2.
C646 prevents specific p53 lysines from acetylation. A, Top, schematic diagrams of p300 protein showing conserved domains and point substitutions that inactivate acetyltransferase catalytic activity. Bottom, p53 protein showing conserved transactivation (TAD1 and TAD2), DNA binding (DBD), tetramerization (TET), and regulatory domains (REG). Lysine acetylation sites and their respective acetyltransferases are displayed. The p537KR-mutant protein contains lysine-to-arginine substitutions at positions indispensable for transcription. B, Protein levels of U2OS cells transfected with nontargeting control (siNC) or p300 (siP300) siRNA, followed by combinations of C646 and doxorubicin. The corresponding acetyl-lysines (AcK) were detected from immunoprecipitated p53. C-G, Endogenous p300-depleted U2OS cells were transfected with control, p300WT–, or p300HAT– plasmids, followed by combinations of C646 and doxorubicin. C, Protein levels at 48 hours posttreatment. D, Colony assays of cells stained with Crystal violet 10 days after treatment. For cell densities, see Supplementary Fig. S2C. E, Graph shows proportion of viable, apoptotic, and necrotic cells as determined by FACS analysis. For contour plots, see Supplementary Fig. S2D. F, Graph shows proportion of viable, apoptotic, and necrotic cells. For contour plots, see Supplementary Fig. S2E. Bars, mean ± SD; n ≥ 3; t test (ns, no significant difference; *，P < 0.05; **，P < 0.01).
Inhibiting p53 Acetylation Mitigates Chemotoxicity

To examine our hypothesis that C646 prohibited the p300-catalyzed acetylation of p53 lysine residues, we performed site-directed mutagenesis on a p53WT plasmid. The engineered p53KR mutant possessed seven lysine-to-arginine substitutions at residue positions reported to be indispensable for p53 activity (Fig. 2A, third and bottom; ref. 15). These plasmids were transfected into p53-null H1299 cells and the immunoblot results showed that C646 treatment in p53WT-expressing cells resulted in a similar downregulation of p53 targets as nontreated p53KR-expressing cells (Fig. 2F). Correspondingly, these conditions offered comparable protection against doxorubicin-induced toxicity as shown in our colony assays (Fig. 2G) and flow cytometry analyses (Fig. 2H and Supplementary Fig. S2A–S2D).

Previous studies reported that the p300-mediated acetylation of p53 may prevent its ubiquitination by Mdm2 (25). To investigate how C646 affected the interplay between p300, p53, and Mdm2, we performed a co-immunoprecipitation (Co-IP) experiment under doxorubicin-treated conditions (Fig. 3A), and found that although C646 did not interrupt binding between p53 and p300, it caused a slight increase in p53–Mdm2 interaction (Fig. 3A, lane 6), which coincided with a drop in p53 K382 acetylation. The complex formation between Mdm2 and the transactivation domain (TAD) of p53 has been shown to repress its transcriptional activity (26), providing a clue as to how C646 inhibits the activation of p53 target genes. The E3 ubiquitin ligase Mdm2 is known to monoubiquitinate, but not polyubiquitinate, p53 in vitro (27), whereas p300 is reported to function as an E4 ubiquitin ligase in some cases (28). Taking these points into consideration, we performed an ubiquitination assay to see how C646 could affect p53 ubiquitination (Fig. 3B). The proteasome inhibitor MG132 was added to impede degradation of ubiquitinated p53 so as to provide us with a more accurate picture of relative ubiquitination levels. Under these conditions, doxorubicin was shown to decrease p53 ubiquitination (Fig. 3B, lane 7), an effect that was reversible by C646 (Fig. 3B, lane 8). Because the ubiquitination level of a protein is related to its degradation rate and steady-state levels (29), we performed protein stability assays using cycloheximide, an inhibitor of translational elongation (Fig. 3C–E and Supplementary Fig. S3A–S3E). Again, the doxorubicin-induced stabilization of p53 protein was antagonized by C646. Overall, these observations highlight the role of C646 in promoting p53 ubiquitination and protein destabilization.

Addressing the safety concerns of C646

A concern surrounding p53 inhibition is whether doing so may increase a person’s risk of developing cancer. In a study by Gerald Evan's group (30), the team proposed that removal of p53’s apoptotic effects did not equate to the abolishment of its tumor suppressive function and demonstrated that restoration of p53 after a brief period of its inhibition was sufficient to prevent tumorigenesis. This meant that any carcinogenic risk posed by p53 inhibition can be minimized through a controlled dosing regimen. Therefore, as a premise, the reversibility of the p53 inhibitor must be established. Although C646 has been previously described as a reversible inhibitor of p300 acetyltransferase activity (16), whether this reversibility extended to p53 inhibition remained untested. To investigate, MCF7 cells were incubated with C646 for an hour before its removal and then treated with doxorubicin at different postremoval time points. Immunoblotting was subsequently performed to visualize the p53 total protein and AcK382 levels (Fig. 4A). When doxorubicin was added immediately after C646 removal...
Reversible effects of C646 treatment. A, MCF7 cells were initially incubated with or without C646. An hour later, the culture media in lanes 1–4 were retained while those in lanes 5–9 were replaced with fresh media in the absence of C646. Doxorubicin was added at the indicated time points, expressed in minutes, after C646 removal. Immunoblot shows relative protein levels 24 hours posttreatment. B, T22 cells were separated into two pools, one exposed to nontargeting control siRNA (siNC) and the other to siRNA targeted at p53 (siP53). From both pools, cells were treated under the corresponding conditions as in A. Con (lane 1), C646 (lane 2), Dox (lane 3), C-D (lane 4), and C-C+D (lane 9). Graph shows relative fluorescence intensities expressed as fold change over mock at 16 hours posttreatment. C–F, MCF7 cells were treated as in B. C, Graph shows signal intensities of the early apoptosis marker caspase-3/7 expressed in relative luminescence units (RLU). D, Histogram shows proportion of cells stained with FITC-Annexin V and subjected to flow cytometry. E, Graph shows percentages of viable and nonviable cells as determined by FACS analysis. F, Graph shows CellTiter-Glo signal intensities expressed in relative luminescence units. Bars, mean ± SD; n ≥ 3; t test (ns, no significant difference; *, P < 0.05; **, P < 0.01).

(Fig. 4A, lane 5), it did not result in a visible p53-AcK382 signal. This implied that one hour of incubation with C646 was sufficient to inhibit p53 acetylation. When doxorubicin was added at longer periods after C646 removal (Fig. 4A, lanes 6–9), the p53-AcK382 increased gradually, implying that the inhibitory effects of C646 had waned over time. To confirm this, we performed a T22 reporter assay and again saw that the removal of C646 for an hour was sufficient to reverse its inhibitory effects on p53 activation (Fig. 4B). Expanding on our inquiry, we performed a series of caspase 3/7 assays (Fig. 4C), Annexin V flow cytometry analyses (Fig. 4D and E), and CellTiter-Glo ATP assays (Fig. 4F), all of which showed that C646 removal resulted in the reversal of doxorubicin-induced growth arrest or apoptosis in a p53-dependent manner. Overall, our results support the hypothesis that C646 acts as a reversible inhibitor of doxorubicin-induced p53 activity.

Improving the safety profile of doxorubicin using C646

Once C646 had been established as a de facto p53 inhibitor, our next goal was to evaluate its potential as a chemoprotective
Using immuno-fluoro-microscopy, we observed that C646 prevented doxorubicin-induced acetylation of p53 K382 in human bone marrow cells (Fig. 5A). By showing that C646 functioned in living cells, we confirmed that C646 was able to cross the plasma membrane, thus excluding the possibility that C646 worked only in cell lysates. We treated human bone marrow cells with C646, followed by a range of doxorubicin concentrations, and performed a series of cell viability assays. Inhibiting p53 Acetylation Mitigates Chemotoxicity.
assays at different time points, showing that C646-treated bone marrow cells were able to tolerate higher concentrations of doxorubicin in a dose-dependent manner (Fig. 5B and Supplementary Fig. S4A). Earlier studies have claimed that doxorubicin was effective at killing colorectal cancer cells (31) and that C646 was able to inhibit their growth (32).

Although our cell viability assays hinted a slight p53-dependent protection of HCT116 cells by C646 against doxorubicin (Fig. S4C and S4D). Besides protecting normal cells from toxicity, it was crucial for doxorubicin to retain its cancer-killing function. Our previous studies have described the Rb–E2F1 pathway as a major p53-independent mechanism behind doxorubicin-induced cell death (Fig. 6A; ref. 6). In our immunoblotting (Fig. 6B) and RT-qPCR (Fig. 6C and Supplementary Fig. S5A) experiments, we not only showed that doxorubicin could activate E2F1 and its apoptotic target genes, but also found that C646 had no toxic effect in normal cells to that in cancer cells (33), and determined that C646 could increase the safety window of doxorubicin by a wider margin in p53-positive rather than in p53-negative colorectal cancers (Fig. 5E and Supplementary Fig. S4C and S4D).

Figure 6.
C646 treatment does not affect p53-independent apoptosis. A, Flow chart illustrates the two canonical pathways of doxorubicin-induced cell death. B-E, Various normal and cancer cell lines were treated with combinations of C646 and doxorubicin. B, Protein levels of p53, E2F1, and their transcriptional targets, with cleaved PARP (cPARP) as apoptosis marker and actin as loading control. C, Graph shows mRNA levels of indicated genes as determined by RT-qPCR. D, Graph shows signal intensities of cells subjected to caspase-3/7 assay expressed in relative luminescence units (RLU). E, Human bone marrow cells were harvested at the indicated time points, stained with propidium iodide, and counted by an ADAM-MC automatic cell counter. Graph shows live cell counts relative to mock treatment. Bars, mean ± SD; n ≥ 3, t-test (ns, no significant difference; *, P < 0.05; **, P < 0.01). F, Protein levels in HCT116 p53−/− cells treated with nontargeting control (siNC) or siRNA against p73 (siP73).

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effect on their regulation. Interestingly, doxorubicin triggered the activation of E2F1 apoptotic targets in cancer but not normal cells, underscoring a differentiation factor that may work in the patient's favor. It is worth mentioning that genetic aberrations affecting the Rb–E2F1 pathway are present in a majority of cancers but are normally absent in normal tissues (34). A deregulated Rb–E2F1 pathway allows cancer cells to overcome cell-cycle checkpoints and proliferate uncontrollably. In contrast, E2F1 activity is kept low and tightly regulated in normal cells. This oncogenic addiction of cancer cells to the Rb–E2F1 pathway allows the differential activation of pro-apoptotic genes in cancer cells while sparing normal cells from apoptosis. Results from our caspase 3/7 assays (Fig. 6D and Supplementary Fig. S5B) and live cell counts (Fig. 6E and Supplementary Fig. S5C) also supported that protection by C646 was mainly p53-dependent, suggesting that the p300 inhibitor would work most effectively in patients with p53-inactive cancers. Previous studies have shown that p300/CBP may also acetylate p53 family members such as p73 (35). To test the degree of p73 influence on the observed C646 regulation on p53 target genes, we repeated the immunoblot experiment under p73-depleted conditions and found no visible difference in p21WAF1, Puma, and Noxa levels (Fig. 6F, lanes 3 and 7). Moreover, HCT116 p53−/− cells expressed acetylated p73 and cleaved PARP only when treated with 2.0 μmol/L of doxorubicin, but not at 0.4 μmol/L (Supplementary Fig. S5D), suggesting that p73 may play an insignificant role in regulating apoptosis under the conditions used in this study.

Taking our study further, we wanted to test whether C646 could protect bone marrow in vivo while allowing doxorubicin to retain its antitumor efficacy. To determine the effective dosages of doxorubicin and C646, we titrated their dose responses in extracted mouse bone marrow cells (Fig. 7A and B and Supplementary Fig. SSE–SSG). Next, we injected C646 into mice intraperitoneally and observed the effects of doxorubicin on their bone marrows. Biopsies taken from the mice revealed that C646 reduced doxorubicin-induced acetylation of p53 (Fig. 7C), demonstrating the distribution of the small molecule C646 into the bone marrow. We then tested the protocol in mice carrying dominant-negative mutant p53R273H tumors. Strikingly, mice cotreated with C646 continued to receive benefits from doxorubicin's antitumor effects (Fig. 7D and E), but their red and white blood cell counts did not fall as much compared to those treated with doxorubicin alone (Fig. 7F and G). Immunoblot results showed that the background levels of E2F1 in mouse bone marrow were much lower than those in HT29 (Supplementary Fig. S5H), resulting in a lower activation of E2F1 targets and PARP cleavage. When these cells were extracted and treated with siRNA, E2F1 depletion caused a significant decrease in doxorubicin-induced apoptosis in HT29 but not mouse bone marrow cells (Fig. 7H), highlighting an important difference between the molecular backgrounds of cancer versus normal cells. According to a study in E2F1 acetylation by Tony Kouzarides' group (36), the intracellular acetylation of E2F1 was largely attributed to the acetyltransferase activity of P/CAF rather than p300/CBP. Although the authors reported a weak acetylation of purified E2F1 by p300/CBP in vitro, the signal was barely detectable even under long exposure, suggesting that the acetyltransferase activity of p300/CBP might have little functional significance in catalyzing the acetylation of endogenous E2F1. Together, these results suggest that C646 protected mice from doxorubicin-induced anemia and neutropenia, but allowed doxorubicin to retain its cancer-specific lethality through p53-independent mechanisms.

Discussion

In this study, we reviewed whether C646 could act as an antagonist of p53. The basis of our hypothesis stems from the heavy influence of lysine acetylation on p53 transcriptional activity and the fact that many of these key lysine residues are regulated by p300-mediated acetylation (37). Because the acetylation levels of p53 have been shown to significantly increase under stressful conditions (38), using three different inducers of p53 acetylation: cisplatin, actinomycin D, and doxorubicin, we showed with some degree of certainty that C646 was able to prevent p300-mediated acetylation of p53. We further showed that C646 inhibited the acetyltransferase activity of p300, resulting in a lack of acetylation that destabilized the p53 protein and lowered its transcriptional activity. This was consistent with previous studies that established positive correlations between p53’s level of acetylation, its stability, and transcriptional activity (13, 39). The study performed by Gerald Evan’s lab established that the development of tumors in p53-suppressed mice after irradiation could be prevented by the temporarily restoration of p53 activity (30). Our data indicated that the suppressive effects of C646 on p53 activity could be undone in a time-dependent manner, fitting well with its description as a reversible p300 inhibitor (16). This property is essential as the carcinogenic risks associated with p53 inhibition requires that the mode of action be temporary and reversible. Although previous studies by Rhoda M. Alanis’s (32) and Takashi Kohno’s (40) groups demonstrated the cancer-killing properties of C646 at 10 and 15 μmol/L respectively, we discovered that a low dose of 1 μmol/L C646 could protect normal cells from p53-dependent apoptosis. This suggests that the dose of C646 may affect the types of substrates regulated by p300/CBP, resulting in contextual functional effects.

Clinical studies have shown that patients with p53-mutant tumors tend to have faster disease progression and poorer survival compared to those with p53 wild-type tumors (10). A reason could be due to the narrow therapeutic window caused by unwanted p53 activation in normal tissues. Although there are no available p53 inhibitors in the clinic, our data showed that C646 was able to selectively protect bone marrow cells from p53-dependent toxicity, mitigate anemia, and neutropenia. A study by Carlos Caldas’ group (41) showed that colorectal cancer cells periodically accumulate mutations that lead to loss of heterozygosity, leaving only one copy of a functioning p300 allele. The lower p300/CBP activity may account for the decreased toxicity protection in HCT116 cells and thus improve the therapeutic index of doxorubicin. This study provides a proof of concept that a C646-like p53 inhibitor may improve the clinical outcome of patients undergoing chemotherapy, especially for those with p53-mutant tumors. In a recent clinical study, patients who received a low dose of arsenic trioxide, which transiently blocked p53 activation, showed resistance to myelosuppression after four or more cycles of chemotherapy (42).

Insofar, the most widely described small molecule p53 inhibitor is the pifithrin (PFT) class of compounds. PFTx has been reported to protect mice from acute radiation syndrome (43),
supporting our hypothesis that p53 inhibition is a viable strategy to protect hematopoietic cells from toxicity. However, obstacles to the development of pifithrin-class compounds include solubility issues (44), unclear mechanism of action, and p53-independent off-target effects (45). In contrast, C646 has a well-defined molecular target with a high-resolution crystal structure, making it suitable for future chemical development. Furthermore, a well-developed p53 inhibitor drug could potentially treat other p53-driven pathologies, such as stroke (46), Alzheimer’s (47), or Parkinson’s disease (48). Our work serves as a milestone for

Figure 7. C646 protects bone marrow function while preserving doxorubicin efficacy. A, Bone marrow cells extracted from femurs of p53WT and p53KO mice were treated with 1 μmol/L of C646, followed by a range of doxorubicin concentrations, then subjected to CellTiter-Glo assay. Plot shows doxorubicin dose–response signal intensities expressed in relative luminescence units (RLU) at 72 hours posttreatment. For 24- to 48-hour timepoints and IC50, see Supplementary Fig. S5E and S5F. B, Bone marrow cells from p53WT and p53KO mice were treated with a range of C646 concentrations, followed by a fixed concentration of 0.4 μmol/L doxorubicin, then subjected to CellTiter-Glo assay. Plot shows C646 dose–response signal intensities expressed in relative luminescence units at 72 hours posttreatment. For 24- to 48-hour timepoints, see Supplementary Fig. S5G. C, Mice were intraperitoneally injected with vehicle control or 1 mg/kg of C646, followed by intravenous injection of control or 4 mg/kg of doxorubicin, daily for 7 days. Immunohistochemical micrographs show bone marrow biopsies labeled with antibodies against p53-K381. Scale bars, 50 μm. D–H, Mice were inoculated with HT29-Luc2 cells expressing mutant p53R273H and were monitored for 10 days until tumor growth became detectable (Day 0). They were then subjected to a treatment protocol of 3 days of PBS or C646 (1 mg/kg, i.p.), followed by 6 days of vehicle control, C646, doxorubicin (4 mg/kg, i.p.), or both. Images show mice at 0, 6, and 11 days since the start of the protocol. E, Chart shows tumor sizes of mice determined by bioluminescence intensity expressed in photons per second. Blood count analyses were performed at the end of experiment. Graphs shows red blood cell (RBC; F) and white blood cell (WBC; G) counts of treated mice. H, Graph shows caspase-3/7 assay signal intensities of cells treated with nontargeting control (siNC) or siRNA against E2F1 (siE2F1). Bars, mean ± SD; n ≥ 3; t test (ns, no significant difference; *, P < 0.05; **, P < 0.01).
This page contains references from a scientific manuscript discussing the role of p53 acetylation in cancer treatment. The references are cited in the text to support the claims made in the manuscript. The manuscript is published in Cancer Research, Volume 77, Issue 16, August 15, 2017, with the article number 4353.


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