

Supplemental Information

Figure S1, related to Figure 1. Inauhzin Induces p53 Level and Activity in Wild Type p53-harboring Cancer Cell Lines.

Cells were treated with different doses of INZ as indicated and harvested 18 hrs post treatment for immunoblotting analyses as described in Figure 1. * indicates residual signals of p53.

Figure S2, related to Figure 1. Depletion of p53 Prevents Growth Suppression Effect of Inauhzin.

(A) A549 cells were transfected with scrambled siRNA or p53 siRNA, and 16hrs prior to cell harvesting, cells were treated with 2 μ M INZ. Protein levels were measured by IB. * indicates residual p53K382Ac antibody-reacting bands.

(B) A549 cells transfected with scrambled siRNA or p53 siRNA followed by exposure to INZ for 72hrs and evaluated by WST cell growth assays. The IC₅₀ value of INZ in the scrambled siRNA and p53 siRNA transfected cells are $4.4 \pm 0.5 \mu$ M and $18.7 \pm 1.7 \mu$ M, respectively (Mean \pm SD, n=3).

(C-D) H460 cells transfected with scrambled siRNA (Scr) or p73 siRNA (Si-p73) were treated with 2 μ M INZ, and IB (C), qPCR (D) and cell viability (E) were assessed. Data in (E) represent average values from four identically treated samples and are expressed as a percentage of surviving cells relative to untreated controls. The dashed line corresponds to 50% cell killing.

Figure S3, related to Figure 3. Inauhzin Does Not Affect the Auto-ubiquitylation of MDM2 and Not Directly Inhibit MDM2 Ubiquitin Ligase Activity.

(A) In vivo ubiquitylation assay was performed as described in Figure 3E. Ubiquitylated MDM2 was separated by 8% SDS-PAGE, and detected by immunoblotting.

(B) In vitro ubiquitylation assays were performed with purified His-MDM2 and GST-p53. Ubiquitylation reactions were terminated after 30 minutes, and the extent of p53 ubiquitylation was evaluated by immunoblot analysis with anti-p53 antibody.

Figure S4, related to Figure 4. Inauhzin Induces High Level of p53 without Causing Dramatic Formation of Foci.

(A) The representative images of γ -H2AX foci. H460 cells were treated with 2 μ M of Inauhzin (INZ), 10 μ M Cisplatin (Cis) for 18hrs or 2mM Hydroxyurea (HU) for 8hrs, and analyzed by monoclonal anti-p53 antibodies (red) and polyclonal anti- γ -H2AX antibodies (green). Bar, 20 μ m.

(B) The number of γ -H2AX foci per nuclei is shown (mean \pm SD). INZ treated cells (1.3 ± 0.2 foci per cell, n = 394); Cis treated cells (8.0 ± 1.2 foci per cell, n = 375); HU treated cells (9.7 ± 0.6 foci per cell, n = 314); DMSO treated cells (0.28 ± 0.1 foci per cell, n = 395).

Figure S5, related to Figure 5. SIRT1 Knockdown Leads to Increased Cell Death.

(A) IB analyses of HCT116^{+/+} cells transfected with SIRT1 siRNA, using antibodies as indicated.

(B) Inductions of p21 and MDM2 mRNA levels in HCT116^{+/+} cells transfected SIRT1 siRNA as assessed by qPCR (mean \pm SD, n=3)

(C-E) FACS analysis of HCT116 and H460 cells transfected with SIRT1 siRNA for 72 hrs. Presented in the (D) and (E) is the percentage of apoptotic cells in the population (sub-G1 DNA content). The results as shown here are representatives of three independent experiments. Values represent means \pm SD (n=3), **p<0.01.

(F-G) Survival curves of HCT116^{+/+} cells transfected SIRT1 siRNA or scrambled siRNA for the indicated days (F), or H460 cells transfected with SIRT1 siRNA followed by exposure to the

indicated concentrations of INZ for 72hrs (G). Surviving cells are determined by WST cell viability assays shown as the absorbance at 450nm. Each data points represent the average value from six samples. Error bars represent standard deviation.

Figure S6, related to Figure 6. Biotinylated Inauhzin Binds to SIRT1 *in vitro*.

(A) Recombinant SIRT1 and SIRT7, produced in *E. coli* BL21-CodonPlus (DE3)-RIPL, and purified through Ni-His columns and stained with Coomassie brilliant blue.

(B) Deacetylation activity of purified SIRT1 was measured by analyzing in the level of Ac-Lys382-p53 with acetylated p53 as a substrate, which was isolated via immunoaffinity purification. Proteins were detected by IB with antibodies as indicated.

(C) Chemical structure of biotinylated Inauhzin (Biotin-INZ).

(D) Biotinylated Inauhzin induces p53 level and acetylation in H460 and HCT116^{+/+} cells as measured by IB.

(E) Purified SIRT1 was incubated at indicated concentrations with biotinylated Inauhzin or biotin overnight at 4 °C. After incubation, each mixture was subjected to Native-PAGE analysis, followed by blotting to PVDF. The blot was probed with NeutrAvidin Protein-Horseradish Peroxidase Conjugated (1:1000; Pierce) and anti-SIRT1 antibodies.

Figure S7, related to Figures 5 and 6. Biochemical Characterization of Inauhzin Inhibition of SIRT1 Activity *in vitro*.

(A) Effect of Inauhzin and its analogues on SIRT1 deacetylase activity *in vitro* using acetylated p53 as a substrate. The inhibitory effect of the Inauhzin analogues on SIRT1 activity was calculated as described in the “Experimental Procedure”.

(B) Inauhzin inhibits the deacetylase activity of SIRT1, but not SIRT2, SIRT3 or HDAC8 *in vitro*. *In vitro* deacetylase assays were conducted by Fluor-de-Lys Fluorimetric Assays as

described in the Supplementary Information. The curve fitting and IC₅₀ determination of INZ were performed using Igor Pro 4.01A.

(C) Inauhzin induces acetylation of Histone H3 at K9 in H1299 cells. H1299 Cells were treated with the amounts of INZ as indicated for 18hrs and harvested for IB with antibodies as indicated.

(D) Inauhzin has a little effect on HDAC1 deacetylase activity. The assay was performed with Flag-HDAC1 and acetylated p53 proteins in the presence of 0, 5 and 25μM of INZ or 1 μM TSA, and acetylated p53 and protein loading were analyzed by IB with antibodies as indicated.

(E-F) In vitro SIRT1 inhibition assay was performed with FdL acetylated peptide substrates and NAD⁺ in the presence of different amounts of INZ, ▲ 25 μM, ■ 12.5 μM, ▣ 6.25 μM, ▤ 3.125 μM, ● 1.5625 μM, ● 0.78125 μM, ● 0 μM. (E) Each curve represents the FdL acetylated substrate titration (2-200 μM) with a constant NAD⁺ concentration of 25μM and various concentration of INZ. The % Km and % Vmax values are shown in the Table below. (F) Each curve represents NAD⁺ titration (10-4000 μM) with a constant substrate concentration of 10μM and various concentration of INZ. The Y-axis of the charts represents arbitrary fluorescent units (AFU). The % Km and % Vmax values are shown in Table below. In all panels, each data represents the mean ± SD (n=3).

Figure S8, related to Figure 6. Inauhzin Is More Effective than Cambinol and Salermide in Inhibiting Cell Growth and Less Toxic than Tenovin-6 to Primary Human Cells.

(A-B) HCT116^{+/+} and H460 cells were treated with INZ, Cambinol, Salermide, and Tenovin-1 or Tenovin-6 at the indicated concentrations for 18hrs and harvested for IB analyses. (C) The inhibitory effect of the compounds on cell growth as evaluated by WST cell growth assays and IC₅₀ values were presented as mean ± SD (n=3).

Figure S9, related to Figure 7. Inauhzin Effectively Distributes to Tumors After i.p. Administration and Potently Represses the Growth of H460 and HCT116 Xenograft Tumors.

(A-B) The plasma and tumor concentrations of Inauhzin were determined by HPLC-MS/MS after administration of 30mg/kg by i.p. injection to mice bearing H460 xenografts (mean \pm SD, n=2).

(C) Representative pictures of HCT116^{p53^{-/-}} and HCT116^{p53^{+/+}} tumors as shown in Figure 7A-B.

(D) Analysis of apoptosis in the normal tissues following INZ treatment. Tissues were collected 21 days after INZ administration and stained with H&E or DAPI and TUNEL. Bar, 50 μ m.

Supplemental Experimental Procedures

Cell Culture and Plasmids.

Human lung non-small-cell carcinoma H460, A549, H1299 and HT29 cells as well as human colon cancer HCT116, human breast cancer MCF7, human embryonic fibroblast WI-38, human osteosarcoma U2OS and SJSA, human glioma U87 and U373, and human ovarian cancer A2780, IGROV1, and SKOV3 cells were used in the study. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin, except SJSA cells, which were grown in RPMI-1640 medium containing glucose. The adenovirus-shRNA for SIRT1 was from X. Charlie Dong's Lab (Indiana University) (Wei et al, 2010). Flag-p53 was provided by Wei Gu (Columbia University).

Antibodies

Antibodies for immunoblotting were as follows: mouse monoclonal anti-p53 (DO-1), rabbit anti-SIRT1 (H300), rabbit anti-p21 (M19), mouse anti-p21 (F5) from Santa Cruz. Anti-p53-Acetylated (lys382), Cleaved PARP, PARP (9542), Puma, rabbit AMPK α , rabbit Phospho-

AMPK-alpha (Thr172) (40H9), rabbit Acetyl-Histone H3 (K9), rabbit Ac-alpha-tubulin (K40), mouse phosphor-p53 (ser46), and phosphor-p53 (ser15) were from Cell Signaling Technologies. 2A10 monoclonal anti-MDM2 antibodies were described previously (Zeng et al, 1999). Antibodies for immunostaining were as follows: rabbit polyclonal anti-p53 (FL-393, Santa Cruz), mouse monoclonal anti-BrdU (IIB5, Santa Cruz).

Immunoblotting

Cells were seeded in 6-well plates. All compounds were dissolved in DMSO and diluted directly into the medium to the indicated concentrations; 0.1% DMSO was used as a control. After incubation with the compounds for the indicated times, cells were harvested and lysed in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 supplemented with 2 mM DTT and 1 mM PMSF. An equal amount of protein samples (50 µg) was subjected to SDS-PAGE and transferred to a PVDF membrane (PALL Life Science). The membranes with transferred proteins were probed with primary antibodies followed by horseradish-peroxidase-conjugated secondary antibody (1:10,000; Pierce). The blots were then developed using an enhanced chemiluminescence detection kit (Thermo Scientific), and signals were visualized by Omega 12iC Molecular Image System (UltraLUM).

FACS Analysis

Logarithmically growing cells were incubated with either Inauhzin or DMSO for the time points as indicated. Cells were harvested, fixed in 70% ethanol overnight and analyzed by propidium iodide (PI) staining and flow cytometry (FACS Calibur, Becton Dickinson) as previously described (Riccardi & Nicoletti, 2006).

RNA Interference

Control scrambled siRNA, or siRNA specific to p53, p73, or SIRT1 were purchased from Santa Cruz Biotechnology. These siRNAs (20 to 120 nM) were introduced into cells using METAFECTENE® SI following the manufacturer's protocol (Biontex). Cells were treated with INZ for IB, qRT-PCR and cell viability assays.

Senescence-Associated β -galactosidase Staining

Cells (3×10^4) were plated in 3.5-cm-diameter plates and treated for 1 week with 2 μ M Inauhzin or 10 μ M nutlin-3 (synthesized by the IUPUI chemical core facility). Senescence-associated β -galactosidase staining was performed by using Senescence- β -Gal Staining Kit (Cell Signaling Technology) and following the manufacturer's instructions. β -Galactosidase activity was measured by the absorbance of 5, 5'-dibromo-4, 4'-dichloro-indigo at 650 nm generated by the β -Galactosidase staining using a Microplate Reader (Molecular Device, SpectraMax M5e).

Reverse Transcription and Real-Time PCR Analyses.

Total RNA was isolated from cells using Qiagen RNeasy Mini kits (Qiagen, Valencia, CA) and treated with DNase I (Invitrogen). Quantitative real-time PCR was performed on an ABI 7300 real-time PCR system (Applied Biosystems) using SYBR Green Mix (Applied Biosystems) as described previously (He et al, 2007). All reactions were carried out in triplicate. The relative gene expression was calculated using the Δ Ct method following the manufacturer's instruction. The primers for p53, p21, MDM2 and GAPDH were described (Sun et al, 2007). The primers that amplify the mir-34a pri-miRNA, mir-24 pri-miRNA were designed with Primer Express software: hsa-pri-mir-34a, 5'-CTTCACCTACAGATGCCAACTTTG-3' and 5'-CCGGTGACTTTGGTCCAATT-3'; hsa-pri-mir-24, 5'-CCGGGCTGTTCGATTGG-3' and 5'-GACTCCTGTTCTGCTGAACTGA-3'.

In Vitro Ubiquitylation Assay.

His-MDM2 and GST-p53 were purified from *Escherichia coli*. The *in vitro* p53 ubiquitylation reactions were performed at room temperature (20-22°C) in the ubiquitylation buffer: 25mM Hepes (pH 7.4), 10mM NaCl, 3mM MgCl₂, 0.05% TritonX-100, 2mM ATP, 200ng Ubiquitin Activating Enzyme E1 (rabbit, Boston Biochem), 50 ng E2 (UbcH5a, human recombinant, Boston Biochem), 10ng ubiquitin (human recombinant, Boston Biochem) and 50ng purified His-MDM2 were incubated with Inauhzin for 20 min. The reaction was initiated by adding the substrate, 50ng of purified GST-53 and quenched by the addition of SDS loading buffer. Ubiquitinated p53s were detected by immunoblotting with the anti-p53 (DO-1) antibody (Li et al, 2002).

DNA Binding Assay.

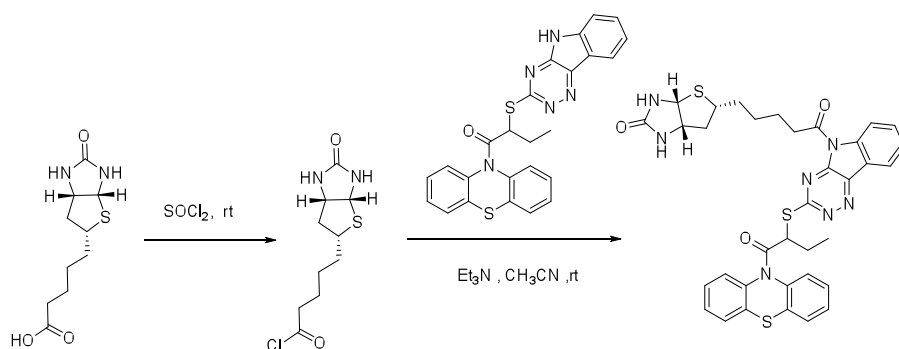
To determine whether Inauhzin direct binds to DNA, Fluorescence Intercalator Displacement Analyses were performed as described previously (Goodwin et al, 2006). 2.5µM ethidium bromide and 1.25 µM DNA (calf thymus DNA, Sigma) was mixed with Inauhzin or Actinomycin D in the buffer (50mM Tris, pH7.4, 100mM NaCl). Fluorescence signals were detected after incubation for 15 min at room temperature using a Microplate Reader (Molecular Device, SpectraMax M5^e).

In Vitro Deacetylation Assays

Fluor-de-Lys Fluorimetric Assays for SIRT1 (BML-SE239, ENZO), SIRT2, SIRT3 and HDAC8 were performed in 384-well plates using Fluor-de-Lys Drug Discovery Kit (BML-AK555, AK556, AK518, ENZO) (Lain et al, 2008). Both SIRT2 and SIRT3 use the same Fluor-de-Lys (FdL) substrate, 317-320 of p53 acetylated on lysine 320. SIRT1 requires a different substrate, 379-382 of p53 acetylated on lysine 382 (BML-KI177, ENZO). In this assay, enzyme was added at a concentration of 0.5units/well for SIRT1 and HDAC8, and 1.0 units/well for SIRT2 and

SIRT3. FdL substrates and NAD^+ used in the assay were at $10\mu\text{M}$ and $25\mu\text{M}$, respectively. Assays were incubated for 45 min at $37\text{ }^\circ\text{C}$. Resulting fluorescence was measured after further incubation for 15 min at $37\text{ }^\circ\text{C}$ with Fluor-de-Lys Developer II (BML-KI176, ENZO) using a Microplate Reader (Molecular Device, SpectraMax M5^e) with excitation set at 360 nm and emission measured at 460 nm. IC_{50} data were analyzed using Igor4.01 (Lake Oswego, Oregon, USA).

Preparation and Characterization of Biotinylated Inauhzin



Biotin (100 mg, 0.410 mM) was placed in a 10 ml reaction flask and cooled to $0\text{ }^\circ\text{C}$. 2.7 ml SOCl_2 was added to the flask and allowed to room temperature. The mixture was stirred for 1 h and excess SOCl_2 was evaporated. The residue was co-evaporated with 5 ml anhydrous toluene for three times to give the biotin acid chloride. Crude acid chloride was dissolved in 5ml anhydrous CH_3CN . INZ (65 mg, 0.138 mM) was dissolved in 3 ml anhydrous CH_3CN and injected to the above solution through syringe. The mixture was cooled to $0\text{ }^\circ\text{C}$, and $100\text{ }\mu\text{l}$ Et_3N (0.717 mM) was dropped to the mixture. The solution was then allowed to room temperature. TLC was used to monitor the reaction. After 11 hrs, TLC indicated that the reaction was completed. The reaction mixture was diluted with 30 ml ethyl acetate and washed twice by saturated NaCl . The organic phase was separated and dried by anhydrous Na_2SO_4 . The organic phase was filtered, concentrated by vacuum and purified through a silica gel column and eluted

with DCM/CH₃OH (55:1). The product was obtained in viscous oil (45 mg, 47 % over two steps). Biotinylated Inauhzin was confirmed by ¹HNMR analysis. Its ¹HNMR spectrum is as follows (500Mz, CDCl₃): δ 8.69(d, *J* = 8.5, 1H), 8.40 (d, *J*=7.5, 1H), 7.92(br, 1H), 7.75-7.72(m, 1H), 7.67(d, *J*=7.0, 1H), 7.59-7.56 (m, 1H), 7.53(d, *J*=3.0, 1H), 7.40 (br, 1H), 7.35-7.29(m, 3H), 7.18(br, 1H), 5.60(d, *J*=39.5, 1H), 5.42-5.38 (m, 1H), 5.14(s, 1H), 4.56-4.53 (m,1H), 4.41-4.37(m, 1H), 3.48-3.41(m, 1H),3.35-3.26(m,1H),3.25-3.23(m,1H), 2.98-2.95(m, 1H), 2.76(d, *J*=12.5, 1H), 1.90-1.83(m, 4H), 1.79-1.75(m, 1H), 1.70(br, 1H),1.61-1.58(m,2H), 0.98-0.90(m,3H).

Immunofluorescence.

Tissues were collected in 4% paraformaldehyde, fixed overnight and embedded in paraffin. Antigen retrieval was performed in a hot water bath for 15 min in 10 mM sodium citrate (pH 6.0), cooled for 20 min at 25°C, and washed with PBST (PBS, 0.1% Tween 20). Tissues were permeabilized by incubating the slides in 1% Triton X-100 in PBS for 10 min and then washed again three times in PBST. After blocking for 1 hr at 25°C in blocking buffer (PBS containing 0.2% BSA and 0.2% Triton X-100), slides were incubated overnight in a humidity chamber with a mouse anti-BrdU monoclonal antibody and anti-p53 rabbit polyconal (FL393) diluted 1:100 in blocking buffer. BrdU and p53 were detected with Alexa Fluor 488 (green) goat anti-mouse antibody and Alexa Fluor 546 (red) goat anti-rabbit antibody (Invitrogen), respectively. The slides were then stained with DAPI for nuclei and the images were captured under a Zeiss Axiovert 200M fluorescent microscope. The images were analyzed using AxioVision 4.7.2.0 software from Zeiss.

Reference

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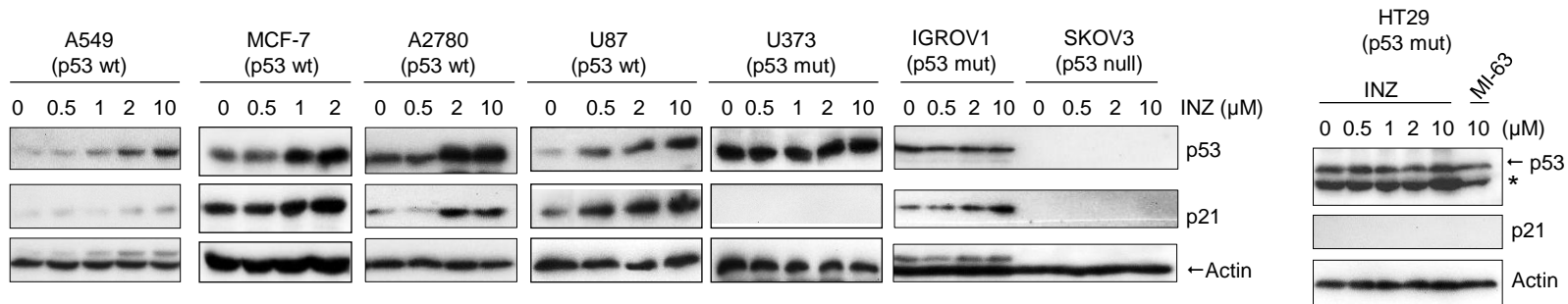


Figure S1, related to Figure 1

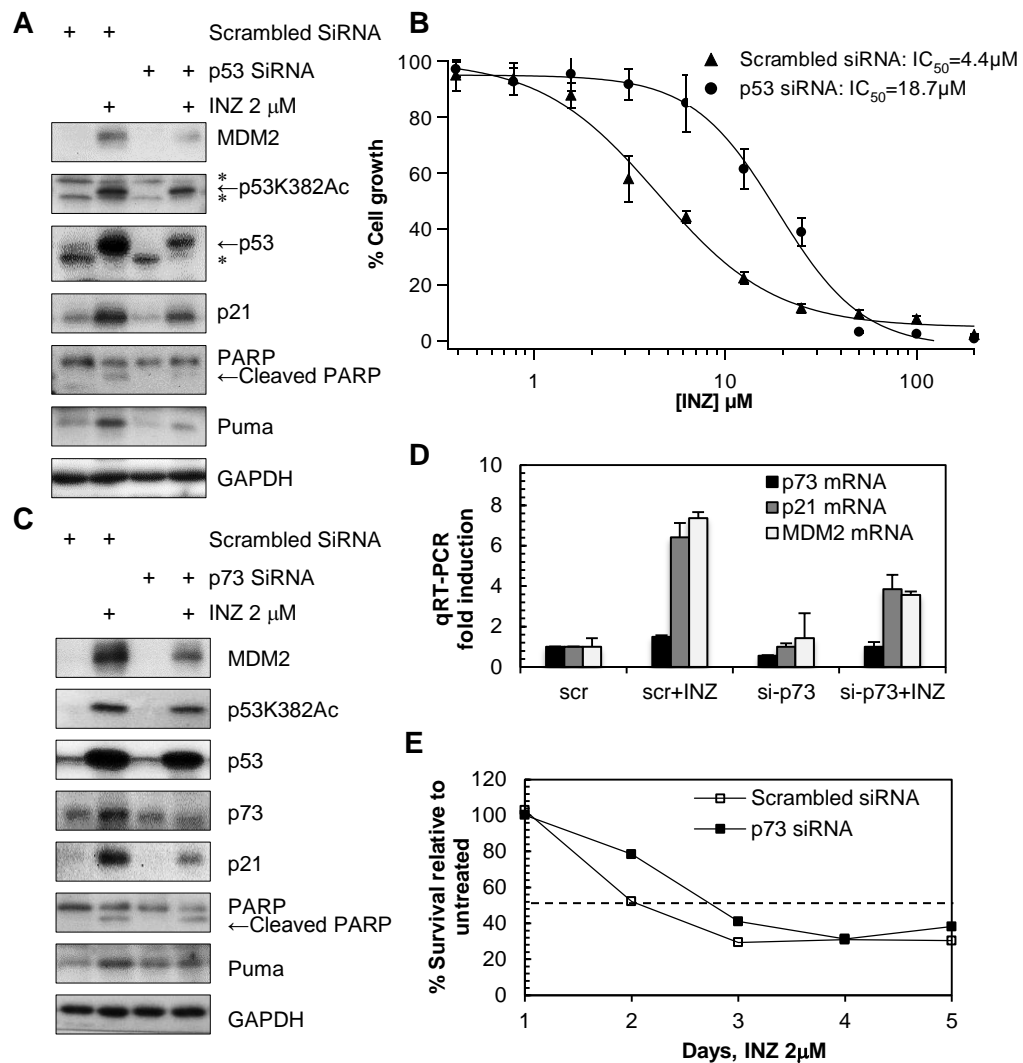


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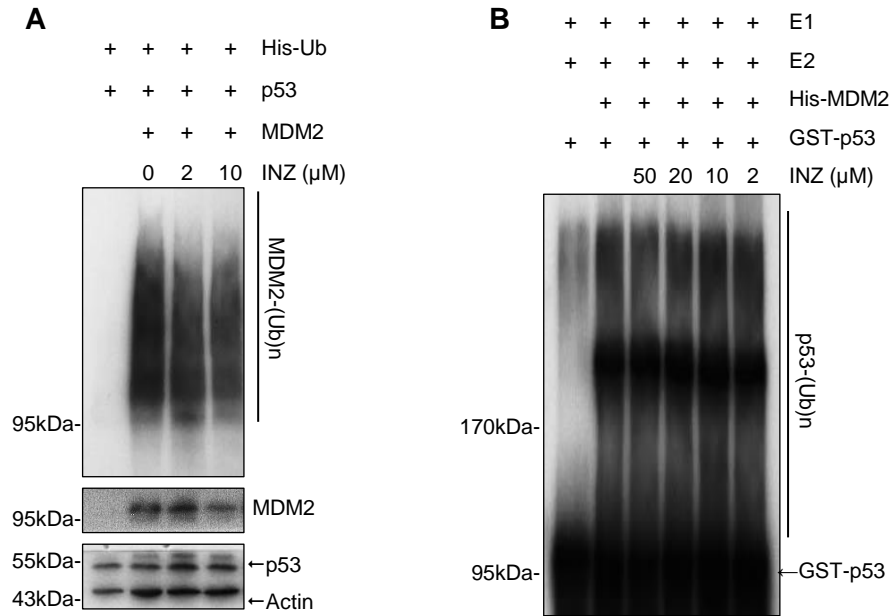


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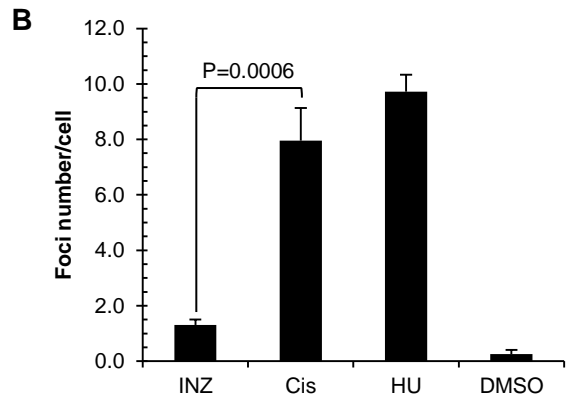
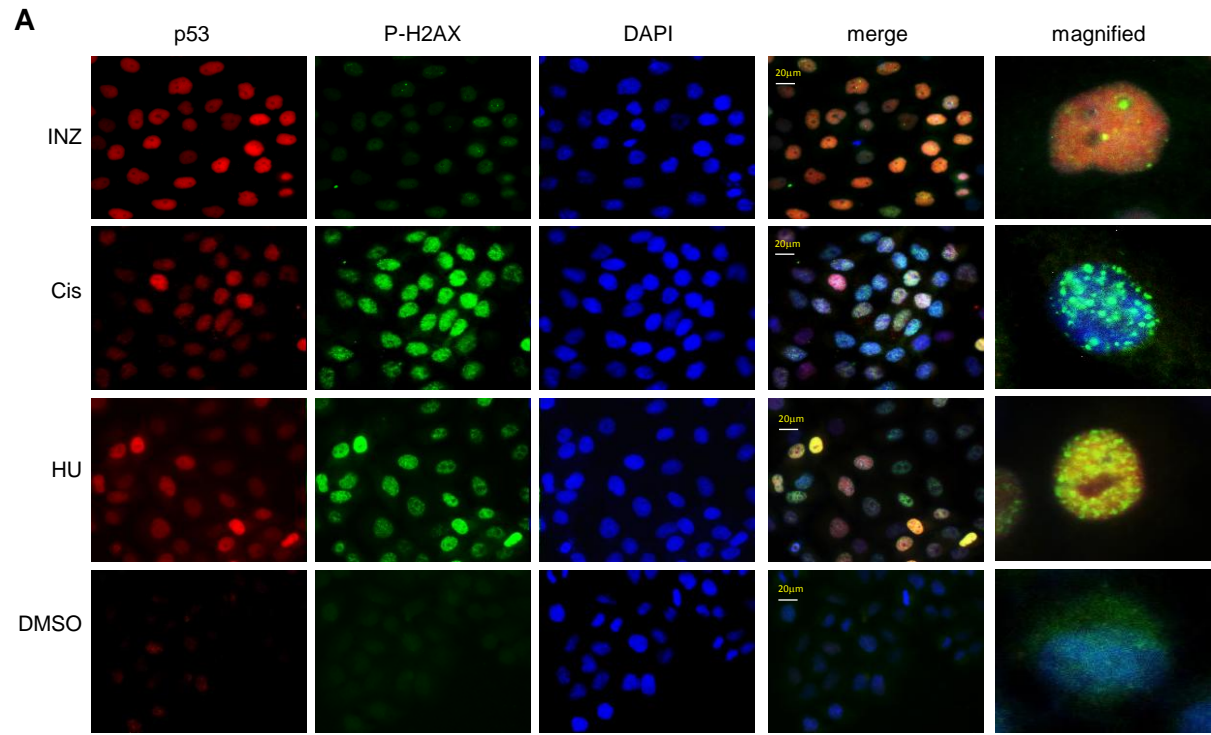


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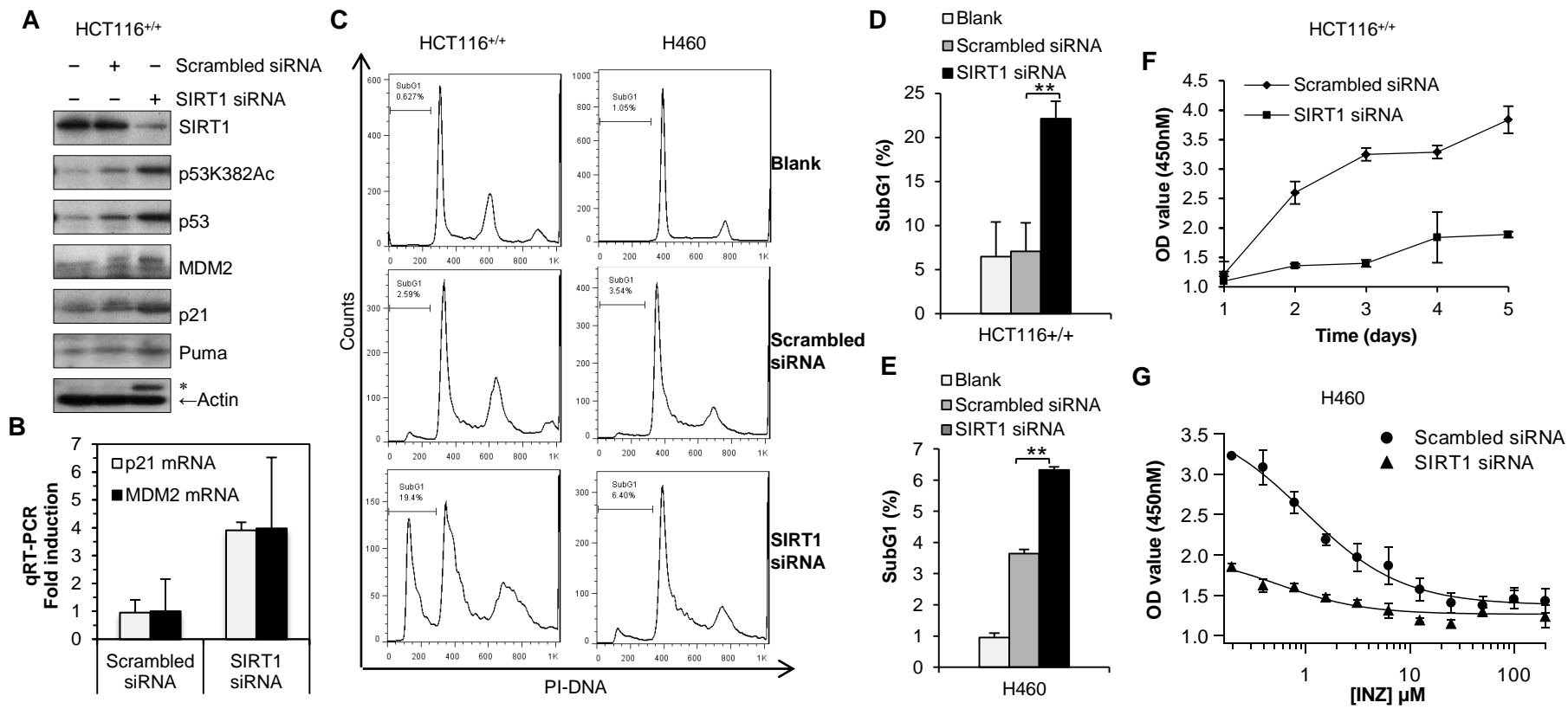


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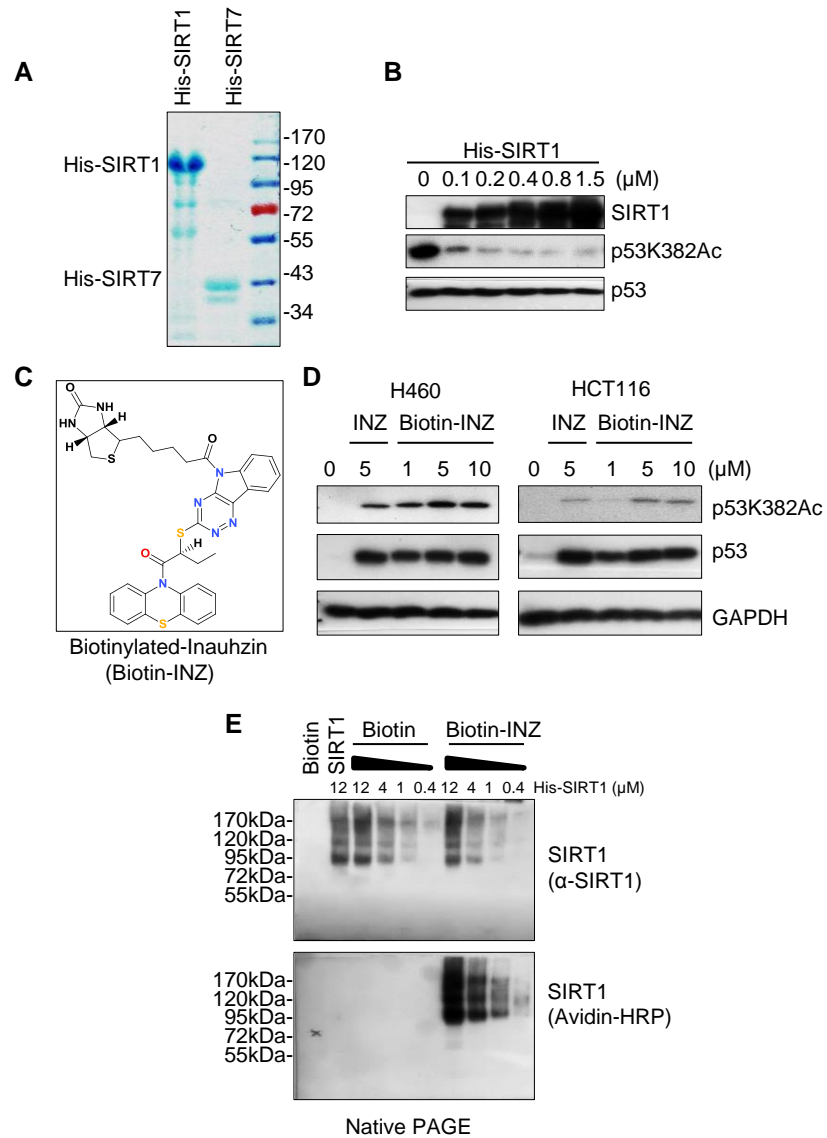


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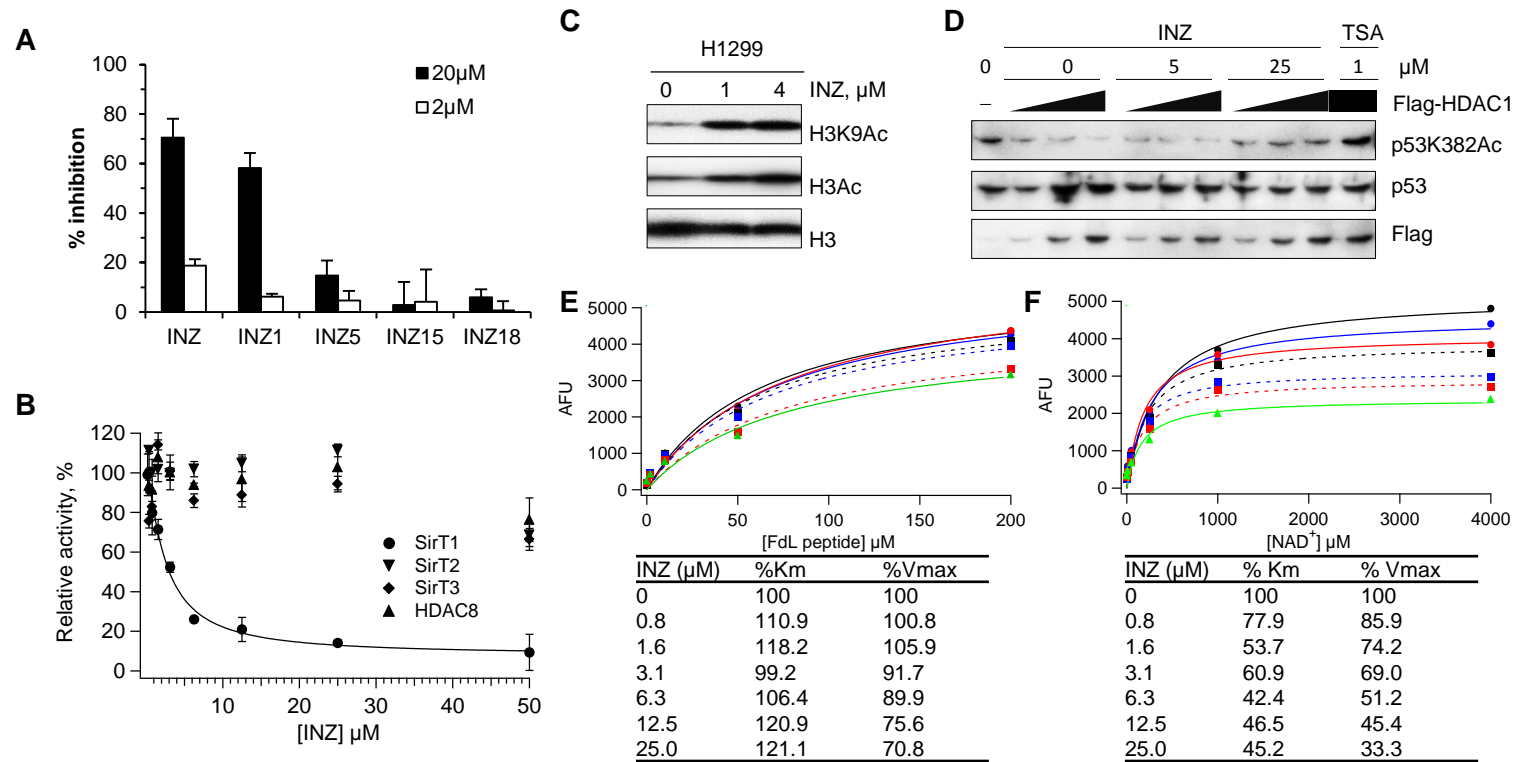
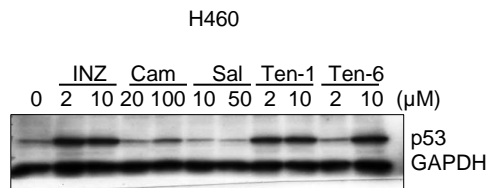
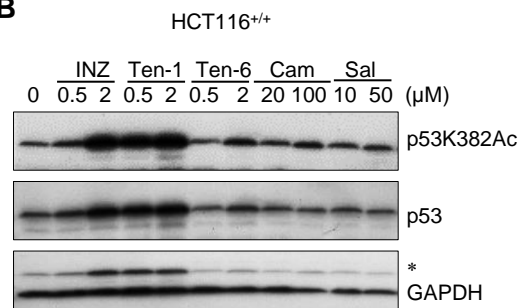


Figure S7, related to Figure 5 and 6

A**B****C**

Structure	Compound	IC ₅₀ (μM) ^a						
		H460	HCT116	SJSA	A549	A2784	NHF	WI38
	Inauhzin	5.4	2.0	30	3.2	1.67	>100	>100
	Cambinol	~100	>100	15	>100	>100	>100	>100
	Salermide	55	>100	> 50	30	24.7	54	49.5
	Tenovin-6	3.2	1.3	> 50	3	1.36	8.5	6.8

^a Relative standard deviations are <20% in all values.**Figure S8, related to Figure 6**

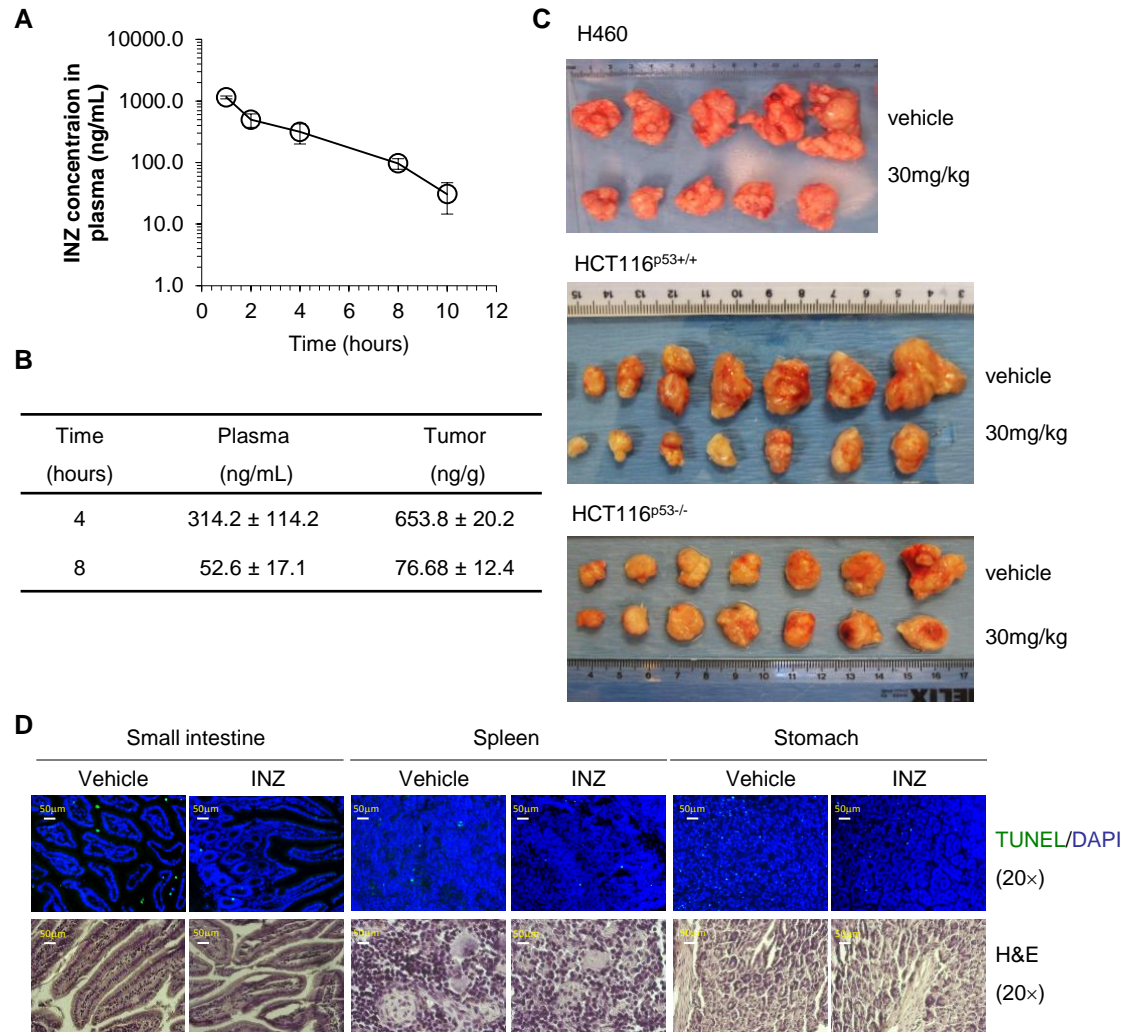


Figure S9, related to Figure 7