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Supplemental Information

Identification of Small Molecules that Modulate Mutant p53 Condensation

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Transparent Methods:

Cell culture

All cell lines were obtained from American Type Culture Collection. Cells were cultured in RPMI1640 (Gibco by Thermo Fisher, Waltham, MA, USA). All media were supplemented with 10% fetal calf serum (PAA Laboratories by GE Healthcare, Little Chalfont, UK) and 1% Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at 37 °C in a 5% CO_2 and 95% air incubator.

p53 activity reporter cell lines

Based on 13 repeats of the canonical activating p53 response element (Wang et al. 2009)) combined with a minimal CMV promoter a luciferase based p53 activity reporter (13xp53RE-luciferase, please see below for sequence) co-expressing the neomycin resistance gene was generated and transduced via lentivirus into different cell lines containing either $p53^{WT}$ (U2OS), $p53^{R175H}$ (Detroit, Cal-33) , $p53^{Y220C}$ (Huh7), $p53^{-/-}$ (Saos2, Calu1, H358, PC3) or $p53^{R273H}$ (MDA-MB-468). Presence of the p53 activity reporter construct was selected for by adding 500µg/ml Geneticin to the media. The presence of p53 WT in U2OS and $p53^{R175H}$ mutants in Cal-33 and Detroit 562 was confirmed by sequencing (data not shown). Calu1 and H358 cells containing the p53 activity reporter were additionally co-transduced by lentiviral transduction with a CMV driven $p53^{R175H}$ expression construct, H358 cells containing the p53 activity reporter additionally with constructs for the CMV driven expression of $p53^{R282W}$ or $p53^{Y220C}$. Presence of the mutant p53 expression constructs was selected for by adding 1µg/ml puromycin to the media and expression of mutated p53 was validated by immunofluorescence against p53 (data not shown). Luciferase activity was measured using the luminescence kit Steady-Glo (Promega, Fitchburg,

WI, USA) and a luminescence plate reader (PHERAstar(BMG Labtech, Ortenberg, Germany)) according to the manufacturer's instructions. Briefly, cells were plated at 3000 cells/5µl in white 384-wellsmall volume plates (Greiner) using a liquid dispenser (Multidrop Combi, ThermoScientific), 5µl compounds were added and cells were either incubated for 16 to 24 h. 5µl SteadyGlo(Promega) was then added to cells and incubated for 30 min in the dark. Subsequently, luminescence intensity was measured in a plate reader. Normalization, quality control, and fitting curves for IC_{50} determination of tested compounds were performed with Genedata Screener® for high-content screening and Genedata Condoseo modules (Genedata AG, Basel, Switzerland).

13xp53RE-luc2 reporter sequence (p53 activity reporter):

ACCACTTTGTACAAGAAAGCTGGGTCTCGAGGGGCATGCCCGGGCATGTCCACGTATGCAGACT
TTACGGGCATGCCCGGGCATGTCCACGTATGCAGACTTTACGGGCATGCCCGGGCATGTCCACG
TATGCAGACTTTACGGGCATGCCCGGGCATGTCCACGTATGCAGACTTTACGGGCATGCCCGGG
CATGTCCACGTATGCAGACTTTACGGGCATGCCCGGGCATGTCCACGTATGCAGACTTTACGGG
CATGCCCGGGCATGTCCACGTATGCAGACTTTACGGGCATGCCCGGGCATGTCCTAGCAGTAGC
TCATATCGGGCATGCCCGGGCATGTCCTAGCAGTAGCTCATATCGGGCATGCCCGGGCATGTCC
TAGCAGTAGCTCATATCGGGCATGCCCGGGCATGTCCTAGCAGTAGCTCATATCGGGCATGCCC
GGGCATGTCCTAGCAGTAGCTCATATCGGGCATGCCCGGGCATGTCCTAGCAGTAGCTCATATC
GGTAGGCGTGTACGGTGGGAGGCCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCTT
AAGGCCACC**ATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCATTCTACCCAC**
TCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCATGAAGCGCTACGCCCTG
GTGCCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTACGCC
GAGTACTTCGAGATGAGCGTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAAT
ACAAACCATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGT
TGGGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGC
GCGAGCTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCGTGAGCAAGA
AAGGGCTGCAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAAGATCA
TCATCATGGATAGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGAC
TTCCCATTTGCCACCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAGCTTCGACCGG
GACAAAACCATCGCCCTGATCATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGC
GTAGCCCTACCGCACCGCACCGCTTGTGTCCGATTCAGTCATGCCCGCGACCCCATCT
TCGGCAACCAGATCATCCCCGACACCGCTATCCTCAGCGTGGTGCCATTTCACCACG
GCTTCGGCATGTTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGTCGTGCTCAT
GTACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGCAAGACTATAAGATTCAATCT
GCCCTGCTGGTGCCCACACTATTTAGCTTCTTCGCTAAGAGCACTCTCATCGACAAGT
ACGACCTAAGCAACTTGCACGAGATCGCCAGCGGCGGGGCGCCGCTCAGCAAGGAG
GTAGGTGAGGCCGTGGCCAAACGCTTCCACCTACCAGGCATCCGCCAGGGCTACGG
CCTGACAGAAACAACCAGCGCCATTCTGATCACCCCCGAAGGGGACGACAAGCCTG
GCGCAGTAGGCAAGGTGGTGCCCTTCTTCGAGGCTAAGGTGGTGGACTTGGACACCG
GTAAGACACTGGGTGTGAACCAGCGCGGCGAGCTGTGCGTCCGTGGCCCCATGATCA
TGAGCGGCTACGTTAACAACCCCGAGGCTACAAACGCTCTCATCGACAAGGACGGCT
GGCTGCACAGCGGCGACATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGG
ACCGGCTGAAGAGCCTGATCAAATACAAGGGCTACCAGGTAGCCCCAGCCGAACTG
GAGAGCATCCTGCTGCAACACCCCAACATCTTCGACGCCGGGGTCGCCGGCCTGCCC
GACGACGATGCCGGCGAGCTGCCCGCCGCAGTCGTCGTGCTGGAACACGGTAAAAC
CATGACCGAGAAGGAGATCGTGGACTATGTGGCCAGCCAGGTTACAACCGCCAAGAA
GCTGCGCGGTGGTGTTGTGTTCGTGGACGAGGTGCCTAAAGGACTGACCGGCAAGTT
GGACGCCCGCAAGATCCGCGAGATTCTCATTAAGGCCAAGAAGGGCGGCAAGATCG
CCGTGAAT*TCTCACGGCTTCCCTCCCGAGGTGGAGGAGCAGGCCGCCGGCACCCTGC*
CCATGAGCTGCGCCCAGGAGAGCGGCATGGATAGACACCCTGCTGCTTGCGCCAGCG
*CCAGGATCAACGTC*TAA

13 repeats of a p53 response element
Luc2 coding region
hPEST coding region

Wang, B., Xiao, Z. and Ren, E. C. (2009) Redefining the p53 response element. *Proc Natl Acad Sci U S A, 106*(34), pp. 14373-8.

Fluorescent mutant p53 cell lines

Fluorescent mutant p53 expression vectors were generated (Sirion Biotech, please see below for sequence) and transduced via lentivirus into Saos2 cells. Presence of the mutant p53 expression constructs was selected for by adding 1µg/ml puromycin to the media. Imaging was performed using a Phenix confocal spinning disc microscope system (PerkinElmer, Waltham, MA, USA) with a 20× or 40x air objective. Image analysis and quantification was performed using Harmony (PerkinElmer, Waltham, MA, USA) or custom written scripts on MetaXpress (Molecular Devices, Sunnyvale, CA, USA).

Fluorescent mutant p53 constructs:

tagRFP-p53, same architecture for all p53 point mutants

ATGGTTTCCAAGGGCGAAGAACTGATCAAAGAAAACATGCACATGAAGCTGTACATGGAAGGC
ACCGTGAACAACCACCACTTCAAGTGCACCAGCGAAGGCGAGGGCAAGCCTTATGAGGGCAC
CCAGACCATGCGGATCAAGGTGGTGGAAGGTGGCCCTCTGCCTTTCGCCTTTGATATCCTGGC
CACCAGCTTTATGTACGGCAGCCGGACCTTCATCAATCACACCCAGGGCATCCCCGATTTCTTC
AAGCAGAGCTTCCCCGAGGGCTTCACCTGGGAGAGAGTGACCACATACGAGGATGGCGGCGT
GCTGACAGCCACACAGGATACAAGTCTGCAGGACGGCTGCCTGATCTACAACGTGAAGATCCG
GGGCGTGAACTTCCCCAGCAATGGCCCCGTGATGCAGAAGAAAACCCTCGGCTGGGAAGCCA
ACACCGAGATGCTGTATCCTGCCGATGGTGGACTGGAAGGCAGATCCGATATGGCCCTGAAGC
TCGTTGGCGGCGGACACCTGATCTGCAACTTCAAGACCACCTACAGAAGCAAGAAGCCCGCCA
AGAACCTGAAGATGCCCGGCGTGTACTACGTGGACCACCGGCTGGAAAGAATCAAAGAGGCC
GACAAAGAGACATACGTCGAGCAGCACGAAGTGGCCGTGGCCAGATACTGTGACCTGCCTTCT
AAGCTGGGCCACAAGCTGAACAGCGGCCTGAGATCTGGATCTGGCGGAGGATCTGCTTCTGG
CGGCTCTGGCTCT**ATGGAAGAACCCCAGAGCGACCCCTCTGTGGAACCTCCTCTGAGCCAAGA**
GACATTCAGCGACCTGTGGAAGCTGCTGCCCGAGAACAATGTGCTGAGCCCACTGCCTAGCCA
GGCCATGGATGATCTGATGCTGTCCCCTGACGACATCGAGCAGTGGTTCACCGAGGATCCCGG
ACCTGATGAGGCCCCTAGAATGCCTGAAGCTGCTCCTCCTGTTGCTCCCGCTCCTGCTGCTCCT
ACACCAGCTGCTCCAGCTCCAGCACCTTCTTGGCCTCTGTCTAGCAGCGTGCCCAGCCAGAAA
ACATACCAGGGCAGCTACGGCTTCCGGCTGGGCTTTCTGCATTCTGGCACAGCCAAGAGCGTG
ACCTGCACATACAGCCCCGCTCTGAACAAGATGTTCTGTCAGCTGGCCAAGACATGCCCCGTG
CAGCTGTGGGTTGACAGCACACCTCCACCTGGCACAAGAGTCAGAGCCATGGCCATCTACAAG
CAGTCCCAGCACATGACCGAGGTCGTGCGGAGATGTCCTCACCACGAGAGATGCAGCGATAG
CGACGGACTGGCTCCTCCTCAGCACCTGATTAGAGTGGAAGGCAACCTGAGAGTGGAATACCT
GGACGACCGGAACACCTTCAGACACAGCGTGGTGGTGCCTTACGAGCCTCCTGAAGTGGGCA
GCGATTGCACCACCATCCACTACAACTACATGTGCAACAGCAGCTGCATGGGCGGCATGAACA
GAAGGCCCATCCTGACCATCATCACCCTGGAAGATAGCAGCGGCAACCTGCTGGGCAGAAAC
AGCTTCGAAGTCCGCGTGTGTGCCTGTCCTGGCAGAGACAGAAGAACCGAGGAAGAGAACCT
GCGGAAGAAGGGCGAGCCACACCACGAACTTCCACCAGGCAGCACCAAAAGAGCCCTGCCTA
ACAACACCAGCAGCAGCCCTCAGCCTAAGAAGAAGCCTCTGGACGGCGAGTACTTCACACTG
CAGATTCGGGGCAGAGAACGCTTTGAGATGTTCAGAGAGCTGAACGAGGCCCTGGAACTGAA
GGATGCCCAGGCCGGAAAAGAGCCTGGCGGATCTAGAGCCCACAGCAGCCACCTGAAGTCCA
AGAAGGGCCAGAGCACCAGCCGGCACAAGAAACTGATGTTCAAGACAGAGGGCCCCGACAG
CGACTGA

tagRFP sequence
linker sequence
p53 sequence

Immunofluorescence

After formaldehyde fixation with 4% PFA, cells were permeabilized with 0.1% Triton-X100 (Sigma-Aldrich) and unspecific binding sites were blocked using 1% BSA. Mouse anti-human p53 (DO-1, Thermo Scientific; DO-7, BD Biosciences), anti-PML (abcam ab179466), anti-Coilin (abcam ab87913) were used as primary antibodies and appropriate secondary antibodies conjugated with Alexa-Fluor 488 (Jackson ImmunoResearch) were used. Cell nuclei were stained with Hoechst 33342 (Life Technologies). Images were acquired by an Opera or Phenix confocal spinning disc microscope system (PerkinElmer, Waltham, MA, USA) with a 20x or 40× water objective. Quantification was carried out with the Harmony (PerkinElmer, Waltham, MA, USA) or MetaXpress software (Molecular Devices).

siRNA

To generate gene knockdown cells, Cal-33 cells were incubated with 10 nM siRNA and Lipofectamine RNAiMAX (Thermo Fisher, Waltham, MA, USA, 1:1000) or control (lipid only) in 12- or 384-well plates and incubated for 2 days at 37 °C and 21% O_2. p53 and p73 siRNA were purchased from Dharmacon (ON-Targetplus SMARTpool Human TP53) and (ON-Targetplus SMARTpool Human TP73).

Protein production

P53 wild type (S94-T312) and two mutants (construct Y220C and R175H) were cloned into a vector for His-tagged overexpression in Escherichia coli. Proteins were expressed in BL21(DE3) cells following IPTG induction at 17°C. For purification cell pellets were thawed and resuspended in buffer A (20 mM TRIS pH 7.4, 100 mM NaCl, 10 mM Imidazole, 2 mM DTT, 5% glycerol and Complete Inhibitor plus Benzonase). Cells were lysed using Microfluidizer and the cell debris pelleted by

centrifugation. Lysates were applied to a Ni-NTA washed with two column volumes of wash buffer A and eluted with the buffer A plus 300 mM imidazole. A final protein purification was performed by size exclusion chromatography using HiLoad 35/600 Superdex 75 column at 1ml/min in buffer A without imidazole/complete inhibitor collecting the monomeric peak.

nDSF

Thermal melting experiments were carried out using a Prometheus NT.48 instrument (NanoTemper Technologies). Proteins were prepared in 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 % Glycerol, 2 mM DTT and 0.5 mg/mL was used as a final concentration in 50 µL volume. For binding experiments, ligands were added to the mixture at a final concentration of 100 µM, 1% DMSO was used as control. Compounds were tested in triplicates. The temperature gradient was 2 °C per min from 20 to 90°C. The intrinsic protein fluorescence at 330 and 350 nm was recorded. Data were analyzed using Prometheus NT.48 software. Ligands, which show a +ΔTm greater than six standard deviations from the mean of the control, were considered as hit (stabilizer).

Western blot analysis

For SDS-PAGE and subsequent western blotting cells were harvested in 1x RIPA lysis buffer supplemented with complete mini protease inhibitor cocktail (Roche). After the total protein concentration was determined with a Bradford protein assay, total cell lysates were boiled for 10 min at 99 °C with 1x sample loading buffer and loaded onto a 4-12% SDS-PAGE gel. For immunoblotting proteins were transferred to a nitrocellulose membrane (0.2 µm pore size, Schleicher & Schuell) and blocked with 5 % milk, incubated with primary antibodies anti-p53 (1:500, ThermoFisher Scientific, DO-1), anti-p73 (1:500, ThermoFisher Scientific, PA5-35368) and anti-β-actin (1:1000, Cell Signaling, #4967), and subsequently incubated

with secondary antibodies IRDye 680 or IRDye 800 (1:10,000; LI-COR Biosciences). Infrared signal was detected using the Odyssey imaging system (Licor).

Intact mass analysis:

25 µM of p53H175R and p53Y220C protein were incubated with 100 µM BAY 1892005. Compound dilution were done in sodium acetate from a 10 mM stock solution in DMSO resulting in a final DMSO concentration of 1 %. Reaction were stopped after 1 h via adding 2 µl of 4 % TFA to 20 µl reaction volume.

Samples were analysed on a Waters nanoAcquity coupled to a Waters SYNAPT G2-S with ESI source. The Waters nanoAcquity were equipped with a Waters Mass Prep C4, 2.1 x 5mm to desalt the samples. The nanoAcquity settings was as followed: Temperature; 65 °C, flow rate; 100 µL /min, buffer A; water / 0.1 % formic acid, buffer B; ACN / 0.1 % formic acid, run time; 6 min, gradient: in 3 min from 20% buffer B to 80%.

The Waters SYNAPT G2-S were operating in ESI positive mode with the following settings: Source temperature; 80 °C, desolvation temperature; 150 °C with a desolvation gas flow rate of 500 L/hour, capillary voltage; 3 kV and cone voltage; 40V. The mass data was collected at a range of 150 m/z – 2200 m/z. Raw data were deconvoluted using MaxEnt1 from Waters.

Cell proliferation assays

Cell viability was determined by means of the AlamarBlue® reagent (Thermo Fisher Scientific) in a Victor X3 Multilabel Plate Reader (Perkin Elmer). The Cal-33 (German Collection of Microorganisms and Cell Cultures GmbH (DSMZ)), Detroit 562 (American Type Culture Collection (ATCC)), Huh7 (Japanese Collection of Research Bioresources Cell Bank (JCRB)), Saos2 (DSMZ), H1299 (ATCC), Calu1 (CLS Cell

Lines Service), HMC-1-8 (JCRB), U2OS (ATCC), were seeded at a concentration of 4000 cells/well in 100 µl of growth medium (RPMI1640, 20% FCS) on 96-well microtiter plates. The SK-BR-3, Au-565, H358 and MCF7 (all from ATCC), were seeded at a concentration of 5000 cells/well in 100 µl of growth medium (RPMI1640, 20% FCS) on 96-well microtiter plates. The plates were treated with various substance dilutions (3E-5 M, 1E-5 M, 3E-6 M, 1E-6 M, 3E-7 M, 1E-7 M, 3E-8 M, 1E-8 M) and incubated at 37°C for 96 hours. The IC50 values (substance concentration needed for 50% inhibition of cell proliferation) were calculated from fluorescence values of treated vs. untreated cells.

Supplemental Data:

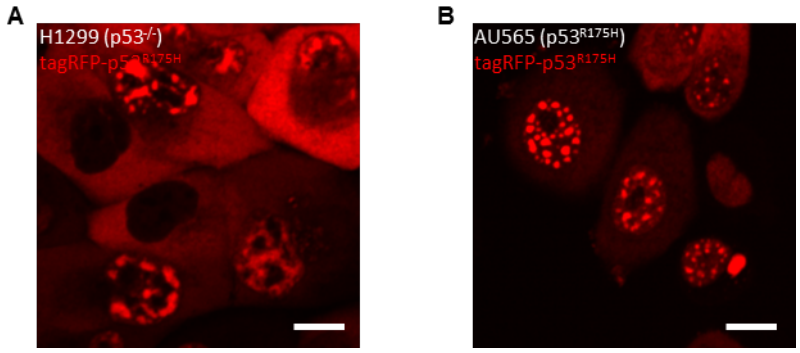


Figure S1: fluorescent p53 reporter, related to Figure 1

A) H1299 and B) AU565 cells expressing fluorescently tagged structural mutant p53^{R175H} show condensate-like structures in the nuclei. Scale bars = 10µm

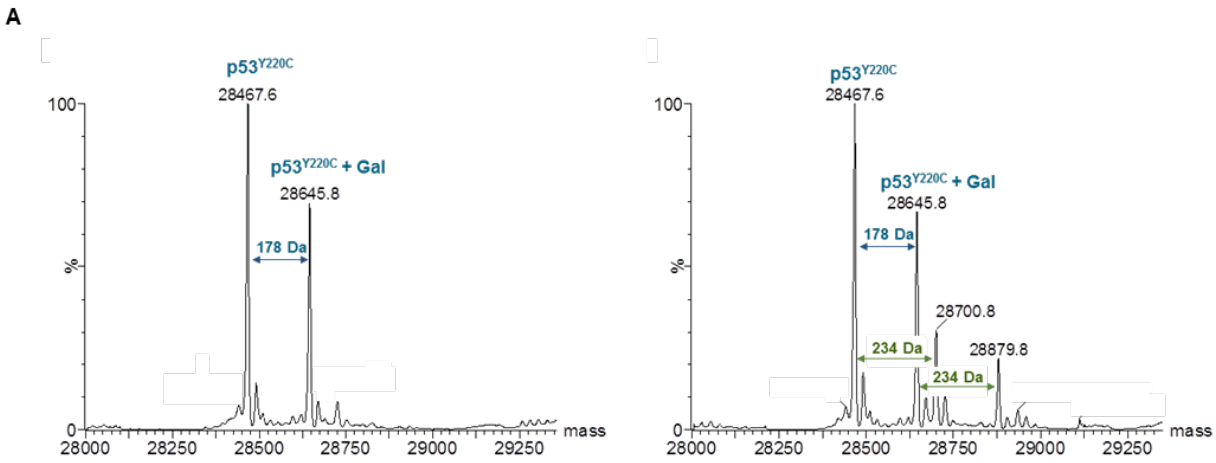


Figure S2: Deconvoluted spectra of intact mass analysis of p53 shows covalent binding of BAY 1892005 to p53Y220C, related to Figure 2. DMSO control shows two peaks of p53^{Y220C} protein, one for the expected mass of 28508 Da and one of 28685 Da representing N-term gluconoylated (Gal) p53^{Y220C} (blue arrows). Incubation with BAY 1892005 showed mass shifts of 234 Da to both the apoprotein and the glyconoylated p53^{Y220C} (green arrows), indicative for covalent binding of BAY 1892005. An additional mass shift of 234 Da indicates a partial two fold binding of BAY 1892005 to p53^{Y220C}.

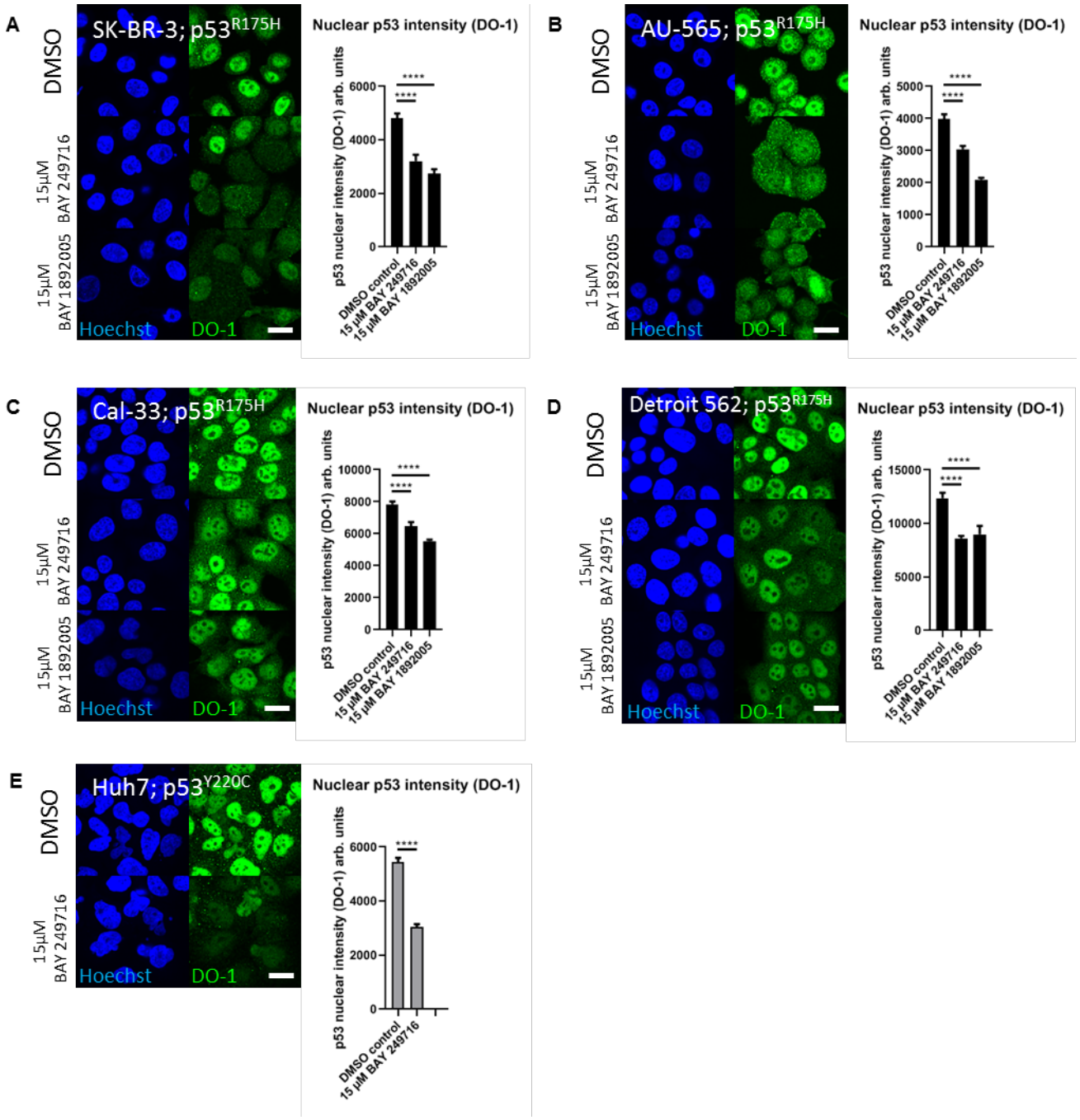


Figure S3: Aminothiazoles lead to reduction of nuclear accumulation of endogenous structural p53 mutants, related to Figure 5

$p53^{R175H}$ cell lines A) SK-BR-3 B) AU-565 C) Cal-33 D) Detroit 562 and E) $p53^{Y220C}$ cell line Huh7 were stained by IF for p53 (DO-1) and the intensity of the staining in the nucleus was quantified. ****p-value < 0.0001. Scale bar = 10µm. Bars show mean with SD (n≥5). Exemplary data of multiple experiments are shown ($n \geq 2$).

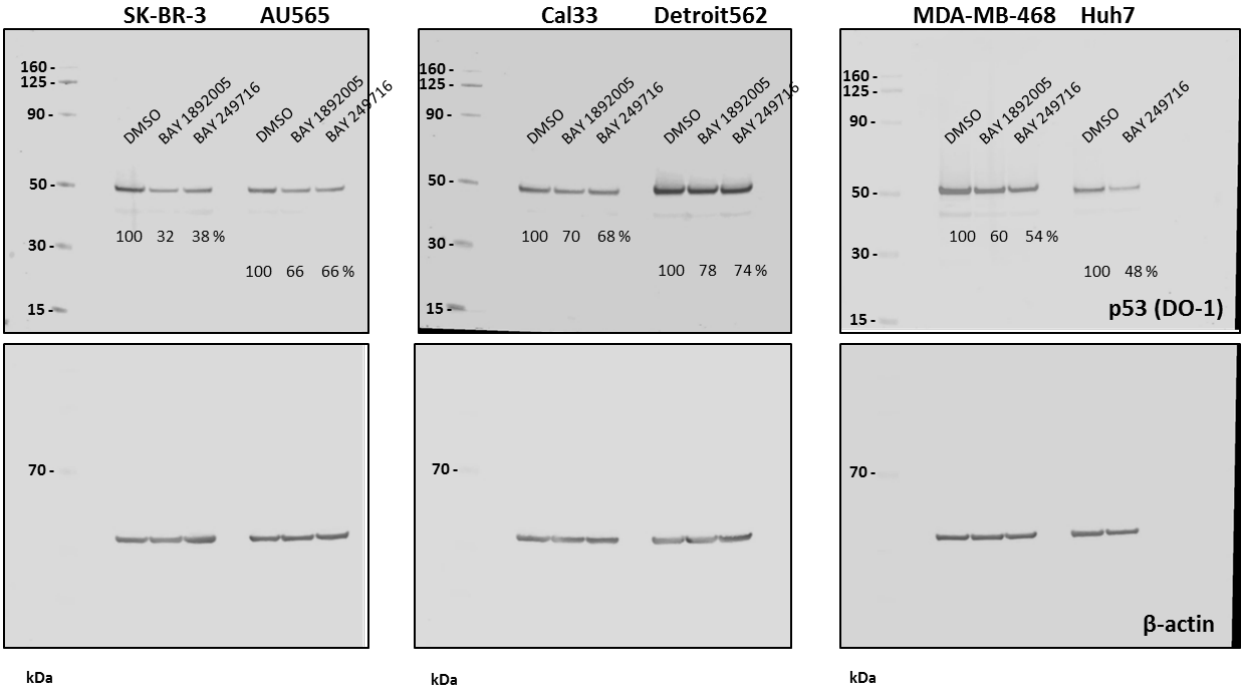


Figure S4: Western Blot quantification shows slight reduction of protein levels in different cell lines after compound treatment, related to Figure 5

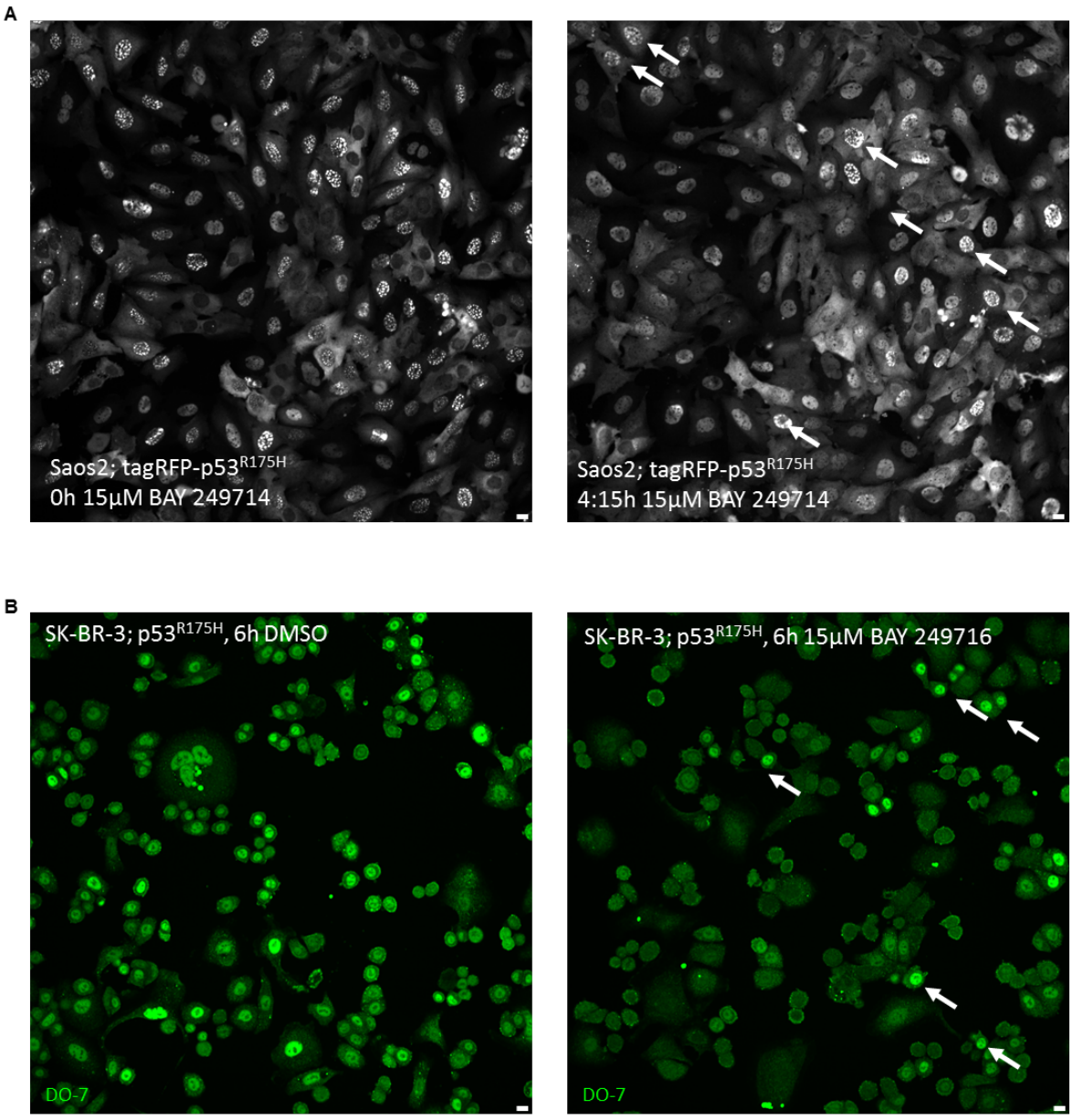
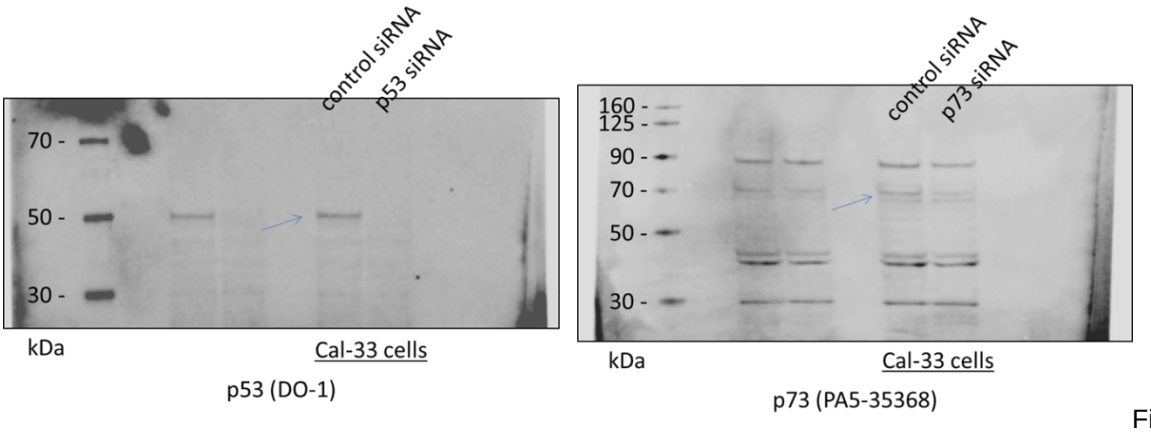


Figure S5: Effect of aminothiazole on cell subpopulations, related to Figure 3 and discussion

A) Saos-2 cells expressing fluorescently tagged structural mutants show p53 condensates in the nuclei which are dissolved after compound treatment with BAY 249716. The overview shows cells displaying dissolution of structural mutant p53 condensates in most cells, while others show less response (arrows). B) SK-BR-3 cells ($p53^{R175H}$) show a decrease in nuclear staining for endogenous p53 accumulation in the nucleus after treatment with BAY 249716, some cells show less reduction (arrows). Scale bars = 10µm. Exemplary data of multiple experiments are shown ($n \geq 2$).



Figure

Figure S6: Western Blot after p53 or p73 siRNA. Related to Figure 6.