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Therapeutic targeting of mutant p53 in pediatric acute lymphoblastic leukemia

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ABSTRACT

Alterations of the tumor suppressor gene *TP53* are found in different cancers, in particular in carcinomas of adults. In pediatric acute lymphoblastic leukemia (ALL), *TP53* mutations are infrequent but enriched at relapse. As in most cancers, mainly DNA-binding domain missense mutations are found, resulting in accumulation of mutant p53, poor therapy response, and inferior outcome. Different strategies to target mutant p53 have been developed including reactivation of p53's wildtype function by the small molecule APR-246. We investigated *TP53* mutations in cell lines and 62 B-cell precursor ALL samples and evaluated the activity of APR-246 in *TP53*-mutated or wildtype ALL. We identified cases with *TP53* missense mutations, high (mutant) p53 expression and insensitivity to the DNA-damaging agent doxorubicin. In *TP53*-mutated ALL, APR-246 induced apoptosis showing strong anti-leukemia activity. APR-246 restored mutant p53 to its wildtype conformation, leading to pathway activation with induction of transcriptional targets and re-sensitization to genotoxic therapy *in vitro* and *in vivo*. In addition, induction of oxidative stress contributed to APR-246-mediated cell death. In a preclinical model of patient-derived *TP53*-mutant ALL, APR-246 reduced leukemia burden and synergized strongly with the genotoxic agent doxorubicin, leading to superior leukemia-free survival *in vivo*. Thus, targeting mutant p53 by APR-246, restoring its tumor suppressive function, seems to be an effective therapeutic strategy for this high-risk group of *TP53*-mutant ALL.

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Introduction

Although most pediatric patients diagnosed with acute lymphoblastic leukemia (ALL) have a favorable prognosis, achievement of long-term survival remains a major clinical challenge, particularly at relapse.[1] Alterations of cell death programs cause treatment failure and resistance in many cancers including leukemia. The nuclear phosphoprotein p53 is a transcription factor that controls cellular responses to stress, including DNA damage. Originally identified more than three decades ago,[2,3] p53 was characterized as a tumor suppressor negatively regulating cell cycle and growth, inhibiting the cancer cell's oncogenic potential.[4,5] The gene coding for p53 (*TP53*) is localized on the short arm of chromosome 17 (17p13) and it is the most frequently mutated gene across different cancers.[6,7] Both deletions and point mutations have been described and mutations often co-occur with loss of the corresponding wildtype allele.[8,9] The majority are *TP53* missense mutations found within the DNA-binding domain coding region (codons 100-300, exons 5-8) and

affect the structural integrity and DNA-binding ability of p53, leading to accumulation of dysfunctional p53 protein and increased oncogenic potential.[10-13]

TP53 mutations are found frequently, in up to 95% of carcinomas, typically in older patients.[7,8] In ALL, recent studies identified alterations of *TP53* in subsets of up to 16%, with higher rates in T-ALL, at relapse, and in elderly patients.[14-18] Moreover, more than 90% of ALL cases with a low hypodiploid karyotype (including loss of chromosome 17) carry somatic *TP53* alterations[19,20] and *TP53* germline mutations confer a high risk for hypodiploid ALL.[21] In pediatric ALL, *TP53* alterations are associated with poor response to chemotherapy and an inferior outcome, particularly at relapse, identifying *TP53*-mutant B-cell precursor (BCP)-ALL patients as a high-risk subgroup with a particular need for alternative therapies.[14,16-18,22]

Different strategies to interfere with the p53 pathway have been evaluated. Inhibition of the interaction of p53 and its negative regulator, mouse double minute 2 (MDM2), leads to sustained p53 transcriptional activity, but requires the presence of wildtype p53.[23] Therefore, direct targeting of mutant p53 has been investigated, identifying small molecules that reactivate p53 function.[24] In line, anti-tumor activity has been observed in murine lymphoma and liver cancer models upon genetic restoration of p53, supporting the principle of p53 reactivation as a therapeutic strategy.[25,26] APR-246 (PRIMA-1^{Met}), the structural analog of PRIMA-1 (p53 reactivation and induction of massive apoptosis) is a small molecule, identified in a screen for mutant p53-dependent growth suppression in sarcoma cells, showing activity on both structural and DNA-binding mutants.[27] APR-246 is a prodrug that is converted into methylene quinuclidinone, which binds covalently to the core domain of mutant p53 interacting with thiol groups of cysteines, restoring p53 wildtype conformation and function.[28,29] In addition, induction of oxidative stress has been reported as a second activity of APR-246, deriving from glutathione depletion, thioredoxin reductase inhibition and other effects.[30-33]

APR-246 demonstrated preclinical antitumor activity and synergism with DNA-damaging drugs in different cancers[32,34-39] and showed very moderate side effect profiles in a first-in-human phase I/IIa clinical trial in patients with refractory prostate cancer, acute myeloid leukemia, chronic lymphocytic leukemia, multiple myeloma and lymphoma.[40] Accordingly, APR-246 is currently being investigated in ovarian and esophageal cancer, myeloid neoplasms and melanoma in phase II clinical trials (*ClinicalTrials.gov*).[41] However, mutant p53 has so far not been addressed as a target for therapeutic intervention in ALL.

In this study, we investigated a large cohort of patient-derived pediatric BCP-ALL primograft samples identifying *TP53*-mutated cases and analyzed the effects of APR-246 in *TP53*-mutated (*TP53*mut) and *TP53*-wildtype (*TP53*wt) BCP-ALL. We identified strong and selective antileukemia activity of APR-246 in *TP53*mut ALL providing the basis to develop personalized therapy regimens for this high-risk subgroup of ALL.

Methods

Additional detailed information is provided in the *Online Supplementary Data*.

Sixty-two patient-derived xenograft samples established by transplantation of patients' ALL cells onto NOD.CB17-Prkdcscid/J mice[42] and six BCP-ALL cell lines were studied. Leukemia samples were obtained from pediatric BCP-ALL patients at diagnosis or relapse upon informed consent from the children and/or their legal guardians in accordance with the institution's ethical review boards. All animal experiments were approved by the appropriate authority (Regierungspräsidium Tübingen) and carried out following the national animal welfare guidelines. *TP53* mutations were analyzed by denaturing high-performance liquid chromatography and confirmed by Sanger sequencing, 17p deletions were assessed by fluorescence *in situ* hybridization. Mutation information was matched to the IARC-*TP53* database.[43] The sensitivity of leukemia samples to doxorubicin, APR-246 (kindly provided by Aprea Therapeutics, Stockholm, Sweden) or the combination was assessed after incubation of ALL cells with increasing drug concentrations, analyzing cell death by flow-cytometry according to forward- and side-scatter criteria. Data from three independent experiments performed in triplicate (cell lines) or of one experiment performed in triplicate (primografts) were analyzed by *t*-test, and differences of half maximal inhibitory concentrations (IC_{50}) titrations by F-test. *P* values ≤0.05 were considered statistically significant. Synergies of drug combinations were assessed calculating combination indices (CI), indicating strong synergism (CI 0.1-0.3), synergism (CI <1), an additive effect (CI=1) or antagonism (CI>1). Apoptosis was analyzed assessing annexin-V-FLUOS positivity and caspase-3 activity. Proteins (p53, PUMA, p21, NOXA, GAPDH) were detected by western blot analysis using the respective antibodies. The wildtype conformation of p53 was detected by immunoprecipitation using a conformation-specific anti-p53 wildtype antibody (PAb1620) followed by western blot analysis with an anti-p53 (total) antibody (DO-7). An immunoglobulin light chain-specific peroxidase conjugated binding protein was used for western blot analyses carried out following immuno-precipitation. Depletion of p53 was achieved by lentiviral shRNA-mediated knockdown or siRNA-mediated downregulation in *TP53*mut or *TP53*wt ALL cells. For *in vivo* treatment, transplanted recipients showing >5% human ALL cells in peripheral blood were randomized and treated (for 3 weeks) with solvent, APR-246 (days 1-5), doxorubicin (day 1), or the combination (APR-246 days 1-5, doxorubicin day 5) and sacrificed at the end of treatment for analysis of leukemia loads. For survival analyses, recipients were followed up after treatment until onset of leukemia-related morbidity and sacrificed. High loads of human ALL cells were detected in bone marrow and spleen in all cases, confirming reoccurrence of manifest leukemia.

Results

Identification of *TP53* mutations in B-cell precursor acute lymphoblastic leukemia

We investigated 62 patient-derived pediatric BCP-ALL samples, which were established in our NOD/SCID/huALL xenograft model from patients at diagnosis (n=53) or relapse (n=9). *TP53*mut cases were identified by denaturing high-performance liquid chromatography and confirmed by Sanger sequencing (exons 4-10). Four *TP53*mut cases were found, one derived from a patient at second relapse (*TP53*mut-1) and three at diagnosis (*TP53*mut-2, -3, -4) (*Online Supplementary Table S1*). In parallel, we characterized six BCP-ALL cell lines and identified two *TP53*mut (RS4;11, KOPN-8) and four *TP53*wt (MUTZ-5, EU-3, UoCB-6 and NALM-6) lines. All samples carried missense mutations previously described (p53.iarc.fr),[15,43] localized

within the region encoding the DNA-binding domain, suggesting loss of p53's tumor suppressive function (Figure 1A, Table 1). In the *TP53*mut samples, the second allele carried a nonsense mutation (*TP53*mut-1), was absent (loss of 17p, *TP53*mut-2, -3), or carried the same missense mutation (*TP53*mut-4) (Table 1). Somatic and germline *TP53*mut are associated with (low) hypodiploid ALL.[15,19-21] One primograft sample (*TP53*mut-3) showed a hypodiploid karyotype with 44 chromosomes (Table 1). In line with disrupted degradation and accumulation of mutant p53 protein, *TP53*mut cases showed higher p53 protein levels compared to *TP53*wt leukemias (Figure 1B).

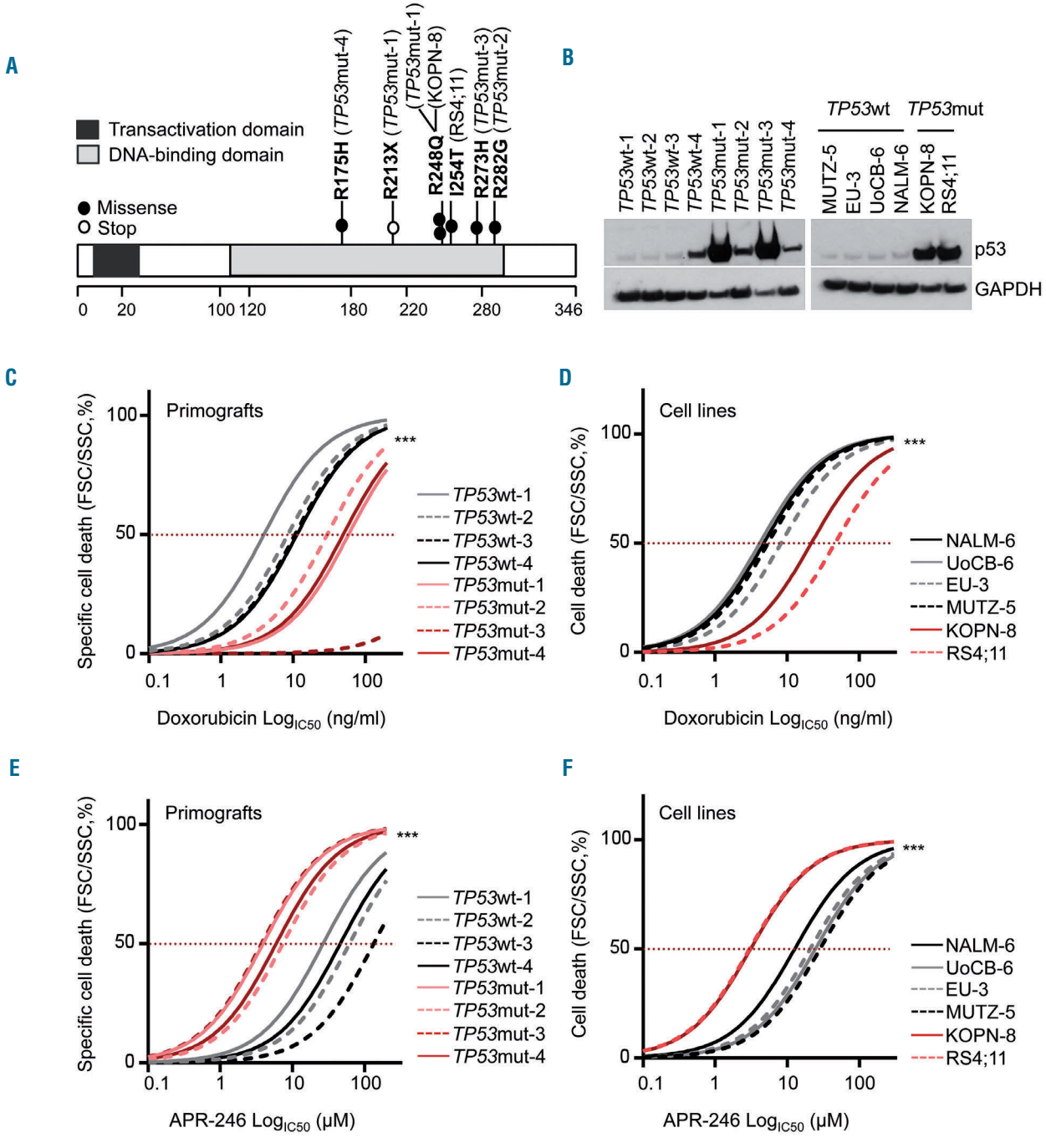

Figure 1. *TP53*-mutated acute lymphoblastic leukemias are DNA-damage resistant but sensitive to APR-246. (A) All *TP53*mut B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) primograft and cell lines harbor missense mutations (filled circles) localized in the DNA-binding domain of *TP53*. The primograft sample *TP53*mut-1 carries an additional stop mutation (R213X, open circle). See also Table 1. (B) Increased p53 protein expression in *TP53*mut compared to *TP53*wt ALL in primograft (left) and cell line (right) leukemia samples. Western blot, anti-p53 antibody (total, clone DO-7) with GAPDH as a loading control. (C-F) Significantly higher half maximal inhibitory concentrations (IC_{50}) for doxorubicin in *TP53*mut (red curves) primograft (C) and cell line (D) BCP-ALL, and significantly lower IC_{50} values for APR-246 in *TP53*mut primograft (E) and cell line (F) samples, indicating insensitivity to the DNA-damaging agent doxorubicin but sensitivity to APR-246 in *TP53*mut BCP-ALL. Dose-response curves reflect cell death induction in response to increasing concentrations summarizing one (primografts, 24 h; C, E) or three (cell lines, 48 h; D, F) independent experiments, each performed in triplicate. Comparison of sensitivities of *TP53*wt and *TP53*mut leukemias, *F*-test, ***$P<0.001$. See also *Online Supplementary Table S2*.

TP53-mutated leukemias are sensitive to APR-246 but not to genotoxic therapy

In response to genotoxic agents and stress, wildtype p53 suppresses cellular viability and proliferation. However, dysfunctional, mutant p53 fails to mediate tumor-suppressive functions such as induction of cell death. Therefore, we analyzed cell death in *TP53*mut and *TP53*wt ALL primografts (*TP53*mut n=4, *TP53*wt n=4) and cell lines (*TP53*mut n=2, *TP53*wt n=4) in response to increasing concentrations of the DNA-damaging agent doxorubicin, a standard genotoxic drug regularly used in ALL treatment protocols, and to APR-246. All *TP53*mut primografts and cell lines showed, as expected, insensitivity to doxorubicin indicated by significantly higher IC_{50} values, in contrast to doxorubicin-sensitive *TP53*wt leukemias (Figure 1C, D; *Online Supplementary Table S2A, B*). An opposite effect was observed upon exposure to APR-246 with high sensitivity and cell death induction in all *TP53*mut leukemias, but low APR-246 sensitivity in *TP53*wt ALL (Figure 1E, F; *Online Supplementary Table S2C, D*). Interestingly, diagnosis- (*TP53*mut-2, -3, -4) or relapse-derived (*TP53*mut-1) primograft samples did not show differences in APR-246 or doxorubicin sensitivity.

Activation of the p53 pathway results in apoptosis induction. Along with cell death, APR-246 led to annexin-V/propidium iodide positivity and caspase-3 activation indicating apoptosis induction in *TP53*mut ALL. In contrast, apoptosis was induced by doxorubicin but not APR-246 in *TP53*wt cells (Figure 2 and *Online Supplementary Figure S1*).

Thus, all identified *TP53*mut leukemias carried missense mutations in the DNA-binding domain, showed accumulation of p53 indicative of dysfunctional mutant p53, resistance to the genotoxic agent doxorubicin, and were highly sensitive to APR-246-induced apoptosis.

APR-246 restores p53's wildtype conformation reactivating tumor suppressive functions

We further addressed the mode of action of APR-246 in *TP53*mut ALL and examined the conformation of p53 and activation of the pathway in response to APR-246. Using a p53 wildtype conformation-specific antibody (PAb1620), larger amounts of p53 with wildtype conformation were immunoprecipitated from lysates of *TP53*mut ALL cells exposed to APR-246, indicating reconstitution of p53 wildtype conformation in *TP53*mut ALL by APR-246 (Figure 3A). However, this effect was not observed in *TP53*wt leukemia cells (*Online Supplementary Figure S2*). Next, we assessed expression of the p53 transcriptional targets PUMA (P53-Upregulated Modulator of Apoptosis), p21 (Cyclin Dependent Kinase Inhibitor 1A, CDKN1), and NOXA upon APR-246 or doxorubicin treatment in *TP53*mut (KOPN-8, RS4;11) and *TP53*wt (NALM-6, UoCB-6) ALL lines. In *TP53*mut ALL, APR-246 led to induction of all p53 targets (Figure 3B, C and *Online Supplementary Figure S3*). In contrast, an opposite picture of activation of p53 transcriptional targets in *TP53*wt but not in *TP53*mut leukemias was observed upon incubation with doxorubicin (Figure 3D, E and *Online Supplementary Figure S3*). Thus, APR-246 induces restoration of mutant p53 to wildtype conformation, transcriptional target expression, and apoptosis in *TP53*mut ALL.

Induction of oxidative stress contributes to APR-246-mediated cellular death

Induction of oxidative stress has been described as a second activity of APR-246 in different cancers.[30-32] APR-246 was reported to interfere with different regulators of the cellular redox system, such as thioredoxin reductase, thioredoxin and glutathione, and with the transcription factor NRF2, leading to induction of reactive oxygen species (ROS).[28,30-33,44-47] Given the activity of APR-246 in *TP53*mut ALL, we addressed whether *TP53*mut and *TP53*wt ALL display distinct sensitivities in response to ROS generation. Upon treatment with 3-morpholinosydnonimine, a spontaneous generator of reactive oxygen and nitrogen species, and the oxidant tert-butyl hydroxyperoxide, increased ROS levels were observed in both

Table 1. ***TP53*** **mutations in acute lymphoblastic leukemia cell lines and primograft samples.**

Sample	Exon	Mut. (bp)	Mut. (aa)	Region	Mut. type	del (17p)	Genotype	Number of chr.s.
*TP53*mut-1	exon 6 exon 7	c.637C>T c.743G>A	p.R213X p.R248Q	DBD	stop missense	-	heterozygous	47
*TP53*mut-2	exon 8	c.844C>G	p.R282G	DBD	missense	del	hemizygous	47
*TP53*mut-3	exon 8	c.818G>A	p.R273H	DBD	missense	del	hemizygous	44
*TP53*mut-4	exon 5	c.524G>A	p.R175H	DBD	missense	-	homozygous	46
RS4;11	exon 7	c.761T>C	p.I254T	DBD	missense	-	heterozygous	47
KOPN-8	exon 7	c.743G>A	p.R248Q	DBD	missense	-	heterozygous	46

Mut: mutation; bp: base pair; aa: amino acid; del: deletion; DBD,: DNA-binding domain; Chr.s: chromosomes.

Table 2. ***TP53*** **mutations in primary samples from patients with acute lymphoblastic leukemia.**

Sample	Exon	Mut. (bp)	Mut. (aa)	Region	Mut. type	del (17p)	Genotype	Diploidy
Patient-1	exon 4	c.375G>A	p.T125T	DBD	splice site	del	hemizygous	diploid
Patient-2	exon 7	c.743G>A	p.R248Q	DBD	missense	-	heterozygous	diploid
Patient-3	exon 7	c.743G>A	p.R248Q	DBD	missense	-	heterozygous	diploid
Patient-4	exon 7	c.733G>C	p.G245R	DBD	missense	-	heterozygous	diploid

Mut: mutation; bp: base pair; aa,: amino acid; del: deletion; DBD: DNA-binding domain.

*TP53*mut and *TP53*wt ALL cells, leading to similar cell death rates (*Online Supplementary Figure S4*).

Next, we investigated whether induction of oxidative stress is involved in APR-246-mediated cell death in *TP53*mut ALL. Importantly, methyl quinuclidinone, the active drug spontaneously formed from APR-246, binds covalently to cysteine residues in the core domain of p53, but also to cysteines in the widely used antioxidant and ROS inhibitor N-acetylcysteine (NAC).[28,44] Thus, NAC directly blocks APR-246 activity and cannot be used to investigate the role of ROS in APR-246-mediated cell death. Therefore, the synthetic antioxidant compound and ROS inhibitor superoxide dismutase mimetic Mn (III) tetrakis (5, 10, 15, 20-benzoic acid) porphyrin (MnTBAP) was used. Cell death was analyzed together with ROS levels in *TP53*mut (KOPN-8 and RS4;11) and *TP53*wt (NALM-6 and UoCB6) leukemia cells exposed to APR-246 with or without NAC or MnTBAP. Similar ROS levels were observed upon APR-246 treatment in both *TP53*mut and *TP53*wt ALL (*Online Supplementary Figure S5A, C, E, G*), however induction of cell death was only seen in *TP53*mut cells (*Online Supplementary Figure S5F, H*) but not in *TP53*wt cells (*Online Supplementary Figure S5B, D*). Interestingly, ROS inhibition by MnTBAP partially inhibited APR-246-induced cell death in *TP53*mut ALL, indicating that ROS contribute to APR-246- induced cell death. It was also interesting that, even in the presence of MnTBAP, i.e. in the absence of ROS, APR-246 retained a statistically significant cytotoxic effect (*Online Supplementary Figure S5F, H*). In line with previous reports,[44] the activity of APR-246 was completely blocked by NAC.

Taken together, these data show that induction of oxidative stress might contribute to APR-246-mediated cell death in ALL, in line with previously reported data of a dual mode of action of APR-246 in other malignancies.[28,30-33,44-46]

APR-246 activity depends on mutant p53

Activity of APR-246 was observed in *TP53*mut but not *TP53*wt ALL. Therefore, we analyzed the effect of APR-246 in *TP53*mut and *TP53*wt cell lines upon lentiviral shRNA-mediated knockdown of p53 (Figure 4A-C). In both *TP53*mut lines (KOPN-8 and RS4;11) depletion of p53 led to APR-246 insensitivity and cell death resistance, in contrast to dose-dependent cell death induction in control-transduced cells (Figure 4D, E). However, *TP53*wt cells with p53 depletion were unresponsive to APR-246, like the corresponding control transduced cells (Figure 4F). A similar result was observed upon siRNA-mediated p53 downregulation with clearly lower cell death induction in

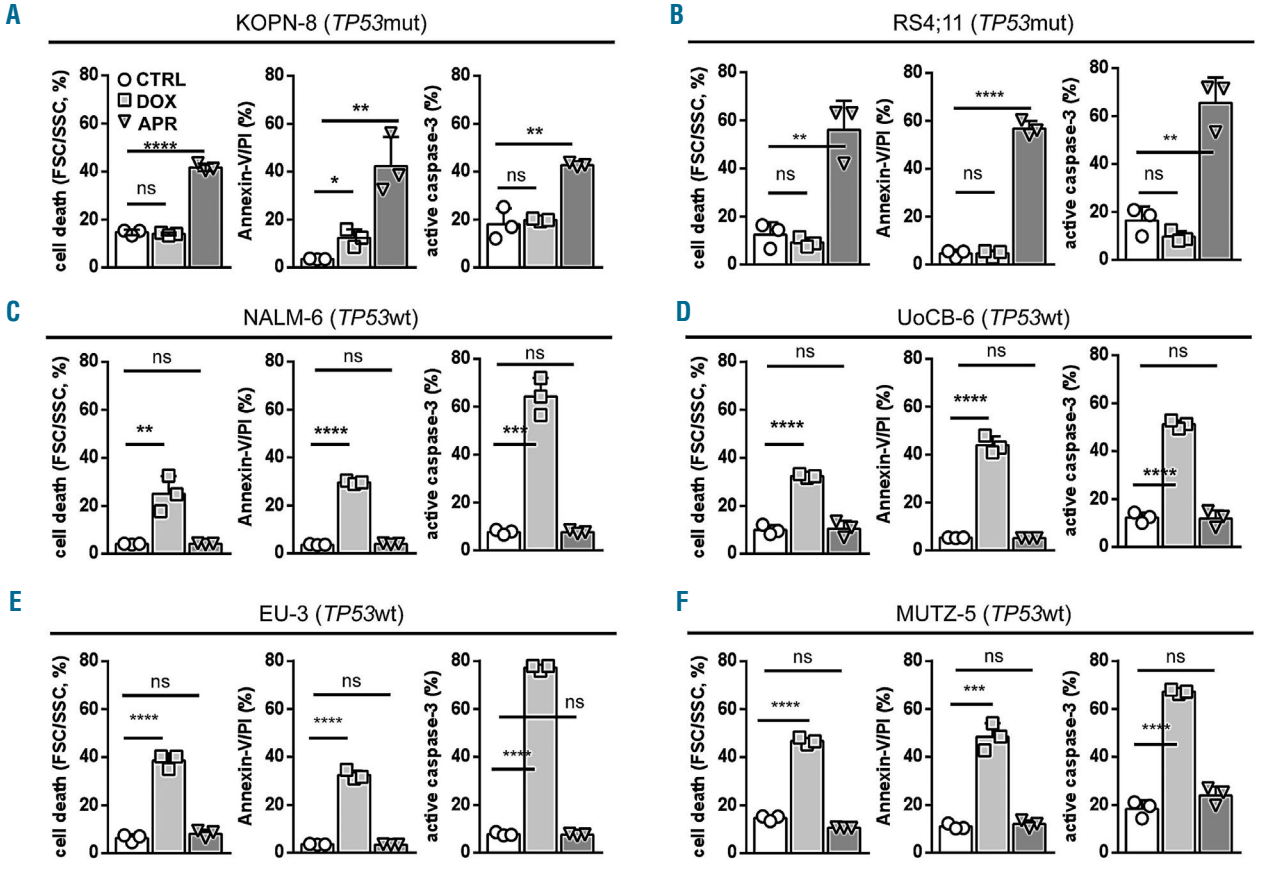

Figure 2. APR-246 induces apoptosis in *TP53*-mutated acute lymphoblastic leukemia. (A-F) Induction of cell death (left diagrams, forward/side scatter criteria, flow cytometry), annexin-V/propidium iodide (PI) positivity (middle diagrams) and caspase-3 activation (right diagrams) by APR-246 in *TP53*mut cell lines KOPN-8 (A) and RS4;11 (B), in contrast to cell death and apoptosis induction by doxorubicin in *TP53*wt lines NALM-6 (C), UoCB-6 (D), EU-3 (E), and MUTZ-5 (F). Proportions of cells after 48 h exposure to solvent (CTRL), APR-246 (APR, 5 μM), or doxorubicin (DOX, 15 ng/mL). Mean values ± standard deviation of three independent experiments, each performed in triplicate. Student *t*-test, ****P<0.0001; ***P<0.001; **P<0.01; *P<0.05; n.s., not significant.

*TP53*mut ALL but no effect in *TP53*wt cells (Figure 4G-J and *Online Supplementary Figure S6*). Together with our observations on APR-246 insensitivity in *TP53*wt ALL (Figure 1E, F) and the absence of p53 transcriptional target expression upon APR-246 treatment in *TP53*wt ALL (Figure 3D, E), these findings indicate that the activity of APR-246 is associated with the presence of mutant p53. Accordingly, distinct sensitivities to APR-246 were found in four primary ALL samples obtained from patients with therapy-resistant disease or relapse carrying different *TP53* mutations (Figure 4L, Table 2). Robust dose-dependent cell death induction was observed in leukemia cells from patients 2, 3, and 4 carrying missense mutations resulting in expression of mutant p53 (Figure 4L, N-P), whereas APR-246 did not induce cell death in ALL cells of patient 1 carrying a hemizygous splice site mutation without detectable expression of p53 protein (Figure 4L, M).

APR-246 re-sensitizes *TP53*-mutated acute lymphoblastic leukemia to doxorubicin

*TP53*mut cancer cells show resistance to DNA damage. Therefore, we analyzed whether reactivation of mutant

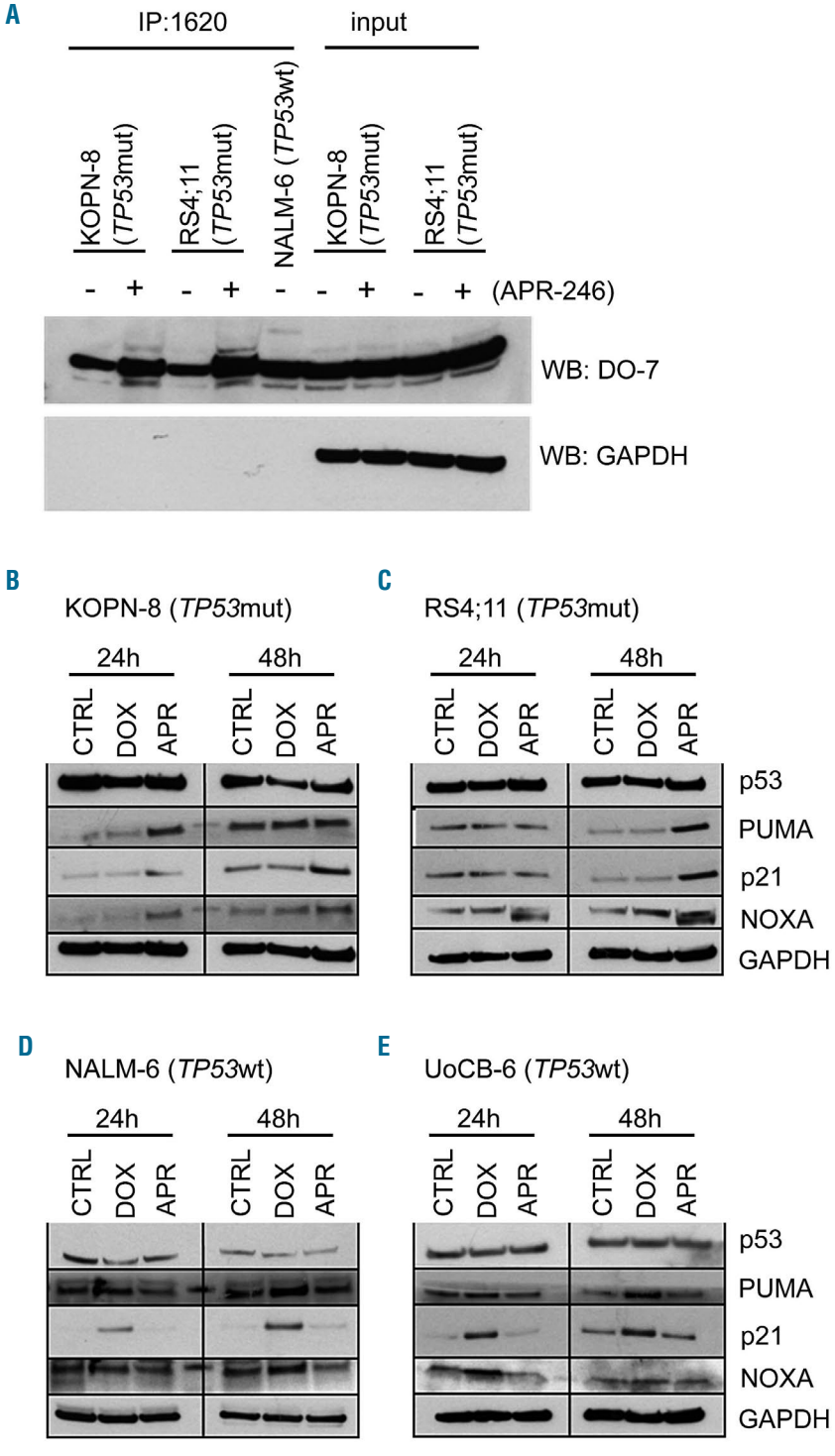

Figure 3. Conformational and functional restoration of mutant p53 by APR-246. (A) Increased levels of p53 with wildtype conformation in *TP53*mut ALL (KOPN-8, mutation R248Q; RS4;11, mutation I254T) upon exposure to APR-246 (5 μM, 24 h). Immunoprecipitation (IP, anti-wt p53 specific antibody PAb1620) and western blot analysis (WB, anti-p53 antibody DO-7, light chain-specific goat anti-mouse peroxidase conjugated binding protein), GAPDH expression in input lysates and absence in precipitates, NALM-6 serves as a wildtype p53 positive control. (B-E) Expression of p53 transcriptional targets PUMA, p21 and NOXA in (B, C) *TP53*mut ALL upon APR-246 treatment and in (D, E) *TP53*wt ALL upon doxorubicin treatment. Western blot, exposure to solvent (CTRL), doxorubicin (DOX, 15 ng/mL), or APR-246 (APR, 5 μM) for the indicated times, with GAPDH as a loading control. The results of one representative out of two independent experiments are shown. See also *Online Supplementary Figures S2 and S3*.

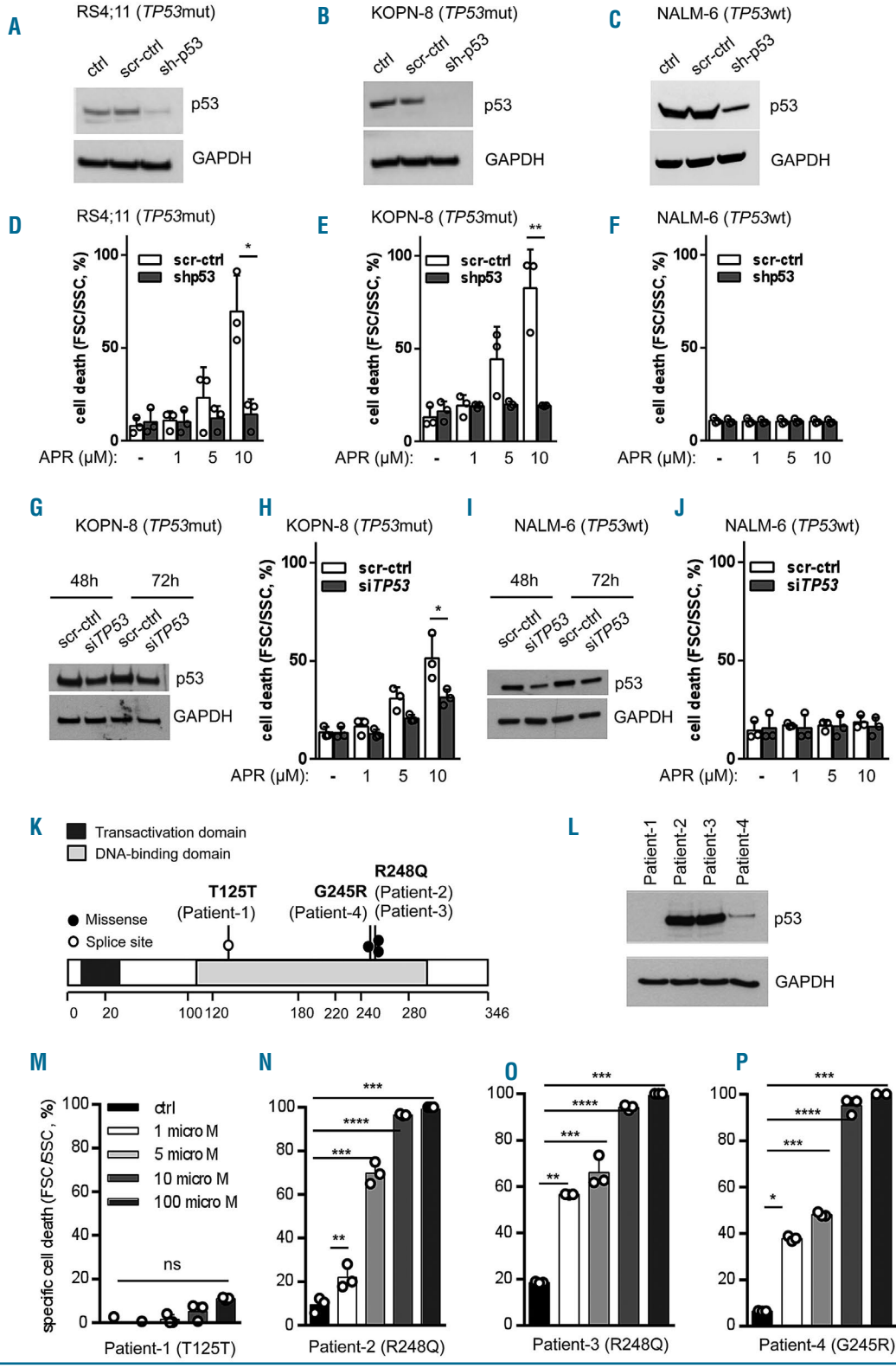

Figure 4. APR-246 activity depends on mutant p53. (A-C) Stable lentiviral shRNA-mediated p53 knockdown in *TP53*mut (RS4;11 and KOPN-8) and *TP53*wt (NALM-6) cell lines. Western blot, anti-p53 antibody DO-7, with GAPDH as a loading control, non-transduced cells (ctrl), cells transduced with scrambled control (scr-ctrl) and *TP53*-specific shRNA (sh-p53). (D-F) Increasing cell death (forward/side scatter criteria, flow cytometry) in control transduced cells and abrogated cell death induction upon p53 knockdown at increasing concentrations of APR-246 (APR, 48 h) in *TP53*mut but not *TP53*wt cells. Mean values ± standard deviation (SD) of three independent experiments, each performed in triplicate. Student t-test, **P*<0.05; ***P*<0.01. (G-J) si-RNA-mediated p53 downregulation in *TP53*mut (KOPN-8), (G) and *TP53*wt (NALM-6), (I) cells leading to clearly lower cell death induction upon p53 downregulation as compared to higher cell death in control cells (H), while p53 downregulation in *TP53*wt cells did not affect cell death induction upon APR-246 treatment (J). Mean values ± SD of three independent experiments. Student *t*-test, **P*<0.05; ***P*<0.01. (K) *TP53* mutations identified in primary samples from patients with acute lymphoblastic leukemia (ALL): the mutations were localized in the DNA-binding domain with one splice site mutation (open circle, Patient-1) and three missense mutations (filled circles, Patients -2, -3, -4). (L) No detectable p53 protein in ALL cells from Patient-1 (western blot, anti-p53 antibody DO-7, GAPDH as a loading control), and (M) no APR-246 activity in these cells (Patient-1), in contrast to cell death induction in cases carrying missense hot spot *TP53* mutations (N, O, P; Patients-2, -3, -4). Mean values ± SD, measurements performed in triplicate. Student *t*-test, *****P*<0.0001; ****P*<0.001; ***P*<0.01; **P*<0.05; n.s., not significant.

p53 re-sensitizes *TP53*mut ALL to the DNA-damaging agent doxorubicin, which is also used in treatment of pediatric ALL. *TP53*mut and *TP53*wt ALL cell lines and primograft samples were exposed to APR-246, doxorubicin, or to combinations of both at increasing concentrations. Strongly increased cell death rates were observed in all four *TP53*mut primografts and two cell lines upon combination treatment with APR-246, as compared to APR-246 or doxorubicin alone, indicating synergistic activity for APR-246 and doxorubicin in *TP53*mut ALL (Figure 5A-F, *Online Supplementary Table S3A)*. In *TP53*wt leukemias however, only doxorubicin showed cell death-inducing activity, which was not increased by adding APR-246 (Figure 5G-L, *Online Supplementary Table S3B*).

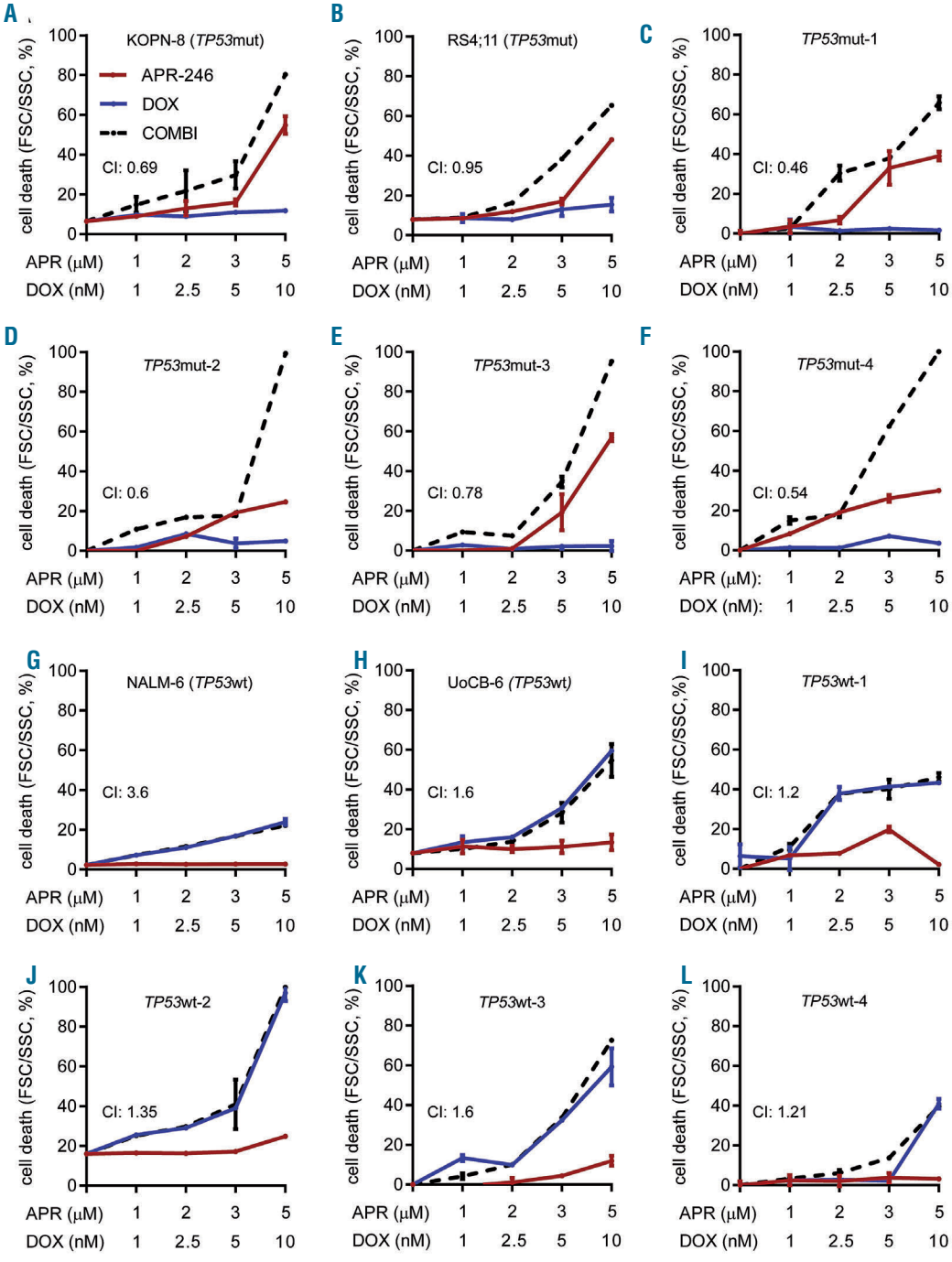

Figure 5. APR-246 synergizes with doxorubicin. Synergistic activity of APR-246 in combination with the DNA-damage-inducing agent doxorubicin in *TP53*mut B-cell precursor (BCP)-acute lymphoblastic leukemia (ALL) cell lines (A, B) and *TP53*mut primograft leukemias (C-F) leading to doxorubicin re-sensitization, in contrast to no increased activity compared to treatment with doxorubicin alone in *TP53*wt cell lines (G, H) and *TP53*wt primograft ALL (I-L). Cell death (forward/side scatter criteria, flow cytometry) after exposure (primografts 24 h, cell lines 48 h) at indicated concentrations of APR-246 (APR), doxorubicin (DOX) or the combination (COMBI, 3 h APR-246 pre-incubation). Mean values ± standard deviation (SD) of three independent experiments, each performed in triplicate (cell lines: A, B, G, H). Mean values ± SD, three measurements (primografts: C-F, I-L). Combination indices (CI) indicating a strong synergistic (CI 0.1-0.3), a synergistic (CI <1), an additive (CI=1) or an antagonistic effect (CI>1) upon combination.

We also addressed whether induction of oxidative stress would increase the antileukemia activity of APR-246. In contrast to clearly increased cell death upon treatment with APR-246 together with the DNA-damaging agent doxorubicin, combining APR-246 with the ROS inducers 3-morpholinosydnonimine and tert-butyl hydroxyperoxide did not lead to clearly increased cell death (*Online Supplementary Figure S7*). Thus, APR-246 effectively synergizes with doxorubicin and re-sensitizes *TP53*mut ALL to DNA-damage-induced cell death, while additional ROS induction did not increase APR-246-mediated leukemia cell death.

Preclinical antileukemia activity of APR-246 and *in vivo* synergy with genotoxic therapy

Based on our findings, we investigated the antileukemia activity of APR-246 in a preclinical setting *in vivo*. Mice were transplanted with the *TP53*mut primograft (*TP53*mut-4; R175H). Upon manifestation of leukemia, as indicated by 5% or more human CD19$^+$ ALL cells in the recipients' peripheral blood, mice were treated with APR-246 (25, 50 or 100 mg/kg) or vehicle until control-treated animals showed signs of leukemia-related morbidity (3 weeks, days 1-5) (Figure 6A). Upon APR-246 treatment, a clear dose-dependent reduction of leukemia loads was observed in all three organ compartments: spleen, bone marrow and central nervous system (Figure 6B-D). Moreover, in leukemia cells isolated from these APR-246-treated animals, dose-dependent increases in mutant p53 with wildtype conformation and expression of PUMA and p21 were detected (Figure 6E, F), indicating restoration of wildtype p53 conformation and function *in vivo*.

Furthermore, APR-246 demonstrated strong *in vivo* antileukemia activity in another *TP53*mut ALL primograft sample (*TP53*mut-1; R248Q/R213X) leading to significantly reduced leukemia loads in the spleen, bone marrow and central nervous system upon therapy of leukemia-bearing recipients (Figure 6G-I).

We also addressed the effects of APR-246 in combination with doxorubicin *in vivo*. Recipients with manifest ALL (*TP53*mut-1; R248Q/R213X; 5% or more human ALL cells in the peripheral blood) were treated with APR-246, doxorubicin, or the combination of both for 3 weeks. After treatment, the animals were followed up and the time until onset of ALL-related morbidity was analyzed for each animal (Figure 6J). Upon sacrifice, high loads of human ALL were detected in the spleen and bone marrow of all recipients, confirming recurrence of manifest leukemia at clinical onset. Importantly, in addition to clear antileukemia activity as a single agent, leading to increased post-treatment survival (P<0.0001), APR-246 synergized strongly with doxorubicin and re-sensitized *TP53*mut ALL to genotoxic therapy *in vivo*, resulting in significantly prolonged survival as compared to APR-246 alone (P=0.0005) (Figure 6K). In all treatment experiments, application of APR-246 was well tolerated and no side effects were observed in the recipients.

Taken together, our findings in ALL carrying *TP53* missense mutations in the DNA-binding domain, which lead to accumulation of dysfunctional p53, indicate that targeting mutant p53 with APR-246 results in refolding of mutant p53 into its native wildtype conformation, induction of p53 transcriptional targets, involvement of oxidative stress, induction of apoptosis, sensitization to DNA damage and, most importantly, preclinical antileukemia activity with significant reduction of leukemia loads, re-sensitization to genotoxic therapy and clearly prolonged survival *in vivo*. Thus, application of APR-246 can provide an effective strategy for directed therapeutic intervention in the high-risk subtype of *TP53*mut BCP-ALL.

Discussion

Investigating a large cohort of 62 patient-derived BCP-ALL samples, all identified *TP53*mut cases showed missense mutations leading to alterations in the DNA-binding domain of p53, high levels of p53 protein and insensitivity to doxorubicin. Interestingly, APR-246 demonstrated robust antileukemia activity in these cases, including induction of apoptosis, effective reduction of leukemia loads, and sensitization to doxorubicin in an *in vivo* model of *TP53*mut ALL. Both *in vitro* and *in vivo* experiments showed that treatment with APR-246 led to restored conformation and activation of mutant p53, and induction of transcriptional targets.

Alterations in *TP53* have been described in diverse cancers at high frequencies of up to 95%.[7] In our cohort, *TP53* mutations were identified in four out of 62 cases (6.5%), in line with reported rates in ALL of 6-16%.[14-16,18] All mutations identified in the primograft and cell line samples were missense mutations localized in the DNA-binding domain, with additional loss of the second allele in some of the cases, consistent with mutational patterns reported throughout different cancer types.[10,43] One *TP53*mut sample showed hypodiploidy, in line with reported associations of hypodiploidy with germline and somatic *TP53* mutations.[19-21]

Mutated dysfunctional p53 results in resistance to therapy-induced DNA damage[48] and poor patient outcome.[14,16-18,22] Correspondingly, increased numbers of *TP53* alterations are seen at ALL relapse[16] and all *TP53*mut leukemias were insensitive to the DNA-damaging agent doxorubicin. Importantly, these *TP53*mut leukemias were sensitive to APR-246, likely by reactivation of high levels of dysfunctional p53 accumulated in the cells. Most importantly, in line with reports in ovarian cancer,[32,39] APR-246 clearly synergized with doxorubicin *in vitro*, *ex vivo* and *in vivo*, re-sensitizing initially resistant *TP53*mut ALL to DNA damage. Therefore, combining functional p53 restoration with genotoxic therapies triggering the p53-mediated DNA-damage response would be the rationale to apply APR-246 together with doxorubicin, a classical DNA-damage-inducing agent used in ALL treatment regimens. Importantly, a favorable pharmacological profile and antitumor effects were observed upon first clinical use of APR-246 in patients with refractory cancers[40] and APR-246 is being tested in combination with anticancer agents, including doxorubicin, in ongoing phase II trials (*ClinicalTrials.gov*).[41]

We addressed the molecular mechanism of action of APR-246 and demonstrated restoration of p53 wildtype conformation, p53 pathway activation with induction of downstream transcriptional targets, and a contribution of oxidative stress leading to apoptosis of *TP53*mut BCP-ALL cells. Importantly, this antileukemia effect was also observed *in vivo* in *TP53*mut ALL, but not in *TP53*wt ALL, upon p53 knockdown or in a patient's sample with a splice site mutation and loss of p53 protein expression. High levels of misfolded mutant p53 were described to be

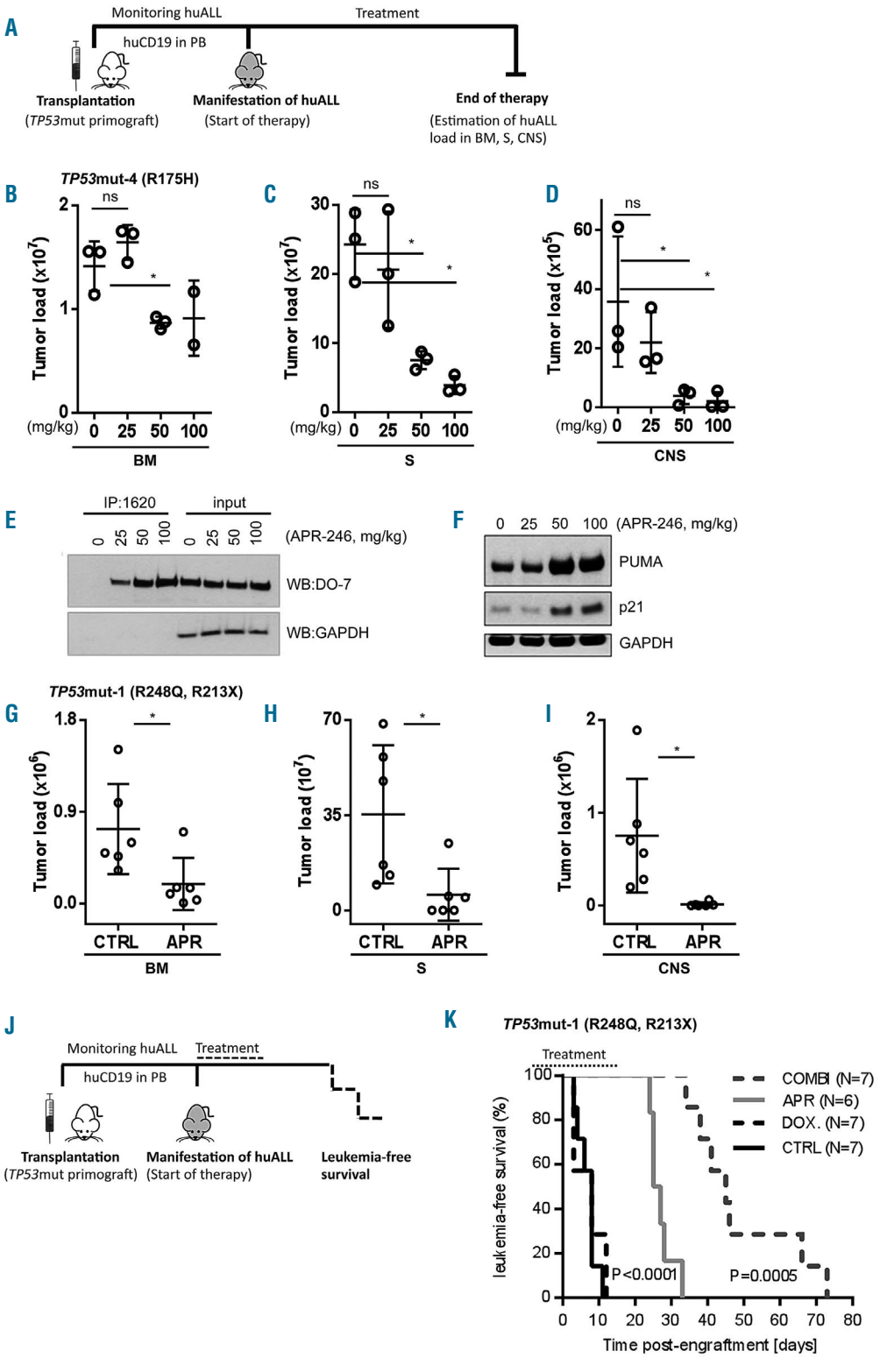

Figure 6. Anti-leukemia activity of APR-246 and synergy with genotoxic therapy in *TP53*-mutated acute lymphoblastic leukemia *in vivo*. (A) Schematic representation of the experimental procedure: endpoint analysis assessing leukemia loads in differently treated recipients. (B-D) Dose-dependent reduction of leukemia load in bone marrow (BM) (B), spleen (S) (C) and central nervous system (CNS) (D) upon treatment of mice bearing *TP53*mut-4 (mutation R175H) acute lymphoblastic leukemia (ALL) with solvent or increasing doses of APR-246 for 3 weeks as indicated (n=3 recipients per group, except n=2 for BM 100 mg/kg). Student t-test, **P*<0.05; n.s., not significant. (E) Restoration of p53 wildtype conformation upon *in vivo* APR-246 therapy, immunoprecipitation (IP: anti-wt p53 specific antibody PAb1620, western blot: anti-p53 antibody DO-7, light chain-specific goat anti-mouse peroxidase conjugated binding protein, GAPDH as a loading control), and (F) dose-dependent induction of p53 transcriptional targets PUMA and p21 (western blot, GAPDH as a loading control). (G-I) Significant reduction of leukemia load in bone marrow (BM) (G), spleen (S) (H) and central nervous system (CNS) (I) upon treatment of *TP53*mut-3 (mutations R248Q, R213X) ALL-bearing mice with APR-246 (APR, 100 mg/kg) or solvent (CTRL) for 3 weeks, n=6 mice per group, Student *t*-test, **P*<0.05. (J) Schematic representation of the experimental procedure: survival analysis. (K) Superior survival of animals treated with APR-246 (APR, 50 mg/kg, 3 weeks, days 1-5; n=6) as compared to doxorubicin (DOX, 2 mg/kg, 3 weeks, day 1; n=7) or vehicle (CTRL, 3 weeks, days 1-5; n=7) (*P*<0.0001); and synergy of the combination of APR-246 and doxorubicin (COMBI, APR-246, 50 mg/kg, days 1-5, and doxorubicin, 2 mg/kg, day 5; n=7) leading to increased survival as compared to APR-246 treatment alone (*P*=0.0005). Kaplan-Meier analysis, log-rank test.

associated with APR-246 sensitivity in cancer cell lines,[38,49,50] consistent with our observation of high APR-246 activity in *TP53*mut ALL with high p53 expression. However, evaluation of larger cohorts of patients together with outcome data would be required to explore the value of the level of expression of p53 as an indicator of APR-246 responsiveness in *TP53*mut ALL.

APR-246 activity has been reported to be mediated independently of p53 by induction of oxidative stress in other types of cancer, including acute myeloid leukemia and multiple myeloma.[30-33] However, we only observed cell death in *TP53*mut ALL, although *TP53*mut and *TP53*wt ALL showed no differences in induction of and sensitivity to oxidative stress. Interestingly, APR-246 activity in *TP53*mut ALL was partially inhibited by ROS neutralization. This suggests that induction of oxidative stress contributes to APR-246-mediated cell death in ALL, in line with reports on a dual mode of action of APR-246,[30-32] which might vary between tumor types and cellular context.

The presence of p53 in a mutated, dysfunctional form, as typically is the case for missense mutations in the DNA-binding core domain, enables binding of the active moiety of APR-246, leading to activity in BCP-ALL.[11,28] This is of clinical relevance, since the majority of *TP53* mutations in BCP-ALL are missense hot spot mutations in the DNA-binding domain[15,43] resulting in accumulation of misfolded p53 protein, which is targeted by APR-246. However, the precise mechanism of activity on DNA contact mutations is not yet known. Importantly, we and others [27] have demonstrated antitumor activity on structural and contact mutants including clear preclinical antileukemia activity on *TP53*mut ALL carrying either a structure (R175H) or contact (R248Q) mutation.

Taken together, our study shows that the small molecule APR-246 exhibits profound antileukemia activity in *TP53*mut BCP-ALL, targeting non-functional mutant p53 resulting from missense mutations in the DNA-binding domain of *TP53*, the most frequent mutation type reported throughout different malignancies. Mechanistically, we showed that APR-246 led to restoration of p53's wildtype conformation, pathway activation with expression of transcriptional targets and induction of apoptosis in *TP53*mut ALL. Moreover, we found a clear synergism between APR-246 and doxorubicin treatment, strongly suggesting that the combination of p53 reactivation and DNA-damage induction could be an effective antileukemia strategy for BCP-ALL patients with *TP53* missense mutations. Hence, targeting mutant p53 appears to be a promising, directed treatment for this high-risk subgroup of *TP53*mut ALL.

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