ARTICLE

Non-genotoxic MDM2 inhibition selectively induces a pro-apoptotic p53 gene signature in chronic lymphocytic leukemia cells

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous hematologic malignancy. In approximately 90% of cases the *TP53* gene is in its wildtype state at diagnosis of this malignancy. As mouse double-minute-2 homolog (MDM2) is a primary repressor of p53, targeting this protein is an attractive therapeutic approach for non-genotoxic reactivation of p53. Since the discovery of the first MDM2 inhibitor, Nutlin-3a, newer potent and bioavailable compounds have been developed. In this study we tested the second-generation MDM2 inhibitor, RG7388, in patient-derived CLL cells and normal cells, examining its effect on the induction of p53-transcriptional targets. RG7388 potently decreased viability in p53-functional CLL cells, whereas p53-non-functional samples were more resistant to the drug. RG7388 induced a pro-apoptotic gene expression signature with upregulation of p53-target genes involved in the intrinsic (*PUMA*, *BAX*) and extrinsic (*TNFRSF10B*, *FAS*) pathways of apoptosis, as well as *MDM2*. Only a slight induction of *CDKN1A* was observed and upregulation of pro-apoptotic genes dominated, indicating that CLL cells are primed for p53-dependent apoptosis. Consequently, RG7388 led to a concentration-dependent increase in caspase-3/7 activity and cleaved poly (ADP-ribose) polymerase. Importantly, we observed a preferential pro-apoptotic signature in CLL cells but not in normal blood and bone marrow cells, including $CD34^+$ hematopoietic cells. These data support the further evaluation of MDM2 inhibitors as a novel additional treatment option for patients with p53-functional CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent B-cell malignancy in adults and is marked by an extremely heterogeneous clinical course.[1-3] CLL is characterized by a clonal expansion of $CD19^+CD5^+$ B cells in the blood, bone marrow and lymphoid tissues.[1-3] Malignant B-lymphocytes accumulate partly due to activation of B-cell receptor (BCR) signaling, leading to increased proliferation and inhibition of apoptosis.[3] In addition to BCR signaling, CLL cells are supported by the tumor microenvironment, including extensive cytokine and chemokine signaling with T cells, myeloid cells, and stromal cells.[4-7]

Although the use of chemo-immunotherapy and BCR antagonists has improved patients' response rates to treatment, CLL remains incurable.[8,9] The identification of new agents that interfere with the survival of CLL cells by promoting apoptosis of these cells is one important approach to improve therapeutic outcomes.[10,11] In fact, several studies have demonstrated that the anti-apoptotic BCL2 protein is highly expressed in CLL and inhibits the activity of pro-apoptotic BH3-only family members, such as p53-upregulated modulator of apoptosis (PUMA).[12-14] Therefore, drugs

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that can enhance expression of these pro-apoptotic BH3-only proteins might represent a clinically relevant therapeutic option for CLL.

The variable clinical course of CLL is driven, at least in part, by molecular heterogeneity which is underscored by the variety of genetic lesions observed, from classical markers of CLL to new genetic lesions uncovered by whole-genome and whole-exome sequencing.[15-19] Among the genetic lesions identified, *TP53* deletions and/or mutations are restricted to ~10% of CLL cases at diagnosis and are associated with decreased survival and clinical resistance to chemotherapeutic treatment.[15,16] Since the prevalence of *TP53* defects at diagnosis is low, the majority of CLL patients retain a functional p53, and in these patients the possibility of activating p53 should be explored as a therapeutic strategy.

Given the central role of p53 in preventing aberrant cell proliferation and maintaining genomic integrity, there is increasing interest in developing pharmacological strategies aimed at manipulating p53 in a non-genotoxic manner, maximizing the selectivity and efficiency of cancer cell eradication.[20,21] The levels and activity of functional p53 are mainly regulated through direct interaction with the human homolog of the murine double-minute 2 (MDM2) protein.[22,23] MDM2 is an E3 ubiquitin ligase which controls the half-life of p53 via ubiquitin-dependent proteasomal degradation.[22] In response to cellular stress, the p53-MDM2 interaction is disrupted and p53 undergoes post-translational modifications on multiple sites to promote transcription of target genes that trigger cell-cycle arrest, apoptosis and/or cell senescence.[20-23] Since the discovery of the first selective small molecule MDM2 inhibitor, Nutlin-3a, newer compounds have been developed with increased potency and improved bioavailability.[24,25] These non-genotoxic compounds bind to MDM2 in the p53-binding pocket with high selectivity and can release p53, leading to effective stabilization of the protein and activation of the p53 pathway.[24,25] Initial preclinical and clinical studies have demonstrated promising efficacy of this class of drugs in a number of p53 wildtype adult and pediatric cancers, as single agents or in combination with other targeted therapies.[26-34] However, the contribution of transcription-dependent pathways to the p53-mediated response in CLL has not been systematically explored, and, importantly, the effect of p53 reactivation and the p53 gene expression signature in normal cells implicated in the dose-limiting hematologic toxicity is yet to be elucidated.

In this study, we compared the effects of a second-generation and clinically relevant MDM2 inhibitor, RG7388, in patient-derived primary CLL cells and normal blood and bone marrow cells, including CD34$^+$ hematopoietic progenitors, and report the contrasting transcriptional induction profile of p53-target genes and consequent preferential pro-apoptotic responses of CLL cells to RG7388 exposure, compared with those of normal hematopoietic cells.

Methods

Patients and cell isolation

Peripheral blood samples (n=55) from CLL patients (*Online Supplementary Table S1*) were collected into EDTA-coated tubes. Informed consent was obtained in accordance with the Declaration of Helsinki, and with approval from the National Health Service Research Ethics Committee. CLL patients' samples were collected and stored under the auspices of the Newcastle Academic Health Partners Biobank (*http://www.ncl.ac.uk/biobanks/collections/nbrtb/*). CLL was diagnosed according to the International Working Group on CLL-164 National Cancer Institute's 2008 criteria.[35]

Normal peripheral blood mononuclear cells (PBMC), bone marrow mononuclear cells (BMMC) and CD34$^+$ hematopoietic stem cells (CD34$^+$ cells) were isolated from six, five and three healthy donors, respectively. Details on the isolation and culture of leukemic and normal cells are provided in the *Online Supplementary Methods*.

Reagents

The small-molecule MDM2 inhibitor RG7388 was custom synthesized as part of the Newcastle University/Astex Pharmaceuticals Alliance and CRUK Drug Discovery Program at the Northern Institute for Cancer Research, Newcastle University. RG7388 was dissolved in dimethylsulfoxide to make a 10 mM stock solution and stored in small aliquots at –20°C.

Nutlin-3a was purchased from Cambridge Bioscience (Cambridge, UK), ibrutinib from Axxora (Enzo Life Sciences, Exeter, UK), and venetoclax (ABT199) from Selleckchem, Absource Diagnostics (Munich, Germany).

Functional assessment of the p53 pathway

The functional status of p53 in CLL samples was determined by observing the modulation of p53 and transcriptional target gene protein products, MDM2 and p21, following short-term exposure to MDM2 inhibitors.[36] The *TP53* mutational status of CLL samples was assessed by next-generation sequencing (using Roche 454 GS FLX and Illumina MiSeq platforms) in 54/55 samples. The presence of a 17p deletion was assessed by fluorescence *in situ* hybridization and/or multiplex ligation-dependent probe amplification analysis in 54/55 samples. In one case (CLL 0255), we were unable to perform DNA analysis; the functional status of p53 for this case was, therefore, evaluated *in vitro* using short-term exposure of the CLL cells to MDM2 inhibitors, and this sample was identified as p53-non-functional.

Ex vivo cytotoxicity assay

Cells (5x10^6/mL) in 100 µL of medium per well of a 96-well plate were exposed to a range of concentrations of RG7388 for 48 h. Cytotoxicity was assessed using an XTT Cell Proliferation Kit II (SigmaAldrich, UK), as detailed in the *Online Supplementary Methods*.

Western blot analysis

Cells (5x10^6/mL) were seeded in 1 mL per well of a 24-well plate and exposed to a range of concentrations of RG7388. Cells were harvested and lysed at 6 h and 24 h. Protein concentration was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, UK). The protocol is described in detail in the *Online Supplementary Methods*.

Real-time reverse transcriptase polymerase chain reaction gene expression analysis

Cells (5x10^6/mL) were seeded in 2 mL per well of a 12-well plate and exposed to a range of concentrations of RG7388 for 6 h and 24 h. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Manchester, UK). The concentration and purity of the RNA were measured using a NanoDrop ND-1000 spectrophotometer. RNA was reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, UK). Relative quantifi-

cation of *BAX, CKDN1A, MDM2, PUMA* (*BBC3*), *FAS, FDXR, GADD45A, TNFRSF10B, ZMAT3, TP53INP1* and *WIP1/PPM1D* mRNA expression was performed by real-time reverse transcriptase polymerase chain reaction (qRT-PCR) based on SybrGreen chemistry using an Applied Biosystems QuantStudio™ 7 Real-Time PCR System (Applied Biosystems, UK). Each sample was analyzed in triplicate using *GAPDH* as a housekeeping control. The relative expression of each gene, expressed as fold-change, was calculated by the $2^{-\Delta\Delta Ct}$ method and the result of each sample was normalized to that of its dimethlysulfoxide-treated matched sample. Validated primer sequences are presented in *Online Supplementary Table S2*. The gene panel selected for this study was based on the results of a recent phase I trial of the MDM2 inhibitor RG7112[29] and published data from our group reporting the effect of MDM2 antagonists in different cancer cell lines.[31,34]

Additional analysis of a panel of anti-apoptotic genes, *BCL2, MCL1* and *BCL2L1* (alias *BCL-XL*), plus the pro-apoptotic genes *PMAIP1* (alias *NOXA*) and *BCL2L11* (alias *BIM*) (*Online Supplementary Table S2*) was also performed on a subset of samples.

Apoptosis assay

Cells ($5x10^5$/well) were seeded in 96-well plates and exposed to increasing concentrations of RG7388 for 24 h. Caspase 3/7 activity (Caspase-Glo® 3/7 Assay, Promega, UK) was assessed as detailed in the *Online Supplementary Methods*. Apoptosis was also determined by examining cleaved poly (ADP-ribose) polymerase (PARP) by western blot.

Co-culture and stimulation of chronic lymphocytic leukemia cells with CD40L-expressing cells

CLL cells were cultured on a monolayer of CD40L-expressing mouse fibroblasts and exposed to RG7388 as detailed in the *Online Supplementary Methods*.

Cell cycle analysis of CD34+ hematopoietic stem cells

$CD34^+$ cells were exposed to RG7388 for 24 h and cell cycle distribution was evaluated as detailed in the *Online Supplementary Methods*.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v6 (GraphPad Software Inc.). Statistical differences between groups were evaluated by a paired Student *t*-test or Mann–Whitney test. Correlations were analyzed by the Pearson rank correlation test. *P*-values <0.05 were considered statistically significant.

Hierarchical cluster analysis of the Euclidean distances of gene expression levels was carried out using the R pheatmap package.[37] The subsequent group comparison of median lethal concentration (LC_{50}) was performed using analysis of variance by parametric tests, applying the Holm-Sidaks correction for multiple comparisons between groups.

Results

TP53 genomic status of chronic lymphocytic leukemia samples

Online Supplementary Table S1 provides details of the *TP53* mutations, including coding region position and amino acid changes as well as 17p deletion status. The mutations detected were mostly (8/9 CLL samples) in the DNA binding domain (amino acids 102-292). The remaining case (CLL273) had a double mutation in the C-terminal tetramerization domain. All mutations were deleterious, leading to loss of function.

The MDM2 inhibitor RG7388 induces functional stabilization of p53 in chronic lymphocytic leukemia cells

We assessed protein expression of p53, as well as p53-regulated downstream targets, in patient-derived CLL cells by western blot, following incubation with RG7388. Inhibition of MDM2 by RG7388 blocked ubiquitin-mediated degradation of p53, leading to its accumulation. In p53-functional CLL samples, RG7388 led to a concentration-dependent stabilization of p53, with subsequent activation of downstream proteins, p21 and MDM2 (Figure 1A). The accumulation of p53 was detectable in all p53-functional CLL samples as soon as 6 h after commencement of treatment and increased at 24 h (Figure 1A). In the 30 p53-functional CLL samples analyzed, RG7388 increased p21 protein expression in 77% of cases and led to a detectable auto-regulatory feedback increase in expression of MDM2 in 85% of cases. The activation of these two downstream targets occurred in a concentration- and time-dependent manner (Figure 1A). Conversely, in p53-non-functional CLL samples, we did not find stabilization of p53 or induction of MDM2 and p21 after treatment with RG7388, even at concentrations of 10 μM (Figure 1B). The increased potency against CLL cells of the second-generation MDM2 inhibitor RG7388 compared with Nutlin-3a is shown in Figure 1C.

RG7388 induces a predominantly pro-apoptotic gene expression signature in chronic lymphocytic leukemia cells

We used qRT-PCR to study the expression of 11 known p53 transcriptional target genes in 26 CLL samples after treatment with RG7388. In p53-functional CLL samples, MDM2 inhibition by RG7388 led to a concentration- and time-dependent upregulation of p53-transcriptional targets (exemplified by CLL 0262 and 0267) (Figure 2A). No change in gene expression was identified in p53-non-functional samples (exemplified by CLL 0261) (Figure 2B).

The results for the 24 p53-functional CLL samples are summarized in Figure 3A, which illustrates the concentration-dependent nature of the fold-change in gene expression. The results for the two p53-non-functional CLL samples are shown in Figure 3B. In p53-functional samples, six genes were induced (≥2-fold expression above baseline) in response to 1 μM RG7388 for 6 h; all of these genes are known to be directly regulated by p53 (Figure 3C). We observed a mean 8.5-fold increase in *PUMA*, 5.1-fold in *MDM2*, 3.8-fold in *BAX*, 2.7-fold in *TNFRSF10B*, 2.6-fold in *FAS*, 2.2-fold in *WIP1*, and 1.6-fold in *CDKN1A* (Figure 3C). Thus, only a slight upregulation of *CDKN1A*, encoding the p21 cyclin-dependent kinase inhibitor, was observed and induction of pro-apoptotic genes dominated. Additional analysis of a panel of anti-apoptotic genes (*BCL2, MCL1* and *BCL2L1* (alias *BCL-XL*), plus the pro-apoptotic genes *PMAIP1* (alias *NOXA*) and *BCL2L11* (alias *BIM*) showed no significant changes in mRNA expression compared with the large change in *PUMA* mRNA (Figure 3D). Western blot analysis confirmed that induction of PUMA protein by RG7388 treatment could be detected in CLL samples (*Online Supplementary Figure S1A*).

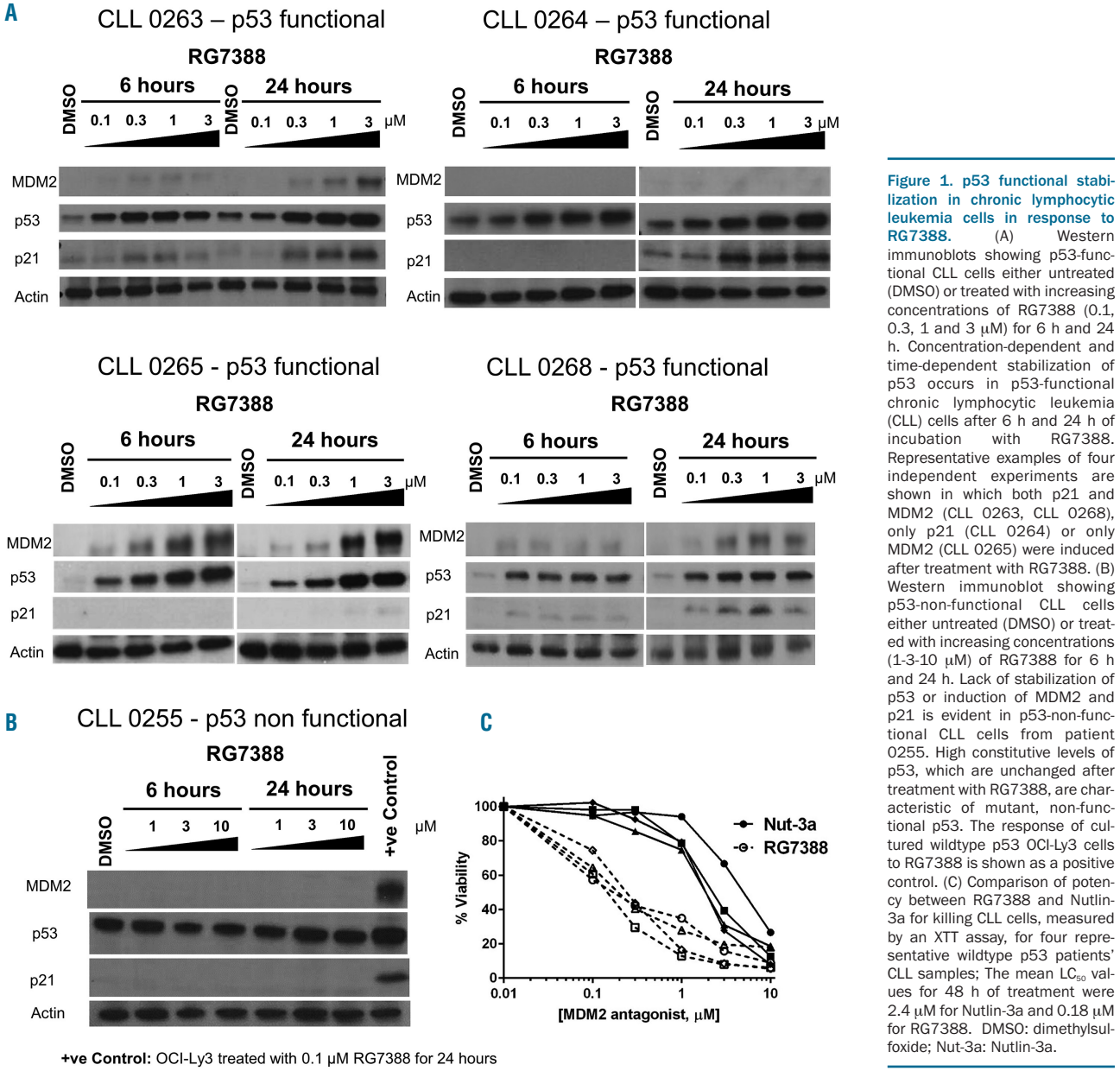
As would be expected on bulk analysis, CLL 0269, harboring a small subclonal 17p deletion (22% of nuclei), but no evidence of a *TP53* mutation, nevertheless showed functional stabilization of p53 by RG7388 (*Online Supplementary Figure S2A*) with subsequent upregulation

of p53 target genes (*Online Supplementary Figure S2B*), apoptosis (*Online Supplementary Figure S2C*) and moderate cytotoxicity (*Online Supplementary Figure S2D*).

To identify functional subgroups based on their gene expression induction after exposure to 1 μM RG7388, we performed unsupervised cluster analysis of CLL samples based on the fold-change of the 11 p53-transcriptional targets. This analysis showed a significant segregation of p53-functional CLL samples into three groups (defined as groups A, B and C), with group A samples showing lower induction of p53 target compared to samples from the other groups, despite the former's wildtype p53 genomic and functional status (Figure 4A). The three groups also showed different mean RG7388 LC_{50} values and, in particular, the mean LC_{50} for group A samples was significantly higher than the mean values for samples in groups B and C (Figure 4B, C).

RG7388 induces a concentration-dependent cytotoxic effect on chronic lymphocytic leukemia cells

To investigate the effect of RG7388 on cell viability, 55 CLL samples (*Online Supplementary Table S1*) were incubated with RG7388 and assayed for viability after 48 h using an XTT assay. Although caspase activity, indicating the triggering of apoptosis, could be seen at 24 h, it took a further 24 h for the loss of viability to become fully evident in the XTT assay (*Online Supplementary Figure S1B*). RG7388 induced a concentration-dependent cytotoxic effect on CLL cells exhibiting functional p53 responses (examples shown in Figure 5A) but not in those without a functional p53 response (Figure 5B). Overall, the median LC_{50} for *TP53* wildtype samples was 0.37 μM (Figure 5C). As expected, CLL samples with mutated/deleted *TP53* were much more drug-resistant (median LC_{50}=4.1 μM) (Figure 5C, which also details the *TP53* mutant allele fre-



+ve Control: OCI-Ly3 treated with 0.1 μM RG7388 for 24 hours

Figure 1. p53 functional stabilization in chronic lymphocytic leukemia cells in response to RG7388. (A) Western immunoblots showing p53-functional CLL cells either untreated (DMSO) or treated with increasing concentrations of RG7388 (0.1, 0.3, 1 and 3 μM) for 6 h and 24 h. Concentration-dependent and time-dependent stabilization of p53 occurs in p53-functional chronic lymphocytic leukemia (CLL) cells after 6 h and 24 h of incubation with RG7388. Representative examples of four independent experiments are shown in which both p21 and MDM2 (CLL 0263, CLL 0268), only p21 (CLL 0264) or only MDM2 (CLL 0265) were induced after treatment with RG7388. (B) Western immunoblot showing p53-non-functional CLL cells either untreated (DMSO) or treated with increasing concentrations (1-3-10 μM) of RG7388 for 6 h and 24 h. Lack of stabilization of p53 or induction of MDM2 and p21 is evident in p53-non-functional CLL cells from patient 0255. High constitutive levels of p53, which are unchanged after treatment with RG7388, are characteristic of mutant, non-functional p53. The response of cultured wildtype p53 OCI-Ly3 cells to RG7388 is shown as a positive control. (C) Comparison of potency between RG7388 and Nutlin-3a for killing CLL cells, measured by an XTT assay, for four representative wildtype p53 patients' CLL samples; The mean LC_{50} values for 48 h of treatment were 2.4 μM for Nutlin-3a and 0.18 μM for RG7388. DMSO: dimethylsulfoxide; Nut-3a: Nutlin-3a.

quency). Interestingly, three samples harboring a subclonal *TP53* mutation (variant allele frequency <50%) in the absence of del17p showed decreased cell viability (RG7388 LC_{50}<1 μM). The LC_{50} values for all other mutant samples, including del17p cases, were >1 μM (Figure 5C). We were unable to perform DNA analysis in CLL 0255 (see Methods). This sample was functionally defective (Figure 1B) and hence included in Figure 5C in the *TP53*-mutant subgroup (LC_{50}=8.4 μM).

Notably, among *TP53* wildtype samples, a small subset showed an intermediate response (1 μM<LC_{50}<10 μM, n=5) or resistance (LC_{50}>10 μM, n=3) to RG7388 (Figure 5D). Importantly, wildtype *TP53* cells from patients in different CLL risk subgroups were similarly sensitive to RG7388. There were no significant differences in LC_{50} between patients with Binet stage A or C (*Online Supplementary Figure S3A*), mutated or unmutated *IGHV* genes (*Online Supplementary Figure S3B*) or cases with high-risk cytogenetic abnormalities such as 11q deletion and trisomy 12 (*Online Supplementary Figure S3C*).

Given the importance of microenvironmental stimuli on survival and activation of CLL cells as well as response to therapy, we next sought to evaluate the effect of RG7388 in CD40L/IL4-stimulated CLL cells. We found that co-culturing CLL cells with CD40L-expressing fibroblasts and interleukin (IL)-4 significantly reduced the spontaneous

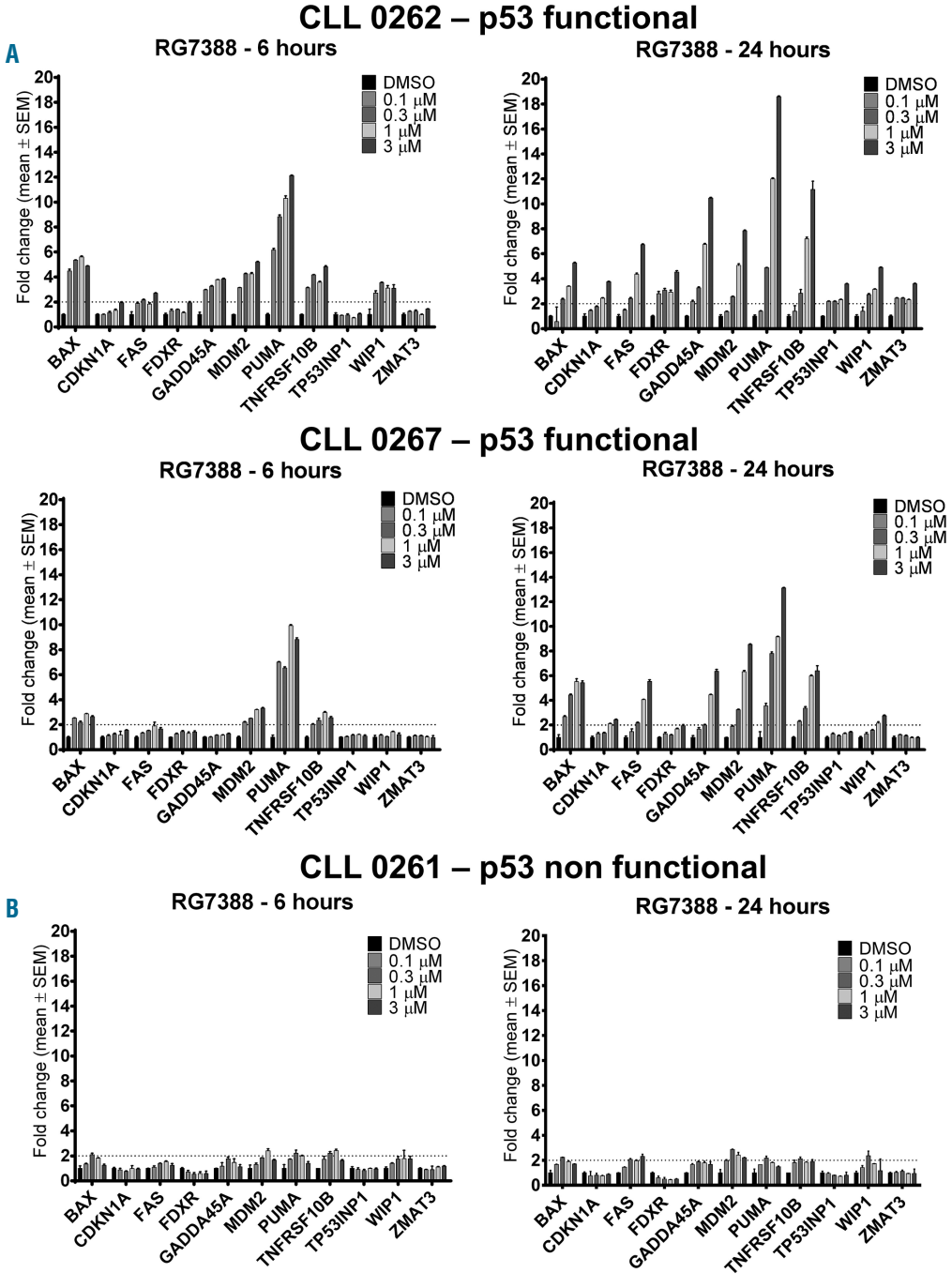


Figure 2. RG7388 induces mRNA upregulation of pro-apoptotic p53 target genes in chronic lymphocytic leukemia cells. (A) Real-time reverse transcriptase polymerase chain reaction (qRT-PCR) plots for two representative p53-functional samples (CLL 0262, CLL 0267) showing preferential induction of *PUMA* after treatment with increasing concentrations (0.1, 0.3, 1 and 3 μM) of RG7388 for 6 h and 24 h. (B) qRT-PCR plots for a representative non-functional p53 sample (CLL 0261) exposed to increasing concentrations (0.1, 0.3, 1 and 3 μM) of RG7388 for 6 h and 24 h. The results are shown as fold-induction relative to that produced by the dimethylsulfoxide (DMSO) solvent control. Genes induced above the cut-off of 2-fold were considered up-regulated by the treatment. Data are presented as mean ± standard error of mean (SEM) of three repeats. LC_{50}: median lethal concentration.

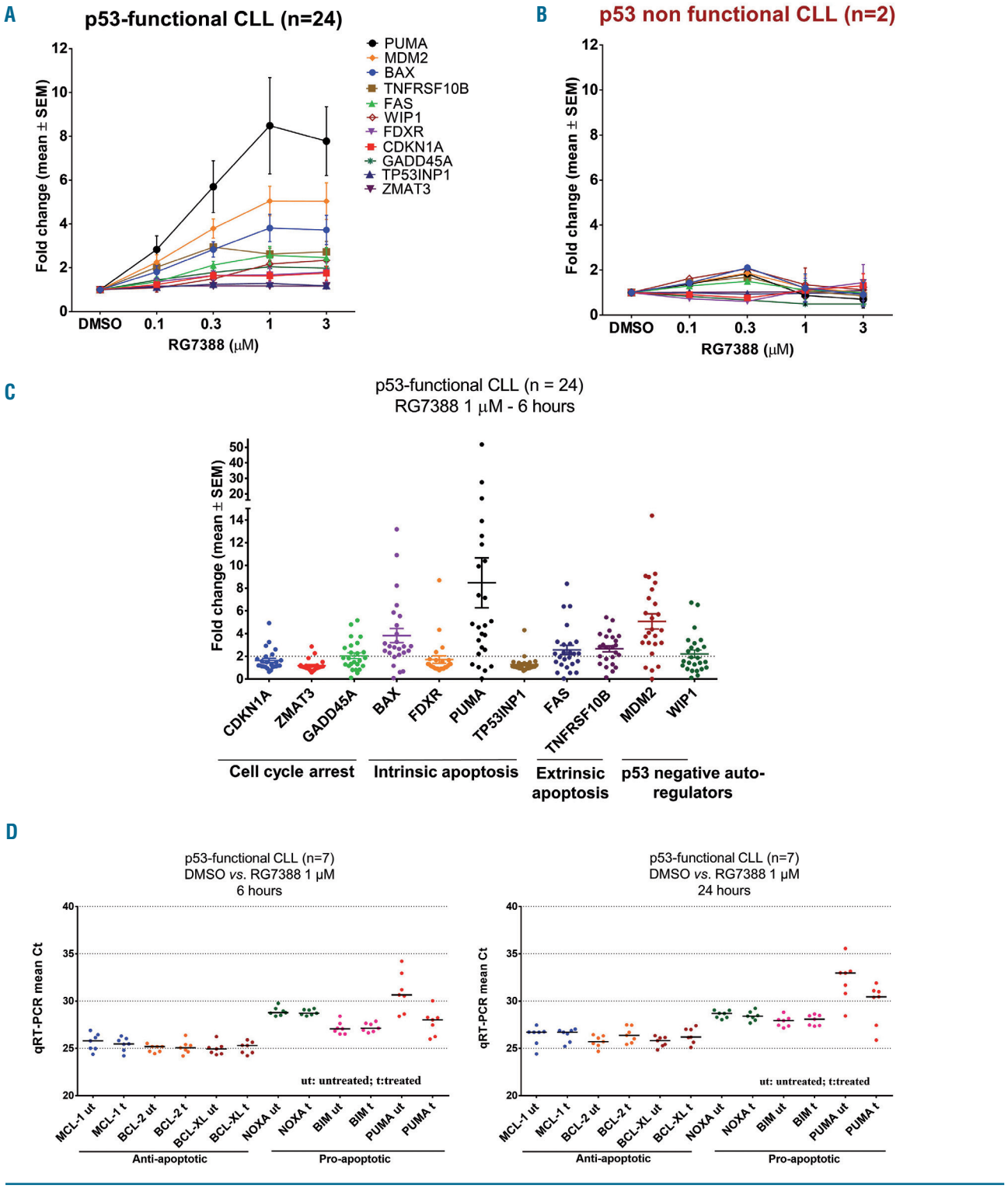


Figure 3. Apoptosis-related gene expression signature induced by RG7388 in primary chronic lymphocytic leukemia cells. Cells from patients with chronic lymphocytic leukemia (CLL) with functional p53 (n=24) were exposed *ex vivo* to RG7388 for 6 h. mRNA expression of genes relating to intrinsic apoptosis (*BAX*, FDXR, *PUMA*, *TP53INP1*), extrinsic apoptosis (*FAS*, *TNFRSF10B*), cell cycle arrest (*CDKN1A*, *ZMAT3*, *GADDA45A*), and p53-negative autoregulation (*MDM2*, *WIP1*) was measured in response to RG7388 relative to treatment with the dimethylsulfoxide (DMSO) solvent control. Genes induced above the cut-off of 2-fold were considered upregulated by the treatment. (A) Expression of p53-target genes in 24 p53-functional samples exposed to increasing concentrations (0.1, 0.3, 1 and 3 μM) of RG7388 for 6 h. Gene induction occurred in a concentration-dependent manner. (B) Expression of p53-target genes in two p53-non-functional samples exposed to increasing concentrations (0.1, 0.3, 1 and 3 μM) of RG7388 for 6 h. No genes were significantly induced by the treatment. (C) Scatter plot showing significant mean induction of *PUMA* (8.5-fold), *MDM2* (5.1-fold), *BAX* (3.8-fold), *TNFRSF10B* (2.7-fold), *FAS* (2.6-fold), and *WIP1* (2.2-fold) in p53-functional CLL samples treated with 1 μM RG7388 for 6 h. A slight upregulation of *CDKN1A* (1.6-fold) was observed. Data are presented as mean ± standard error of mean (SEM). (D) Scatter plot of real-time reverse transcriptase polymerase chain reaction (qRT-PCR) Ct values (cycle number to reach the critical threshold) for anti-apoptotic genes *MCL1*, *BCL2* and *BCL-XL*, plus additional pro-apoptotic genes *NOXA* and *BIM*, in comparison with *PUMA* for patients' CLL samples (n=7), showing no significant change in Ct values and hence mRNA expression between RG7388-treated and untreated (DMSO control) samples except for *PUMA*; Change in Ct for *PUMA* untreated *vs*. *PUMA* treated at 6 h *P*=0.0001, at 24 h *P*=0.0066 (paired *t*-test, n=7).

apoptosis associated with CLL cells and induced their proliferation. Importantly, RG7388 abrogated the protection induced by CD40L/IL4 and inhibited proliferation of stimulated CLL cells (*Online Supplementary Figure S4A*). Proliferating CLL cells cultured on the CD40L-expressing layer for 96 h were exposed to RG7388 and cell counting 48 h after exposure revealed a concentration-dependent suppression of cell growth with half maximal growth inhibitory (GI_{50}) values in the nanomolar range (*Online Supplementary Figure S4B, C*). Furthermore, p53 stabilization and induction of p53 targets were much more pronounced in stimulated CLL cells than in their unstimulated counterparts, suggesting that p53 anti-tumor activity can be rescued even in CLL cells protected by their microenvironment (*Online Supplementary Figure S4D, E*). Interestingly, it was found that the upregulation of *CDKN1A* and *MDM2* was greater in stimulated CLL cells than in unstimulated ones, whereas the induction of *PUMA* was lower in the stimulated CLL cells (*Online Supplementary Figure S4F*), and there was no induction of cleaved PARP (*Online Supplementary Figure S4D, E*), suggesting that RG7388 may elicit a preferential growth-arrest rather than apoptosis in CD40L/IL4-stimulated CLL cells and that it can disrupt the signaling from the microenvironment that leads to *in vivo* CLL cell proliferation.

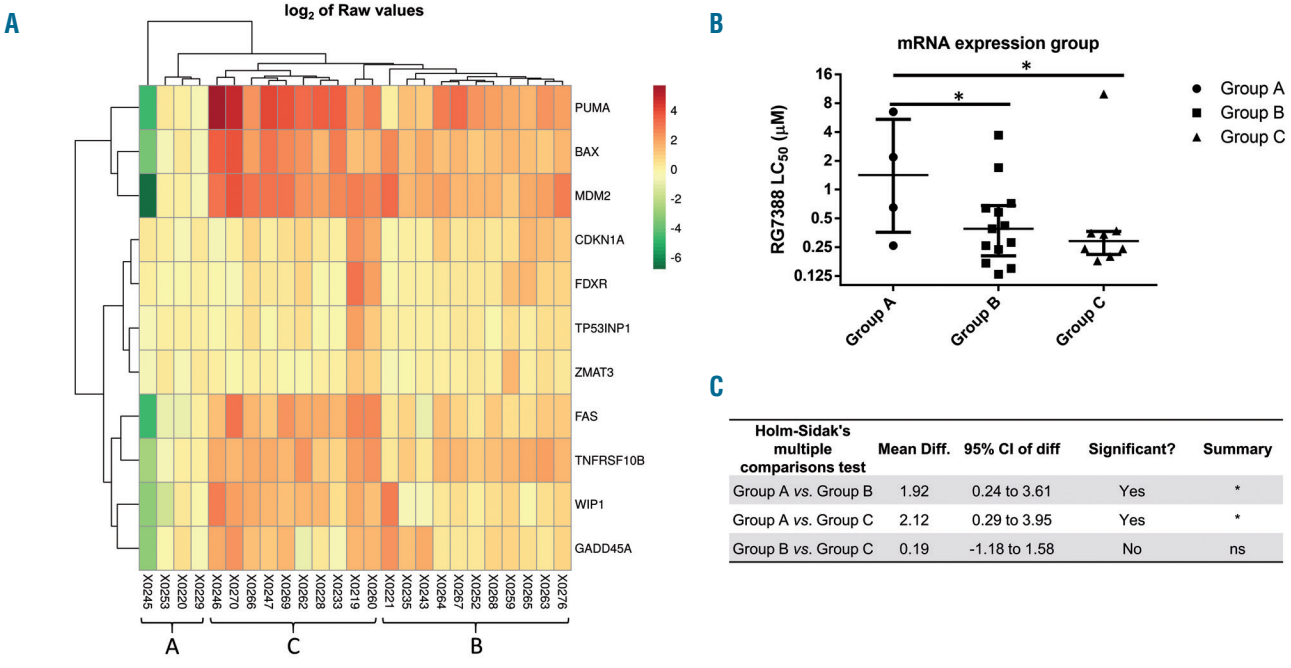
RG7388 induces apoptosis in p53-functional chronic lymphocytic leukemia

To further investigate the mechanism of RG7388 cytotoxicity, induction of apoptosis was assessed by measuring caspase 3/7 activity and cleaved PARP expression. At 24 h, RG7388 increased caspase 3/7 activity in p53-functional cells (Figure 6A), whereas no increase in caspase 3/7 activity was observed in p53-non-functional CLL samples (Figure 6B). To corroborate this, we also measured cleaved PARP expression by western blot and found that RG7388 increased expression of the 89 kDa cleaved PARP isoform in p53-functional CLL samples (Figure 6C) but not in p53-non-functional samples (Figure 6D).

Gene expression signature and response to RG7388 in normal cells and chronic lymphocytic leukemia cells are markedly distinct

One concern about the use of p53-reactivating therapies is their effect on normal cells. It has been suggested that MDM2 inhibitors might activate different cellular responses in normal and tumor cells.[38-41] To investigate this specifically and in more mechanistic detail in the context of CLL, we tested the effect of RG7388 on normal cells implicated in the dose-limiting hematologic toxicity of MDM2 inhibitors. We isolated PBMC, BMMC and $CD34^+$ cells from healthy donors and analyzed the transcriptional profile of p53-target genes and the cytotoxic response to RG7388.

As expected, p53 transcriptional targets were induced by RG7388 in all normal cell types. However, in contrast to p53-functional CLL cells, which displayed a strong pro-apoptotic gene signature (Figure 2), MDM2 inhibition led to a significant and preferential upregulation of *MDM2* in PBMC (Figure 7A), BMMC (Figure 7B) and $CD34^+$ cells (Figure 7C).



C

Holm-Sidak's multiple comparisons test	Mean Diff.	95% CI of diff	Significant?	Summary
Group A *vs.* Group B	1.92	0.24 to 3.61	Yes	*
Group A *vs.* Group C	2.12	0.29 to 3.95	Yes	*
Group B *vs.* Group C	0.19	-1.18 to 1.58	No	ns

Figure 4. RNA profiling of p53-transcriptional targets in chronic lymphocytic leukemia cells identifies subgroups with different sensitivity to RG7388. (A) Unsupervised hierarchical clustering and heat-map of p53 functional chronic lymphocytic leukemia (CLL) samples exposed to 1 μM RG7388 for 6 h, based on fold-change in expression of an 11-gene panel. The 11 selected p53-transcriptional target genes are listed on the right. Group A, columns 1-4; group B, columns 13-25; group C, columns 5-12. (B) Groups (Gp) of CLL patients' samples identified by the hierarchical clustering analysis compared based on the median lethal concentration (LC_{50}) values of RG7388. *P<0.01 (C) Group comparison performed using analysis of variance by parametric analysis and applying the Holm-Sidak correction for multiple comparisons. This analysis showed significant differences in mean RG7388 LC_{50} values between groups A and B and between groups A and C.

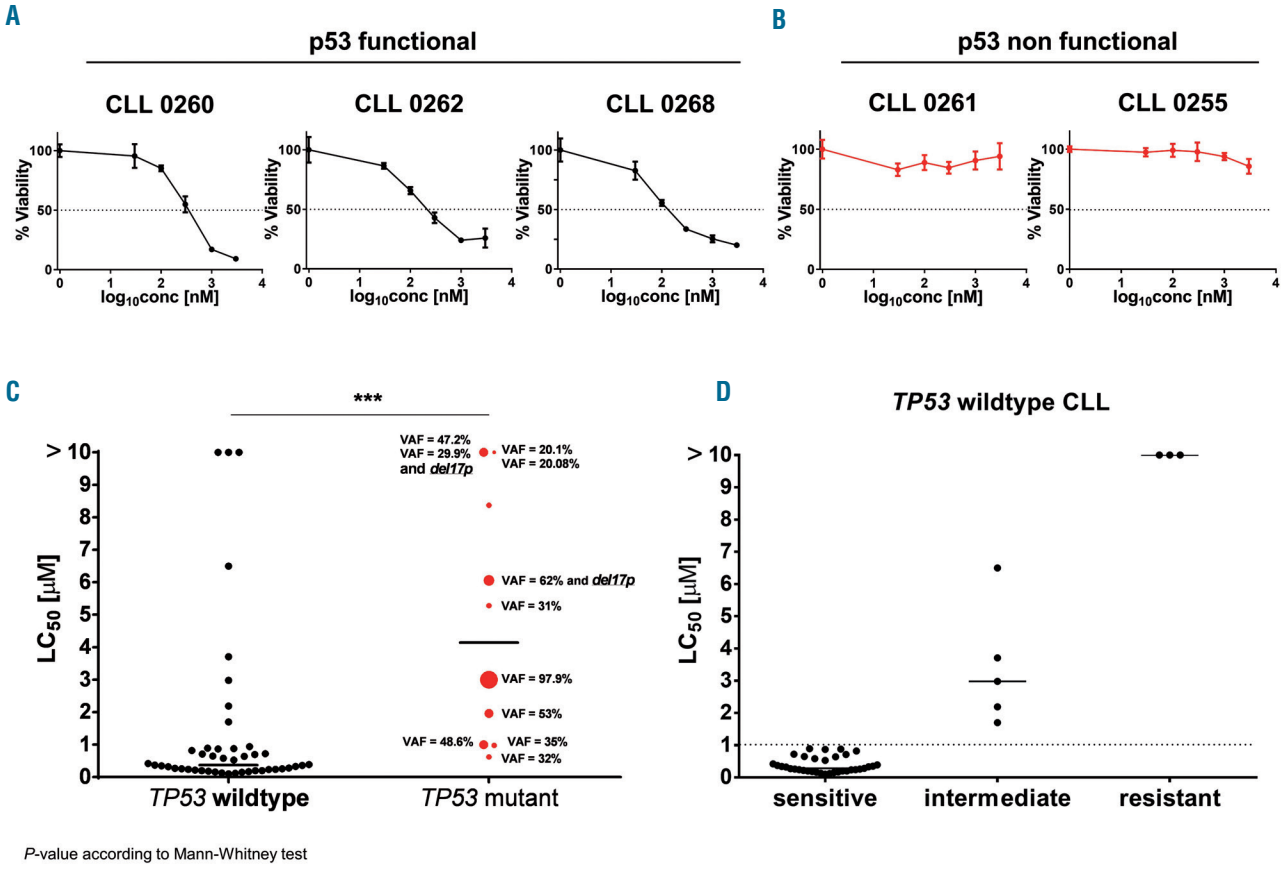


Figure 5. Effect of RG7388 on p53-functional and p53-non-functional chronic lymphocytic leukemia cell viability *ex vivo*. (A) Cytotoxicity curves for three representative p53-functional chronic lymphocytic leukemia (CLL) samples (CLL 0260, CLL 0262, CLL 0268) exposed to increasing concentrations (0.1, 0.3, 1 and 3 μM) of RG7388 for 48 h. RG7388 markedly decreased cell viability, as assessed by an XTT assay. (B) Cytotoxicity curves for two representative p53-non-functional CLL samples (CLL 0261, CLL 0255) exposed to RG7388 for 48 h. RG7388 showed no impact on cell viability. (C) Dot-plot of median lethal concentration (LC_{50}) values for n=45 *TP53* wildtype and n=10 *TP53* mutant CLL samples exposed to RG7388 for 48 h. *TP53* status of these samples was assessed by next-generation sequencing and fluorescence *in situ* hybridization and/or multiplex ligation-dependent probe amplification. The size of the dots indicates the variant allele frequency (VAF). Horizontal bars represent the median. The *P*-value was assessed by the Mann-Whitney test. *** *P* value <0.0001 (D) Dot-plot of LC_{50} concentrations for n=45 *TP53* wildtype CLL samples exposed to RG7388 for 48 h and classified according to their cytotoxic response as sensitive responders (LC_{50} <1 μM), intermediate responders (1 μM <LC_{50} <10 μM) and resistant (LC_{50} >10 μM).

We then compared the data obtained from CLL cells (Figures 3-6) with the effects seen in normal cells. Treatment with 1 μM RG7388 for 6 h induced the pro-apoptotic gene *PUMA* in p53-functional CLL cells but not in p53-non-functional CLL or normal BMMC. Only a relatively small induction of *PUMA* was observed in normal PBMC and $CD34^+$ cells (Figure 8A). However, for *MDM2*, induction was highest in normal $CD34^+$ cells and lower, but comparable, in normal PBMC and p53-functional CLL cells (Figure 8B). Furthermore and strikingly, *MDM2* upregulation dominated over the other target genes in normal cells (Figure 7) in contrast to the dominance of *PUMA* in CLL cells (Figure 2). Of additional importance, the mean induction of *CDKN1A* was higher in normal PBMC than in p53-functional CLL cells (Figure 8C), suggesting that the reactivation of p53 in normal circulating blood cells by MDM2 inhibitors does not activate a cell-death signal.

Importantly, the RG7388 LC_{50} values were always >3 μM for normal PBMC and BMMC, and >2 μM for $CD34^+$ cells (Figure 8D), whereas the LC_{50} values were <0.4 μM for p53-functional CLL cells (Figures 5C and 8D). We also found that when normal BMMC and PBMC were treated with RG7388, the increase of caspase 3/7 activity was significantly lower than that observed in p53-functional CLL cells (*Online Supplementary Figure S5*). The small amount of caspase activity and cell killing induced by RG7388 in PBMC likely represents the effect on the small component of normal B cells, while T cells remain unaffected, as previously reported for the response to Nutlin-3a.[42]

Also of note, positively-selected $CD34^+$ cells (*Online Supplementary Figure S6A, B*) incubated with RG7388 for 24 h showed a reduced proportion of cells in S-phase, together with an increase of those in G0/G1 (*Online Supplementary Figure S6C*). There was also a small increase of cells in the subG1 phase of the cell cycle (*Online Supplementary Figure S6D*).

RG7388 induces cytotoxicity independently of *MDM2* and *PUMA* basal expression or upregulation

MDM2 has been reported to be overexpressed in 50-70% of CLL cases.[43,44] However, the role of MDM2 overexpression in p53 dysfunction remains controversial, and it has been suggested that p53 activation in CLL cells is largely unaffected by variations in basal levels of

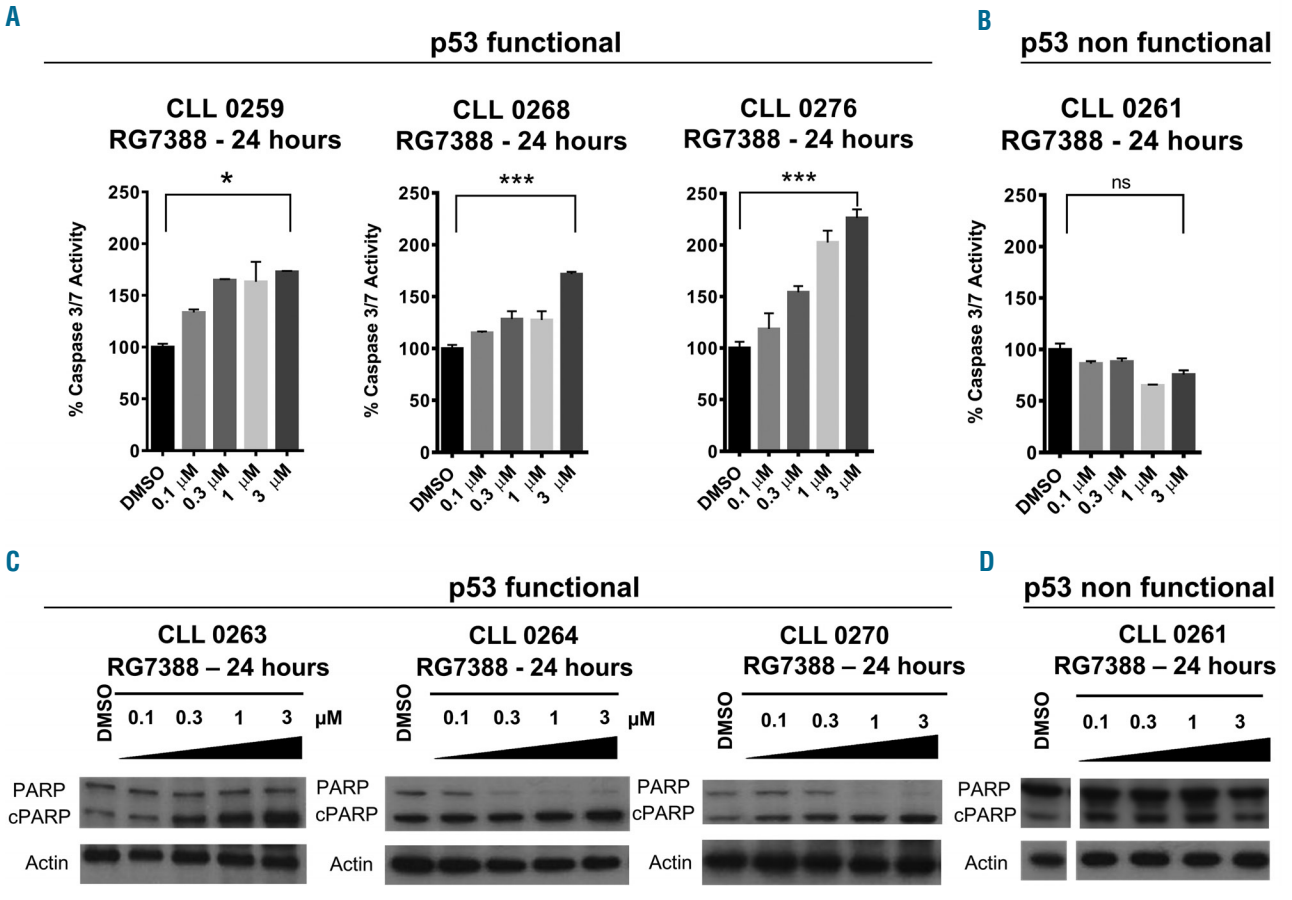


Figure 6. RG7388 leads to a significant dose-dependent increase in apoptosis in p53-functional chronic lymphocytic leukemia cells. (A) Caspase 3/7 activity for three representative p53-functional chronic lymphocytic leukemia (CLL) samples (CLL 0259, CLL 0268, CLL 0276) exposed to increasing concentrations (0.1, 0.3, 1 and 3 µM) of RG7388 for 24 h. *P< 0.01; ***P< 0.0001; according to a paired t test (B) Caspase 3/7 activity of a representative p53-non-functional CLL sample (CLL 0261) exposed to increasing concentrations (0.1, 0.3, 1 and 3 µM) of RG7388 for 24 h. Caspase 3/7 activity was measured by a Caspase 3/7 Glo luminescence-based assay and is represented as percentage change relative to that following exposure to the dimethylsulfoxide (DMSO) solvent control. Data are presented as mean ± standard error of mean (SEM) of three repeats. P-values were calculated by a paired t-test. (C) Western immunoblot for three representative p53-functional CLL samples (CLL 0263, CLL 0264, CLL 0270) showing increased expression of cleaved poly (ADP ribosome) polymerase (PARP) induced by RG7388 treatment for 24 h. (D) Western immunoblot for a representative p53-non-functional CLL sample (CLL 0261) showing no change in either full-length or cleaved PARP (cPARP) expression after exposure to RG7388 for 24 h. Basal levels of cPARP appeared high in this sample (indicative of spontaneous apoptosis) but did not increase with RG7388 treatment. The western immunoblots show the full-length pro-form of PARP (116 kDa) and the cPARP form (89 kDa).

MDM2.[45,46] Moreover, it remains unclear whether basal levels of the crucial apoptotic regulator *PUMA* may serve as a biomarker of response to MDM2 inhibitors. To examine whether *MDM2* or *PUMA* basal expression influences the cytotoxic effect of RG7388, we measured the basal mRNA levels of these two transcripts by qRT-PCR. The basal Ct values of *MDM2* and *PUMA* were generally lower, and hence expression higher, in primary CLL samples than in normal BMMC (*Online Supplementary Figure S7A, B*). However, mean MDM2 basal Ct values were significantly higher in CLL cells than in normal PBMC (*Online Supplementary Figure S7A*), whereas PUMA basal expression was comparable in CLL and normal PBMC (*Online Supplementary Figure S7B*). Basal MDM2 and PUMA Ct values did not differ significantly between CLL samples and $CD34^+$ cells. The basal levels of expression of MDM2 and PUMA were also similar between RG7388-sensitive samples (LC_{50} <1 µM) and intermediate/resistant CLL samples (LC_{50} >1 µM) (*Online Supplementary Figure S7C, D*). Moreover, we found no correlation between basal *MDM2* or *PUMA* expression and RG7388 LC_{50} values (*Online Supplementary Figure S7C, D*), supporting the previous observations that variation in MDM2 expression does not affect the functional activation of p53 and Nutlin 3a-induced cell death in CLL.[45,46]

In our cohort, the fold-changes in *MDM2* and *PUMA* expression induced by 1 µM RG7388 at 6 h also did not, alone, correlate with the LC_{50} values (*Online Supplementary Figure S8A, B*), suggesting that additional factors are important determinants of MDM2 inhibitor-induced cytotoxicity in CLL.

Combination treatments with RG7388

Although not the primary aim of this study, we include some initial data regarding combination treatments. Adding ABT199 (venetoclax) to RG7388 had an additive effect on response, but for *ex vivo* treatment there was no additional benefit of adding ibrutinib to RG7388 (*Online Supplementary Figure S9*).

Discussion

Given the central role of p53 in preventing aberrant cell proliferation and maintaining genomic integrity, as well as in the response to chemotherapy, there is increasing interest in the development of pharmacological strategies aimed at activating p53.[20,21] These strategies include compounds that rely on non-genotoxic activation of p53 by preventing it from being inhibited and targeted for degradation by MDM2, thus stabilizing p53 and activating its transcriptional activity to promote p53-induced apoptosis.[20,21,24,25] Here, we provide a strong rationale for the future evaluation of MDM2 inhibitors in CLL therapy, based on our observations that CLL cells are particularly primed for p53-dependent apoptosis compared with normal PBMC, BMMC and CD34$^+$ hematopoietic stem cells.

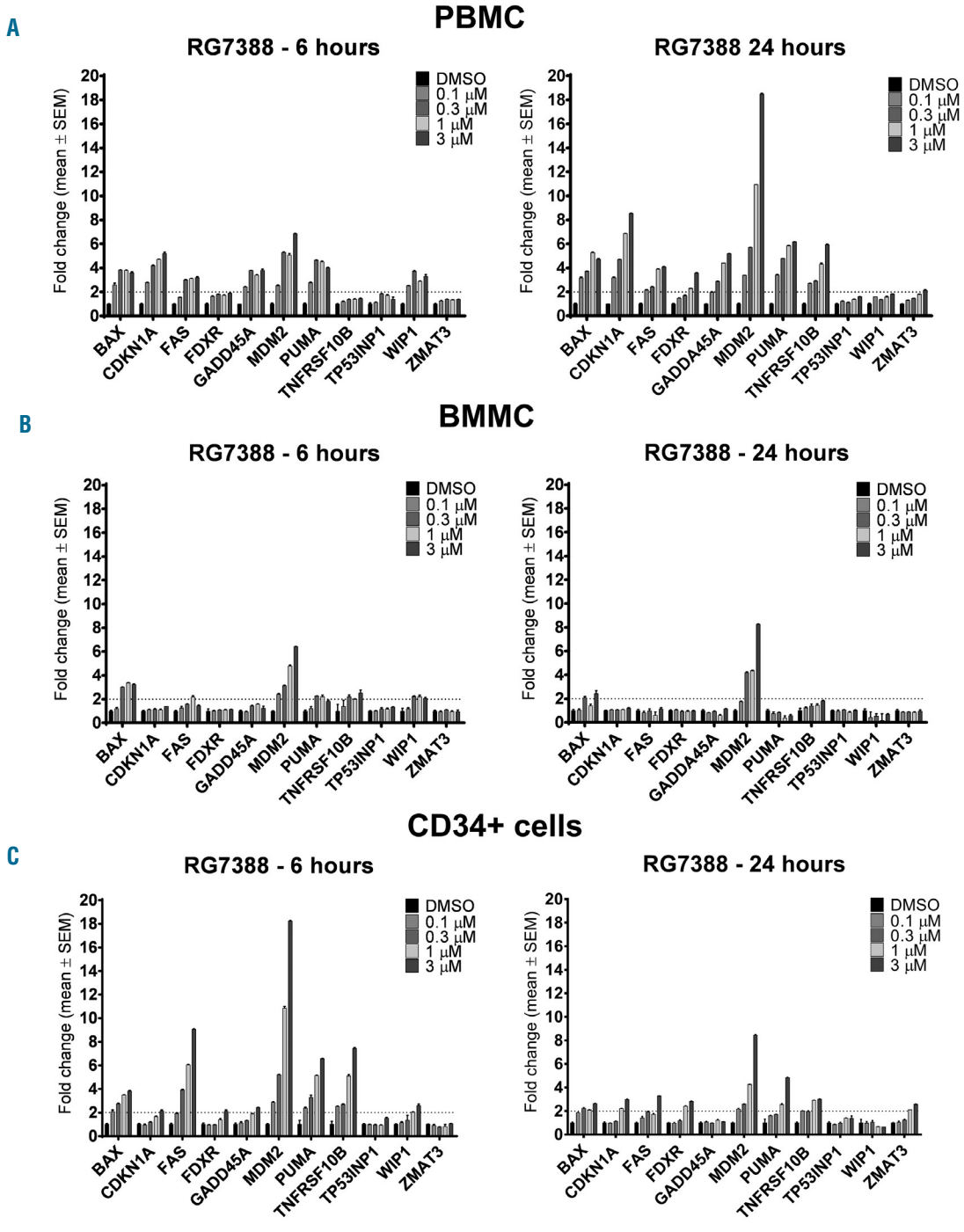


Figure 7. RG7388 preferentially induces *MDM2* mRNA in normal peripheral blood mononuclear cells, bone marrow mononuclear cells and CD34$^+$-selected bone marrow cells from healthy donors. Real-time reverse transcriptase polymerase chain reaction plots for (A) one representative sample of normal peripheral blood mononuclear cells (PBMC) sample, (B) one representative sample of normal bone marrow mononuclear cells (BMMC) sample and (C) one representative sample of normal CD34$^+$ hematopoietic stem cells (CD34$^+$ cells) all showing preferential induction of *MDM2* after treatment with increasing concentrations (0.1, 0.3, 1 and 3 µM) of RG7388 for 6 h and 24 h. Data are presented as mean ± standard error of mean (SEM) of at least three replicates.

We showed that RG7388 activates p53 and restores p53-transcriptional activity, inducing a characteristic dominant pro-apoptotic gene expression signature of p53-target genes selectively in CLL cells. Overall, no significant induction of transcriptional targets was observed in p53-non-functional samples, consistent with the specificity of RG7388 for p53 wildtype cells. However, a CLL sample harboring a subclonal 17p deletion in 22% of nuclei showed functional activation of p53 and induction of cell death in response to RG7388. This suggests that in the presence of low subclonal levels of p53 loss, the predominant p53-functional cell population can still respond to non-genotoxic activation of p53 and patients with subclonal *TP53* abnormalities could still benefit from treatment with new-generation MDM2 inhibitors, especially in combination with other p53-independent targeted therapies.

Moreover, RG7388 triggered apoptosis in CLL cells. This effect was dependent, in the majority of samples, on the presence of functional p53. CLL samples with predominantly mutated, non-functional p53 did not show induction of apoptosis. As a consequence of upregulation of apoptotic genes and activation of apoptosis, RG7388 significantly decreased the cell viability of p53-functional CLL samples, but CLL samples that displayed non-functional p53 on western blot and mutated/deleted *TP53* showed greater resistance. However, in the *TP53*-mutant subgroup, three samples harboring subclonal *TP53* mutations showed LC_{50} values lower than 1 μM, indicating significantly decreased cell viability upon exposure to RG7388. This finding is in line with the results of a recent phase I clinical trial evaluating the effect of the earlier-generation MDM2 inhibitor RG7112 in leukemia.[29] This clinical study included a small number of heavily pre-treated CLL patients and in this subgroup RG7112 showed clinical activity, with evidence of induction of PUMA and apoptosis in a patient with CLL whose white blood count decreased by >50%.[29] Among RG7112-treated patients, the investigators reported two patients with *TP53* mutant leukemic cell samples who exhibited a clinical response.[29]

Interestingly, among *TP53* wildtype CLL samples, we identified a small subset that showed an intermediate response or resistance to RG7388 treatment, suggesting that *TP53* mutational status is not the only determinant of

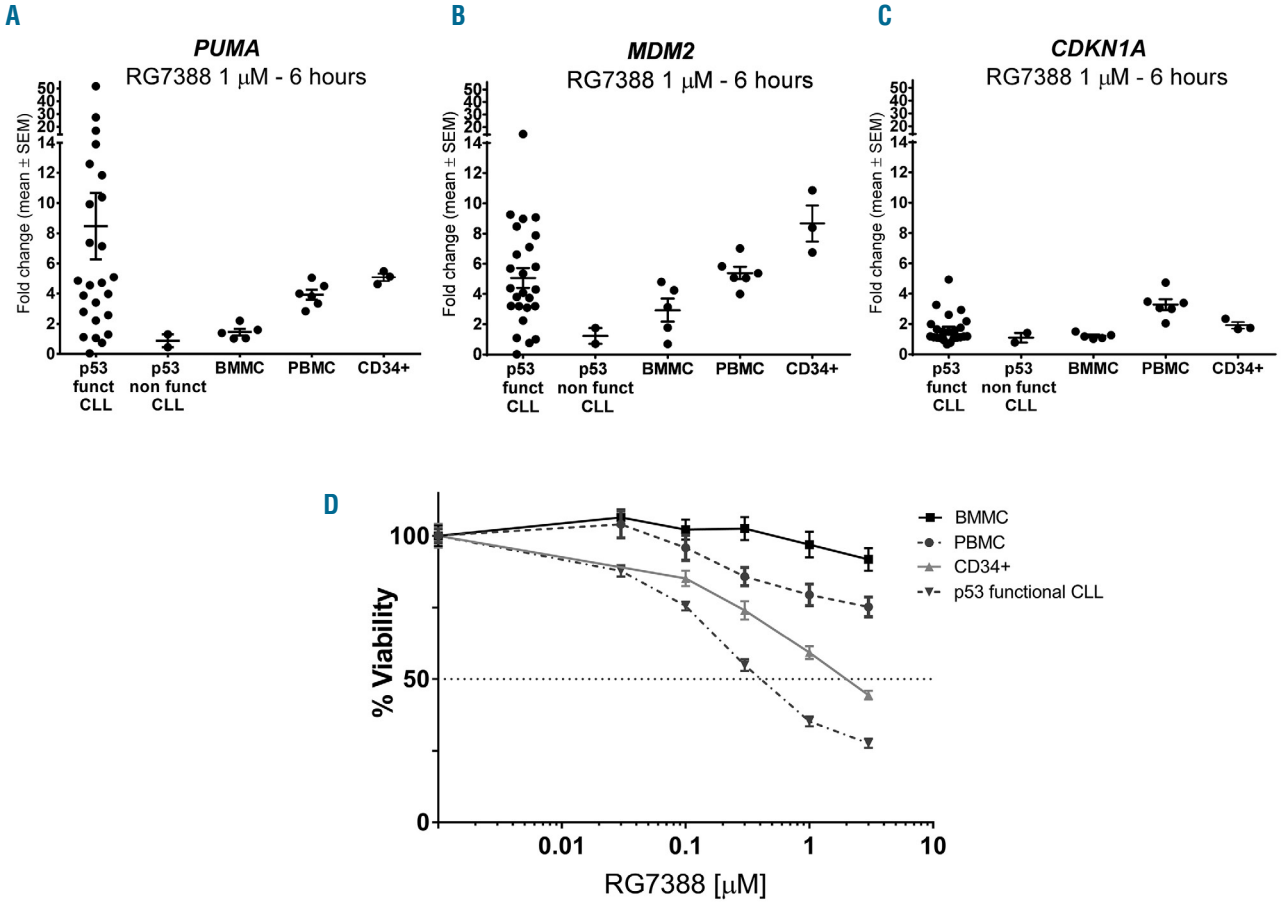


Figure 8. RG7388 selectively induces *PUMA* expression and cell death in chronic lymphocytic leukemia cells compared with normal hematopoietic cells. (A-C) Fold changes in mRNA of *PUMA* (A), *MDM2* (B) and *CDKN1A* (C) measured by real-time reverse transcriptase polymerase chain reaction in p53-functional CLL samples (n=24), p53-non-functional chronic lymphocytic leukemia (CLL) samples (n=2), normal bone marrow mononuclear cells (BMMC) (n=5), normal peripheral blood mononuclear cells (PBMC) (n=6) and normal $CD34^+$ samples (n=3) exposed to 1 μM RG7388 for 6 h. (D) Cytotoxic response comparison of normal BMMC, normal PBMC, normal $CD34^+$ samples and p53-functional CLL samples exposed to RG7388 (0.03, 0.1, 0.3, 1 and 3 μM) for 48 h. RG7388 markedly decreased cell viability, assessed by an XTT assay, of p53-functional CLL cells but to a much lesser extent in normal cells.

response to MDM2 antagonists and other biomarkers should be sought. In fact, in addition to p53 dysfunction resulting from *TP53* mutations and/or deletions, human cancers may display p53 suppression as a consequence of upregulation of MDM2 expression.[47] MDM2, which can enhance tumorigenic potential and resistance to apoptosis, has also been reported to be overexpressed in 50-70% of CLL cases;[43,44] it is, therefore, reasonable to hypothesize that aberrant expression of *MDM2* could be an indicator of response to MDM2 inhibitors. However, in our study the basal mRNA expression of *MDM2* was not significantly different between RG7388-sensitive samples (LC_{50} <1 μM) and more resistant CLL samples (LC_{50} >1 μM). Moreover, we found no significant correlation between basal *MDM2* expression or *MDM2* fold-induction and LC_{50} values, supporting previous observations that MDM2 overexpression does not have an impact on functional activation of p53 or MDM2 inhibitor-induced cytotoxicity in CLL.[45,46] In contrast, a recent study showed that MDM2 protein expression in blasts may identify patients with acute myeloid leukemia likely to exhibit better outcomes to RG7388-based therapy.[33] Quantification of MDM2 basal levels might, therefore, also be clinically relevant in other hematologic malignancies in order to predict sensitivity to MDM2 inhibitors.

The main concern regarding p53-reactivating therapies is their effect on normal cells. The activation of functional p53 by MDM2 inhibitors could elicit different cellular responses in tumor cells compared to normal cells. However, there is a paucity of data on the effect of new-generation MDM2 antagonists on normal cells, especially $CD34^+$ hematopoietic stem cells in which drug-induced cytotoxicity can result in the dose-limiting cytopenia that has been reported in early clinical trials of these agents. Although some initial studies (using Nutlin-3 and MI-219) suggested that MDM2 inhibition results in different cellular responses in normal and tumor cells,[38-41] the pattern of p53-dependent gene expression induced by MDM2 inhibition in primary CLL cells *versus* normal blood cells has not been investigated.

Here, we show for the first time that the expression of p53-target genes in response to RG7388 in normal peripheral blood and bone marrow cells (including positively-selected $CD34^+$ hematopoietic progenitors) is distinct from that in primary CLL cells. Induction of the pro-apoptotic *PUMA* gene after RG7388 treatment was the dominant response in CLL cells. This contrasted with the response of normal blood cells and $CD34^+$ hematopoietic stem cells, in which activation of apoptosis was weak or absent and upregulation of the negative feedback regulator *MDM2* dominated over that of pro-apoptotic target genes. Interestingly, the induction of *CDKN1A* was also higher in normal PBMC than in p53-functional CLL cells, suggesting that reactivation of p53 in normal, circulating blood cells by MDM2 inhibitors fails to elicit the predominant cell-death signal seen in CLL cells. In $CD34^+$ cells, gene expression and cell cycle distribution changes also suggest that cell-cycle arrest and an effective re-establishment of the MDM2-p53 negative feedback loop, rather than apoptosis, might be the main effects elicited by RG7388. These findings provide a mechanistic rationale for observations on the use of first-generation MDM2 antagonists that have suggested a predominant, reversible growth arrest as a primary response of normal cells to MDM2 inhibition.[38-41] Consistent with this, activation of caspase 3/7 and cytotoxicity upon exposure to RG7388 were significantly less in normal blood and bone marrow cells than in primary CLL cells.

Although p53 is activated by MDM2 inhibitors in both normal and tumor cells with functional p53, the gene expression signature and the cytotoxic effect induced by p53 activation in these two settings are markedly distinct, which translates into different cell fates and provides a therapeutic index with significant implications for the potential applications of MDM2 inhibitors as new anti-cancer agents. Of additional importance, RG7388 also effectively blocked proliferation signals provided externally to CLL cells *in vitro* to model the microenvironment (CD40L and IL4), which are crucial *in vivo* stimuli for proliferation of leukemic cells in lymph nodes and bone marrow.

IgM stimulation of BCR signaling has been reported to increase protein levels of MCL1, but not BCL2, and to promote the survival of CLL cells.[48] Because of the importance of BCR signaling in CLL it would be interesting to explore the effect of IgM and/or IL4 stimulation on the response of CLL cells to MDM2 inhibitors, with and without specific inhibitors of BCL2 and MCL1. IgM stimulation of BCR signaling would also provide a potential *ex vivo* model simulating the lymph node microenvironment for investigation of combination treatments with ibrutinib.

We cannot rule out that conformational changes in BAX may be important, although *BAX* expression changed little compared to the clear large changes in *PUMA* expression. A transcription-independent role of p53 in CLL cell apoptosis, involving direct interactions of p53 with mitochondrial anti-apoptotic proteins such as BCL2, has been suggested.[42] We favor a model in which p53 transcription-dependent and -independent mechanisms work hand in hand. Stabilization of p53 and upregulation of p53 transcriptional target genes, including predominantly pro-apoptotic genes, particularly *PUMA*, are the earliest and necessary events in the response of CLL cells to MDM2-p53 binding interaction inhibitors. Gene knockout mouse studies show that PUMA is necessary for apoptosis and p53 induction on its own is not sufficient. Studies on *BAX* nullizygous mice concluded that PUMA provides the critical link between p53 and BAX and is both necessary and sufficient to mediate DNA damage-induced apoptosis.[49] Furthermore *PUMA* knockout studies in mice show recapitulation of virtually all apoptotic deficiency in *p53* knockout mice.[50] It is therefore reasonable to link the major induction of *PUMA* by MDM2 inhibitor treatment of CLL cells with an important role in their sensitivity to the induction of apoptosis by these compounds. The absence of any marked downregulation of BCL family anti-apoptotic gene expression in our current study ruled out suppression of the transcriptional expression of these genes as a major contributory mechanism to the response to MDM2 inhibitors.

In considering the therapeutic potential of MDM2 inhibitors in CLL, it should also be emphasized that, despite improvements in patients' response rate using chemo-immunotherapy combinations or BCR-antagonists, none of the current therapeutic regimens is curative.[8,9] They are subject to limitations, including the evolution of drug resistance mechanisms. Resistance as a result of mutations in the venetoclax-binding domain of BCL2 has been reported in a high proportion of patients who relapse after treatment with venetoclax.[51] Similarly, a high

incidence of clonal evolution leading to ibrutinib resistance due to mutations in *BTK* and *PLCG2* have been reported in patients progressing on treatment.[52]

Continued preclinical studies to develop innovative therapeutic strategies for CLL therefore remain a high priority. In particular, new agents promoting CLL cell apoptosis with limited toxicity to normal cells represent an attractive therapeutic strategy for CLL, which is a disease of elderly patients who would benefit from the use of compounds with a therapeutic window associated with minimal effects on normal cells. Moreover, given the clinical heterogeneity of CLL, there is a constant need to identify treatment strategies that can be effective also in the most aggressive subtypes of this disease. In our cohort, RG7388 significantly decreased the viability of CLL cells isolated from patients in different poor prognosis subgroups, including cases with advanced-stage disease, cases with unmutated *IGHV* genes and cases with 11q deletion and trisomy 12, which are usually more prone to progression. This indicates that inhibiting the p53-MDM2 interaction is a promising treatment strategy to explore for high-risk CLL patients with functional p53.

Taken together, our data demonstrate that MDM2 inhibitors induce a pro-apoptotic response in cells from patients with both low- and high-risk subtypes of CLL, at doses which show a lesser effect on normal blood cells and hematopoietic stem cells. This therapeutic window supports the clinical evaluation of new-generation, non-genotoxic MDM2 inhibitors, used in combined treatment strategies with other targeted therapies for the treatment of CLL.

Acknowledgments

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