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

Carmela Ciardullo,1 Erhan Aptullahoglu,1 Laura Woodhouse,2 Wei-Yu Lin,1 Jonathan P Wallis,1 Helen Marr,2 Scott Marshall,6 Nick Bown,5 Elaine Willmore1 and John Lunec1

1Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne; 2Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne; 3Department of Haematology, Freeman Hospital, The Newcastle upon Tyne NHS Foundation Trust, Newcastle upon Tyne; 4Department of Haematology, City Hospitals Sunderland NHS Trust, Sunderland and 5Northern Genetics Service, Institute of Genetic Medicine, Newcastle upon Tyne, UK

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.

Correspondence:
JOHN LUNEC
john.lunec@ncl.ac.uk

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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. 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. 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 cell cycle arrest, apoptosis and/or cell senescence.2,3 Since the discovery of the first selective small molecule MDM2 inhibitor, Nutlin-3a, newer compounds have been developed with increased potency and improved bioavailability. 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. 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. 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/nabrb). CLL was diagnosed according to the International Working Group on CLL-164 National Cancer Institute’s 2008 criteria. 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), irinotecan 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. 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 RNAesy 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−ΔΔCt method and the result of each sample was normalized to that of its dimethylsulfoxide-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 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 (5x10⁵/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 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.35 The subsequent group comparison of median lethal concentration (LC₅₀) was performed using analysis of variance by parametric tests, applying the Holm-Sidak’s 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 S2A).

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 LC50 values and, in particular, the mean LC50 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 S4) 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 LC50 for TP53 wildtype samples was 0.37 μM (Figure 5C). As expected, CLL samples with mutated/deleted TP53 were much more drug-resistant (median LC50=4.1 μM) (Figure 5C, which also details the TP53 mutant allele fre-
quency). Interestingly, three samples harboring a subclonal TP53 mutation (variant allele frequency <50%) in the absence of del17p showed decreased cell viability (RG7388 LC50<1 μM). The LC50 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 (LC50=8.4 μM).

Notably, among TP53 wildtype samples, a small subset showed an intermediate response (1 μM<LC50<10 μM, n=5) or resistance (LC50>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 LC50 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 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, GADD45A), 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 (GI50) 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).

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 (LC50) 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 LC50 values between groups A and B and between groups A and C.
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 LC50 values were always >3 μM for normal PBMC and BMMC, and >2 μM for CD34+ cells (Figure 8D), whereas the LC50 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...
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\textsubscript{50} <1 μM) and intermediate/resistant CLL samples (LC\textsubscript{50} >1 μM) (Online Supplementary Figure S7C, D). Moreover, we found no correlation between basal MDM2 or PUMA expression and RG7388 LC\textsubscript{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. \cite{45,46}

In our cohort, the fold-changes in MDM2 or PUMA expression induced by 1 μM RG7388 at 6 h also did not, alone, correlate with the LC\textsubscript{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 \textit{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. 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. 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 LC50 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. 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%. Among RG7112-treated patients, the investigators reported two patients with TP53 mutant leukemic cell samples who exhibited a clinical response.

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 MDM2 inhibition in chronic lymphocytic leukemia.
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. MDM2, which can enhance tumorigenic potential and resistance to apoptosis, has also been reported to be overexpressed in 50-70% of CLL cases; 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 (LC50 <1 μM) and more resistant CLL samples (LC50 >1 μM). Moreover, we found no significant correlation between basal MDM2 expression or MDM2 fold-induction and LC50 values, suggesting previous observations that MDM2 overexpression does not have an impact on functional activation of p53 or MDM2 inhibitor-induced cytotoxicity in CLL. 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. 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 can be 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, 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. 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 anticancer 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. 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. We favor a model in which p53 transcription-independent and -dependent 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. Furthermore PUMA knockout studies in mice show recapitulation of virtually all apoptotic deficiency in p53 knockout mice. 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. 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. Similarly, a high
incidence of clonal evolution leading to ibrutinib resistance due to mutations in BTK and PDL1G2 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 unmaturated 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.

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