Targets for the treatment of drug resistant chronic lymphocytic leukemia
Tonino, S.H.

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Induction of TAp73 expression by platinum-based compounds to overcome drug resistance in p53 dysfunctional chronic lymphocytic leukemia

Sanne H. Tonino
Chantal E. Mulkens
Guangli Suo
Marinus H.J. van Oers
Eric Eldering
Jean Y. Wang
Arnon P. Kater

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Abstract

The activity of platinum-based compounds such as cisplatinum (CDDP) has been linked to the induction of the p53 family member TAp73 in solid tumours. In CLL, loss of function of p53 is highly associated with fludarabine (F-ara-A) refractory disease and poor outcome.

In p53 dysfunctional CLL cells isolated 24 and 48 hours after in vivo treatment with CDDP, a clear induction of TAp73 was observed. We further studied the role of TAp73 in CDDP-induced apoptosis in the p53 dysfunctional pro-lymphocytic B cell line MEC1, as a model for p53 dysfunctional B cell malignancies. CDDP treatment resulted in cell cycle arrest and sensitized MEC1 cells to FAS- and F-ara-A-induced cell death. At the molecular level, treatment with CDDP induced c-Abl dependent protein expression of TAp73 and its downstream targets p21, Bid, Puma and CD95. TAp73 RNA interference markedly decreased sensitivity to CDDP/ F-ara-A. In vitro treatment of quiescent peripheral blood derived CLL cells with CDDP did not result in increased TAp73 expression levels. Yet, a clear induction of TAp73 was seen after stimulation of CLL cells with CD40-ligand. Moreover, increased expression levels of TAp73 were found in lymph node derived CLL cells. Our data indicate that the activity of CDDP in chemo-refractory CLL might be mediated by induction of TAp73.
Introduction

Although considerable advances have been made in upfront treatment strategies for chronic lymphocytic leukemia (CLL), patients are not cured and many experience relapsing disease requiring renewed treatment. Most cytotoxic drugs, including fludarabine (F-ara-A), depend on intact p53 function for their activity and repeated cycles of therapy may eventually result in drug resistance due to acquired cytogenetic alterations, mainly affecting genes involved in the p53 response \(^1\). Fludarabine refractory disease infers a very poor prognosis with a median life expectancy of less than one year \(^2;3\). Therefore, the identification of alternative drug targets, independent of p53, is urgently needed.

In recent years the p53 family member TA\(\text{p}73\) has received increasing attention. TA\(\text{p}73\) shares many functions with p53, but it is rarely mutated in cancer \(^4\). Its role in apoptosis regulation and response to drug therapy has been studied mainly in various solid tumours. Much less is known about its function and regulation in lymphoid malignancies such as CLL. However, the functional relevance of TA\(\text{p}73\) in the apoptotic response in CLL has been demonstrated by adenoviral overexpression studies of TA\(\text{p}73\), which restored F-ara-A sensitivity in p53 dysfunctional CLL cells \(^5\).

Platinum-based compounds like cisplatinum (CDDP) are among the most active antitumour agents, and are mainly used in the treatment of solid malignancies. Their primary mechanism of action is the formation of adducts and cross-links between DNA strands, thereby blocking DNA replication and transcription. Furthermore, apoptotic programmes are initiated in which the role of TA\(\text{p}73\) is being increasingly appreciated, especially in the absence of functional p53. Treatment with CDDP results in upregulation of TA\(\text{p}73\) in multiple human solid tumour cell lines \(^6;8\). Furthermore, TA\(\text{p}73\) upregulation in response to CDDP induced DNA damage was found to be mediated by c-Abl \(^6\).

Clinically, platinum-based combination regimens are active both in relapsed diffuse large B cell lymphoma \(^9;11\) and in chemorefractory CLL \(^12;13\). Whether treatment with platinum-based compounds exerts its effects through TA\(\text{p}73\) induction in (p53 dysfunctional) CLL is unknown.

Targeting the TA\(\text{p}73\) pathway may provide a means to overcome drug resistance in CLL. We recently observed induction of TA\(\text{p}73\) protein expression in samples derived from a p53 dysfunctional patient following in vivo CDDP treatment. The expression of TA\(\text{p}73\) correlated with clinical response. This prompted further in vitro analyses on the mechanisms and consequences of TA\(\text{p}73\) induction in p53 dysfunctional leukemia cells.
Materials and Methods

Patient material
Peripheral blood mononuclear cells (PBMCs) from patients with CLL were isolated and subsequently frozen and stored as previously described 14. Approval for these studies was acquired from the Amsterdam Academic Medical Center Medical Ethical Committee. Informed consent was obtained in accordance with the Declaration of Helsinki. All samples included in these studies contained > 90 % CD5+CD19+ cells. P53 dysfunction (as assessed by RT-MLPA) was defined as the absence of upregulation of mRNA expression levels of Puma, Bax and p21 upon 5 Gy radiation, as previously described 15.

Lymph node (LN) material, diffusely infiltrated by CLL (unmutated), was freshly frozen in liquid nitrogen directly after surgical removal. Immunohistochemical analysis of these LNs revealed that greater than 90% of the tissue consisted of tumour cells 16.

Cells culture and drugs
Cells were cultured in Iscove modified Dulbecco medium (IMDM; Gibco Life Technology, Paisley, United Kingdom) supplemented with 10% (vol/vol) heat inactivated fetal calf serum (FCS; ICN Biomedicals, Meckenheim, Germany), 100 μg/mL gentamycin, and 5 mM L-glutamine (Invitrogen, Carlsbad, CA) at 37°C degrees.

For CD40 stimulation, the fibroblast cell line HeLa (ATCC, Manassas, VA) and the CD40-ligand transfected cell line HeLa-CD154 were used, as described previously 5. In the synergy experiments the following drugs and reagents were used: CDDP (Mayne Pharma, Brussels, Belgium), F-ara-A (Sigma-Aldrich, St Louis, MO), imatinib (Novartis, Basel, Switzerland), and CH11 (Beckham Coulter Company, Marseille, France).

Analysis of apoptosis, cell cycle and surface marker expression
For assessment of apoptosis MitoTracker Orange (Molecular Probes, Leiden, the Netherlands) staining was used. Cells were stained with MitoTracker 200nM at 37°C for 30 minutes and acquired using a FACS Calibur flow cytometer and analysed with CellQuest software (Becton Dickinson (BD) Biosciences, San Jose, CA, USA).

Cell cycle progression was analyzed by propidium iodide staining. After incubation with CDDP as indicated, cells were washed in PBS and fixated in cold 70% ethanol. After 30 minutes on ice, the cells were washed twice in Phosphate-citrate buffer (192 parts 0.2M disodium phosphate, 8 parts 0.1M citric acid with pH 7.8) and ribonuclease (Sigma-Aldrich) was added. After addition of propidium iodide (PI; 50 μg/ml) cells were analyzed by FACS Calibur flow cytometry and analyzed using CellQuest software.

Analysis of drug interactions
The interaction between drugs was analyzed as described previously 17. For each condition, expected viability after treatment with a combination of drugs was calculated by multiplication of the measured viability of the samples treated with the drugs of
interest separately (corrected for base-line viability). Expected survival is plotted against measured viability of the sample treated with the combination of both drugs. Measured viability lower than expected viability indicates synergy between the drugs.

**Protein isolation and Western blot**

CLL cells, incubated with drugs as indicated, were collected, washed once in ice-cold phosphate buffered saline (PBS), and lysed by sonification in radioimmunoprecipitation (RIPA) buffer as previously described. For separated nucleus/cytoplasm samples, cells were first lysed in NP40 buffer and the remaining cell-pellet (consisting mainly of nuclear components) in l-amli buffer, as previously described. Protein content was measured using the BCA protein assay kit. Thirty to 100 μM of protein lysate was loaded onto each lane of a 7.5%, 10% or 13% gradient SDS–polyacrylamide gel electrophoresis (PAGE) gel (Biorad, Hercules, CA) and transferred onto a polyvinylidene fluoride microporous membrane (PVDF-FL, Millipore, Billerica, MA). Membranes were probed with antibodies against β-actin (clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-α-tubulin (Cell signaling, Beverly, MA), TA-p73 (IMG-246, Imgenex, San Diego, CA), polyclonal anti-p21 (Santa Cruz Biotechnology), polyclonal anti-Puma (Cell Signaling), polyclonal anti-Bid (Cell Signaling) or mouse anti-c-ABL (clone 8E9; BD Pharmingen, San Jose, CA, USA). IRDye 680 donkey anti-rabbit IgG, IRDye 800 donkey anti-goat IgG or IRDye 800 donkey anti-mouse IgG (Westburg, Leusden, the Netherlands) were used as secondary antibody and blots were scanned on the Odyssey imager (LI-COR Biosciences, Lincoln, NE).

**Lentivirus-mediated RNA interference targeting c-Abl and TAp73**

One shRNA target set designed for c-Abl (sequence: CCGGGCTGAAATCCACC-AAGCTTTTCTCGAGAAAGGCTTTGAGATTCTGCTTTTGT) and two designed for TAp73 (sequence:CCGGCCCGCTTTGAGAAACTCTACTCGAGAGTCTTTTCTCAA GAGCAGGTTTTTT and sequence:CCGGCCAAGGGTTACAGACATTCTTACTCGAGTAA ATGCTCTGTAACCCCTCCGTTTTT respectively) were inserted in the pLKO.1 lentiviral vector. As control for transfection efficiency pLKO-TurboGFP was used. The plasmids were prepared with plasmid maxi kit and transfected in HEK293FT cells (Invitrogen) with the transfection reagent (GeneTran, Biomiga, San Diego, CA) to produce lentiviral particles as previously described. Lentivirus titer determination was performed according to the manufacturer’s instructions (Open Biosystems, Huntsville, AL). Primary cultures of MEC1 cells were infected with concentrated lentiviral stocks at 20 multiplicity of infection (MOI) in the presence of 8 μg/ml polybrene. After 24 hours cells were washed and suspended in IMDM medium supplemented with puromycin (1 μg/ml) for 6 days to select for cells stably infected with the lentivirus. The transfected MEC-1 cells were cultured in fresh medium for 5 days after puromycin selection before qRT-PCR, immunoblotting and functional experiments.
RNA extraction and qRT-PCR

Total RNA was extracted using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA was reverse transcribed using the SuperScript II kit (Invitrogen) according to the manufacturer’s instructions. Polymerase chain reaction containing first-strand cDNA (1:10 dilution), SYBR Green PCR Master Mix, and forward and reverse primers was performed using a 79HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Cycling conditions were as follows: initial step 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 min and 60°C for 1 min. All reactions were run in triplicates. The primers for the PCR amplification of TAp73, c-Abl, p21, Bid and Puma genes were designed using the Primer Express software (version 2.0). The relative expression levels of genes were expressed in arbitrary units, where the Ct value of the gene of interest was normalized to that determined for GAPDH RNA, a housekeeping gene, to correct for differences in concentrations of the cDNA templates. Then, fold induction was calculated by dividing CDDP treated samples by medium control.

Results

In vivo induction of TAp73

A 36-year old male patient was referred to our clinic with fludarabine resistant CLL according to the iwCLL criteria. He had previously been treated with chlorambucil, CVP (cyclophosphamide, vincristin, prednisone), FCR (fludarabine, cyclophosphamide, rituximab), alemtuzumab and R-CHOP (cyclophosphamide, adriamycin, vincristin, prednisone, rituximab). Cytogenetic analysis revealed deletion of 17p in 87% of the cells. At the functional level, the leukemia cells of this patient proved to be p53 dysfunctional as p53 and its downstream targets p21 and Puma were not induced following 5 Gy irradiation (supplemental figure 1A). After initiation of R-DHAP combination therapy (CDDP 100 mg/m² day 1, cytarabine 2 gr/m² bid day 2, dexamethasone 40 mg day 1-4 and rituximab 375 mg/m² day 3), the peripheral blood (PB) lymphocyte count decreased rapidly after a remarkable initial rise (Figure 1A). A similar pattern was seen in a 70-year old female with F-ara-A resistant CLL/ SLL (supplemental figure 1B). In addition to a sharp rise in the lymphocyte count 1 day after administration of CDDP, she developed an overt tumourlysis syndrome for which she was treated with rasburicase. Furthermore, a significant decrease in lymphadenopathy was apparent already within one week.

We studied protein levels in samples of the first patient taken before and 24 and 48 hours after start of treatment. Already after 24 hours an induction of Puma and Bid was seen, which is striking as both proteins are p53 response genes. In agreement with earlier findings that both the expression of Bid and Puma can be regulated by TAp73, we found an induction of TAp73, already after 24 hours of treatment (Figure 1B).
CDDP induces p53 independent cell death and cell cycle arrest

The role of TAp73 in apoptosis induction in p53 dysfunctional cells was further studied using the p53 dysfunctional cell line MEC1 23 (supplemental figure 2). First, the sensitivity of MEC1 cells to CDDP was tested in dose-response experiments. Cell death was measured after 24 and 48 hours. As shown in Figure 2A, after 48 hours of treatment, significant cell death was seen at concentrations of 20 μM or higher. To avoid interference by apoptosis, a sublethal concentration of CDDP was used in further experiments. Also, such concentrations are in the range of drug levels achieved in plasma in vivo 24.

First, the effect of CDDP treatment on the intrinsic (mitochondrial) apoptosis pathway was studied. In accordance with the lack of functional p53, MEC1 cells are not sensitive to F-ara-A. However, a clear synergy was observed upon treatment with low-dose CDDP in combination with F-ara-A (Figure 2B). Next, we analyzed the effects on the extrinsic (death-receptor mediated) apoptosis pathway. Upon treatment with CDDP, surface expression of FAS was upregulated (data not shown) and enhanced soluble FAS-ligand induced cell death was observed (Figure 2C). Finally, we analyzed CDDP mediated changes in cell cycle transition. As shown in Figure 2D, CDDP causes a G2/M phase cell cycle arrest. At higher concentrations cells accumulate in the S phase, but this may reflect a non-specific effect due to the formation of DNA-adducts.

C-Abl dependent upregulation of TAp73 upon treatment with CDDP

Next, we studied whether the functional effects of CDDP in this p53 dysfunctional MEC1 cell line could be linked to induction of TAp73. Already after 24 hours of CDDP treatment, induction of TAp73, and its downstream targets p21 25 and Bid could be detected, even at concentrations as low as 5 μM (Figure 3A). In accordance with previous data on the regulation of TAp73 26, induction occurred only at the protein level, as no induction of
Figure 2. Functional consequences of treatment of MEC1 cells with CDDP (A) Apoptosis induced by increasing doses of CDDP as indicated, assessed by Mitotracker staining; mean +/- standard error of mean (SEM) of three experiments. (B) Viability of MEC1 cells after treatment with CDDP (5 μM open symbols; 10 μM solid symbols) and F-ara-A (▼ 5 μM, ▲ 10 μM, ● 25 μM, ■ 50 μM). Measured viability is plotted against expected viability (based on activity of the separate drugs; see Methods section); the area below the diagonal represents synergism. (C) Apoptosis assessed by Mitotracker staining upon treatment with CDDP for 24 hours followed by FAS-ligand (FAS-L; CH11) for 24 hours as indicated; mean + SEM of three experiments. (D) Analysis of cell cycle transition by Propidium Iodide (PI) staining after 48 hours of CDDP-treatment in ascending doses as indicated.
TAp73 RNA was seen (data not shown). Induction of TAp73 in solid tumour cell lines depends on post-translational modification mediated by c-Abl. Likewise, upregulation of TAp73 after CD40-ligand stimulation in CLL cells required c-Abl. We therefore repeated the experiment in the presence of imatinib, a specific c-Abl kinase inhibitor. Indeed, upregulation of TAp73, p21 and Bid was strongly inhibited in the presence of imatinib (Figure 3A). Next we studied the subcellular localization of c-Abl after treatment with CDDP, because it has recently been shown that nuclear entry of c-Abl contributes to DNA damage-induced activation of the intrinsic apoptotic pathway. As shown in Figure 3B, following CDDP treatment, c-Abl translocates to the nucleus, further supporting a role for c-Abl (and TAp73) in CDDP-induced apoptosis in MEC1 cells.

Figure 3. c-Abl-dependent upregulation of TAp73 upon treatment of MEC1 cells with CDDP (A) Protein levels of TAp73, p21 and Bid analyzed by Western blot after 48 hours of treatment with CDDP and/or imatinib as indicated. Actin was used as a loading control. (B) Nuclear and cytoplasmatic protein levels of c-Abl, 3 and 6 hours of treatment with CDDP as indicated. Actin was used as a loading control. Tubulin was used as control for adequate division of the nuclear and cytoplasmatic compartment.
Knockdown of both c-Abl and TAp73 results in impaired CDDP-induced upregulation of p21, Puma and Bid

To confirm an essential role of c-Abl in CDDP-induced TAp73 induction, protein expression levels of TAp73 following CDDP treatment were measured in mock transfected MEC1 cells and MEC1 cells transfected with a c-ABL-specific shRNA construct. As depicted in Figure 4A, knockdown of c-Abl prevented upregulation of TAp73.

RNA expression levels of the reported TAp73 target genes Bid, p21 and Puma were measured after knockdown of c-Abl and TAp73 respectively. Knockdown of either c-Abl or TAp73 prevented upregulation of p21 and Puma upon CDDP treatment (Figure 4B). RNA expression levels of Bid are relatively high in MEC1 cells (data not shown). Although a modest effect on Bid RNA expression levels was observed following CDDP treatment of wild-type MEC1 cells, this was not seen in the knockdown cells. These data not only demonstrate that these proteins are downstream targets of TAp73 (and c-Abl), but also confirm that in the absence of functional p53, transcription is mediated by TAp73. At the functional level, apoptosis upon combined treatment with CDDP and F-ara-A was

![Figure 4. Effects of knockdown of c-Abl and TAp73 in MEC1](image)

(A.) Protein levels of c-Abl and TAp73 analyzed by Western blot upon 48 hours of treatment with 10 μM CDDP as indicated after lentiviral transduction of MEC1 cells with a shRNA targeting c-Abl and TAp73 (two constructs). (B.) RNA expression levels, assessed by qRT-PCR, of p21, Bid, Puma and Noxa upon treatment of lentiviral transduced MEC1 cells with CDDP 10 μM for 48 hours. Plotted is fold induction compared to untreated cells; dotted line represents ‘no induction’ (fold induction = 1). (C.) Apoptosis in MEC1, mock knockdown (NO KD) and TAp73 knockdown cells (p73 KD – 2) following treatment with 5 μM CDDP and 10 μM F-ara-A as indicated; mean + SEM of three experiments
diminished in TAp73 knockdown cells (Figure 4C), indicating that the response to this combination of drugs depends, at least in part, on TAp73. In c-Abl knockdown cells, high rates of spontaneous apoptosis were observed (data not shown), which implies that c-Abl also has an important role in maintaining viability.

**TAp73 is expressed in CLL cells residing in the lymph nodes**

To translate the results of our experiments in MEC1 cells to patient derived CLL cells, we treated PB derived CLL cells with increasing doses of CDDP *in vitro*. In contrast to MEC1 cells, circulating primary CLL cells are in G0/ early G1-arrest (reviewed by Bertilaccio et al 28). After 48 hours of CDDP treatment, no induction of TAp73 was seen, nor of its downstream target Bid. Even after 96 hours of treatment with CDDP at concentrations up to 100 μM, no induction of TAp73 was seen (data not shown). In contrast, CD40-ligand stimulation of these PB derived CLL cells did result in upregulation of TAp73 protein levels (Figure 5A).

*In vitro* stimulation of CLL cells with CD40-ligand is applied as a model for the proposed interactions between activated T cells and CLL cells residing in the LN 16. Such interactions with the microenvironment are thought necessary for cell cycle induction

![Figure 5](image-url)

**Figure 5. Differential expression of TAp73 in PB versus LN derived CLL cells** (A) Protein levels of TAp73 and Bid assessed by Western blot in CLL cells with dysfunctional p53 after treatment CDDP for 48 hours as indicated. Actin was used as loading control (blot representative of 8 patients tested). (B) Protein levels of TAp73, p21 and Bid in CLL cells cocultured with CD40-ligand expressing fibroblasts for 24 hours with our without imatinib. Cells were washed and lysed after an additional 24 hours of culture. Actin was used as loading control (blot representative of 11 patients tested). (C) Protein levels of TAp73 and Bid in peripheral blood (PB) and lymph node (LNN) derived CLL cells. Actin was used as loading control.
To investigate whether induction of TAp73 expression upon CD40-ligation indeed relates to TAp73 expression in LN derived CLL cells, we compared TAp73 protein expression levels in PB derived CLL cells of untreated patients with expression levels in LN derived CLL cells. Strong TAp73 expression was detected in 4 of 7 LN derived CLL samples, whereas very little or no expression was seen in peripheral blood derived CLL cells (Figure 5). Furthermore, the downstream target Bid was consistently upregulated in the LN samples, whereas no expression of Bid was seen in PB derived cells.

Discussion

In this study we investigated the role of the pro-apoptotic protein TAp73 in apoptosis regulation in p53 dysfunctional leukemia cells treated with CDDP, following the observation of increased expression of this protein in p53 dysfunctional CLL cells derived from a patient treated with a regimen containing CDDP. In the p53 dysfunctional cell line MEC1, CDDP treatment induced c-Abl dependent upregulation of TAp73 and of its downstream targets, in accordance with findings in solid tumour cell lines. This resulted in increased sensitivity to both F-ara-A- and FAS-induced cell death. No upregulation of TAp73 was seen in PB derived CLL cells treated with CDDP, but increased expression of TAp73 was found in LN derived CLL cells, suggesting that this transcription factor may have a role in apoptosis regulation in cells residing in the secondary lymphoid tissue.

The p53 family member p73 was first described in 1997. Over the years many isoforms have been identified, which are represented in two major groups of proteins with opposing functions. TAp73 induces cell cycle arrest and apoptosis, whilst the oncogenic (N-terminal truncated) deltaNp73 inhibits both TAp73 and p53 induced apoptosis (reviewed by Ozaki et al and Rufini et al). Although the role of the p73 family in human cancer is much less well-defined than that of p53, its role in hematological oncogenesis is being increasingly appreciated. Specifically, inactivation of the TAp73 gene by epigenetic silencing or deletion is a common finding in malignant lymphoproliferative disorders. TAp73 is inactivated in about 35% of acute lymphoblastic leukemia (ALL) and about one-third of non Hodgkin lymphomas (NHLs).

In CLL, increased expression of both TAp73 and deltaNp73 was found, and the pattern of overexpression of different isoforms has been linked to clinical behaviour in CLL. A functional role of TAp73 in apoptosis regulation and response to drug treatment in CLL was first described by Dicker et al. Upon CD40-ligation, TAp73 was upregulated in a c-Abl-dependent manner, CD95 and Bid were induced and cells were sensitized to F-ara-A treatment, notably independent of p53 function. Based on these and previous results, in a phase I trial, autologous CLL cells transduced to express CD40-ligand were infused in CLL patients. This resulted in a comparable pattern of protein expression, including increased expression of TAp73. Furthermore, clinical responses were seen, also in patients with documented 17p deletion. In addition, recently the activity of
some novel drugs for the treatment of CLL has been described to involve upregulation of TAp73, including HDAC-inhibitors \(^{21}\), lenalidomide \(^{39}\) and forodesine \(^{40}\).

Although we found clear expression of TAp73 in LN derived CLL cells, and in CD40-ligand stimulated PB derived CLL cells, no induction was seen upon CDDP treatment of PB derived CLL cells. The cause of this difference is not known, but it is conceivable that it relates to the proliferative state of the clone and/or interactions with the microenvironment as discussed below.

First, TAp73 regulation has been shown to primarily take place at the level of protein degradation. Normally, levels of TAp73 are maintained at low levels because of constitutive activity of the ubiquitin E3 ligase Itch, which targets TAp73 for poly-ubiquitinylation and degradation via the ubiquitin-proteasome pathway. Upon DNA-damage Itch is degraded and expression levels of TAp73 are stabilized \(^{26}\). Itch was recently found to be negatively regulated by the microRNA miR106b \(^{21}\). It was postulated that silencing of specific miRNA-regulated pathways may characterize quiescent tumours such as CLL \(^{21};^{41}\), which, in this case would imply impaired protein stabilization of TAp73.

Secondly, the intracellular events linking CD40-ligation to expression of TAp73 in CLL are not known. Stabilization of TAp73 is mediated by c-Abl, as was shown in solid tumour cell lines \(^{6}\), but also in the present study. Upregulation of TAp73 upon CD40-ligation in CLL was also dependent upon c-Abl \(^{5}\). Furthermore, we have described that other downstream effects of CD40-ligand stimulation, namely the induction of anti-apoptotic proteins, could be blocked by inhibitors of c-Abl \(^{14}\). Possibly, activation/phosphorylation of c-Abl depends on the replicative state of the cell. Indeed, in contrast to p53, TAp73 has been shown to be regulated along the cell cycle in various cell lines. TA73 was found to be target of transcription factor E2F1 during the S phase of the cell cycle, and TAp73 proteins accumulated in cells in S phase \(^{42}\). E2F-1 regulates TAp73 expression either directly \(^{42};^{43}\) or via microRNAs like miR106b \(^{21}\). Furthermore, expression of E2F-1 was linked to CD40-ligation in a B cell lymphoma cell line and also in mouse B cells, as CD40-ligation allows for release of repression of this transcription factor \(^{44};^{45}\). It is highly likely that additional interactions with the environment contribute to the mechanisms available to the cell to respond to cytotoxic insults.

Although CD40 activation in vitro and presumably also in vivo (in the secondary lymphoid tissue) induces expression of TAp73 and its downstream targets, CD40 activation also results in NFκ-B mediated expression of anti-apoptotic molecules, like Bcl-xL and A1/Bfl-1 \(^{46}\), which probably tips the balance towards an anti-apoptotic, pro-survival profile. The observation of TAp73 expressing cells in the PB following CDDP treatment might reflect a shift in the apoptotic balance (mediated by TAp73), causing cells to detach from the LN environment and go into apoptosis. This hypothesis is supported by the initial rise in the PB leukocyte count observed in patients who have been treated with CDDP (Figure 1A) and also by the major LN responses induced by the R-DHAP regimen \(^{13}\).
We have demonstrated a functional role of TAp73 in the response to CDDP treatment in a p53 dysfunctional B cell lymphoma cell line. As we found induction of TAp73 both in in vivo treated CLL cells and in LN derived CLL cells, we expect that TAp73 has a role in the response to treatment in CLL. Further exploring the role and regulation of TAp73 in CLL may provide clues for the development of novel treatment strategies for p53 dysfunctional and chemorefractory CLL.
Reference List


Supplemental figure 1. p53 dysfunctional CLL (A) Protein levels of p53 and the downstream targets p21 and Puma after 5 Gy irradiation as indicated, in a p53 dysfunctional (left panel) and a p53 functional (right panel) CLL patient. (B) Leukocyte number (×10⁹/L) following treatment with R-DHAP in a patient with F-ara-A refractory CLL.

Supplemental figure 2. MEC1 is a p53 dysfunctional human pro-lymphocytic cell line (A) May-Grunwald-Giemsa staining (500x) (B) Upper panel: fluorescent in situ hybridization (FISH) of MEC1 showing trisomy 7 (CEP7) and 1 x deletion of 17p (p53). Lower panel: western blot showing lack of p53 induction upon 5 Gy radiation. I-83, a p53-functional CLL cell-line, is used as control. (C) Puma RNA-levels (assessed by RT-MLPA) and expression of CD95 (FAS, assessed by FACS-staining) upon 5 Gy radiation, indicating lack of p53 function in MEC1. The I-83 cell line is used as control; figure A and B in full colour on page 174.