Targets for the treatment of drug resistant chronic lymphocytic leukemia
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ROS-mediated upregulation of Noxa overcomes chemoresistance in chronic lymphocytic leukemia

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Abstract

In recent years, considerable progress has been made in treatment strategies for chronic lymphocytic leukemia (CLL). Yet, the disease remains incurable due to the development of chemoresistance. Strategies to overcome resistance mechanisms are therefore highly needed.

At least 2 mechanisms contribute to the development of resistance to drugs; acquired mutations resulting in a dysfunctional p53 response and shifts in the balance between apoptosis regulating proteins. Platinum-based compounds have been successfully applied in relapsed lymphoma and recently also in high-risk CLL. In this study we investigated the efficacy and mechanism of action of cisplatinum (CDDP) in chemorefractory CLL.

Independent of p53 functional status, CDDP acted synergistically with fludarabine (F-ara-A). The response involved generation of reactive oxygen species (ROS), which led to specific upregulation of the pro-apoptotic BH3-only protein Noxa. Induction of Noxa resulted in cell death by apoptosis since inhibition of caspase activation completely abrogated cell death. Furthermore, drug resistance upon CD40-ligand stimulation, a model for the protective stimuli provided in lymph nodes, could also be overcome by CDDP/F-ara-A. ROS accumulation resulted in Noxa upregulation mainly at the transcriptional level and this was, at least in part, mediated by the mitogen-activated protein kinase (MAPK) p38. Lastly, Noxa RNA-interference markedly decreased sensitivity to CDDP/F-ara-A, supporting a key role for Noxa as mediator between ROS signaling and apoptosis induction. Our data indicate that interference in the cellular redox-balance can be exploited to overcome chemoresistance in CLL.
Introduction

The clinical course of chronic lymphocytic leukemia (CLL) is highly variable; however, most patients eventually develop symptomatic disease requiring treatment. Although important advances have been made in first and second line treatment strategies, cures are not attained. Various biological markers correlate with poor prognosis and response to treatment, such as IgVH mutational status, expression levels of CD38 and/or ZAP-70 and cytogenetic abnormalities (reviewed by Van Bockstaele et al.1). Especially deletion or mutation of the p53 locus (17p13) is associated with a very poor outcome 2. Most drugs, including alkylating agents and nucleoside analogs, rely on intact p53 function for their activity and treatment with these compounds results in selection and evolution of clones containing cytogenetic changes affecting the p53 response 3. A second important contribution to chemoresistance in CLL is made by shifts in the balance between pro- and anti-apoptotic proteins. Increased expression levels of Bcl-2 family members like Bfl-1/A1 4 and in particular Mcl-1 5 have been associated with resistance to chemotherapy. Shifts in the apoptotic balance presumably arise in CLL cells residing in secondary lymphoid tissue. In these niches, cells receive stimuli from the microenvironment inhibiting apoptosis and are consequently protected from the action of cytotoxic drugs 6. Indeed, we found increased expression of Mcl-1, Bcl-xL and Bfl-1/A1 in lymphoid tissue-derived leukemia cells in comparison with peripheral blood-derived CLL cells 7. It is postulated that clones harboring acquired mutations originate from these niches 6.

The prognosis for patients with fludarabine (F-ara-A) refractory disease is extremely poor, with a median survival of less than a year 8. Therefore it is highly necessary to develop therapeutic strategies that circumvent resistance mechanisms to cytotoxic agents in order to obtain sustained responses in these patients.

Several novel drugs are currently under investigation in clinical and preclinical studies for this category of patients (reviewed by Kater and Tonino 9), but although responses have been observed, when used as single agent these drugs generally do not induce long-term disease free survival.

An approach to overcome drug resistance could be to alter the cellular apoptotic balance, independent of p53, in order to sensitize cells to alkylating agents and nucleoside analogs. Regimens containing platinum-based compounds have shown activity in CLL, importantly also in p53 dysfunctional cases. In a small phase I-II trial, the OFAR-regimen (oxaliplatin, F-ara-A, cytarabine, and rituximab) induced a response in 7 of 20 patients with documented 17p-deletion 10. We recently found marked activity of the R-DHAP regimen (rituximab, dexamethasone, cytarabine and CDDP) in 8 of 10 treated patients with F-ara-A refractory disease, including patients with proven p53 dysfunction 11. Synergy between F-ara-A and platinum-based compounds was studied in in vitro studies and was found to result from inhibition of nucleotide excision repair by F-ara-A of DNA-damage induced by platinum-based compounds in both a cell-free system and in CLL cells derived from patients with early-stage disease (in which p53 dysfunction is found in less than 5%
of cases) \(^{12,13}\). CLL cells exhibit high DNA repair capacity and this was found to correlate with resistance to cytotoxic drugs \(^{14,15}\). However, to what extent these repair pathways are involved in response to therapy in CLL, how these processes are regulated and how cell fate is decided upon after DNA-damage is not well known. Since p53 is the central player in the regulation of DNA repair pathways \(^{16}\) as well as in the initiation of the ensuing apoptotic response (reviewed by Rich et al. \(^{17}\)), the above described synergy very likely depends on a functional p53 pathway. However, clinical effectiveness in p53 dysfunctional patients suggests that an alternative, p53 independent, mechanism is in play in the activity of the combination of CDDP and F-ara-A.

As elucidating p53 independent apoptosis pathways may yield useful information for the development of treatment strategies for chemorefractory patients, we studied how platinum-based compounds abrogate F-ara-A -resistance in CLL. We found synergy between the two classes of drugs, irrespective of p53 functional status. Resistance resulting from CD40-ligation was also overcome. The apoptotic response towards CDDP/F-ara-A combination treatment was mediated by an increase in reactive oxygen species (ROS) and required upregulation of the pro-apoptotic BH3-only protein Noxa. These data stress the potential clinical relevance of targeting the redox-balance to overcome drug resistance in CLL.

### Material and methods

#### Patient material and cell culture

Peripheral blood mononuclear cells (PBMCs) of patients with CLL, collected during follow-up visits at the department of Hematology, were isolated and subsequently frozen and stored as previously described \(^{18}\). Patient characteristics are summarized in table 1. 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 described \(^{19}\).

Cells were cultured in Iscove modified Dulbecco medium (IMDM; Invitrogen, Carlsbad, CA), supplemented with 10\% (vol/vol) heat-inactivated fetal calf serum (FCS; ICN Biomedicals, Meckenheim, Germany), 100 \(\mu\)g/mL gentamycin, and 5 mM L-glutamine (Invitrogen). Where indicated, CLL cells (1.5 – 2 \(*\) 10\(^6\)/ml) were stimulated with CD40-ligand for 48 hours prior to drug treatment as previously described \(^{18}\). In the synergy experiments the following drugs and reagents were used: CDDP (Mayne Pharma, Brussels, Belgium), Oxaliplatin, F-ara-A, CCCP (Sigma-Aldrich, St Louis, MO), H\(_2\)O\(_2\) (Merck KGaA, Darmstadt, Germany), p38-i (SB202190), JNK-i (Sp600125; Enzo Life Sciences Inc, Farmingdale, NY), Q-VD-OPh (R & D systems, Minneapolis, MN),
Z-IETD-fmk, N-acetylcysteine, Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate), butylated hydroxyl-anisole (BHA) (all Sigma-Aldrich).

Assessment of apoptosis and ROS-production

Apoptosis was assessed by annexin-V (IQ Products, Groningen, the Netherlands)/propidium iodide (PI) staining (Sigma-Aldrich) or MitoTracker Orange (Molecular Probes, Leiden, The Netherlands) staining as previously described 7 and analyzed by flow cytometry. Where indicated specific apoptosis is presented: (% apoptosis treated cells - % apoptosis untreated cells)/ % viable untreated cells. For the measurement of cellular ROS content, cells which had been incubated with drugs for the indicated period of time, were harvested and washed with pre-warmed phenol-red free Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) and incubated with 10 μM carboxy-H2-DCFDA (C2938, Invitrogen) dissolved in warm DMEM at 37 °C. After 30 minutes, cells were washed and analyzed by flow cytometry.

Table 1. Patient characteristics.

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1 Mutational status of the immunoglobulin variable heavy chain; M = mutated, UM = unmutated, poly = polyclonal. 2 as analyzed by FISH (fluorescence in situ hybridization); ND = not performed. 3 P53-function as assessed by RT-MLPA as described 9 and in Methods. 4 Patient sample was designated p53-dysfunctional based on deletion of 17p and F-ara-A resistance in vitro.

Z-IETD-fmk, N-acetylcysteine, Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate), butylated hydroxyl-anisole (BHA) (all Sigma-Aldrich).
mRNA isolation and RT-MLPA

CLL cells were incubated with Q-VD-OPh for 30 minutes at 37 °C before the addition of drugs as indicated. After the indicated period of time, cells were harvested and total mRNA was isolated using the GenElute mammalian total mRNA miniprep kit (Sigma-Aldrich). A reverse transcription multiplex ligation-dependent probe amplification assay (RT-MLPA, apoptosis-kit R011-B1, MRC-Holland, Amsterdam, the Netherlands) was performed as described previously \(^{20}\). Expression levels were normalized for the expression of the housekeeping gene β2-microglobulin. For data presentation gene expression in treated cells was compared to expression in untreated cells. Genes that showed at least two-fold increase or decrease in mean relative expression level upon treatment were subjected to statistical testing for significance.

Protein isolation and Western blot

CLL cells, incubated with drugs as indicated, were washed once in ice-cold phosphate buffered saline (PBS), and lysed by sonification in radioimmunoprecipitation (RIPA) buffer as previously described \(^{21}\). 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), p73 (IMG-246, Imgenex, San Diego, CA), Noxa (IMG-349, Imgenex), Puma (polyclonal, Cell signaling, Beverly, MA), Bax and Mcl-1 (polyclonal, Pharmingen, Franklin Lakes, NJ), and Bcl-2 (polyclonal, Alexis, Farmingdale, NY). 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). Where indicated protein expression levels were quantified using LI-COR Odyssey software version 3.0.

Nucleofection

CLL cells were transfected using the Amaxa nucleofection technology (Lonza, Cologne, Germany) according to the manufacturers’ recommendations and as described \(^{22}\). In short, CLL cells (5x10^6) were left to recuperate from thawing for 4 to 6 hours and subsequently spun down at 1300 rpm for 5 minutes, resuspended in 100 μl of amaxa buffer and transfected with 3 μl of siRNA (stock concentration 100 μM) using programme X-01. Cells were immediately transferred into warm medium and after 1 hour of incubation at 37°C, drugs were added as indicated. The Noxa siRNA (s10709) and negative control siRNA 1 were used (Ambion, Austin, TX).
Statistics

The Shapiro-Wilk normality test was performed to assess normal distribution of data sets. In case of Gaussian distribution of the data, a two-sided t test was used to analyze differences between data sets. If there was no Gaussian distribution, a two-tailed Mann-Whitney-U test was used to analyze differences between groups of unpaired samples and a Wilcoxon matched paired test to analyze differences between paired samples. For the comparison of more than 2 sets of data the Kruskal-Wallis test was used, with a post-hoc Dunns test in case of statistical difference. Statistic significance was set at a p- value < 0.05.

Results

Platinum-based compounds sensitize CLL cells to caspase-dependent apoptosis induction by F-ara-A, independent of p53 function

The response of CLL cells to increasing doses of CDDP (1-100 μM) was assessed after 24 and 48 hours. After 48 hours, cell death was seen only in doses above 50 μM. As the C_{max} of CDDP in vivo is within the range indicated by the shaded area in figure 1A, and does not exceed 10 μM, CDDP is not expected to be active as a single agent in CLL (Figure 1A). As expected, p53 functional CLL cells were sensitive to F-ara-A in increasing doses, whereas p53 dysfunctional CLL samples were resistant, even in doses much higher than those that can be achieved in vivo (Figure 1B, C). Addition of 10 μM CDDP, which by itself did not induce cell death, not only enhanced F-ara-A induced cell death in p53 functional samples, but also synergistically induced cell death in p53 dysfunctional CLL. Significant synergy was already observed with 5 μM F-ara-A. Assessment of apoptosis by measuring both loss of mitochondrial membrane potential (MitoTracker staining) as well as phosphatidylserine-exposure (annexin-V/ PI staining) yielded comparable results (presented are results from annexin-V/ PI staining, Figure 1B, C left graph).

Oxaliplatin (DACH oxalate-platinum), a third generation platinum-based compound with improved pharmacokinetics and toxicity-profile in comparison with CDDP, is currently tested in chemorefractory CLL as component of the OFAR-regimen. A similar synergistic effect was found of combining oxaliplatin with F-ara-A, indicating that the observed synergistic effect applies to platinum-based compounds in general (Figure 1C right graph).

To analyze whether the observed cell death is caspase-dependent, synergy experiments were repeated in the presence of the general caspase inhibitor Q-VD-OPh. Addition of Q-VD-OPh significantly abrogated cell-death in p53 functional (Supplemental figure 1) as well in p53 dysfunctional cells (Figure 1D). Cell death could not be inhibited by the caspase-8 inhibitor Z-IETD-fmk (data not shown), indicating that the mitochondrial, rather than the extrinsic apoptosis pathway is involved.
Figure 1. Synergism between F-ara-A and CDDP in CLL is independent of p53-functional status.

(A) CLL cells of 8 patients (pt no 2, 3, 5, 8, 11, 15, 18, 22) were incubated for 48 hours with CDDP at increasing doses (1-100 μM). Cell death was assessed by annexin-V/PI staining (as described in the Methods section). The shaded area presents the concentration-range attained upon CDDP treatment in vivo. (B) CLL cells with functional p53 (n=5; pt no 11, 12, 18, 21, 22) were treated with increasing doses of F-ara-A (5-50 μM), with or without 10 μM CDDP, for 48 hours. Cell death was assessed by annexin-V/PI staining. To correct for variation in base-line apoptosis levels (see Figure 1A), specific apoptosis is depicted (as described in the Methods section). Presented is mean + standard error of mean (SEM; *p < .05; Mann-Whitney-U test). (C) CLL cells with dysfunctional p53 were treated with increasing doses of F-ara-A (5-50 μM), with or without 10 μM CDDP (n=6; pt 1-6) or 10 μM oxaliplatin (n=4; pt no 2, 3, 5, 6), for 48 hours. Cell death was assessed by annexin-V/PI staining. Presented is mean + SEM (*p < .05, **p < .01; Mann-Whitney-U test). (D) Left panels: Cells of pt no 5 were treated with 10 μM CDDP and 10 μM F-ara-A (C+F) with or without pre-incubation with 20 μM Q-VD-OPh. Apoptosis was assessed after 48 hours using annexin-V/PI staining. Right graph: summarized data of 5 p53 dysfunctional CLL patients (pt no 1-3, 5, 6). Bars represent mean + SEM (**p < .01; Mann-Whitney-U test).
Co-treatment of CLL cells with CDDP and F-ara-A results in upregulation of Noxa

We have described earlier that F-ara-A induced apoptosis relies upon p53 dependent upregulation of the BH3-only molecule Puma. To study whether the combination of CDDP and F-ara-A results in alterations in the balance between pro- and anti-apoptotic proteins, the mRNA expression level of relevant apoptosis-regulating genes was assessed by RT-MLPA. Treatment with 10 μM CDDP did not result in significant modulation of gene expression in p53 functional and p53 dysfunctional CLL samples, in accordance with the absence of an apoptotic response. As expected, since the activity of F-ara-A depends on p53, treatment with this drug induced significant upregulation of the p53 target genes Puma, p21 and Bax in p53 functional samples, whereas no upregulation of Puma and Bax was detected in p53 dysfunctional samples. Combination treatment with CDDP and F-ara-A induced significant changes in expression levels of only two genes: p21 and Noxa, irrespective of p53 function (Figure 2A). To test protein expression levels, cells were treated as indicated in the presence of Q-VD-OPh to prevent caspase-mediated breakdown of proteins. Noxa protein levels were clearly increased upon combination treatment, whereas no induction occurred upon treatment with CDDP or F-ara-A as single drugs. Puma protein was upregulated in a p53-dependent fashion (Figure 2B,C). Levels of the anti-apoptotic Bcl-2 family-member Mcl-1, the principal binding partner of Noxa, remained stable, as did protein levels of anti-apoptotic Bcl-2 and Bfl-1/A-1 and pro-apoptotic Bax and Bim (Figure 2B,C and data not shown).

CD40 ligation does not protect against the cytotoxic effect of CDDP and F-ara-A

Next to the functional status of p53, protective stimuli derived from the microenvironment in secondary lymphoid tissue constitute a major determinant of drug-resistance in CLL. The protective microenvironment can in part be mimicked in vitro by stimulating CLL cells with CD40-ligand, which results in increased expression levels of Bcl-xL, Bfl-1/A1 and Mcl-1 and decreased expression levels of Noxa. To analyze whether combination treatment with CDDP and F-ara-A can also overcome chemoresistance due to these protective signals, CLL cells were co-cultured with CD40-ligand expressing fibroblasts for 48 hours and subsequently treated with CDDP and F-ara-A for 48 hours. As shown in figure 3A, cells co-cultured with control fibroblasts were sensitive to F-ara-A, whereas CD40-ligand stimulation resulted in complete resistance to F-ara-A. Addition of CDDP to F-ara-A resulted in a significant increase in apoptosis levels despite CD40 ligation. We have shown earlier that F-ara-A mediated induction of Puma expression is not abrogated by CD40-ligand stimulation. Analysis of protein expression levels upon combination treatment revealed strong upregulation of Noxa (figure 3B). Since Noxa selectively binds and inhibits Mcl-1, these data suggest that induction of Mcl-1 expression is an important determinant of CD40-ligand mediated chemoresistance. The
decrease in Mcl-1 protein expression level in CD40-ligand stimulated cells treated with CDDP and F-ara-A may be secondary to caspase activation 26.

Activity of CDDP is not correlated with p73 expression in CLL

The observed synergy between CDDP and F-ara-A in p53 dysfunctional CLL samples implies activation of p53 independent apoptosis pathways. Previous studies in various solid cancers have indicated that the activity of CDDP involves p53 independent upregulation of p73, a member of the p53 family 27. Since Noxa is reported to be a response gene of p73 28, we studied whether p73 was upregulated by CDDP in CLL cells and whether the observed apoptosis was mediated by this protein. We first tested this hypothesis in the p53 dysfunctional pro-lymphocytic cell-line Mec-1. Upon CDDP-

Figure 2. Co-treatment of CLL cells with F-ara-A and CDDP induces upregulation of Noxa. (A) Cells of 3 p53 functional (pt no 11, 18, 22) and 3 p53 dysfunctional (pt no 2, 4, 5) CLL patients were treated with 10 µM CDDP, 10 µM F-ara-A or the combination in the presence of Q-VD-OPh. The mRNA expression level of 30 apoptosis regulating genes was assessed by RT-MLPA (as described in the Methods section). Gene expression upon treatment was related to gene expression in untreated cells. The resulting matrix was imported in the program MultiExperiment Viewer (www.tigr.org/software/tm4), and values were assigned green or red colors; green for values between 0 and 1 indicating downregulation and red for values >1 indicating upregulation. The CLL samples are ordered as indicated below the matrix. In the right hand column, the genes are ordered by functional category (HKG= house-keeping genes; β2M= β-2-microglobulin). Significant changes in expression are indicated with (*) for p53+ samples and (†) for p53- samples (p < .05, Kruskal Wallis test with post-hoc Dunns-test). (B) CLL cells were treated with 10 µM CDDP, 10 µM F-ara-A or the combination as indicated for 48 hours in the presence of Q-VD-OPh to prevent caspase-dependent breakdown of proteins. Protein lysates were analyzed for Mcl-1, Puma and Noxa expression by Western blot. Actin was used as loading control. Shown is a representative blot of both a p53 functional (pt no 20) and a p53 dysfunctional CLL sample (pt no 3). (C) Summarized data of the relative expression (compared to control) of Mcl-1, Puma and Noxa in 4 p53 functional (pt no 18-20, 22) and 4 p53 dysfunctional (pt no 1-3, 5) CLL samples upon treatment as described in (B). Protein expression was quantified using Licor Odyssey software and corrected for the expression of actin. Bars represent mean + SEM (* p < .05, Kruskal Wallis test with post-hoc Dunns-test); matrix in full colour on page 175.

Figure 3. CD40 ligation does not rescue CLL cells from apoptosis induced by CDDP/ F-ara-A combination treatment (A) Cells of 6 p53-functional CLL patients (pt no 9, 10, 13, 14, 17, 21) were stimulated with CD40-ligand for 48 hours, harvested and treated with 10 µM CDDP, 10 µM F-ara-A or the combination for 48 hours. As control, cells were cocultured with 3T3 fibroblast. Apoptosis was assessed by annexin-V/ PI staining. Bars represent mean + SEM (* p < .05, *** p < .001; Mann-Whitney-U test). (B) Cells were treated as in (A), lysed and tested for expression levels of Mcl-1, Noxa and actin (pt no 18; representative blot of 4 patients tested).
treatment upregulation of p73 was seen in a time- and dose-dependent fashion (data not shown). Next, we analyzed expression levels of p73 upon treatment with CDDP in p53 functional and dysfunctional CLL cells. No upregulation of p73 protein levels were found when cells were treated with doses up to 100 μM (Figure 4A) and incubation up to 96 hours (data not shown). CD40 ligation was used as a control, as this was demonstrated to induce upregulation of p73 protein levels in CLL 21. Also treatment with F-ara-A or the combination of these drugs had no effect on p73 levels in CLL (Figure 4B).

**N-acetylcysteine abrogates apoptosis as well as Noxa-upregulation**

The mechanism of action of platinum-based compounds is classically described to be mediated by DNA-damage and the ensuing death response 29. However, CDDP binds to many cellular components; in fact only 10-15% is found in the nuclear compartment, whereas 75-85% is bound to proteins 30. Once in the cell, platinum-based compounds are aquated and become highly reactive. In this form they rapidly bind cytoplasmatic proteins, among which anti-oxidant proteins like reduced glutathione 31 and constituents of mitochondria causing mitochondrial dysfunction 32. We hypothesized that in CLL cells, disruption of the cellular redox-balance and the generation of ROS, rather than direct

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**Figure 4. CDDP treatment does not induce p73 in CLL** (A) CLL cells of 2 patients with functional (pt no 19, 20) and 2 with dysfunctional p53 (pt no 2, 3) were treated with 10 μM or 100 μM CDDP; after 48 hours p73 protein expression levels were assessed by Western blot. As control, CLL cells were cocultured with CD40-ligand expressing fibroblasts for 24 hours and analyzed for p73 expression after an additional 24 or 48 hours (pt no 20). Lysates were obtained in the same experiment and loaded on separate gels. As antibody control a lysate of HEK293 cells transfected with a construct encoding HA-tagged p73 was used (* denotes the endogenous p73 band; ** the HA-tagged p73 band in transfected cells). (B) CLL cells were co-cultured with CD40-ligand expressing or control 3T3 fibroblasts for 48 hours and treated with 10 μM CDDP and 10 μM F-ara-A as indicated for 48 hours. P73 and actin protein expression levels were analyzed by Western blot (pt no 18; representative blot of at least 6 patients tested).
DNA-damage, might contribute to the apoptotic response upon treatment with CDDP. To study whether the activity of the combination of CDDP and F-ara-A involves generation of ROS, experiments were performed in the presence of the free radical scavenger N-acetylcysteine (NAC). As shown in figure 5A, apoptosis upon CDDP/ F-ara-A combination treatment in p53 dysfunctional cells was almost completely abrogated by NAC, whereas sensitivity to F-ara-A in p53 functional samples was unaffected by the addition of NAC. To confirm that cell death was abrogated by NAC through its ROS-scavenging properties, experiments were repeated with the alternative scavengers Tiron and BHA (butylated hydroxyl-anisole). These experiments yielded comparable results, but data analysis was hampered by toxicity of these compounds after 48 hours (Supplemental figure 2A). Upregulation of Noxa protein-levels was abrogated by co-treatment with NAC, whereas expression-levels of Puma remained unaffected (Figure 5B), which supports a functional role for Noxa in apoptosis induction upon CDDP/F-ara-A combination treatment. Furthermore, cell death upon combination treatment in CD40-ligand stimulated cells could be diminished by the addition of NAC (and Tiron and BHA), indicating that also in this setting, death is mediated by ROS (figure 5C and supplemental figure 2B).

To further test our hypothesis, we performed direct assessment of both cellular glutathione as well as ROS levels. A significant but only partial decrease was found in glutathione levels within 6 hours of treatment, not only in cells treated with high dose CDDP (100 μM), as expected, but also in cells treated with 10 μM CDDP in combination with 10 μM F-ara-A, whereas treatment with the drugs at this dose level alone, did not result in a significant modulation of glutathione levels (Supplemental Figure 2C). Direct assessment of cellular ROS content revealed an increase in ROS levels within 6 hours of combination treatment (Figure 5D upper left panels + right graph). This occurred well before loss of mitochondrial membrane potential (upper left panels) and phosphatidylserine exposure (not shown), which supports a pivotal role for ROS in the induction of apoptosis. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a potent uncoupler of oxidative phosphorylation and inducer of ROS, was used to confirm effects of ROS accumulation in CLL (Figure 5D lower left panels).

**Accumulation of ROS results in upregulation of Noxa, primarily on a transcriptional level**

We further studied the mechanism by which accumulation of ROS results in upregulation of Noxa. First we determined what source of ROS is most likely accountable for induction of Noxa protein levels. Cells were treated with either CCCP or with hydrogen peroxide (H₂O₂, a common ROS intermediate generated by various extra- and intracellular ROS producing sources). Treatment with 100 μM CCCP (and 300 μM H₂O₂; data not shown) strongly induced apoptosis within 24 hours independent of p53 function and this could be completely abrogated by the addition of NAC (assessed by annexin-V/ PI-staining; Figure 6A). However, accumulation of Noxa protein was seen after 24 hours of treatment
**A**

p53 functional CLL

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p53 dysfunctional CLL

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**B**

- **p53 functional CLL**
  - Mcl-1
  - Puma
  - Noxa
  - Actin
  - Drugs: M, C, F, CF
  - Apoptosis %: 30, 29, 80, 90

- **p53 dysfunctional CLL**
  - Mcl-1
  - Puma
  - Noxa
  - Actin
  - Drugs: M, C, F, CF
  - Apoptosis %: 35, 39, 40, 70

**C**

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<td>+ NAC</td>
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**D**

- **p53 functional CLL**
  - Mcl-1
  - Puma
  - Noxa
  - Actin
  - Drugs: M, C, F, CF
  - Apoptosis %: 35, 35, 86, 85

- **p53 dysfunctional CLL**
  - Mcl-1
  - Puma
  - Noxa
  - Actin
  - Drugs: M, C, F, CF
  - Apoptosis %: 39, 40, 43, 45

**E**

- **control**
  - Apoptosis %: 0
  - + NAC
  - Apoptosis %: 75

**F**

- **control**
  - Apoptosis %: 0
  - CDDP/ F-ara-A
  - Apoptosis %: 75
with 100 μM CCCP (Figure 6B), but not with H₂O₂. The proteasome inhibitor bortezomib was used as positive control. These data suggest that mainly ROS derived from a mitochondrial source are required for the upregulation of Noxa.

Next, we determined the level at which ROS-dependent Noxa induction is regulated. In accordance with our findings upon CDDP/F-ara-A treatment (Figure 2 A), treatment with CCCP resulted in upregulation of Noxa mRNA levels, irrespective of p53 functional status (Figure 6C and Supplemental Figure 4A), which was abrogated by the addition of NAC.

Apart from the p53 family, the MAPK signaling pathways play an important role in deciding cell fate upon cellular stress, including oxidative stress (reviewed in McCubrey et al. 33). Of these, mainly the JNK en p38 pathways orchestrate the apoptotic response upon cellular stress and are hence grouped together and referred to as stress-activated protein kinases (SAPK). To investigate the role of SAPK we treated cells with CCCP in the presence of specific pharmacological inhibitors of p38 en JNK. As shown in figure 6D, p38 inhibition abrogated Noxa upregulation upon CCCP treatment, whereas JNK inhibition did not.

As recently has been shown in CLL cells, Noxa protein has a short half life trough rapid proteasomal dependent degradation 34. We measured proteasome activity in CLL cells treated with CCCP or bortezomib. Although some decrease was observed upon CCCP treatment, effects were very limited, especially when compared to the decrease of proteasomal activity following treatment with bortezomib (decrease in activity 15-20% versus > 95%) (data not shown).
Knockdown of Noxa attenuates CDDP/ F-ara-A induced cell death

The functional relevance of Noxa upregulation in the apoptotic response upon treatment was further investigated by means of RNAi. Cell viability was not affected by the nucleofection procedure (not shown). Nucleofection with Noxa siRNA, but not a scrambled control (scr) siRNA, resulted in a 50-60% decrease in Noxa protein expression...
Figure 6. (A) P53 dysfunctional (n=5; pt no 2-6) and p53 functional (n=5; pt no 11, 19-22) CLL cells were treated with 100 μM CCCP for 24 hours with or without pre-incubation with 5 mM NAC for 30 minutes. Apoptosis was assessed by annexin-V/PI staining. Presented is mean + SEM (** p < .01; Mann-Whitney-U test). (B) CLL cells were treated with 300 μM H2O2, 30 nM Bortezomib (B) or 100 μM CCCP (as indicated) for 24 hours in the presence of Q-VD-OPh to prevent caspase-dependent breakdown of proteins. Protein lysates were analyzed for Noxa expression by Western blot. Actin was used as loading control. Shown is a representative blot (pt no 23) of 3 independent experiments. (C) Cells of 3 p53 functional (pt no 11, 18, 22) CLL patients were treated with 100 μM CCCP with or without pre-incubation with 5 mM NAC for 30 minutes in the presence of Q-VD-OPh. The mRNA expression level of 30 apoptosis regulating genes was assessed by RT-MLPA (as described in the Methods section). Presented are the mRNA expression levels of Noxa normalized for β2-microglobulin. (* p < .05, Students T-test). For complete results of the assay supplemental figure 3. (D) Left panels: CLL cells (pt no 24) were treated with 100 μM CCCP with or without 10 μM of the MAPK-inhibitors p38-i (SB202190) or JNK-i (Sp600125; as indicated) for 24 hours in the presence of Q-VD-OPh to prevent caspase-dependent breakdown of proteins. Protein lysates were analyzed for Noxa expression by Western blot. Actin was used as loading control. Right graph: summarized data of the relative expression (compared to control) of Noxa in 3 CLL samples upon treatment as described in (B). Protein expression was quantified using Licor Odyssey software and corrected for the expression of actin. Bars represent mean + SEM.

Figure 7. Apoptosis induced by CDDP/ F-ara-A combination treatment is mediated by Noxa. (A) Protein expression levels of Noxa and actin in untransfected cells and in cells nucleofected with Noxa or control (scr) siRNA, untreated (left panels) or treated with 10 μM CDDP and 10 μM F-ara-A for 48 hours (right panels), were analyzed by Western blot for control of knockdown of Noxa. Apoptosis was assessed by MitoTracker staining (pt no 1). (B) Protein expression levels in 4 patients (pt no 1-3, 5), treated as described in (A) were quantified using Licor Odyssey software and the Noxa/ Mcl-1 ratio was calculated. Noxa/ Mcl-1 ratio of untransfected cells was set to 1. Bars represent mean + SEM (* p < .05, Mann-Whitney-U test). (C) Cells of p53-dysfunctional CLL patients were transfected with Noxa RNAi or control scrambled (Scr) RNAi and treated with 10 μM CDDP and 10 μM F-ara-A for 48 hours, 100 μM CCCP for 24 hours or left untreated. Apoptosis was assessed using annexin-V/PI staining (n=4; pt no 1-3, 5). Bars represent mean + SEM (* p < .05, Mann-Whitney-U test).
levels upon combination treatment (Figure 7A and B). Knockdown of Noxa significantly diminished sensitivity to CDDP/F-ara-A combination treatment and also to CCCP (Figure 7C). These data indicate a functional role for Noxa in the apoptotic response upon drug-induced, p53 independent ROS accumulation in CLL cells.

**Discussion**

In this study we show that the combination of CDDP and F-ara-A induces apoptosis in CLL cells, independent of p53 functional status. The activity of this combination is mediated by the generation of ROS. The BH3-only protein Noxa was identified as an important player in the ensuing apoptotic response. Noxa-mediated apoptosis induction in p53 dysfunctional cells by CCCP confirms the potency of ROS-signaling in p53 independent apoptosis induction. Furthermore, our data suggest that the MAPK p38 links ROS-accumulation to Noxa induction.

Initially we investigated whether apoptosis was mediated by p73, but although this protein mediates responses towards CDDP in various cancer types, this seems not to apply in CLL, as no upregulation of p73 upon CDDP (and/or F-ara-A) treatment was found. Activity of both drugs is classically described to be based on infliction of DNA damage \(^{29,35}\), and the subsequent activation of the DNA repair machinery in which p53, and possibly p73, play key roles. However the large majority of CLL cells, especially those derived from peripheral blood, are in G1 arrest. Active DNA-replication does not prevail and in this respect CLL differs from other cancer types. It is therefore conceivable, that alternative mechanisms of action of both drugs play a more prominent role in CLL.

Studies in various cancer types have shown that malignant cells are under increased oxidative stress due to enhanced ROS-formation \(^{36}\). Therefore, the balance between ROS and anti-oxidant mechanisms is especially critical in cancer cells, and hence presents an attractive target for therapeutic intervention (reviewed by Fruehauf and Meyskens Jr.\(^{37}\)). Most likely, this also applies to CLL, since in comparison to normal lymphocytes, CLL cells are under enhanced oxidative stress, which is associated with oxidative damage to nuclear and mitochondrial DNA \(^{36,38,39}\).

We found that responses to CDDP/F-ara-A combination treatment in CLL were mediated by the generation of ROS. Cellular ROS content increased within hours after the addition of drugs. Synergy between these drugs could furthermore be abrogated by the addition of ROS-scavengers. Platinum-based compounds have been shown to stress the cellular redox-system in various ways. CDDP induces mitochondrial dysfunction and an increase in ROS production by damaging mitochondrial DNA and disruption of the respiratory chain \(^{32,40}\). Furthermore, CDDP reacts with thiol-containing molecules like glutathione \(^{31,41}\), thereby depleting intracellular glutathione levels \(^{42}\) and also inhibits thioredoxin reductase, an important component of the alternative cellular reducing machinery \(^{40}\). We found a decrease in cellular glutathione levels upon combination
treatment, whereas treatment with single drugs (in clinically relevant doses) did not significantly affect glutathione levels. However, as we found Noxa upregulation upon treatment with the uncoupler of oxidative phosphorylation CCCP, but not upon treatment with H\textsubscript{2}O\textsubscript{2}, accumulation of ROS from a mitochondrial source may be required to induce upregulation of Noxa. Clinically attainable doses of CDDP alone did not induce ROS-accumulation in our experiments. It could be that the cellular anti-oxidant system is capable of neutralizing CDDP in the used dose within the time-period of the experiments. Indeed, when we used supra-physiological concentrations of CDDP (50 – 100 μM), a decrease in glutathione levels, ROS-production, Noxa upregulation and apoptosis were observed, which could be abrogated by the addition of NAC (data not shown). Although F-ara-A as a single agent does not seem to induce ROS-production directly \textsuperscript{43}, it has been found to enhance ROS production in leukemia cells when combined with other agents by a yet unrevealed mechanism \textsuperscript{44}.

We found that apoptosis was the primary mechanism of cell death induced by combination treatment, as blocking caspase activation almost completely abrogated death. The BH3-only protein Noxa played an important role in this process. As the knockdown achieved in our nucleofection experiments was partial, it is conceivable that the role of Noxa in the apoptotic response may be underestimated. We have previously shown that in CLL mRNA expression levels of Noxa are high, independent of p53 functional status \textsuperscript{20}. Although the Noxa/ Mcl-1 balance was found to be decisive for cell fate in cells residing in the LN (and upon CD40 stimulation as its in vitro counterpart \textsuperscript{7}), the role and regulation of Noxa in CLL specific responses to drug treatment are not well known. Noxa is a p53 response gene in many cell-types \textsuperscript{45}, but this is not the case in CLL \textsuperscript{20}. In this study we show that, at least in CLL, Noxa is the only BH3-only member that is induced upon ROS signaling. These findings are of particular interest as they constitute an apoptosis pathway in CLL which does not depend upon p53. We found that ROS-mediated Noxa upregulation is mainly transcriptional. As inhibition of the MAPK/SAPK p38 abolished Noxa upregulation, this pathway may play an important role in the cellular reaction, including induction of Noxa, to oxidative stress. Although in recent years, it has been increasingly appreciated that MAPK activation may be the major component deciding cell fate upon CDDP treatment (reviewed in Brozovic and Osmak \textsuperscript{46}), we here describe a novel link between treatment induced p38 activation and Noxa induction in CLL. Interestingly, in a very recent report, in keratinocytes Noxa upregulation upon UVB irradiation was also found to be orchestrated by p38 MAPK in a p53 independent manner \textsuperscript{47}.

The potential relevance of ROS dependent apoptosis in drug responses is supported by the recent observation that F-ara-A resistant cells are highly sensitive to beta-phenylethyl isothiocyanate (PEITC), a compound that induces ROS accumulation by disabling the glutathione system \textsuperscript{38} and data in various other reports that link the response to drugs, including bendamustine and HDAC-inhibitors \textsuperscript{36;48-51}, to p53 independent ROS production. However, whether ROS production is required for the initiation of
apoptosis, or is a consequence of this could not be conclusively determined for all of these compounds.

Since major advances have recently been made in first and second line therapy for patients with CLL, it is expected that increasing numbers of patients, among which a substantial number of p53 dysfunctional cases, will eventually need third line therapy. Designing strategies that target the distinguishing biochemical features of malignancy may be a promising approach. The altered redox balance in cancer represents one of such features, as ROS adaptation may play a critical role in drug resistance. Our data indicate that pharmacological induction of ROS or abrogation of cellular adaptive mechanisms to ROS may be a sensible strategy in high-risk, chemoresistant CLL patients.

Acknowledgements

The authors would like to thank M. Baou and G. M. Cohen of the MRC Toxicology Unit, University of Leicester, United Kingdom for sharing their experience with nucleofection-procedures.
Reference List


Supplemental figure 1. Cells of p53 functional (n=6; pt no 12, 18-22) CLL were incubated with the pan-caspase inhibitor Q-VD-OPh for 30 minutes before the addition of 10 μM CDDP, 10 μM F-ara-A or the combination. Apoptosis was assessed after 48 hours using annexin-V/PI-staining. Bars represent mean ± SEM (*** p < .001; Mann-Whitney-U test).
Supplemental figure 2. (A) p53 dysfunctional CLL cells (pt no 1 and 4) were pre-incubated with 5 mM NAC, 5 mM Tiron or 100 μM BHA for 30 minutes and subsequently treated with 10 μM F-ara-A and 10 μM CDDP (C+F) as indicated for 48 hours. Apoptosis was assessed by annexin-V/PI staining. (B) CLL cells (pt no 16 and 21), which have been co-cultured with CD40L-expressing or control 3T3 fibroblast for 48 hours, were pre-incubated with 5 mM NAC, 5 mM Tiron or BHA 100 μM for 30 minutes and subsequently treated with 10 μM F-ara-A and 10 μM CDDP (C+F) as indicated for 48 hours. Apoptosis was assessed by annexin-V/PI staining. (C) CLL cells were treated with 10 μM CDDP and/or 10 μM F-ara-A or 100 μM CDDP (as indicated) and lysed after 3 (n=3), 6 (n=4) or 16 hours (n=2). Cellular glutathione content was assessed as described in the Methods section. Presented is glutathione content, corrected for protein content of the sample, relative to the glutathione content of untreated cells. Only two samples were available for the 16 hour time-point, so statistical analysis could not be performed. X: no reliable measurement of glutathione levels possible, as considerable cell death was seen. Bars represent mean + SEM (* p < .05; Mann-Whitney-U test).
Supplemental figure 3. Cells of 3 p53 functional (pt no 11, 18, 22) and 3 p53 dysfunctional (pt no 2, 4, 5) CLL patients were treated with 100 μM CCCP in the presence of Q-VD-OPh. The RNA expression level of 30 apoptosis regulating genes was assessed by RT-MLPA (as described in the Methods section). Frames indicate genes which show at least two-fold induction or decrease in expression level.