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Nucleoside analogs induce proteasomal down-regulation of p21 in chronic lymphocytic leukemia cell lines

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ABSTRACT

Nucleoside analogs (NAs) represent an important class of anticancer agents that induce cell death after conversion to triphosphate derivatives. One of their most important mechanisms of action is the activation of p53, leading to apoptosis through the intrinsic pathway. Classically, the activation of p53 also induces p21 accumulation, which leads to cell cycle arrest at the G1/S transition. In previous work, we observed that 2-chloro-2′-deoxyadenosine (CdA), a NA with high activity in lymphoid disorders, including chronic lymphocytic leukemia (CLL), promotes the G1/S transition in the CLL cell line EHEB at cytotoxic concentrations. This finding led us to investigate the p21 response to NAs in these cells. We show here that CdA, but also fludarabine, gemcitabine, and cytarabine, strongly reduced the p21 protein level in EHEB cells as well as in JVM-2 cells, another CLL cell line. This p21 depletion occurred despite induction of p53 and increase of p21 mRNA and was prevented by proteasome inhibitors. Increase of proteasomal degradation caused by NAs appeared to be ubiquitin-independent. Also, NAs induced in these cells an increase of cyclin-dependent kinase (Cdk2) activity and a monoubiquitination of cell proliferating nuclear antigen (PCNA), two processes that are negatively regulated by p21. These changes were not observed with other p53 activators, like etoposide and nutlin-3a that increased the p21 protein level. In conclusion, our study reveals that NAs can induce an alternative pattern of cellular response in some cell models.

1. Introduction

The purine nucleoside analogs (NAs) cladribine (2-chloro-2′-deoxyadenosine, CdA) and fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine-5′-monophosphate) are effective drugs in indolent B-cell malignancies, including chronic lymphocytic leukemia (CLL) [1–3]. To exert their antileukemic effects, purine NAs require intracellular conversion to triphosphate compounds. This process takes place mainly in lymphoid cells that have a high ratio of deoxycytidine kinase to cytosolic 5′-nucleotidase. Once activated, the purine NAs induce cell death by apoptosis.

Several mechanisms have been put forth to explain the antileukemic activity of CdA and fludarabine. One mechanism is the inhibition of DNA synthesis, including replication and repair, leading to an accumulation of DNA strand breaks and activation of the p53 pathway [4,5]. This process leads to the induction of pro-apoptotic genes, release of cytochrome c, and activation of the intrinsic pathway of apoptosis. In addition, purine NAs can promote apoptosis by p53-independent mechanisms. The triphosphate derivatives can bind to proteins located in the mitochondrial membrane, disrupting its permeability and causing the release of cytochrome c and apoptosis inducing factor (AIF) [6]. They also cooperate with cytochrome c and apoptosis protein-activated factor-1 (Apaf-1) to initiate the intrinsic pathway of apoptosis [7,8]. Lastly, purine NAs, mainly fludarabine, inhibit RNA and protein synthesis, which could contribute to their cytotoxicity by reducing the synthesis of proteins that are essential for cell survival [9,10]. The diversity of these effects can explain why purine NAs are efficient towards both proliferating and quiescent cells.

We previously investigated the effect of CdA in EHEB cells, a continuous cell line derived from CLL lymphocytes [11] with low sensitivity to CdA [12]. Analysis of the cell cycle showed that CdA accelerated the progression from G1 to S phase before inducing cell death [12,13]. This cell response to CdA was unexpected because purine analogs are known to activate the transcription factor p53, which results in the accumulation of its target p21, inhibition of cyclin-dependent kinase 2 (Cdk2) and G1/S-phase arrest [5,14].

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As preliminary data indicated a lack of p21 accumulation in EHEB cells treated with CdA in spite of p53 induction [13], we further investigated the p53–p21 axis in the CLL cell lines EHEB and JVM-2. We clearly demonstrate that not only CdA, but also fludarabine and pyrimidine analogs, can induce p21 depletion, while p53 is up-regulated. Furthermore, we show that this decrease in p21 protein upon NA treatment is due to an increased proteasomal degradation, which had already been reported after selected DNA-damaging conditions, particularly UV irradiation [15–18], but never after NAs. We finally focus on the possible impact of this decrease in p21 protein level.

2. Materials and methods

2.1. Materials

CdA was synthesized and supplied by Prof. J. Marchand (Laboratory of Organic Chemistry, Université catholique de Louvain, Louvain-la-Neuve, Belgium). Stock solutions of CdA were prepared in 150 mM NaCl in ethanol (v/v). The nucleoside form of fludarabine (F-Ara-A, referred later to as fludarabine), gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC), cytarabine (1-β-D-arabinofuranosylcytosine, Ara-C), doxorubicin, cycloheximide, carbobenzol-L-leucyl-L-leucyl-L-leucinal (MG-132), N-acetyl-leu-leu-norleucinal (LLnL), histone H1, nutlin-3a, etoposide, and β-actin antibody (A5441) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-fmk) was purchased from Alexis Biochemicals (San Diego, CA, USA). Antibodies other than β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal calf serum (FCS) was from Lonza (Basel, Switzerland). RPMI-1640 and all tissue culture reagents were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). [γ^{32}-P]ATP (10 mCi/ml) was purchased from PerkinElmer (Waltham, MA, USA). The protein A/G sepharose beads were from Oncogene Research Products (Cambridge, MA, USA).

2.2. Cell culture and incubations

The EHEB and JVM-2 cell lines were purchased from DSMZ-German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). Cells were cultured in RPMI-1640 medium with Glutamax supplemented with 10% heat-inactivated FCS at 37 °C in humidified air containing 5% CO_2. Peripheral blood from CLL patients was obtained after written informed consent in accordance with the hospital ethics committee. CLL lymphocytes were isolated and incubated as previously reported [19]. Inhibitors (MG-132, LLnL, Z-VAD-fmk) were added 30 min before NAs. Stock solutions of inhibitors were prepared in DMSO.

2.3. Cell lysis

After incubation, (2–4) × 10^6 cells (or 1 × 10^7 cells for Cdk2 assay) were pelleted by centrifugation at 1500 × *g* for 5 min and washed twice in ice-cold PBS. Cell pellets were resuspended in 70–250 μl of lysis buffer containing 20 mM HEPES (pH 7.9), 150 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA (pH 8), 1% Nonidet P-40, 0.1% SDS, 50 mM NaF, and freshly added protease inhibitors (5 mM benzamidine, 1 mM *p*-toluenesulfonyl fluoride, 5 μg/ml leupeptin and antipain) and 1 mM orthovanadate, and lysed for 20 min on ice. Cell lysates were centrifuged at 16,000 × *g* for 10 min at 4 °C, and the supernatants were stored at −20 °C until use. Protein concentrations were determined by Bradford's method [20], using BSA as the standard.

For proliferating cell nuclear antigen (PCNA) analysis, cell pellets (4 × 10^6 cells) were resuspended in 125 μl buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors) and incubated for 10 min on ice with gentle shaking. After centrifugation at 7200 × *g* for 5 min and removal of the supernatant containing soluble PCNA, the remaining insoluble fraction was solubilized by resuspension in 50 μl buffer B (20 mM HEPES pH 7.9, 50 mM KCl, 400 mM NaCl, 10 mM dithiothreitol, 1% Triton X-100, and protease inhibitors) and incubation for 30 min on ice. The supernatant obtained after centrifugation at 16,000 × *g* for 10 min was used to analyze PCNA monoubiquitination with anti-PCNA antibody that recognizes both PCNA and monoubiquitinated PCNA as described [18,21].

2.4. Western blot analysis

Protein expression was analyzed by Western blot as previously described [19]. Briefly, aliquots of cell lysate (20–30 μg protein) were subjected to SDS-PAGE in 12% (w/v) acrylamide gels and transferred to Immobilon-P Transfer Membranes (PVDF) (Millipore, Billerica, USA). The membranes were blocked in 5% skim milk for 1 h at room temperature and incubated sequentially with a primary antibody (sc-126 for p53, sc-6246 for p21, or sc-56 for PCNA) overnight at 4 °C and a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The protein bands of interest were detected using (advanced) enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK). Membranes were reprobed with β-actin antibody to assess equal loading of protein. Quantitative Western blot data were obtained using ImageJ 1.42q software (http://rsb.info.nih.gov./ij).

For the p21 ubiquitination analysis, 500 μg of protein extract was mixed with 2 μg of anti-p21 antibody (sc-397) on a rocker for 3 h at 4 °C. Immune complexes were captured by protein A/G sepharose beads. After centrifugation and washing with lysis buffer, the beads were boiled for 5 min in 2× Laemmli sample buffer. The supernatant obtained after centrifugation was subjected to SDS-PAGE in 10% (w/v) acrylamide gels. Western blot was performed as described above using anti-ubiquitin (sc-8017) and anti-p21 (sc-6246) as the primary antibodies.

2.5. Cdk2 activity

For each assay, 500 μg of protein extract was subjected to immunoprecipitation with 2 μg of anti-Cdk2 antibody (sc-163) and protein A/G sepharose beads. The beads were washed twice with lysis buffer and twice with kinase buffer (20 mM Tris–HCl pH 7.5, 0.1 mM EGTA pH 7.0, 10 mM $MgCl_2$, and 1 mM dithiothreitol). Kinase reactions were carried out in 30 μl kinase buffer containing 1 μg of histone H1, 20 μM ATP, and 6 μCi of [γ-^{32}P]ATP, as described in [22]. After 30 min at 37 °C, 30 μl of 2× SDS sample loading buffer was added to terminate the reaction. The mixture was boiled for 5 min and centrifuged. Histone H1 was resolved on a 12% SDS-polyacrylamide gel and analyzed by autoradiography. In some experiments, the bands corresponding to histone H1 were revealed by Coomassie blue staining, cut out from the gel, and subjected to counting by Cerenkov radiation.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

Total cellular RNA was prepared using TRIzol (Gibco/Invitrogen). One microgram of RNA was reverse transcribed with random hexamers according to the Fermentas (St. Leon-Rot, Germany) protocol, and the cDNA was subsequently amplified by qPCR. The specific primers for p21 were from Eurogentec (Ougree, Belgium). All reactions were performed in duplicate in MyiQ or iCycler iQ systems (Bio-Rad, Hercules, CA, USA). The amplification was performed as recommended by the manufacturer (Eurogentec, Ougree, Belgium). PCR product specificity and genomic DNA

contamination were verified by melting curve analysis and/or agarose gel electrophoresis. The relative quantification of p21 gene expression was performed using the $2^{-\Delta\Delta CT}$ method [23]. 18S expression was used as an endogenous control to normalize expression within each sample.

2.7. Data analysis

The results of experiments repeated three times in the same conditions are given as the means ± SEM. Significance was estimated by the paired two-tailed Student's *t*-test. Changes were considered significantly different when $P < 0.05$.

3. Results

3.1. CdA reduces the p21 protein level in EHEB cells despite functional integrity of the p53–p21 pathway

First, we analyzed the effect of CdA at concentrations ranging from 1 to 10 μM (i.e. around the IC_{50} of CdA in EHEB cells [12]) after a 24-h incubation (Fig. 1A). In agreement with our preliminary data [13], we observed that CdA reduced the p21 protein level in a dose-dependent manner, this effect being detectable at the lowest concentration tested, whereas p53 was up-regulated. Analysis of the time effect at 10 μM CdA showed that the depletion of p21 did not start immediately after adding the nucleoside (Fig. 1B), but required about 4 h to become apparent. Densitometric quantification showed that p21 depletion could reach 80% after a 24-h incubation with CdA.

To probe the signaling from p53 to p21 in EHEB cells, we analyzed the p21 protein level in cells treated with nutlin-3a, a non-genotoxic agent that activates p53 by antagonizing its negative regulator MDM2 [24,25]. Nutlin-3a up-regulated p53 and increased the p21 protein level in contrast to CdA that induced p21 depletion (Fig. 1C). We also tested the effect of various non-nucleosidic DNA-damaging agents known to activate p53, including UV irradiation, which was shown in several reports to down-regulate p21 regardless of p53 accumulation [15–18]. We found that the p21 level was strongly reduced 8 h after UV irradiation, but increased after incubation with etoposide, a topoisomerase inhibitor, or doxorubicin, an anthracycline (Fig. 1D). These results demonstrate that p21 is able to increase above its basal level in EHEB cells, though certain genotoxic stress conditions elicit p21 depletion.

3.2. p21 depletion is triggered by various nucleoside analogs in the CLL cell lines EHEB and JVM-2

Next, we investigated whether other NAs can induce p21 depletion in EHEB cells. We examined the effect of fludarabine, another purine analog used in CLL. Like CdA, fludarabine caused a marked depletion of p21 protein, which was almost maximal at 5 μM, despite evident accumulation of p53 (Fig. 2A). We then analyzed the effect of the pyrimidine analogs gemcitabine (dFdC) and cytarabine (Ara-C), which are drugs used in the treatment of solid and hematological tumors. Gemcitabine and cytarabine, at concentrations found to be cytotoxic for EHEB cells (unpublished results), also induced dose-dependent p21 depletion despite p53 activation (Fig. 2B).

We then examined if this peculiar effect of NAs might be observed in other cell lines. We selected JVM-2 cells, a CLL cell line with intermediate sensitivity to CdA (IC_{50} ~0.5 μM). CdA, fludarabine (Fig. 2C), gemcitabine, and cytarabine (Fig. 2D) were found to reduce the level of p21 protein in JVM-2 cells, like in EHEB cells, though p53 was activated. In contrast, in lymphocytes freshly isolated from CLL patients, the p21 protein level increased after CdA treatment (Supplementary data, Fig. S1), as reported previously [26].

3.3. Purine analogs promote proteasomal degradation of p21 in CLL cell lines

Impaired p21 accumulation after p53 induction could be due to multiple cellular mechanisms, including a failure to up-regulate mRNA synthesis, as observed after treatment with hydroxyurea or aphidicolin [27], or enhanced proteolysis, as observed in some UV irradiation conditions [15–17].

The influence of purine analogs on p21 gene transcription was analyzed using qPCR. EHEB cells were incubated for 24 h with 10 μM nutlin-3a as a positive control and CdA or fludarabine at two concentrations (Fig. 3). The expression of p21 mRNA was up-regulated by more than 4-fold in cells treated with nutlin-3a and dose-dependently, up to 4-fold, in the presence of CdA or fludarabine. These results demonstrate that the expression of p21 mRNA is enhanced by purine analogs in EHEB cells and,

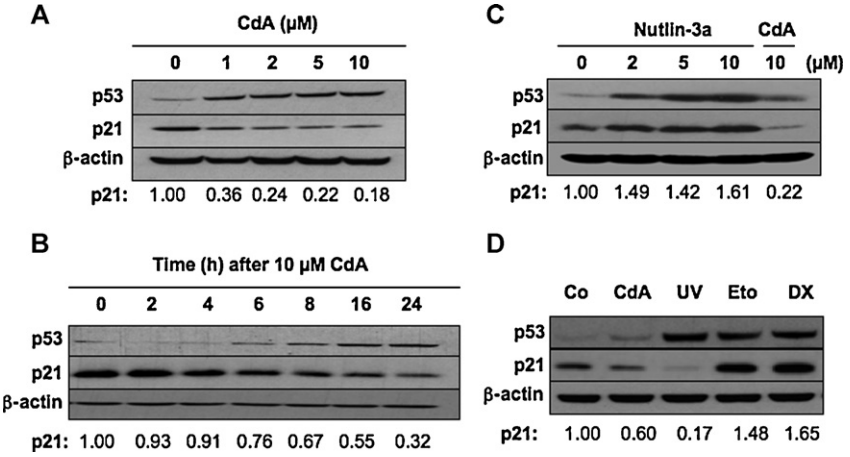

Fig. 1. Comparison of the effects of CdA and other p53 activators on p21 protein level in EHEB cells. (A) Cells were incubated for 24 h in the absence or presence of CdA at the indicated concentrations or (B) for various times with 10 μM CdA. (C) Cells were incubated for 24 h in the absence or presence of nutlin-3a at the indicated concentrations or with 10 μM CdA. (D) Cells were incubated for 8 h without (Co) or with 10 μM CdA, 20 μM etoposide (Eto), or 1 μM doxorubicin (DX), or were UV-C-irradiated (50 J/m^2) before the 8-h incubation. The p53 and p21 protein levels were analyzed by Western blot. β-Actin served as a loading control. Densitometric quantification of p21 normalized to β-actin is given below immunoblots.

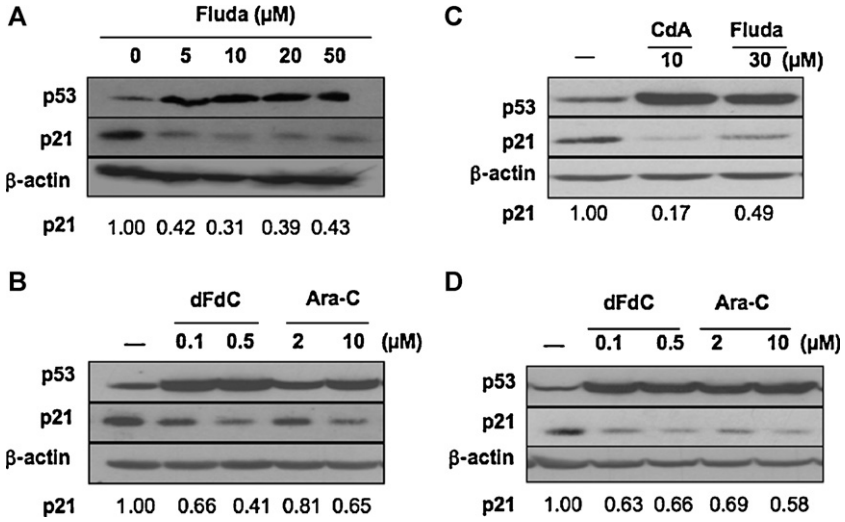

Fig. 2. Effect of various nucleoside analogs on p53 and p21 protein levels in EHEB (A and B) and JVM-2 cells (C and D). (A) EHEB cells were incubated for 24 h in the absence or presence of fludarabine (Fluda) at the indicated concentrations. (B) EHEB cells were incubated for 24 h in the absence (−) or presence of gemcitabine (dFdC) or cytarabine (Ara-C) at the indicated concentrations. (C) JVM-2 cells were incubated for 24 h in the absence (−) or presence of CdA or fludarabine (Fluda) or (D) in the absence (−) or presence of gemcitabine (dFdC) and cytarabine (Ara-C) at the indicated concentrations. The p53 and p21 protein levels were analyzed by Western blot. β-Actin served as a loading control. Densitometric quantification of p21 normalized to β-actin is given below immunoblots.

therefore, that the p21 depletion observed under these conditions does not result from inhibited gene transcription, but rather increased protein degradation.

To verify this hypothesis, we measured the p21 protein half-life. EHEB cells were pre-incubated with or without CdA or fludarabine for 4 h and then treated with 10 μM cycloheximide to prevent new protein synthesis. The p21 protein level, which was analyzed at different time points after the addition of cycloheximide, decreased more rapidly in the presence of CdA or fludarabine than in their absence (Fig. 4A and B), confirming that purine analogs enhance p21 turnover in EHEB cells. Densitometric quantification of p21 allowed us to calculate that the half-life of p21 protein was reduced from approximately 3 h in the absence of NAs to approximately 1 h in the presence of CdA or fludarabine (Fig. 4B).

The p21 protein is known to be regulated by the proteasome system [28]. To verify that NAs promote proteosomal turnover of p21, we analyzed the effect of proteasome inhibitors MG-132 and LLnL on the p21 depletion induced by CdA or fludarabine in EHEB cells. Inhibiting proteasome activity led to an increase in the basal level of p21 protein, but also prevented the depletion of p21 observed after exposure of the cells to CdA or fludarabine (Fig. 5A). The same results were obtained in JVM-2 cells (data not shown).

Because p21 can be cleaved by caspase-3 in cells that undergo apoptosis [29,30], we tested whether this protease plays a role in the NA-induced degradation of p21. Previous work showed that caspase-3 activity increases in EHEB cells after a 24-h incubation with 10 μM CdA [12]. However, the pan-specific caspase inhibitor Z-VAD-fmk was unable to prevent the CdA-induced depletion of p21 (Fig. 5B). All of these data indicate that the depletion of p21 induced by CdA and fludarabine in CLL cell lines results from enhanced degradation through the proteasome pathway.

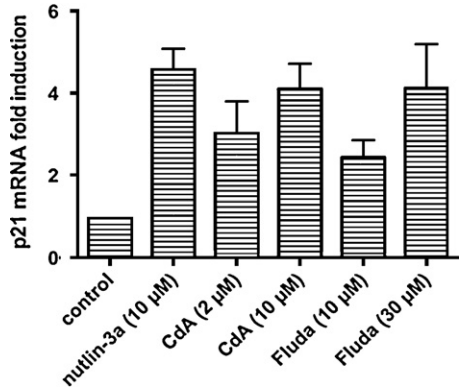

Fig. 3. Analysis of p21 mRNA expression after incubating EHEB cells with nutlin-3a, CdA, and fludarabine. Cells were incubated for 24 h with nutlin-3a, CdA, or fludarabine (Fluda) at the indicated concentrations. The level of p21 expression was assessed by qPCR and normalized against the level of 18S ribosomal RNA. The data (means ± SEM of three independent experiments) are represented as the fold increase in expression relative to untreated cells.

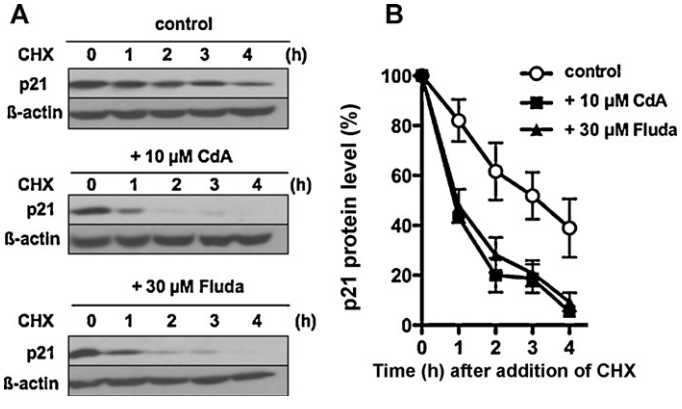

Fig. 4. Influence of CdA and fludarabine on p21 half-life in EHEB cells. (A) Cells were incubated for 4 h in the absence (control) or presence of 10 μM CdA or 30 μM fludarabine (Fluda) before the addition of 10 μM cycloheximide (CHX). The p21 protein levels were then assessed at the indicated time points using immunoblot analysis. β-Actin served as a loading control. (B) The density of the p21 bands obtained in the immunoblots from three independent experiments was quantified and plotted on a graph. The signals obtained at 0 time for untreated and treated cells were not significantly different and set to 100%. Data are presented as means ± SEM.

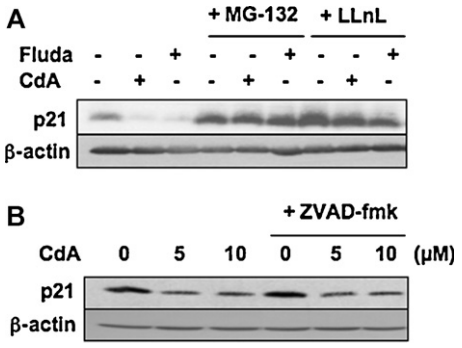

Fig. 5. Influence of proteasome and caspase inhibitors on the CdA and fludarabine-induced p21 decrease in EHEB cells. (A) Cells were pre-treated with 0.5 μM MG-132, 10 μM LLnL, or nothing, and then incubated for 24 h in the absence or presence of 10 μM CdA or 30 μM fludarabine (Fluda). (B) Cells were pre-treated with 20 μM Z-VAD-fmk or nothing, and then incubated for 24 h in the absence or presence of 5 or 10 μM CdA. The p21 protein levels were analyzed by Western blot. β-Actin served as a loading control.

3.4. p21 degradation induced by nucleoside analogs is ubiquitin-independent

The proteasomal degradation of p21 has been reported to occur by both ubiquitin-dependent and ubiquitin-independent pathways [31–34]. Therefore, we analyzed the effect of NAs on p21 ubiquitination by immunoblotting with an anti-ubiquitin antibody after immunoprecipitating p21. We first investigated the effect of 10 μM CdA on p21 ubiquitination in EHEB cells incubated with or without the proteasome inhibitor MG-132. After 8-h incubation with or without CdA, p21 ubiquitination was not detected in the absence of MG-132, even after a long film exposure (Fig. 6A). In contrast, p21 ubiquitination was easily detectable in cells incubated with MG-132 but not further increased by CdA. The effects of CdA, fludarabine, gemcitabine, and cytarabine on p21 ubiquitination in cells incubated with MG-132 are compared in Fig. 6B. Analyses performed after a 5-h incubation showed that none of the NAs increased the level of ubiquitinated p21 compared to control cells. The same results were obtained after 8-h and 16-h incubations (data not shown). These data indicate that NA-induced p21 degradation is ubiquitin-independent.

3.5. p21 depletion induced by nucleoside analogs is associated with Cdk2 activation and PCNA monoubiquitination

The first recognized, and one of the most studied, role of p21 is its ability to induce cell cycle arrest after DNA damage. This effect is primarily mediated through the inhibition of Cdk2, the major kinase that allows progression from G1 to S-phase [35]. Our previous observation that CdA did not block the cell cycle at the G1/S transition in EHEB cells, but promoted the entry of cells into S-phase [12,13], and the present finding that the p21 level was reduced by CdA (Fig. 1) strongly suggested that Cdk2 activity might be enhanced in EHEB cells following CdA treatment. We assessed the Cdk2 activity by measuring its ability to phosphorylate histone H1. As predicted, we observed that CdA induced a time- and dose-dependent (Fig. 7A and B) increase in Cdk2 activity in EHEB cells. We also found that the Cdk2 activity was augmented in EHEB cells incubated for 24 h with fludarabine, gemcitabine, or cytarabine, though to a lesser extent than the change in activity observed with CdA (Fig. 7C). On the contrary, Cdk2 activity was completely abolished in EHEB cells incubated with etoposide, doxorubicin and nutlin-3a, which induced strong p21 accumulation (data not shown). CdA and fludarabine also up-regulated Cdk2 activity in JVM-2 cells (Supplementary data, Fig. S2).

In addition to associating with Cdk2 and other Cdks, p21 can bind to PCNA, a homotrimeric protein complex that forms a ring around double-stranded DNA and plays an essential role in both DNA replication and repair [36,37]. The interaction of p21 with PCNA has been shown to inhibit several PCNA functions in vitro, notably PCNA-dependent replication and the resynthesis step of nucleotide excision repair (NER) [18,38–40]. In line with the latter finding, p21 depletion after UV irradiation has been shown to promote PCNA-dependent DNA repair and suggested to play a critical role in the cellular response to UV-induced DNA damage [15]. Though the role of p21 in NER in vivo is still being debated, recent reports indicate that p21 down-regulation may promote DNA repair by translesion DNA synthesis (TLS), a process that allows DNA lesions to be bypassed [18,40,41]. In particular, p21 was demonstrated to be a negative regulator of PCNA mono-ubiquitination after UV irradiation [18]. This posttranslational modification is essential for the interaction of PCNA with DNA polymerase η, a TLS-specific polymerase that can replicate past damaged templates and trigger TLS [42]. Therefore, we sought to analyze whether the NA-induced depletion of p21 might elicit PCNA monoubiquitination. Monoubiquitinated PCNA can be detected by SDS-PAGE as a slowly migrating form of PCNA, the molecular weight of which is increased by approximately 10 kDa, corresponding to a single ubiquitin bound to PCNA [21]. In accordance with our hypothesis, we found that treatment with CdA induced a higher molecular weight form of PCNA in a time- and

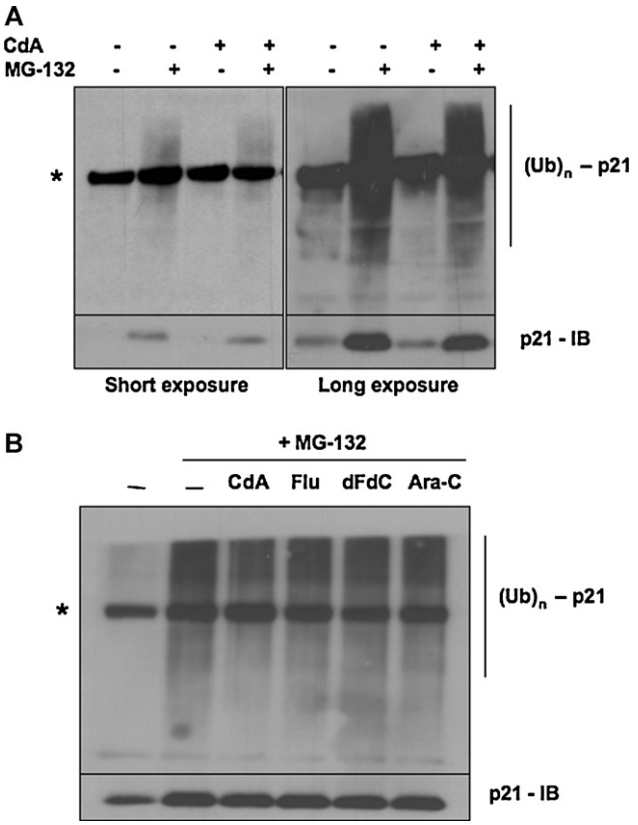

Fig. 6. Influence of nucleoside analogs on p21 ubiquitination in EHEB cells. (A) Cells were pre-treated with 0.5 μM MG-132 or nothing, and then incubated for 8 h in the absence or presence of 10 μM CdA, as indicated. p21 was immunoprecipitated and the level of its ubiquitination analyzed using anti-ubiquitin antibody. Immunoprecipitated p21 protein was immunoblotted as an input control. Two exposure times are compared. (B) Cells were left untreated (–) or were pre-treated with 0.5 μM MG-132 and then incubated for 5 h in the absence (–) or presence of 10 μM CdA, 30 μM fludarabine (Flu), 0.5 μM gemcitabine (dFdC), or 10 μM cytarabine (Ara-C). p21 ubiquitination was analyzed as described in (A). *Immunoglobulin heavy chain bands.

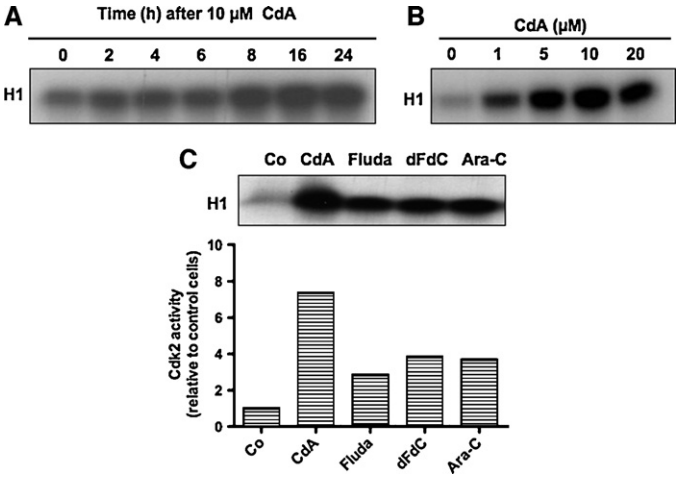

Fig. 7. Influence of nucleoside analogs on Cdk2 activity in EHEB cells. (A) Cells were incubated for various times in the presence of 10 μM CdA or (B) for 24 h in the presence of increasing concentrations of CdA. (C) Cells were incubated for 24 h in the absence (Co) or presence of 10 μM CdA, 30 μM fludarabine (Fluda), 0.5 μM gemcitabine (dFdC), or 10 μM cytarabine (Ara-C). Cdk2 activity was analyzed using histone H1 as the substrate. The radioactivity of histone H1 was detected by autoradiography after SDS-PAGE. In panel C, the bands corresponding to histone H1 were also cut out from the gel and counted, allowing quantification of Cdk2 activation (means of duplicate experiments).

dose-dependent manner (Fig. 8A and B), indicating that PCNA was monoubiquitinated. Similarly to CdA, fludarabine, gemcitabine, and Ara-C also caused PCNA monoubiquitination (Fig. 8C). In contrast, PCNA was not monoubiquitinated after exposing the cells to nutlin-3a or etoposide. The same results were observed in JVM-2 cells (Fig. 8D). We verified in additional experiments that PCNA was also monoubiquitinated after UV-irradiation (50 J/m^2) consistently with previous reports [18,21], but not after doxorubicin (1 μM), in both EHEB and JVM-2 cells (data not shown).

4. Discussion

The present study demonstrates that CdA as well as other NAs can down-regulate p21 in CLL cell lines, which constitutes a still unexplored response to this class of chemotherapeutic agents. The down-regulation of p21 by CdA in EHEB cells was unexpected because CdA and also fludarabine have previously been shown to induce p21 accumulation in response to p53 activation in various cell types, including freshly isolated CLL lymphocytes [22,26,43–45]. However, our reproducible results with four different NAs in two different cell lines show that this p21 depletion is not a fortuitous effect. Therefore, NAs can be added to the list of genotoxic agents that, as recently emphasized by Soria and Gottifredi [46], do not comply in some cell models with the dogma stating that DNA damage activates p53 to trigger p21 accumulation. Among these agents are UV-irradiation, cisplatin, and methyl methane sulfonate, which all block replication forks [46], but also ionizing radiation, which induces DNA double-strand breaks [47].

Since p21 mRNA is clearly increased after NA treatment, NA-induced p21 depletion cannot result from a failure to up-regulate p21 transcription. Instead, as shown by the results obtained in the presence of proteasome inhibitors, NAs appear to promote the degradation of p21 via the proteasome pathway. Proteasomal p21 degradation can occur through both ubiquitin-dependent and independent pathways, under basal as well as stress conditions [40,46,48,49]. Although the pathway implicated in the UV-induced p21 depletion is debated [15,16], this process is now thought to be ubiquitin-dependent because it has been shown to require CRL4^{Cdt2} E3 ubiquitin ligase activity [50,51]. We were not able to detect increased p21 ubiquitination after incubating the cells with NAs, suggesting that NA-induced proteasomal p21 degradation occurs via an ubiquitin-independent pathway. This could be mediated by direct interaction between p21 and the C8α-subunit of the 20S proteasome [32], a process that can been promoted by MDM2 and/or MDMX [52–54], as well as by 14-3-3τ as recently discovered [55]. Phosphorylation also is known to regulate the stability of the p21 protein [56]. Accordingly, UV-induced p21 proteolysis appears to be mediated by phosphorylation at Ser114 by the kinase GSK-3β activated by the ATR signaling pathway [17]. In our study, we did not observe any effect of various ATR or GSK-3β inhibitors on NA-induced p21 degradation (data not shown). Therefore, the signal pathway by which NAs trigger proteasomal p21 degradation remains to be elucidated.

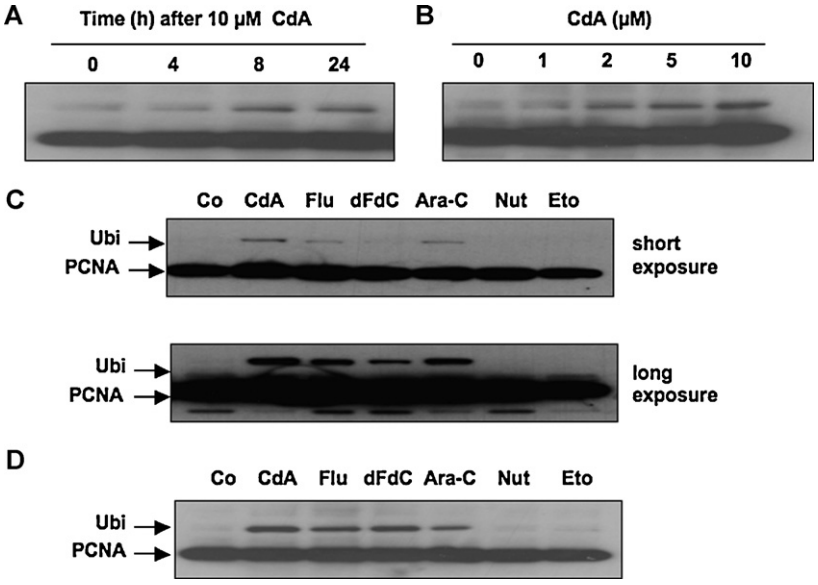

Fig. 8. Influence of nucleoside analogs and other p53 activators on PCNA monoubiquitination. (A) EHEB cells were incubated for various times in the presence of 10 μM CdA or (B) for 24 h in the presence of CdA at the indicated concentrations. (C) EHEB cells or (D) JVM-2 cells were incubated for 24 h in the absence (Co) or presence of 10 μM CdA, 30 μM fludarabine (Flu), 0.5 μM gemcitabine (dFdC), 10 μM cytarabine (Ara-C), 10 μM nutlin-3a (Nut), or 20 μM etoposide (Eto). After Triton extraction, PCNA was analyzed by Western blot using anti-PCNA antibody. In (C), two exposure times are shown to confirm the lack of PCNA monoubiquitination after nutlin-3a or etoposide treatment.

As a rule, the cell cycle is arrested after DNA damage as the result of p53 induction, transcription of its target gene p21, accumulation of the p21 protein, and the inhibition of Cdk2 activity [35]. Accordingly, fludarabine has been shown to inhibit Cdk2 in ML-1 cells following induction of p53 [22]. In contrast, our results consistently showed that NAs, particularly CdA, increase Cdk2 activity in CLL cell lines. Nevertheless, even if p21 depletion releases a block [57], we cannot currently attest that this event is enough to trigger Cdk2 activation. Indeed, a number of other factors could be involved in the activation of Cdk2, including changes in the phosphorylation state or in the binding to cyclins or p27 [58]. Studies are in progress to examine which event really determines NA-induced Cdk2 activation in our cell models. The physiological implication of Cdk2 activation by NAs also remains to be elucidated. This activation likely explains our previous observation that CdA promotes G1/S progression in EHEB cells [12,13], but a role in apoptosis [59] or DNA repair [60] cannot be excluded.

In addition to its role in cell cycle control, p21 can assume both pro- or anti-apoptotic functions in response to anti-tumor agents, depending on the cell type and cellular context (reviewed in [61]). Previous studies have shown that HCT116 cells, in which the p21 gene has been deleted through homologous recombination, are ~4-fold less sensitive to CdA than wild-type HCT116 cells [43]. These data suggest that the NA-induced depletion of p21 in CLL cell lines may contribute to decrease cell sensitivity to NAs. Our observation that p21 depletion is accompanied by PCNA monoubiquitination supports this hypothesis. Indeed, PCNA monoubiquitination stimulates the recruitment of polymerase η, a polymerase involved in DNA damage tolerance at stalled DNA replication forks. This recruitment promotes TLS, a process that avoids the replication block during S-phase and favors DNA repair and cell survival. TLS has been consistently implicated as a potential mechanism of resistance to cisplatin treatment [62]. More recently, studies conducted with polymerase η-deficient fibroblasts have shown that these cells are more sensitive to chemotherapeutic agents, including gemcitabine and cytarabine, indicating that polymerase η, and thus TLS, can indeed modulate cell sensitivity to NAs [63]. Further work is needed to determine whether PCNA monoubiquitination could play a role in the clinical resistance to NAs, an almost universal phenomenon in patients suffering from CLL.

To conclude, we identified two distinct cellular stress reactions in response to DNA damaging agents in the CLL cell lines EHEB and JVM-2 with on one hand, p21 depletion, Cdk2 activation and PCNA monoubiquitination triggered by NAs, and on the other hand p21 accumulation, Cdk2 inhibition and no change in PCNA monoubiquitination after etoposide and doxorubicin. These opposite patterns of cell behavior towards p53 inducers might be related to the different nature of DNA lesions. Importantly, potential discrepancies in p53 and p21 protein up-regulation should be taken into account in the assays that measure p21 protein expression as a tool to probe the functionality of the p53 pathway [64].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.12.009.

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