ELSEVIER

Biochemical Pharmacology 65 (2003) 121–129

Biochemical Pharmacology

Influence of p53 and p21^{WAF1} expression on sensitivity of cancer cells to cladribine

Carlos María Galmarini*, Nathalie Voorzanger, Nicole Falette, Lars Jordheim, Emeline Cros, Alain Puisieux, Charles Dumontet

INSERM 453. 8, Avenue Rockefeller, 69373 Lyon Cedex 08, France

Received 19 March 2002; accepted 27 August 2002

Abstract

The present study was performed to gain insight into the role of p53 and p21^{WAF1} on the cytotoxicity of the purine analogue cladribine (2-CdA) on cancer cells. Drug sensitivity, cell cycle distribution and drug-induced cell death were compared in three lines derived from the colorectal carcinoma HCT116: the p53+/+ cell line containing wild-type p53 and the p53−/− and p21^{WAF1}−/− lines, in which both alleles of *p53* or *p21^{WAF1}* were deleted by homologous recombination, respectively. p53−/− and p21^{WAF1}−/− cells were significantly more resistant to the cytotoxic effects of 2-CdA than the p53+/+ cells. p53+/+ cells and p21^{WAF1}−/−, but not p53−/− cells, displayed wt-p53 protein accumulation and arrested in S-phase after exposure to 2-CdA. mRNA analysis of the transporter hENT1 and of enzymes involved in drug metabolism did not show alterations which might explain a drug-resistant phenotype in the p53−/− or p21^{WAF1}−/− cells. Exposure of p53+/+ cells to 2-CdA resulted in expression of p21^{WAF1} mRNA and protein, enhanced expression of uncleaved PARP-1, and a higher degree both of apoptosis and necrosis than in p53−/− and p21^{WAF1}−/− cells exposed to 2-CdA. Addition of the specific PARP-1 inhibitor 3-AB to 2-CdA-treated cells rendered p53+/+ cells resistant to this drug. Bax levels were reduced in the p53−/− while they increased in the p53+/+ line and remained stable in the p21^{WAF1}−/− cells. We conclude that p53 and p21^{WAF1} status of cancer cells influences their sensitivity to 2-CdA cytotoxicity. This may involve alterations in the apoptotic cascade as well as in PARP-1-dependent cell death.

Keywords: Protein p53; Drug resistance; Antimetabolites; Cell death; Cladribine; Apoptosis

1. Introduction

2-Chloro-2′-deoxyadenosine (2-CdA[1]) [1], has achieved an important role in the clinic [2,3]. This compound is a deoxyadenosine analogue with one chlorine substitute for the hydrogen atom in the 2′-position of the purine ring. This modification makes the compound resistant to deamination by adenosine deaminase. 2-CdA activity is dependent on the formation of a triphosphorylated metabolite (2-CdATP) that is subsequently incorporated into DNA, thereby inducing DNA damage [4] and cell death [5].

2-CdA enters cells via a nucleoside transporter system and is essentially phosphorylated into 2-CdAMP by dCK [6]. It is subsequently phosphorylated to its active 5′-triphosphate form, 2-CdATP, by the tandem action of AMP kinase and nucleoside diphosphate kinase [7]. Inactivation of 2-CdA can occur by dephosphorylation of 2-CdAMP back to the nucleoside by a cytosolic 5′-nucleotidase activity [8].

2-CdATP inhibit DNA synthesis by incorporation into the A sites of the growing DNA strand [9,10] inducing an S phase-specific apoptosis [11]. 2-CdA metabolites also inhibit DNA replication indirectly through their inhibitory action on ribonucleotide reductase causing a subsequent reduction of the dNTP pools required for DNA synthesis [10]. It was also shown that 2-CdA interferes with the proper repair of DNA strand breaks, which activate PARP-1 with consequent NAD+ depletion leading to cell death [12].

* Corresponding author. Tel.: +33-478-7772-36; fax: +33-478-7770-88.
E-mail address: fgalma@rockefeller.univ-lyon1.fr (C.M. Galmarini).

Abbreviations: 2-CdA, cladribine; 3-AB, 3-aminobenzamide; AFC, 7-amino-4-trifluoromethyl coumarin; cN-II, "high-Km" 5′-nucleotidase; dCK, deoxycytidine kinase; DEVD, caspase 3-specific Asp-Glu-Val-Asp tetrapeptide; dNTP, deoxynucleotide triphosphate; hENT1, human equilibrative nucleoside transporter 1; IC_{50}, inhibitory concentration 50; RNR, ribonucleotide reductase; RNR-M2, ribonucleotide reductase M2 subunit; mtPTP, mitochondrial permeability transition pore; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PARP-1, poly-(ADP-ribose) polymerase; PI, propidium iodide.

0006-2952/02/$ – see front matter © 2002 Published by Elsevier Science Inc.
PII: S0006-2952(02)01448-X

Resistance to 2-CdA has been mainly ascribed to an insufficient intracellular concentration of 2-CdA triphosphates because of reduced levels of the activating enzyme dCK [13], or to an increased degradation by increased 5′-nucleotidase activities [8,14]. However, the role of genes regulating apoptosis such as *p53* or cell cycle arrest such as $p21^{WAF1}$ has not yet been clearly established.

After treatment with DNA damaging agents, *p53* is up-regulated and blocks the cell cycle at the G1/S checkpoint or leads to apoptosis of the damaged cells. The G1/S phase arrest involves activation of $p21^{WAF1}$ [15], which functions by associating with cyclin–CDK complexes. Induction of apoptosis by *p53* involves anti-apoptotic genes such as *Bcl-2* and pro-apoptotic genes such as *Bax* [16]. Tumor cells with inactivated *p53* have generally displayed a greater degree of resistance to DNA toxic agents [17]. This resistance can be explained by abolished G1/S checkpoints and reduced sensitivity to apoptosis.

Previous work has demonstrated the role of functional p53 protein in cellular response to 2-CdA treatment in non-dividing chronic lymphocytic leukemia cells [18]. The present study was designed to investigate whether inactivation of p53 and $p21^{WAF1}$ proteins affects sensitivity to 2-CdA cytotoxicity in dividing cells, and to determine their role in the downstream biochemical signals that lead to biological effects induced by 2-CdA.

2. Material and methods

2.1. Reagents

Stock solutions of cladribine (Leustatine®) were prepared in distilled water and fresh dilutions were prepared before each experiment. Propidium iodide, MTT, 3-AB and antibodies against β-actin were purchased from Sigma. Antibodies against Bax (B-9) and $p21^{WAF1}$ (sc-187) were purchased from Santa Cruz Biotechnology Inc.; antibodies against p53 (DO7) and Bcl-2 were purchased from DAKO. Antibodies against PARP-1 were purchased from Boehringer Mannheim; this antibody recognizes full length PARP-1 and the large kDa PARP-1 fragment (89 kDa) generated by caspases. Peroxidase-conjugated secondary antibodies were purchased from Covalab. Enhanced chemiluminescence Western blot detection reagents (ECL system) were purchased from Amersham.

2.2. Cell lines

HCT116 cells are derived from a human colorectal carcinoma. HCT116 p53+/+ (p53+/+) cells contain wt-p53 and $p21^{WAF1}$ while in HCT116 p53−/− (p53−/−), both alleles of p53 have been deleted through homologous recombination [19]; in HCT116 $p21^{WAF1}$−/− cells, $p21^{WAF1}$ genes have been deleted through homologous recombination [19] but this cell line contains wt-p53. All cell lines were maintained in monolayer cultures on 75 cm^2 flasks in Dulbecco's minimum Essential medium containing 10% fetal calf serum, 1% L-glutamine and 2% penicillin–streptomycin. All cell lines were kindly provided by Vogelstein.

2.3. Cytotoxicity assays

Cell viability was determined using the MTT assay as previously described [20]. Briefly, asynchronously growing cells were transferred into 96-well cultures plates (Costar®, Corning Inc.) in 100 μL of medium with a final cell concentration of 3×10^3 cells per well and incubated in media for 24 hr. Corresponding drug concentrations were then added to each plate. After 72 hr of drug exposure, 20 μL of MTT reagent (5 mg/mL) was added to each well. Cell viability was expressed as the percent of absorbance of treated wells relative to the untreated control wells. The IC_{50} was defined as the drug concentration resulting in 50% loss of cell viability relative to untreated cells. Assays were performed in triplicate in at least three separate experiments. Based on this information, a concentration of 325 nM 2-CdA (IC_{75} median value of p53+/+ cells [IC95%: 136–457]) was chosen for drug exposure experiments. For PARP-1 inhibition experiments, p53 cells were treated with 2-CdA as previously described with or without 3-AB (10 mM; $\cong IC_{25}$ for 3-AB on HCT116 cell cultures in the absence of 2-CdA) for 72 hr and cell viability was detected by MTT.

2.4. rt-PCR and quantitative real-time PCR

The level of mRNA expression of biological factors involved in 2-CdA resistance was assessed by quantitative real-time PCR at baseline in all cell lines. Cellular RNA extraction and cDNA synthesis were performed as previously described, in a Perkin-Elmer 9600 thermal cycler [8]. Quantitative real-time PCR was performed in a Light-cycler detection system (Roche) as previously described [21]. Briefly, cDNA (5 μL) was mixed with primers (300 nM each), LightCycler-FastStart DNA Master SYBR Green I (Roche) (hENT1 and RNR-M2) or LightCycler-FastStart DNA master hybridization probes (Roche) (18S, dCK and cN-II), and probes (130 nM; if necessary) in a total volume of 20 μL for 40 cycles. Primers and probes sequences are published elsewhere [21]. The data was expressed as C_t, which is the PCR cycle number at which the accumulated fluorescent signal in each reaction crosses a threshold above background. Mean C_t values were then normalized to the expression level in reference to a 18S ribosomal RNA: ΔC_t: sample mean C_t − control mean C_t. The results were then expressed as $2^{-(\Delta C_t)}$. For each sample, a ratio between the studied gene $2^{-(\Delta C_t)}$ values and 18S ribosomal $2^{-(\Delta C_t)}$ values were calculated and considered as final amount of mRNA. All samples were analyzed in three separate experiments.

2.5. Flow cytometric detection of cell cycle and apoptosis

For analysis of DNA content and cell cycle distribution, unfixed cells were treated with 2-CdA (325 nM) for 24 hr. After drug-exposure, 10^6 cells/mL were resuspended in 2 mL of PI solution (50 μL/mL), incubated at 4° overnight and then analyzed by flow cytometry [22]. For apoptosis and necrosis determination, we used the Annexin-V-Fluos Staining Kit (Boehringer Mannheim). Briefly, 10^6 cells/mL cells were incubated with Annexin-V-fluorescein in a HEPES buffer containing PI for 10 min and analyzed by flow cytometry. Annexin-V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phospholipids. This protein can hence be used as a sensitive probe for phospholipid exposure upon the outer leaflet of the cell membrane and is therefore suited for apoptosis detection. The simultaneous application of PI which is used for dye exclusion tests allows the discrimination of necrotic cells (Annexin-V positive, PI positive stained cell cluster) from the apoptotic cells (Annexin-V positively, PI negative stained cell cluster) [23].

Flow cytometry was performed on a FACScalibur (Becton Dickinson). For determination of apoptotic and necrotic fractions, analysis was performed using CellQuest™ software (Becton Dickinson). Cell cycle distribution and DNA ploidy status were calculated after exclusion of cell doublets and aggregates on a FL2-area/FL2-width dot plot using Modfit LT 2.0™ software (Verity Software Inc).

2.6. Northern blots

For Northern blot analysis, total RNA was extracted using Tri-Reagent (Sigma). This procedure is an improvement of the single-step method reported by Chomczynski and Sacchi for total RNA isolation [24]. RNA samples (10 μg) were separated by electrophoresis through a denaturing formaldehyde agarose gel and transferred to nylon membranes (Hybond-N+; Amersham). Membranes were labeled with $p21^{WAF1}$ cDNA probe.

2.7. Western blots

Protein expression was determined by Western blot analysis in untreated cells and after 24 hr incubation with 2-CdA (325 nM). Briefly, protein was extracted from cells with cold lysis buffer (20 mM Tris–HCl pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP40, soybean trypsin inhibiteur (STI) 1 mg/mL, leupeptin 100 μg/mL, aprotinin 100 μg/mL, benzamidine 30 mg/mL, *N*-Tosyl-L-phenylalanine chloromethyl (TPCK) 1 mg/mL and phenylmethylsulfonyl potassium (PMSK) 5 mg/mL). Equal amounts of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using gels of 8% (for PARP-1) or 12% acrylamide, and transferred onto nitro-cellulose membrane (Hybond-ECL, Amersham Corp.). The blots were incubated with appropriate dilution of primary antibody (DO7, 1/1000; sc-187, 1/5000; Bcl-2, 1/500; B-9, 1/500; PARP-1, 1/2000), followed by incubation with peroxidase-conjugated secondary antibody. Protein signals were detected by chemiluminiscence and exposure to Kodak film (Eastman Kodak Company). Horizontal scanning densitometry was performed by utilizing the Kodak Digital Science 1D image analysis software. Adequate protein loading and blotting was controlled by staining of membranes with Rouge Ponceau, as well as immunoblotting with an anti-actin antibody (data not shown).

2.8. Assays for caspase 3 activity

Determination of activation of caspase 3 at baseline and after 24 hr of 2-CdA incubation were performed as previously described [25]. Untreated and treated cells were resuspended in the lysis buffer (5× buffer Cold Spring Harbor (CSH), Triton 0.01%, orthovanadate 1× and protease inhibitor 1×) at 4° for 30 min and centrifuged at 4° for 15 min at 13,000 *g*.

The DEVD tetrapeptide labeled with a fluorogenic substrate (AFC), DEVD-AFC, and the inhibitor (DEVD-CHO) (Tebu) were used at 50 μmol/L. For each cell lysate three measures were performed using three groups: the negative control: cell lysates were preincubated at 30° for 3 hr with DEVD-CHO in the reaction buffer (50 mmol/L HEPES, 10% sucrose, 0.1% CHAPS (pH, 7.5)) and 10 mmol/L dithiothreitol (dTT) before addition of the DEVD-AFC substrate; the blank group: DEVD-AFC substrate incubated in the reaction buffer only at 30° for 1 hr; the assay group: cell lysates were directly incubated 1 hr at 30° with DEVD-AFC substrate in the reaction buffer and dTT.

Fluorescence measurements were performed after 1 (t_0) and 2 hr (t_{60}) of incubation. Caspase 3 activity was detected by AFC release monitored on a spectrofluorometer (Kontron Analytical SFM25) using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase 3 activity was calculated with the following formula: $1\ \text{unit} = (\text{dFU/min}) \times (\text{calibration curve slope})^{-1} \times (1\ \text{U/L} \times 10^{-6}\ \text{mmol AFC/min})$, where dFU is the difference of fluorescence units: (FU of the assay group at t_{60} − FU of the blank group at t_{60}) − (FU of the assay group at t_0 − FU of the blank group at t_0).

3. Results

3.1. Detection of p53 protein by Western blot

We analyzed p53 expression after treatment with 2-CdA (325 nM) by Western blot at different time intervals. In p53+/+ and $p21^{WAF1}$−/− cells, we observed significant wt-p53 accumulation in a time-dependent manner after drug treatment with maximal effects obtained after 18 hr

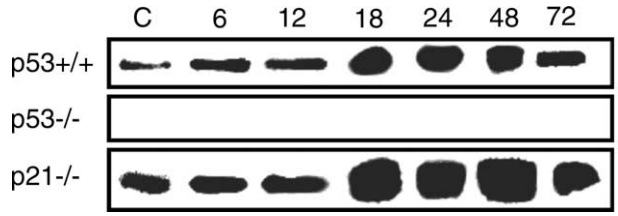

Fig. 1. Kinetics of p53 protein induction by 2-CdA in HCT116 cells. Cells were exposed to 2-CdA (325 nM) for different time intervals (6–72 hr). Western blots were performed on 50 μg of total cell protein with DO7 anti-p53 antibody. p53+/+: HTC116 p53+/+; p53−/−: HCT116 p53−/−; p21: HCT1116 $p21^{WAF1}$−/−.

exposure to 2-CdA (Fig. 1). p53 protein accumulation was detected up to 72 hr after exposure to 2-CdA. These data indicate that 2-CdA can initiate p53 induction in tumor cells. In p53−/− cells, no p53 protein expression was detected (Fig. 1).

3.2. Detection of $p21^{WAF1}$ by Northern and Western blots

We then examined whether 2-CdA induced $p21^{WAF1}$ expression at the mRNA and protein level. For this purpose, we analyzed $p21^{WAF1}$ mRNA expression by Northern blot and protein expression by Western blot in HCT116 cells treated with 2-CdA (325 nM) at different time intervals. In p53+/+ cells, drug treatment led to an increase in $p21^{WAF1}$ mRNA expression (Fig. 2A), with maximum levels after 6 hr of exposure to 2-CdA. At the protein level (Fig. 2B), maximal $p21^{WAF1}$ expression was detected at 48 hr after drug exposure. The kinetics of $p21^{WAF1}$ mRNA and protein expression were thus correlated. The delay between $p21^{WAF1}$ RNA induction and $p21^{WAF1}$ protein accumulation may be related to an earlier beginning of

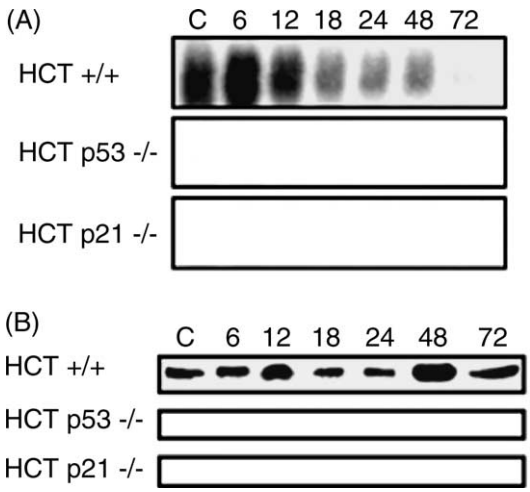

Fig. 2. Effect of 2-CdA on $p21^{WAF1}$ mRNA (A) and protein (B) expression of HCT116 cell lines. $p21^{WAF1}$ RNA levels were determined by Northern blotting and protein levels by Western blotting in cells exposed to 2-CdA (325 nM) at different time intervals up to 72 hr. Data shown are representative of three experiments. p53+/+: HTC116 p53+/+; p53−/−: HCT116 p53−/−; p21: HCT1116 $p21^{WAF1}$−/−.

Table 1
Chemosensitivity of HCT116 cell lines exposed to graded concentrations of 2-CdA for 72 hr (50% inhibitory concentration (IC_{50}) values[a] were obtained from dose response curves assessed by MTT assay)

Drug (nM)	p53+/+[b]	p53−/−	p21−/−
2-CdA	31.7 ± 16	520 ± 368	141 ± 52

[a] IC_{50} values are means of three separate experiments each of which was performed in triplicate; mean ± SD.
[b] p53+/+, HCT116 p53+/+; p53−/−, HCT116 p53−/−; p21−/−, HCT1116 $p21^{WAF1}$−/−.

mRNA synthesis. As expected, there was no detectable $p21^{WAF1}$ induction in cells lacking either p53 or $p21^{WAF1}$.

3.3. Effect of p53 and $p21^{WAF1}$ status on chemosensitivity to 2-CdA

To determine sensitivity of HCT116 cells to 2-CdA, cell lines were incubated with this drug and the effects on cell growth were measured using the MTT assay, as described previously. As shown in Table 1, p53−/− cells were 16.4-fold more resistant to 2-CdA than p53+/+ cells. Similarly, $p21^{WAF1}$−/− cells were 4.4-fold more resistant to 2-CdA than p53+/+ cells.

3.4. Analysis of factors involved in 2-CdA metabolism and drug targets by rt-PCR

To test whether resistance of p53−/− and $p21^{WAF1}$−/− cells was due to different mechanisms associated with 2-CdA resistance, we analyzed by quantitative real-time PCR the mRNA levels of biological factors involved in 2-CdA metabolism and drug targets in the absence of drug exposure. As shown in Fig. 3, hENT1 transporter, dCK, and cN-II were highly increased in $p21^{WAF1}$−/− cells. p53−/− cells expressed lower levels of dCK while there were no major differences in the mRNA expression of hENT1 and

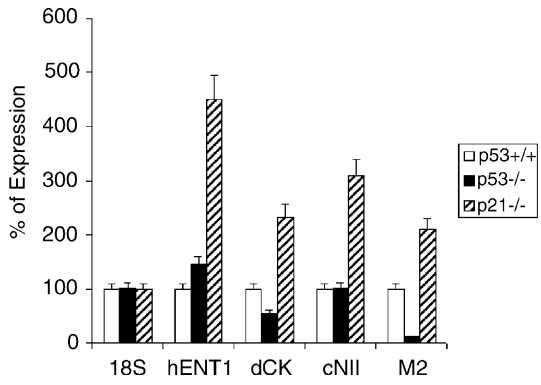

Fig. 3. Analysis of mRNA expression of biological factors possibly involved in resistance to 2-CdA by quantitative real-time PCR. 18S: 18S ribosomal RNA; ENT1: human equilibrative nucleoside transporter 1; dCK: deoxycytidine kinase; cN-II: high Km 5′-nucleotidase; M2: ribonucleotide reductase M2 subunit. Quantitative rt-PCR was performed as described in material and methods. p53+/+: HTC116 p53+/+; p53−/−: HCT116 p53−/−; p21: HCT1116 $p21^{WAF1}$−/−.

Table 2
Analysis of cell cycle distribution and apoptosis in HCT116 cells by flow cytometry before and after 24 hr treatment with 2-CdA[a]

	p53+/+		p53−/−		p21−/−	
	Control	24 hr	Control	24 hr	Control	24 hr
G1	61.7 ± 10	43.3 ± 1.4	51.2 ± 14.9	63.3 ± 3.4	63.9 ± 6.4	38.6 ± 9.9
S	30 ± 8.7	56.4 ± 1.3	37.7 ± 12.2	36.1 ± 2.9	30.4 ± 5.5	61.4 ± 9.9
G2/M	8.1 ± 2.7	0.16 ± 0.2	10.8 ± 2.1	0.45 ± 0.8	5.4 ± 1.1	0.2 ± 0.4

[a] The reported values (mean ± SD, N = 3) are expressed as percentage of total cells. Cells were treated with 2-CdA (325 nM).

cN-II between p53−/− cells and p53+/+ cells. Analysis of RNR-M2 (fraction containing the enzyme activity) showed a higher level of mRNA expression in $p21^{WAF1}$−/− cells than in p53+/+ cells (Fig. 3). In contrast, p53−/− cells expressed almost no RNR-M2 in comparison to p53+/+.

3.5. Detection of 2-CdA-induced cell cycle alterations

Flow-cytometric cell cycle analysis of HCT116 cells demonstrated that all cell lines were diploid with a moderate proliferative index (Table 2). After 24 hr of continuous exposure to 2-CdA, a substantial accumulation of p53+/+ cells in S-phase was observed with a decrease in the G0/G1 and G2/M fractions. Similarly, after 24 hr incubation with 2-CdA, $p21^{WAF1}$−/− cells arrested in S-phase while the G0/G1 and G2/M fractions were reduced. Conversely, no S-phase arrest was observed in the p53−/− cells after drug treatment (Table 2). These results suggest that the arrest in S-phase due to 2-CdA requires the presence of p53 protein, but not $p21^{WAF1}$ protein.

3.6. Comparison of apoptotic and necrotic levels after exposure to 2-CdA

To test whether the deficiency of p53 and $p21^{WAF1}$ led to defective apoptotic and necrotic pathways in 2-CdA exposed cells, we used the Annexin-V flow-cytometric approach. Drug exposure caused an strong increase of Annexin-V staining typical of apoptosis in p53+/+ cells (Fig. 4, Table 3). However, the amount of apoptotic cells increased slightly in p53−/− and $p21^{WAF1}$−/− cells after exposure to 2-CdA (Table 3). Similarly, the proportion of necrotic cells after 2-CdA exposure was higher in the p53+/+ cells than in the p53−/− and $p21^{WAF1}$−/− cells. These results demonstrated that cells lacking p53 and $p21^{WAF1}$ were less susceptible to 2-CdA-induced apoptosis and necrosis than p53+/+ cells.

3.7. Analysis of apoptotic pathways

Expression of Bax and Bcl-2 proteins were examined in the three lines after a 24 hr exposure to 2-CdA. At baseline, Bax expression was slightly decreased in p53−/− cells and increased in $p21^{WAF1}$−/− cells in comparison to p53+/+ cells (Fig. 5). After exposure to 2-CdA Bax protein expression strongly increased in p53+/+ cells while the expression of this protein was strongly reduced in p53−/− cells and remained stable in the $p21^{WAF1}$−/− line. Bcl-2 expression levels increased after drug treatment in the three lines.

Caspase 3 activity was similar in the three lines at baseline, and no increase in caspase 3 activity was observed in any line after exposure to 2-CdA (Table 3). Analysis of PARP-1 expression showed an increase in the expression of this protein after exposure to 2-CdA in the three lines (Fig. 6). However, there was no PARP-1 cleavage, consistently with the lack of increase of caspase 3

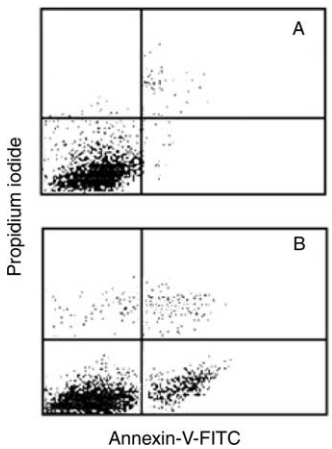

Fig. 4. Induction of apoptosis by 2-CdA treatment in HCT116 p53+/+ cells. Cells were incubated in the presence (B) or absence of 2-CdA (A) (325 nM) for 24 hr and apoptosis was measured using the Annexin-V staining kit as described in Section 2. The simultaneous application of Annexin-V-FITC and propidium iodide (PI) allows the discrimination of apoptotic cells (Annexin-V positive, PI negative stained cell cluster) from the necrotic cells (Annexin-V positive, PI positive stained cell cluster).

Table 3
Apoptotic and necrosis percentage and caspase 3 activity in HCT116 cells exposed 2-CdA

	Apoptosis[a] (%)	Necrosis[a] (%)	Caspase 3 (pmol/min)
p53+/+ (Con)[b]	0.9 ± 0.05	3 ± 0.2	28.8 ± 15.8
p53+/+ (2-CdA)	18 ± 5	9.2 ± 0.9	29.9 ± 19
p53−/− (Con)	3.3 ± 0.5	1.1 ± 0.1	43.9 ± 23.2
p53−/− (2-CdA)	5.3 ± 0.5	3 ± 0.2	36.6 ± 21
p21−/− (Con)	3 ± 1	1.6 ± 0.4	24.9 ± 13.7
p21−/− (2-CdA)	7 ± 2.6	3.1 ± 0.9	24.7 ± 12.3

[a] As measured by the flow cytometric Annexin-V-Fluos staining assay.
[b] Determination of percent apoptosis, necrosis and activation of caspase 3 were performed after 24 hr of culture without (Con) or in the presence of 2-CdA (325 nM) (2-CdA).

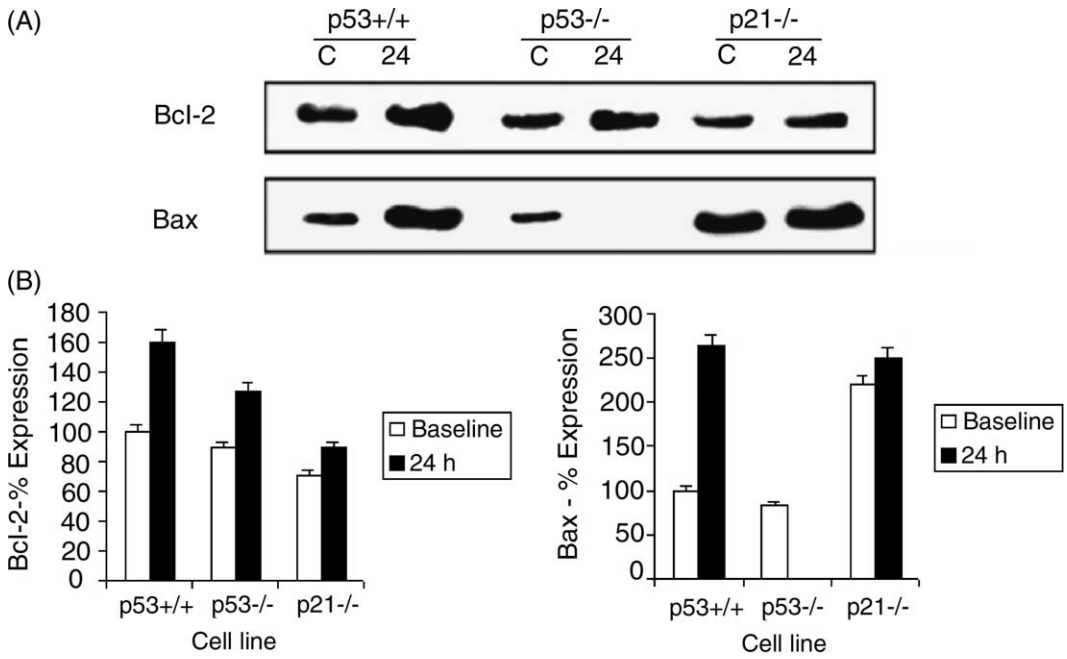

Fig. 5. Effect of 2-CdA on expression of pro- (Bax) and anti-apoptotic (Bcl-2) proteins (B) by Western blot. HCT116 cells were treated with 2-CdA (325 nM) for 24 hr and subsequently lysed, subjected to SDS–PAGE, and immunoblotted with corresponding monoclonal antibody as described in Section 2. Horizontal scanning densitometry was performed by utilizing the Kodak Digital Science 1D image analysis software. The experiments shown are representative of two performed. p53+/+: HTC116 p53+/+; p53−/−: HCT116 p53−/−; p21: HCT1116 $p21^{WAF1}$−/−.

activity (Fig. 6A). The increase in PARP-1 content was most evident in the p53+/+ line. To investigate the involvement of PARP-1 in cell death induced by 2-CdA in p53+/+ cells, we examined the effect of 3-AB, a PARP-1 inhibitor in p53+/+ cells treated with 2-CdA. As shown in Fig. 6B, addition of 3-AB (10 mM) partially reduced the cytotoxic effects of 2-CdA. In these cells, the IC_{50} of 2-CdA was 27.2 ± 2.5 nM in the absence of 3-AB and 155 ± 41 nM in the presence of 3-AB. These results suggest that PARP-1 is involved in 2-CdA-induced cytotoxicity.

4. Discussion

To determine the role of p53 and $p21^{WAF1}$ in the cytotoxicity of 2-CdA on dividing cells, we compared the cytotoxic effects of 2-CdA on HCT colorectal lines that differ in their p53 or $p21^{WAF1}$ protein content. Our results indicate that cells containing a wt-p53 are more sensitive to 2-CdA than p53−/− and $p21^{WAF1}$−/− cells. These results are in keeping with previous reports showing a correlation between *p53* gene abnormalities and 2-CdA resistance. Pettitt *et al*. observed that p53-knockout cells were more resistant to 2-CdA than wild-type cells [26]. Moreover, *p53* gene abnormalities have been correlated with resistance to 2-CdA-induced killing in *in vitro* studies of CLL cells [27,28]. Our results also show that $p21^{WAF1}$ is related to 2-CdA sensitivity.

In our model, p53 but not $p21^{WAF1}$ status influences the ability of cells to arrest at the G1/S checkpoint in response to 2-CdA. p53+/+ and $p21^{WAF1}$−/− cells expressing p53 arrested in S-phase after drug exposure while p53−/− cells continued to proliferate regardless of drug exposure. Of note was that arrest of $p21^{WAF1}$−/− cells at the G1/S-phase checkpoint was obtained in the absence of $p21^{WAF1}$ mRNA or protein expression. Thus, after 2-CdA exposure, cell arrest at the G1/S phase checkpoint is dependent on p53. This indicates that p53-dependent cell cycle regulating factors other than $p21^{WAF1}$ are involved in 2-CdA-induced cell cycle arrest.

We also observed that wt-p53 cells were more susceptible to 2-CdA-induced apoptosis than p53−/− and $p21^{WAF1}$−/− cells. 2-CdA-induced apoptosis has been reported to be associated with activation of caspase 3 [29,30]. However, caspase 3 levels were similar in the three lines, and no increase in caspase 3 activity was observed in either line after exposure to 2-CdA. Other caspases or non-caspase proteases may be involved in 2-CdA-induced apoptosis [31–33]. A number of apoptosis-associated genes have been shown to be activated or regulated by p53. Bcl-2 and Bax are proteins that have opposing effects, with Bcl-2 serving to prolong cell survival and Bax acting as an accelerator of apoptosis [16]. Our results show strongly reduced Bax protein levels in the p53−/− cells after 2-CdA treatment whereas Bax levels in p53+/+ were dramatically increased and did not vary in $p21^{WAF1}$−/− cells. In contrast, Bcl-2 protein slightly increased in the three lines after exposure to 2-CdA. Bax participates in the formation of the mitochondrial permeability transition pore that allows the release of cytochrome *c* and apoptosis inducing factor (AIF) that precedes apoptosis [34]. Thus, it is likely that the higher

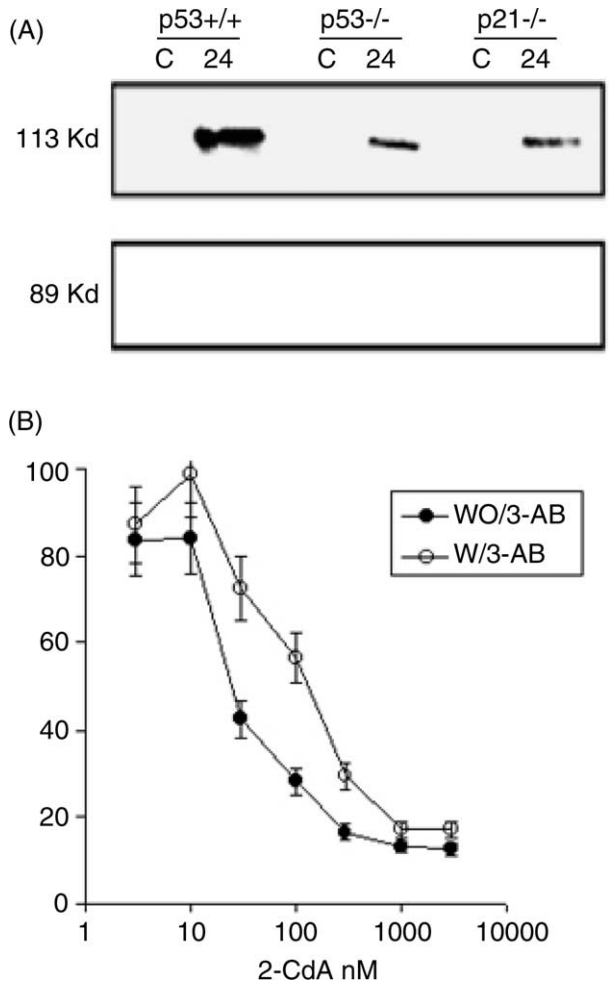

Fig. 6. Western blot analysis of PARP-1 expression after 2-CdA treatment (A) and effects of PARP-1 inhibition on 2-CdA-induced cell lysis (B). For (A), HCT116 cells were treated with 2-CdA (325 nM) for 24 hr and subsequently lysed, subjected to SDS–PAGE, and immunoblotted with PARP-1 monoclonal antibody as described in Section 2. Horizontal scanning densitometry was performed by utilizing the Kodak Digital Science 1D image analysis software. The experiments shown are representative of two performed. p53+/+: HTC116 p53+/+; p53−/−: HCT116 p53−/−; p21: HCT1116 $p21^{WAF1}$−/−. For (B), p53+/+ cells were plated at 3×10^3 cells per well in the presence of increasing concentrations of 2-CdA with (W) or without (WO) 3-AB (10 mM). Cell growth was determined by the MTT assay after 72-hr incubation. The results shown are representative of four performed in triplicate.

anti-apoptotic to pro-apoptotic protein ratio observed in p53−/− cells after exposure to 2-CdA confers a survival advantage to these cells in comparison with the p53+/+ or $p21^{WAF1}$−/− cells, as has previously been suggested [35]. Whether the lower anti-apoptotic to pro-apoptotic protein ratio observed in $p21^{WAF1}$−/− cells explains the slightly higher apoptotic level and the lower resistance observed in this line compared to p53−/− line is not yet clear.

PARP-1 plays a role in DNA repair by signaling genomic damage [36]. PARP-1 activation has also been suggested to be involved in cell killing by 2-CdA [37]. Apparently, inhibition of ongoing DNA repair by 2-CdA favors the accumulation of DNA breaks, inducing PARP-1 activation resulting in consumption of NAD and depletion of total adenine nucleotides [37,38]. ATP depletion predisposes cells to cellular necrosis [39,40]. Moreover, it seems likely that PARP-1-mediated ATP depletion might contribute to the cell membrane disruption that occurs during the late stages of apoptosis [41]. In our model, p53+/+ cells expressed higher levels of uncleaved PARP-1 (113 kDa) after exposure to 2-CdA. In these cells, 2-CdA also induced more apoptosis and necrosis than in p53−/− or $p21^{WAF1}$−/− cells. These results indicate that PARP-1 may contribute to the induction of 2-CdA-induced killing in proliferating cells. Moreover, PARP-1-mediated killing may be related to the absence of $p21^{WAF1}$ but not to p53 deficiency since PARP-1 downregulation was observed in p53−/− cells and in $p21^{WAF1}$−/− cells expressing p53 protein (neither of these cell lines expressed $p21^{WAF}$ protein). Conversely, Pettitt recently published that in resting lymphocytes, PARP-1 may act as p53-independent mechanism of cell killing responsible for cytotoxicity in cases in which both alleles of p53 were mutated [42]. The fact that inhibition of PARP-1 by 3-AB-induced drug resistance in the p53+/+ cells suggests the role of PARP-1 in 2-CdA-induced cytotoxicity and may provide an explanation for the much stronger effects of 2-CdA toward p53+/+ proliferating colorectal cells in comparison to p53−/− and $p21^{WAF1}$−/− cells. However, as 3-AB is likely to inhibit enzymes other than PARP-1 at the high concentration used in this study, our results need confirmation with more specific PARP-1 inhibitors [43].

The analysis of parameters involved in 2-CdA transport or metabolism did not demonstrate alterations in p53−/− or p21−/− cells which could explain a drug-resistant phenotype. We found that p53−/− cells expressed slightly lower levels of dCK and strongly reduced levels of the M2 subunit from RNR. In reference to dCK, it is important to stress that *in vitro* cells having at least 10% normal levels of dCK can activate dCK-dependent compounds such as nucleoside analogues, suggesting that in p53−/− cells 2-CdA activation may be thought to proceed normally.

One of the major limitation of our study is the use of HCT116 cells given that 2-CdA is not known for its therapeutic efficacy against colorectal cancer. These cell lines were chosen because of the availability of p53- and p21-null derivatives. However, the sensitive HCT116 p53+/+ cell line showed IC_{50} values (31.7 ± 16 nM) in a range similar to that reported for leukemia or lymphoma cells treated with 2-CdA (ranging from 10 to 500 nM) and human B-cell lymphocytes (approximately 200 nM) [8,44,45].

It must be also stressed that the use of lines with deleted genes through homologous recombination has a major caveat which is the genetic drift over time with accumulation of chromosomal aberrations which may be involved in chemoresistance mechanisms. One way to avoid this problem is to study inducible p53 or $p21^{WAF1}$ variants, an approach which is currently being developed in our laboratory.

In summary, in wt-p53 cells, 2-CdA elicits a DNA damage response characterized by p53 and $p21^{WAF1}$ activation, cell cycle arrest in G1/S phase and PARP-1 and Bax overexpression. These events induce apoptosis and necrosis. In the absence of $p21^{WAF1}$, 2-CdA also induces a p53 response with cell cycle arrest in G1/S phase. However, $p21^{WAF1}-/-$ cells showed a lower PARP-1 expression and no modifications of the Bcl-2/Bax ratio. These differences were associated with a lower level of apoptosis and necrosis than those observed in p53+/+ cells and a drug-resistant phenotype. On the other hand, the lack of a functional p53 protein allows the cells that eventually accumulate DNA damage to continue proliferating. In p53−/− cells, both apoptotic and necrotic pathways are altered as reflected by the increased levels in the Bcl-2/Bax ratio and the lower PARP-1 expression favoring drug resistance. More work will be necessary to unravel possible connections between p53, $p21^{WAF1}$, PARP-1 regulation and cell death induced by 2-CdA.

Acknowledgments

Carlos M. Galmarini is a recipient of the ''Fondation de France'' grant. Lars Jordheim is a recipient of the ''Ligue contre le Cancer-Comité du Saone et Loire''.

References

[1] Cheson BD. New antimetabolites in the treatment of human malignancies. Semin Oncol 1992;19(6):695–706.
[2] Beutler E. New chemotherapeutic agent: 2-chlorodeoxyadenosine. Semin Hematol 1994;31(1):40–5.
[3] Piro LD. Cladribine in the treatment of low-grade non-Hodgkin's lymphoma. Semin Hematol 1996;33(Suppl 1):34–9.
[4] Matsumoto SS, Yu AL, Bleeker LC, Bakay B, Kung FH, Nyhan WL. Biochemical correlates of the differential sensitivity of subtypes of human leukemia to deoxyadenosine and deoxycoformycin. Blood 1982;60(5):1096–102.
[5] Carson DA, Wasson DB, Esparza LM, Carrera CJ, Kipps TJ, Cottam HB. Oral antilymphocyte activity and induction of apoptosis by 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine. Proc Natl Acad Sci USA 1992;89(7):2970–4.
[6] Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. Leukemia 2001;15(6):875–90.
[7] Spasokoukotskaja T, Arner ES, Brosjo O, Gunven P, Juliusson G, Liliemark J, Eriksson S. Expression of deoxycytidine kinase and phosphorylation of 2-chlorodeoxyadenosine in human normal and tumour cells and tissues. Eur J Cancer 1995;2:202–8.
[8] Dumontet C, Fabianowska-Majewska K, Mantincic D, Callet Bauchu E, Tigaud I, Gandhi V, Lepoivre M, Peters GJ, Rolland MO, Wyczechowska D, Fang X, Gazzo S, Voorn DA, Vanier-Viornery A, Mackey JR. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol 1999;106(1):78–85.
[9] Hentosh P, Koob R, Blakley RL. Incorporation of 2-halogeno-2′-deoxyadenosine 5-triphosphates into DNA during replication by human polymerases alpha and beta. J Biol Chem 1990;265(7):4033–40.
[10] Griffig J, Koob R, Blakley RL. Mechanisms of inhibition of DNA synthesis by 2-chlorodeoxyadenosine in human lymphoblastic cells. Cancer Res 1989;49(24 Pt 1):6923–8.
[11] Lassota P, Kazimierczuk Z, Darzynkiewicz Z. Apoptotic death of lymphocytes upon treatment with 2-chloro-2′-deoxyadenosine (2-CdA). Arch Immunol Ther Exp 1994;42(1):17–23.
[12] Benjamin RC, Gill DM. Poly(ADP-ribose) synthesis *in vitro* programmed by damaged DNA. A comparison of DNA molecules containing different types of strand breaks. J Biol Chem 1980;255(21):10502–8.
[13] Mansson E, Spasokoukotskaja T, Sallstrom J, Eriksson S, Albertioni F. Molecular and biochemical mechanisms of fludarabine and cladribine resistance in a human promyelocytic cell line. Cancer Res 1999;59(23):5956–63.
[14] Schirmer M, Stegmann AP, Geisen F, Konwalinka G. Lack of cross-resistance with gemcitabine and cytarabine in cladribine-resistant HL60 cells with elevated 5′-nucleotidase activity. Exp Hematol 1998;26(13):1223–8.
[15] Avramis VI, Nandy P, Kwock R, Solorzano MM, Mukherjee SK, Danenberg P, Cohen LJ. Increased p21/WAF-1 and p53 protein levels following sequential three drug combination regimen of fludarabine, cytarabine and docetaxel induces apoptosis in human leukemia cells. Anticancer Res 1998;18(4A):2327–38.
[16] Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. Oncogene 1994;9(6):1799–805.
[17] Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, Jacks T. p53 status and the efficacy of cancer therapy *in vivo*. Science 1994;266(5186):807–10.
[18] Gartenhaus RB, Wang P, Hoffman M, Janson D, Rai KR. The induction of p53 and WAF1/CIP1 in chronic lymphocytic leukemia cells treated with 2-chlorodeoxyadenosine. J Mol Med 1996;74(3):143–7.
[19] Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998;282(5393):1497–501.
[20] Galmarini CM, Falette N, Tabone E, Levrat C, Britten R, Voorzanger-Rousselot N, Roesch-Gateau O, Vanier-Viornery A, Puisieux A, Dumontet C. Inactivation of wild-type p53 by a dominant negative mutant renders MCF-7 cells resistant to tubulin-binding agent cytotoxicity. Br J Cancer 2001;85(6):902–8.
[21] Galmarini CM, Clarke ML, Falette N, Puisieux A, Mackey JR, Dumontet C. Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. Int J Cancer 2002;97(4):439–45.
[22] Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 1975;66:188–93.
[23] Guchelaar HJ, Vermes I, Koopmans RP, Reutelingsperger CP, Haanen C. Apoptosis- and necrosis-inducing potential of cladribine, cytarabine, cisplatin, and 5-fluorouracil *in vitro*: a quantitative pharmacodynamic model. Cancer Chemother Pharmacol 1998;42(1):77–83.
[24] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162(1):156–9.
[25] Voorzanger-Rousselot N, Favrot M, Blay JY. Resistance to cytotoxic chemotherapy induced by CD40 ligand in lymphoma cells. Blood 1998;92(9):3381–7.
[26] Pettitt AR, Clarke AR, Cawley JC, Griffiths SD. Purine analogues kill resting lymphocytes by p53-dependent and -independent mechanisms. Br J Haematol 1999;105(4):986–8.
[27] Pettitt AR, Sherrington PD, Cawley JC. The effect of p53 dysfunction on purine analogue cytotoxicity in chronic lymphocytic leukaemia. Br J Haematol 1999;106(4):1049–51.

[28] Johnston JB, Daeninck P, Verburg L, Lee K, Williams G, Israels LG, Mowat MR, Begleiter A. P53, MDM-2, BAX and BCL-2 and drug resistance in chronic lymphocytic leukemia. Leuk Lymphoma 1997;26(5):435–49.
[29] Genini D, Adachi S, Chao Q, Rose DW, Carrera CJ, Cottam HB, Carson DA, Leoni LM. Deoxyadenosine analogs induce programmed cell death in chronic lymphocytic leukemia cells by damaging the DNA and by directly affecting the mitochondria. Blood 2000;96(10): 3537–43.
[30] Leoni LM, Chao Q, Cottam HB, Genini D, Rosenbach M, Carrera CJ, Budihardjo I, Wang X, Carson DA. Induction of an apoptotic program in cell-free extracts by 2-chloro-2′-deoxyadenosine 5′-triphosphate and cytochrome *c*. Proc Natl Acad Sci USA 1998;95(16):9567–71.
[31] Fadeel B, Orrenius S, Zhivotovsky B. The most unkindest cut of all: on the multiple roles of mammalian caspases. Leukemia 2000;14:1514–25.
[32] Liang Y, Yan C, Schor NF. Apoptosis in the absence of caspase 3. Oncogene 2001;20(45):6570–8.
[33] Marzo I, Perez-Galan P, Giraldo P, Rubio-Felix D, Anel A, Naval J. Cladribine induces apoptosis in human leukaemia cells by caspase-dependent and -independent pathways acting on mitochondria. Biochem J 2001;359(Pt 3):537–46.
[34] Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 1999;399(6735):483–7.
[35] Bentley DP, Pepper CJ. The apoptotic pathway: a target for therapy in chronic lymphocytic leukemia. Hematol Oncol 2000;18(3): 87–98.
[36] de Murcia G, Menissier de Murcia J. Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biochem Sci 1994;19(4):172–6.
[37] Seto S, Carrera CJ, Kubota M, Wasson DB, Carson DA. Mechanism of deoxyadenosine and 2-chlorodeoxyadenosine toxicity to nondividing human lymphocytes. J Clin Invest 1985;75(2):377–83.
[38] Carson DA, Carrera CJ, Wasson DB, Yamanaka H. Programmed cell death and adenine deoxynucleotide metabolism in human lymphocytes. Adv Enzyme Regul 1988;27:395–404.
[39] Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 1997;185(8):1481–6.
[40] Eguchi Y, Shimizu S, Tsujimoto Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res 1997;57(10): 1835–40.
[41] Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 1995;182(2):367–77.
[42] Pettitt AR, Sherrington PD, Cawley JC. Role of poly(ADP-ribosyl)ation in the killing of chronic lymphocytic leukemia cells by purine analogues. Cancer Res 2000;60(15):4187–93.
[43] Banasik M, Komura H, Shimoyama M, Ueda K. Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase. J Biol Chem 1992;267(3):1569–75.
[44] Cardoen S, Van Den Neste E, Smal C, Rosier JF, Delacauw A, Ferrant A, Van den Berghe G, Bontemps F. Resistance to 2-chloro-2′-deoxyadenosine of the human B-cell leukemia cell line EHEB. Clin Cancer Res 2001;7(11):3559–66.
[45] Hentosh P, Tibudan M. 2-Chloro-2′-deoxyadenosine, an antileukemic drug, has an early effect on cellular mitochondrial function. Mol Pharmacol 1997;51(4):613–9.