BIOCHEMICAL PHARMACOLOGY 73 (2007) 351–358

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Pharmacological inhibition of the MAPK/ERK pathway increases sensitivity to 2-chloro-2′-deoxyadenosine (CdA) in the B-cell leukemia cell line EHEB

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ARTICLE INFO

Article history:
Received 7 June 2006
Received in revised form
4 October 2006
Accepted 13 October 2006

Keywords:
MAPK/ERK pathway
2-Chloro-2′-deoxyadenosine
EHEB
MEK inhibitors
Caspase-3
Chronic lymphocytic leukemia

ABSTRACT

EHEB leukemic cells, which are derived from a patient suffering B-cell chronic lymphocytic leukemia (B-CLL), display intermediate sensitivity to the purine analogue 2-chloro-2′-deoxyadenosine (CdA). Because the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway can rescue cancer cells from apoptotic signals, we investigated MAPK/ERK signaling in EHEB cells in response to CdA. We observed that CdA, at concentrations around its IC_{50}, dose- and time-dependently increased the phosphorylation state of ERK 1/2 (p-ERK), indicating an activation of the MAPK/ERK pathway. This activation required CdA metabolism and de novo protein synthesis, and was independent on caspase activation. Interruption of ERK signaling, using the specific MEK inhibitors U-0126 and PD-98059, significantly enhanced CdA cytotoxicity, evaluated by the MTT assay. Drug interaction analysis showed synergism in the majority of combinations between CdA and MEK inhibitors tested. MEK inhibitors also dramatically increased apoptosis induced by CdA alone, evaluated by caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage. Collectivelly, these observations show that ERK 1/2 activation elicited by CdA serves as a cytoprotective function and suggest that inhibitors of this pathway could be combined with CdA in the treatment of selected hematological malignancies.

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1. Introduction

EHEB cells, a continuous cell line derived from a patient with B-cell chronic lymphocytic leukemia (B-CLL), display intermediate sensitivity to the nucleoside analogue 2-chloro-2′-deoxyadenosine (CdA) [1]. CdA is a prodrug that must be converted into its triphosphate derivative CdATP as an essential requirement for cytotoxicity. CdATP inhibits various processes involved in DNA synthesis and activates apoptosis pathways [2]. We have reported that resistance toward CdA in EHEB cells can be partly explained by a reduced activity of deoxycytidine kinase (dCK), which catalyzes the first and

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doi:10.1016/j.bcp.2006.10.012

limiting step of the conversion of CdA into CdATP [3]. In addition, although the significance of this observation has not been unravelled, we have observed that CdA induces a paradoxical activation of the cell cycle of EHEB cells at concentrations around 5 μM, the concentration required to kill 50% of the cells (IC_{50}) [1,3]. In the present report, we hypothesized that activation of survival pathways might contribute to resistance to CdA in EHEB cells. We focused our attention on the antiapoptotic mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, which is deregulated and participates to the generation of mitogenic signals in essentially all hematologic malignancies [4]. In various models, activation of ERK may rescue cancer cells from apoptotic signals and induce rapid proliferation [5,6]. In addition, it has been shown that pharmacological inhibition of the ERK pathway can restore chemosensitivity in various leukemic cells [7–11]. We firstly examined the effects of CdA treatment on the MAPK/ERK pathway. We found that CdA dose- and time-dependently increased ERK phosphorylation, indicating activation of the MAPK/ERK pathway. We further investigated whether activation of ERK serves a protective role against the cytotoxic effects of CdA.

2. Materials and methods

2.1. Chemicals

CdA was synthesised and supplied by Prof. J. Marchand (Laboratory of Organic Chemistry, Université Catholique de Louvain, Louvain-la-Neuve). Stock solutions of CdA were prepared in ethanol/150 mM NaCl (v/v). Ficoll-paque plus (density: 1.077), Hybond C-extra membranes, ECL enhanced chemiluminescence kit were from GE Healthcare. FCS and penicillin-streptomycin were purchased from BioWhittaker Europe. RPMI-1640 and all tissue culture reagents were from Gibco/Invitrogen. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), anti-β-actin antibody, and horseradish peroxydase conjugated anti-rabbit (or anti-mouse) antibody were purchased from Sigma–Aldrich. U-0126, anti-phospho ERK-1/ERK-2, anti-ERK-1/ERK-2 antibodies, and BSA for blots were from Promega. PD-98059 and U-0124 were from Biomol. PMA (phorbol-12-myristate-13-acetate) and ZVAD-fmk were from Calbiochem. Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-AMC) and AMC (7-amino-4-methyl coumarin) were purchased from Alexis Biochemicals. Anti-PARP antibody was obtained from Santa Cruz Biotechnology. Other chemicals, materials and reagents were from Sigma, Merck Biosciences or Bio-Rad Laboratories.

2.2. Cell culture and incubation

EHEB cells, a B-cell line established from B-CLL lymphocytes [12], were cultured in RPMI-1640 with Glutamax, supplemented with 10% heat-inactivated FCS at 37 °C in an atmosphere of 5% CO_2 in air. Freshly obtained peripheral blood from B-CLL patients (diagnosis was confirmed by cytological and immunological studies) was fractionated by Ficoll-Paque sedimentation. Mononuclear cells were washed with PBS and resuspended in RPMI-1640 supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Cells were counted, diluted to indicated concentration in RPMI-1640, and incubated at 37 °C in 5% CO_2 in air. Hydrophobic inhibitors were dissolved in DMSO and equal amount of DMSO was also added in control cells. All inhibitors used in this study were added 30 min before CdA (or PMA), except in the MTT assay where they were added at the same time.

2.3. Cytotoxicity and drug interaction analysis

EHEB cells (4×10^4 cells/well) or B-CLL cells (2×10^6 cells/well) were incubated with or without various concentrations of CdA and/or ERK pathway inhibitors in 96-well plates. After 72 h for EHEB cells and 96 h for B-CLL cells, cell viability was measured using the MTT assay as described by Bontemps et al. [13]. Each condition was done in triplicate in the same experiment. The absorbance of each well was measured at 570 nm and 655 nm with a Multiwell Scanning Spectrophotometer (Bio-Rad). Cell viability, expressed as a percentage, was calculated by the equation: (mean absorbance of treated cells/mean absorbance of control cells) × 100. The IC_{50} value was calculated with the following equation: ([% leukemic cell survival (LCS) > 50%] − 50)/([% LCS > 50%] − [LCS < 50%]) × (drug concentration above 50% LCS − drug concentration below 50% LCS) + (drug concentration below LCS 50%).

Interaction between drugs, i.e. synergism, additivity or antagonism, was defined according to the multiplicative and maximum models as previously reported [14,15]. In the multiplicative model, the expected effect of a drug combination is the product of the effect of each single drug. In the maximum model, the expected effect of a combination is equal to that of the most active single drug. These methods were combined to establish the interactions between drugs: synergism when the observed LCS (drug A + drug B) < LCS (drug A) × LCS (drug B); additivity if LCS (drug A) × LCS (drug B) < observed LCS (drug A + drug B) < LCS of the most active single drug (D_{max}); antagonism if observed LCS (drug A + drug B) > LCS D_{max}.

2.4. Western blot analysis

For ERK analysis, 10×10^6 EHEB cells were washed in ice-cold PBS and resuspended in 200 μl of ice-cold lysis buffer containing 20 mM Tris–HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 150 mM NaCl, protease inhibitors (5 mM benzamidine, 1 mM *p*-toluenesulfonyl fluoride, 5 μg/ml leupeptine and antipaine) and 1 mM orthovanadate freshly added. Cells were lysed by sonication during 2 × 7 s, and the lysate was centrifugated at 16,100 × *g* for 10 min at 4 °C. Cell extracts (80 μg of protein) were subjected to SDS-PAGE in gels containing 10% (w/v) acrylamide and transferred to Hybond C-extra membranes. Membranes were blocked with 5% fat-free milk powder in Tris-buffered saline (TBS) for 1 h at room temperature and probed overnight at 4 °C with anti-phospho ERK-1/ERK-2 (1/5000) in TBS-T (Tween 0.1%, w/v) containing 1% of BSA. After extensive washings in TBS-T, the membranes were incubated for 1 h at room temperature with anti-rabbit antibody coupled to horseradish peroxidase (1/10,000) in TBS-T containing 5% fat-free milk powder. After further extensive

washings in TBS-T and a last in TBS, the blots were developed using enhanced chemiluminescence. When indicated, β-actin (1/10,000) was analyzed as a loading control. For total ERK analysis, parallel blots were probed with antibody against ERK-1/ERK-2 (1/5000). Quantification of bands was carried out using National Institute of Health (NIH) image software. The protein content of cell extracts was measured by the method of Bradford, using bovine serum albumin as the standard [16].

For PARP cleavage analysis, 4×10^6 EHEB cells were washed twice in ice-cold PBS and resuspended in 100 μl of a first ice-cold lysis buffer containing 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 0.5% Nonidet-P40, protease inhibitors (5 mM benzamidine, 1 mM *p*-toluenesulfonyl fluoride, 5 μg/ml leupeptine and antipaine) for 10 min. The lysates were then centrifuged at 7200 × *g* for 5 min at 4 °C, and the pellets containing the nuclei were resuspended in 50 μl of a second ice-cold lysis buffer containing 20 mM HEPES, pH 7.9, 1 mM EDTA, 50 mM KCl, 10 mM dithiothreitol, 1% Triton X-100, 400 mM NaCl and protease inhibitors (5 mM benzamidine, 1 mM *p*-toluenesulfonyl fluoride, 5 μg/ml leupeptine and antipaine) for 30 min and centrifugated at 16,100 × *g* for 10 min at 4 °C. Aliquots of supernatants containing the nuclear proteins (12.5 μg of protein) were separated on 7.5% SDS-polyacrylamide gels, transferred to membranes, and probed with a mouse anti-PARP antibody (1/200), which recognizes both the 112 kDa native PARP protein and its 85 kDa cleavage fragment. After removal of the specific antibody, blots were incubated with anti-mouse peroxidase-conjugated secondary antibody (1/5000). To ensure that equal amounts of protein were loaded, the blots were reprobed with anti-β-actin antibody (1/10,000).

2.5. Assay for caspase-3 activity

After incubation, 4×10^6 EHEB cells or 200×10^6 B-CLL cells were washed twice in ice-cold PBS. Cell pellets were resuspended in 125 μl of ice-cold buffer containing 10 mM Tris–HCl, pH 7.4, 10 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 1% nonidet-P40, protease inhibitors (5 mM benzamidine, 1 mM *p*-toluenesulfonyl fluoride, 5 μg/ml leupeptine and antipaine) and lysed for 20 min on ice. Cell lysates were centrifugated at 16,100 × *g* for 10 min at 4 °C and the supernatants were used to determine the activity of caspase-3 by fluorometric assay. Cell extracts (100 μl, containing ~250 μg of protein) were incubated at 37 °C in 50 mM Tris–HCl, pH 7.4, dithiothreitol 5 mM and 37.5 μM Ac-DEVD-AMC, used as a fluorogenic substrate. Release of AMC was measured using a spectrofluorometer (Bio-Rad) with a pair of excitation/emission wavelenght of 360/460 nm. Caspase-3 activity was calculated by converting fluorescence units into micromoles of AMC released per min per mg of protein using a standard curve obtained from free AMC.

2.6. Statistical analysis

Differences in IC_{50} between cells incubated in the presence of CdA alone and cells incubated with CdA and ERK pathway inhibitors were analysed for statistical significance by the two-tailed Student's *t*-test for paired samples, at a level of significance of $P = 0.05$. All means were calculated from at least three independent experiments.

3. Results

3.1. Activation of the MAPK/ERK pathway by CdA

EHEB cells were incubated for 24 or 48 h with or without increasing concentrations of CdA, or for 10 min with PMA, a ERK pathway activator used as a positive control. Cell lysates were subjected to immunoblot analysis for phospho-ERK 1/2 (p-ERK 1/2) and total ERK (Fig. 1(A)). CdA increased the phosphorylation of ERK 1/2 in a time-dependent manner beginning to some extent at 24 h, and becoming obvious after 48 h of treatment with CdA. ERK 1/2 phosphorylation level after 48 h of incubation with CdA was quantified by gel scanning as the ratio of treated to untreated samples in four different experiments (Fig. 1(B)). ERK 1/2 phosphorylation increased after 48-h incubation with 2, 5, and 10 μM CdA, to a mean of 4.3-, 13.8-, and 24.0-fold above basal activity, respectively. These results show that CdA, at concentrations around its IC_{50}, increases the phosphorylation level of p-ERK 1/2 in EHEB cells, indicating activation of the MAPK/ERK pathway. It is worth noting that increase of p-ERK was never accompanied by any significant change in the level of total ERK (Fig. 1(A)), which shows that p-ERK increase does not result from increase in ERK protein level.

Because a delay (24 h) in time was observed before activation of the ERK pathway by CdA, we hypothesized that *de novo* protein synthesis could be required. Experiments were performed in the presence of 3 μM cycloheximide (CHX), which decreases protein synthesis by close to 80% in EHEB cells, while displaying a moderate cytotoxicity (30% decrease in LCS) at this concentration. We found that CHX reduced the level of total ERK and the level of p-ERK1/2 in basal conditions, but also completely abolished its activation by CdA (Fig. 2). To

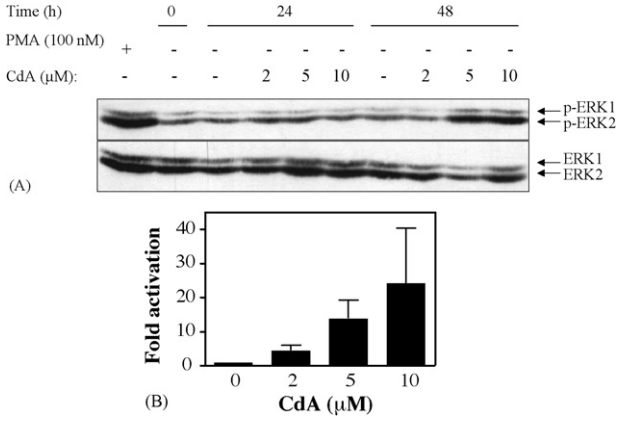

Fig. 1 – Time- and dose-dependent effect of CdA on the levels of p-ERK 1/2 and ERK 1/2 in EHEB cells. (A) EHEB cells were incubated for 24 or 48 h in the absence or in the presence of increasing concentrations of CdA, as indicated. Cell lysates were subjected to immunoblot analysis for p-ERK 1/2 and total ERK with each specific antibody. As a positive control, EHEB cells were incubated for 10 min with 100 nM PMA (first lane). (B) Increases in phosphorylation (*Fold*) of ERK 1/2 after 48 h of incubation with CdA, calculated after scanning of four gels and normalization to the band intensity in the control condition.

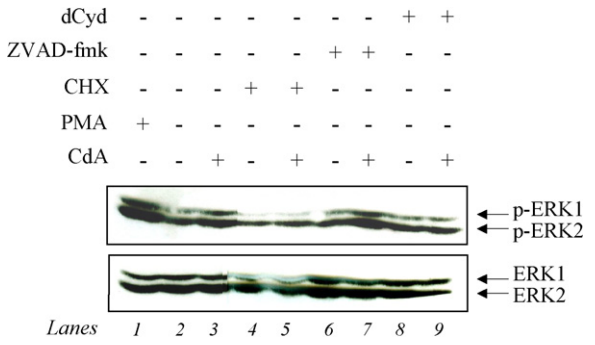

Fig. 2 – Effect of protein synthesis inhibition, caspase inhibition, and dCyd on CdA-induced ERK 1/2 activation. EHEB cells were incubated for 48 h before assay for p-ERK. Lane 1: PMA 100 nM; lane 2: untreated cells; lane 3: CdA 10 μM; lane 4: CHX 3 μM; lane 5: CdA 10 μM with CHX 3 μM; lane 6: ZVAD-fmk 20 μM; lane 7: CdA 10 μM with ZVAD-fmk 20 μM; lane 8: dCyd 100 μM; lane 9: CdA 10 μM with dCyd 100 μM.

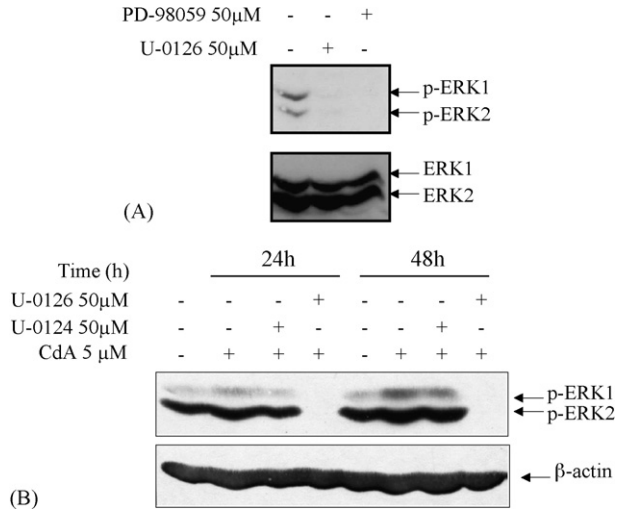

Fig. 3 – Effect of MEK inhibitors on ERK 1/2 phophorylation. (A) EHEB cells were incubated during 2 h with 50 μM PD-98059 or U-0126 as indicated, and assayed for p-ERK 1/2 and total ERK 1/2. (B) Cells were incubated during 24 or 48 h with or without 5 μM CdA, and with or without U-0126 or U-0124 as indicated. β-Actin level was used as a loading control.

determine whether activation of the ERK pathway occurred upstream or downstream of caspase activation, cells were pretreated with ZVAD-fmk at a concentration of 20 μM, which was shown to completely abolish the activation of caspase-3 induced by CdA. This pretreatment did not inhibit CdA-induced activation of ERK, suggesting that caspases do not lie upstream of ERK (Fig. 2). In contrast, preincubation of EHEB cells with 100 μM deoxycytidine, which competes with CdA for phosphorylation by dCK, completely abrogated CdA-induced ERK 1/2 phosphorylation, showing that activation of the ERK pathway by CdA results from CdA metabolism (Fig. 2).

3.2. *Enhanced sensitivity to CdA following ERK 1/2 inhibition*

Because activation of the MAPK/ERK pathway has been associated with anti-apoptotic effects, and occurs after CdA, we examined the possibility that inhibitors of this pathway could modulate sensitivity to this drug. To verify this hypothesis, we used two commercially available and unrelated MEK inhibitors, PD-98059 and U-0126, which function by binding to the dephosphorylated form of MEK-1, the immediate upstream kinase of ERK 1/2, thus preventing its activation [17]. We firstly confirmed that these inhibitors actually inhibited the phosphorylation of ERK 1/2 induced by PMA (results not shown). Incubation with both U-0126 and PD-98059 at 50 μM also completely inhibited ERK 1/2 phosphorylation seen in basal conditions in EHEB cells (Fig. 3(A)). Both inhibitors also prevented the activation of the ERK pathway by

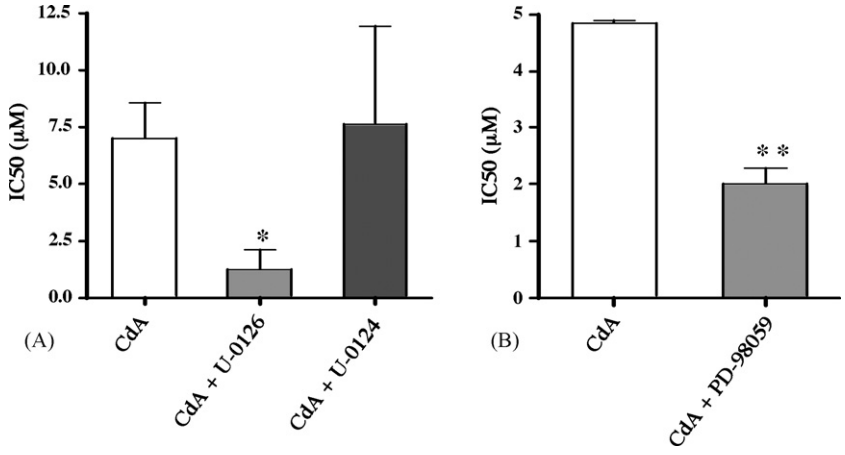

Fig. 4 – Effect of MEK inhibitors on CdA-induced cytotoxicity in EHEB cells. Cells were treated with increasing concentrations of CdA preceded or not by preincubation with 25 μM U-0126, 25 μM U-0124 (A), or 20 μM PD-98059 (B). Cell survival was measured by the MTT assay and the concentration required to kill 50% of cells (IC_{50}) was calculated from the dose–response curve, as explained in Section 2. Values are means ± S.E.M. of three separate experiments; $^{*}P < 0.05$; $^{**}P < 0.01$.

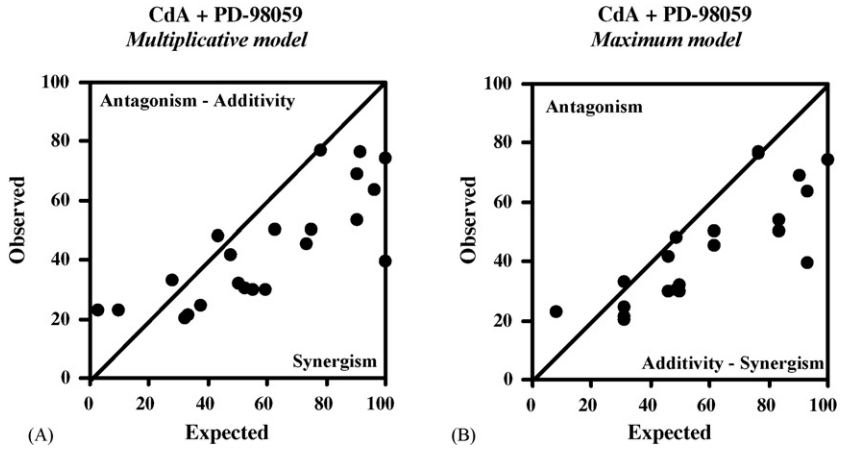

Fig. 5 – Comparisons (n = 22) between observed leukemic cell survival (LCS) and expected LCS values for the combination CdA + PD-98059 in EHEB cells. (A) Expected LCS according to the multiplicative model where LCS (drug A + drug B) = LCS (drug A) × LCS (drug B). (B) Expected LCS according to the maximum model where LCS (drug A + drug B) = LCS of the most active single drug. The continuous line is $x = y$.

CdA, whereas U-0124, a structural analogue of U-0126, devoid of its capacity to bind to MEK-1, did not. Results obtained with U-0126 and U-0124 are shown in Fig. 3(B).

We then sought to investigate the effect of ERK pathway inhibition on CdA-induced cytotoxicity using the MTT assay. EHEB cells were incubated for 72 h with increasing concentrations of CdA in the presence or in the absence of ERK pathway inhibitors. As shown on Fig. 4(A), addition of U-0126 significantly ($P < 0.04$) reduced the IC_{50} of CdA as compared to the IC_{50} of CdA alone. Conversely, the cytotoxicity of CdA was not modified by the inactive analogue U-0124. The IC_{50} of CdA was also significantly ($P = 0.007$) diminished in the presence of 20 μM PD-98059 (Fig. 4(B)). It is important mentioning that PD-98059 alone, at concentration up to 50 μM, did not significantly reduce cell survival. U-0126 at 25 μM alone exhibited a slight, but significant (LCS after 72 h of incubation = 62%, $P = 0.03$) cytotoxicity towards EHEB cells (not shown). Due to their relatively low toxicity, IC_{50} values could not be determined for MEK inhibitors alone.

3.3. *Characterization of in vitro drug interaction*

To differentiate between synergism, additivity and antagonism, LCS were analyzed according to the multiplicative and maximum models as detailed in Section 2. Analysis of three experiments at up to four different concentrations of CdA and two concentrations of PD-98059, resulting in 22 individual comparisons between observed and expected LCS, showed synergistic interaction in 82%, additivity in 4.5%, and antagonism in 13.6% of the samples treated with CdA and PD-98059. This is illustrated in Fig. 5 which shows the comparison between the expected LCS values, as predicted by both the multiplicative and the maximum model, and the observed LCS values for each drug combination. When LCS observed for the combination of CdA and PD-98059 was compared to LCS of the most active single drug in each individual experiment, a highly significant difference was found ($P < 0.001$). The combination of CdA and U-0126 was tested in 14 individual experiments, at up to four different concentrations of CdA and one of U-0126. Synergism was found in 71% of the samples, additivity in 21%, and antagonism in 7% (not shown). The observed LCS for the combination of CdA and U-0126 was significantly lower than the LCS of the most active single drug when these values were compared in each individual experiments ($P < 0.0001$). This shows that, overall, the drug combinations were significantly more toxic than the most active single drug.

3.4. *Characteristics of cell death following incubation with drugs*

At concentrations around the IC_{50}, CdA induces hallmarks of apoptosis in EHEB cells [1]. We sought to investigate whether the synergism in term of cell killing between MEK inhibitors and CdA, resulted in an increase in markers of apoptosis. EHEB cells were incubated for 24 h with or without 2 and 5 μM CdA, and with or without inhibitors of the ERK pathway, and assayed for caspase-3 activity (Fig. 6). Fig. 6(A) shows that co-incubation of cells with CdA and 25 μM U-0126 significantly ($P < 0.05$) enhanced the activation of caspase-3 as compared to CdA alone. Similar enhancement of caspase-3 activity was observed when PD-98059 was added to CdA (Fig. 6(B)). Regarding the effect of MEK inhibitors alone, we noted that both inhibitors induced a small (but significant) activation of caspase-3. Time-course experiments showed that U-0126 not only increased the extent of caspase-3 activation after CdA, but also increased the rate at which it occurred. Activation of caspase-3 was indeed significant at 16 h in the presence of CdA and U-0126 ($P = 0.04$), whereas it was not detected in the presence of U-0126 or CdA alone (not shown). Use of the inactive analogue U-0124 did not lead to any increase in caspase-3 activation above the level induced by CdA alone (not shown). During apoptosis, caspases cause PARP-1 cleavage and inactivation. PARP-1 proteolysis produces a 85 kDa C-terminal fragment, with a reduced catalytic activity, and a 27 kDa N-terminal peptide, which retains the DNA binding domains. We investigated whether cytotoxicity induced by the combination of CdA and MEK inhibitors was associated with

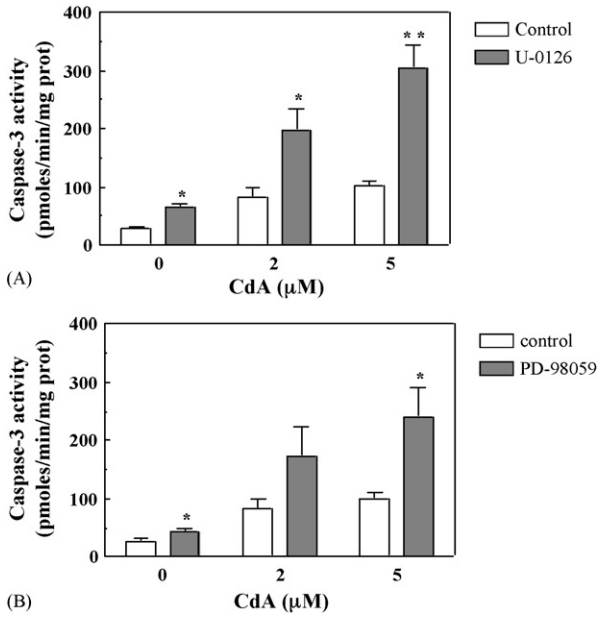

Fig. 6 – Activation of caspase-3. EHEB cells were incubated for 24 h with or without 2 and 5 μM CdA, and with or without 25 μM U-0126 (A), or 50 μM PD-98059 (B), and assayed for caspase-3 activity. Values are means ± S.E.M. of six separate experiments; $^{*}P < 0.05$; $^{**}P < 0.01$.

enhanced PARP cleavage. As shown on a representative gel (Fig. 7), PARP degradation after CdA alone was slightly detectable at 16 and 28 h after starting the incubation. In contrast, when CdA was combined with U-0126, PARP cleavage occurred faster and was more intense than after individual exposure to CdA. Taken together, these results show that MEK inhibitors readily augment CdA-induced markers of apoptosis.

3.5. *Combination of MEK inhibitors and CdA in B-CLL lymphocytes*

Because EHEB cells could constitute a model for B-CLL, we investigated whether the MAPK/ERK pathway was activated by CdA in lymphocytes isolated from B-CLL patients. We were unable to observe activation of ERK 1/2 by CdA at clinically relevant concentrations. In most of the experiments, p-ERK 1/2 was undetectable in basal conditions. However, further experiments showed that ERK 1/2 can be activated in B-CLL cells after addition of PMA. Moreover, PMA-activated ERK 1/2 can be inhibited by U-0126 in B-CLL cells (not shown). We also

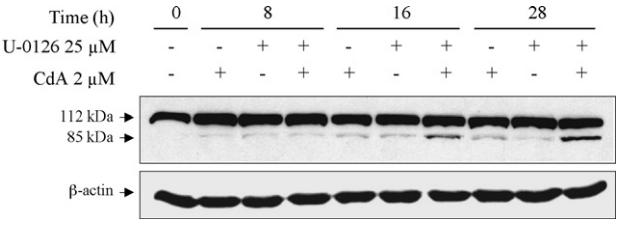

Fig. 7 – PARP-1 proteolysis. EHEB cells were incubated during indicated times with 2 μM CdA, with 25 μM U-0126, or both. β-Actin was used as a loading control.

observed that inhibition of the ERK pathway in B-CLL cells induced loss of cell viability, although variable amongst patients. Mean LCS after 96 h incubation with 50 μM U-0126 and PD-98059 was 41.5 ± 11.3% and 67.683 ± 8.4%, respectively, with few or no evidence of caspase-3 activation. Finally, we did not observe synergism in term of cell killing between CdA and MEK inhibitors in B-CLL samples (not shown).

4. Discussion

In the present report, we first show that CdA, at concentrations below and around its IC_{50}, activates the MAPK/ERK signal transduction pathway in EHEB cells. To assess activation of this pathway, we measured the phosphorylation state of ERK 1/2, using phospho-specific antibodies, a validated method to study ERK activation [18–21]. These antibodies are directed toward the dually phosphorylated region (pTEpY) on active ERK enzyme. Indeed, dual phosphorylation on T183 and Y185 residues is a requirement for maximal activity of both ERK 1 and ERK 2 [4]. We observed that CdA-induced ERK phosphorylation (activation) was time- and dose-dependent. Our results correlate with previous reports showing activation of ERK by other nucleoside analogues such as fludarabine or ara-C, and by other anticancer agents such as cisplatin or daunorubicin [5,8,11,22]. The mechanisms by which CdA activates ERK 1/2 remain speculative. Competition with dCyd shows that phosphorylation of CdA by dCK is needed before ERK activation. The effect of CdA on ERK 1/2 activation appears to depend on protein synthesis, since it is abolished by co-incubation with CHX. Phosphorylation of ERK after CdA is also insensitive to the addition of a pan-caspase inhibitor showing that it hierarchically occurs proximally to caspase activation. Other regulators implied in ERK activation, such as Ras, Raf-1, or PKCζ, were not investigated in the present study.

We have previously reported that EHEB cells have an intermediate sensitivity to CdA, which was partially attributed to a reduced activity of dCK in this cell line, with in corollary a lower accumulation of CdATP and a lower inhibition of ribonucleotide reductase [1,3]. Because activation of the ERK pathway is primarily related to cell survival, our results suggested that activation of this pathway might also contribute to CdA resistance in EHEB cells. To verify this hypothesis, we prevented ERK activation with two specific MEK1 inhibitors, U-0126 and PD-98059. These inhibitors appear to be the most specific ERK pathway inhibitors because they inhibit the fewest non-target kinases in a large panel of kinases. In particular, they have no impact on p38 and JNK 1/2 phosphorylation levels [23]. They function by inhibiting MEK1, through similar allosteric mechanisms, preventing phosphorylation and activation of ERK 1 and ERK 2 [17]. Treatment with these compounds, which actually interrupted ERK 1/2 signalling in our model, led to enhanced cytotoxicity of CdA. The IC_{50} of CdA was indeed reduced by more that 5- and 2.4-fold, in the presence of U-0126 and PD-98059, respectively. Drug interaction analysis showed that combination of CdA with MEK inhibitors resulted in synergistic cell killing. We also observed that caspase-3 activation and PARP cleavage, both hallmark of apoptosis, occurred at earlier time points and more intensively when CdA was

combined with MEK inhibitors, as compared to CdA alone. To explain synergism between the latter, one can hypothesize that ERK 1/2 interruption may lead to down-regulation of targets such as Bcl-2, Bcl-xL, or inhibitor of apoptosis protein (IAP), thus lowering the apoptotic threshold [7,23,24]. It should nevertheless be remembered that both PD-98059 and U-0126 may increase dCK activity by 2–3-fold, which could increase CdATP formation, and in corollary CdA toxicity [25,26]. The latter mechanism could partly explain synergism between CdA and MEK inhibitors. Taken together, our results shed some light on the role of MAPK/ERK signaling in EHEB cells. Although significant, the toxicity of these inhibitors alone is low in term of MTT survival reduction and caspase activation. This indicates that ERK pathway modestly contributes to survival of EHEB cells in steady-state condition, as described in other models [7,24]. Conversely, when activated by CdA, ERK pathway is an intrinsically self-limiting component of action of this drug and in consequence constitutes a molecular target to enhance sensitivity to CdA. Our results accord with previous observations showing that targeting the ERK component of the MAPK pathways may sensitize cancer cells to various cytotoxic approaches, such as paclitaxel, UCN-01, daunorubicin, cisplatin, araC, vinblastine, or Gy-irradiation [8,11,21,27–29].

The current results could have clinical implications in diseases in which nucleoside analogues are active and ERK pathway constitutively activated, such as in a wide range of myeloid and lymphoid leukemias [4,30]. Clinically, MEK inhibition could be achieved using rituximab, an anti-CD20 monoclonal antibody, or small-molecule inhibitors which become available [23,31]. Still, our results indicate that B-CLL might not be a candidate disease for ERK inhibition strategies. Indeed, ERK is only weakly active in B-CLL cells, and, although activable in these cells by PMA, it is not by CdA. Therefore, ERK inhibition does not consistently potentiate CdA cytotoxicity. This accords with reports showing that ERK can be activated in CLL cells, but that its inhibition does not oppose to survival signals, and thus does not constitute a target for pharmacological intervention [4,19,32]. In this disease, other signaling pathways, constitutively activated, such as the phosphatidylinositol-3 kinase (PI-3K) and PKCδ, seem to be more important in mediating cell survival [33]. It is however worth noting that *in vitro* findings may not be strictly applicable to the clinical situation, in which CLL cells are in contact with several survival signals which may activate survival pathways. In conclusion, our results indicate that CdA activates MAPK/ERK signaling in EHEB cells. Interruption of this process by pharmacological inhibition leads to a marked potentiation of CdA toxicity. Our data enlighten the interest of molecular drug targeting and the potential for MEK inhibitors as chemosensitizers in specific cancer therapies.

Acknowledgements

This work was supported in part by grants from the Belgian Fonds National de la Recherche Scientifique (Télévie, FRSM, Crédit au Chercheur), the Bauchau and Jacques Goor Foundation, and the Salus Sanguinis Foundation.

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