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R-Roscovitine (Seliciclib) Affects CLL Cells More Strongly Than Combinations of Fludarabine or Cladribine With Cyclophosphamide: Inhibition of CDK7 Sensitizes Leukemic Cells to Caspase-Dependent Apoptosis

Małgorzata Rogalińska,[1] Jerzy Z. Błoński,[2] Oxana Komina,[3] Paweł Góralski,[4] Jolanta D. Żołnierczyk,[1] Henryk Piekarski,[4] Tadeusz Robak,[2] Zofia M. Kiliańska,[1] and Józefa Węsierska-Gądek[3*]

[1] *Department of Cytobiochemisty, University of Lodz, Lodz, Poland*

[2] *Department of Hematology, Medical University of Lodz, Lodz, Poland*

[3] *Cell Cycle Regulation Group, Department of Medicine 1, Div.: Institute of Cancer Research, Medical University of Vienna, Vienna, Austria*

[4] *Department of Physical Chemistry, University of Lodz, Lodz, Poland*

ABSTRACT

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of malignant, apoptosis-resistant B $CD19^+/CD5^+$ cells. Populations of CLL cells are heterogeneous and consist primarily of quiescent cells with a minor subset of dividing cells. In this study the efficacy of a first-line in vivo therapy was compared with treatment by R-roscovitine (ROSC) alone or by purine analogues (cladribine and fludarabine) combined with maphosphamide for 14 CLL patients under ex vivo conditions. ROSC induced the highest reduction in numbers of living B-cells, coinciding with an increased rate of apoptosis. After 24 h the percentage of apoptotic cells in ROSC-treated cultures was markedly higher than in untreated controls. ROSC also induced strong activation of the apoptosome and effector caspases in CLL cells. During progression of apoptosis the plasma membrane became permeable, resulting in the release of activated caspases into the culture medium. Leukemic cells were more sensitive to ROSC than normal mononuclear cells. Treatment with ROSC did not affect the activating phosphorylation of CDK2 or CDK1. However, ROSC decreased phosphorylation of survivin, CDK7, and RNA-Pol II, resulting in inhibition of transcription elongation and subsequent down-regulation of levels of anti-apoptotic factors, thereby facilitating apoptosis. Unlike ROSC, two other purine analogues barely affected the cellular levels of anti-apoptotic proteins and more weakly activated effector caspases. In addition, the efficacies of in vivo and ex vivo therapies were found to be correlated. Marked between-patient differences in expression patterns of apoptosis-regulating factors in CLL cells were observed, explaining the variations in patients' sensitivity to therapy. J. Cell. Biochem. 109: 217–235, 2010.

KEY WORDS: CDKs; SURVIVIN; INHIBITION OF TRANSCRIPTION; ACTIVATED CASPASES; PHARMACOLOGICAL CDK INHIBITORS; DSC

Abbreviations used: AP, alkaline phosphatase; CLL, chronic lymphocytic leukemia; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; DSC, differential scanning calorimetry; FACS, fluorescence-activated cell sorting; HMC, Hoffman modulation contrast; HRP, horseradish peroxidase; PBMNC, peripheral blood mononuclear cell; PI, propidium iodide; ROSC, R-roscovitine; WCL, whole cell lysate.

Additional Supporting Information may be found in the online version of this article.

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*Correspondence to: Prof. Dr. Józefa Węsierska-Gądek, PhD, Cell Cycle Regulation Group, Department of Medicine I, Div.: Institute of Cancer Research, Vienna Medical University, Borschkegasse 8 a, A-1090 Vienna, Austria.
E-mail: jozefa.gadek-wesierski@meduniwien.ac.at

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The pathogenesis of B-cell chronic lymphocytic leukemia (CLL) is a multistep process in which an imbalance between programmed cell death and cell proliferation plays a significant role [for reviews, see Caligaris-Cappio 2000; Pleyer et al., 2009]. Resistance to apoptosis is especially important in CLL cells, which are mainly non-proliferating G_0/G_1 (quiescent) cells that gradually accumulate, because they survive too long [Gale et al., 1994; Reed, 1998; Reed and Pellecchia, 2005].

The clinical course of this hematological cancer is highly variable and ranges from indolent disease requiring no treatment for years to a highly aggressive form of disease that needs immediate therapy [Dighiero, 2005]. Conventional treatments for B-CLL patients have been improved by the introduction of antibiotics, monoclonal antibodies, and purine analogues (fludarabine and cladribine) administered separately, or in combination with cyclophosphamide [Gandhi and Plunkett, 2001; Robak, 2005; Van den Neste et al., 2005; Kay, 2006; Robak, 2008]. The results of a recent prospective multicenter study showed fludarabine plus cyclophosphamide (FC) to have high efficacy, and this treatment was recommended as a first-line therapy, especially for younger CLL patients [Eichhorst et al., 2006]. Unfortunately, however, all accepted therapy regimens for CLL are neither curative nor associated with prolonged survival [Chanan-Khan and Porter, 2006]. Moreover, the appearance of insensitivity to cytotoxic agents, for example, fludarabine, requires the development of new therapeutic strategies that circumvent resistance of leukemic cells to the drugs.

As recently reported, R-roscovitine (Seliciclib, CYC202, hereafter ROSC), a selective pharmacological inhibitor of cyclin-dependent kinases (CDKs) seems to be a promising drug for treating cancers, including CLL [Hahntow et al., 2004; Alvi et al., 2005; Wesierska-Gadek and Schmid, 2006], and it is in Phase II clinical trials against various B-cell malignancies [Ribas et al., 2006]. Available data indicate that all of the mentioned agents exert their therapeutic effects mainly by inducing apoptosis [Castejon et al., 1997; Bellosillo et al., 1999; Genini et al., 2000; Marzo et al., 2001; Reed and Pellecchia, 2005; Kobylinska et al., 2006], and trigger caspase activation in immature and mature B lymphoid cancer cells via the mitochondrial, but not the death receptor, signaling pathway [Genini et al., 2000; Marzo et al., 2001; Wieder et al., 2001; Kobylinska et al., 2006; Reed, 2008]. In addition, ROSC is known to inhibit the activity of CDK2/cyclin E and, at higher dosage, CDK1/cyclin B complexes [Havlicek et al., 1997]. Since regulation of the activity of CDK1/cyclin B dimers is highly complex, and requires the orchestrated cooperation of several cellular regulators, the evidence indicates that the primary effects of ROSC may be interference with the activities of CDK2.

Interestingly, in addition to its inhibitory action on cell cycle CDKs, ROSC affects the functional status of CDK7, thereby modulating transcriptional regulation, and strongly activates p53 tumor suppressor protein by site-specific phosphorylation via stimulation of HIPK2 [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2007]. Further, the ROSC-mediated activation of wt p53 protein is essential for the therapeutic outcome [Wesierska-Gadek et al., 2008a,b], especially in tumor cells that are resistant to apoptosis [Wesierska-Gadek et al., 2005a]. Moreover, ROSC abolishes phosphorylation of two serine residues of CDK7 that are critical for its activation and subsequent phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II [for a review, see Wesierska-Gadek and Schmid, 2006]. All these properties make ROSC highly suitable for treating CLL cells, since an ideal anti-CLL agent should target both dividing and non-dividing CLL cells.

It is generally known that not all CLL patients respond to therapy. The experiments performed in this study under ex vivo conditions on samples from six patients confirmed the low susceptibility of leukemic B-cells to treatment with purine analogues (cladribine and fludarabine) combined with mafosfamide (an active form of cyclophosphamide) at pharmacological concentrations (hereafter referred standard therapy). In all cases a weak reduction ($<10\%$) in the proportion of leukemic cells and a marginal increase in the apoptosis rate, compared to untreated controls, were observed after treatment for 24 h.

In the light of the observed low efficiency of the standard therapy, which also induces detectable DNA damage, we decided to explore the effect of ROSC and compare its efficiency with that of both tested medicament combinations, and to compare the outcomes of ROSC treatment and the standard therapy administered to CLL patients. This new drug alone was much more effective than purine analogues combined with mafosfamide in all CLL samples. In 11 out of 14 cases ROSC induced the highest reduction in the number of living B-cells, accompanied by an increase in the apoptosis rate, and even after 24 h the rate of caspase-3-dependent apoptosis was markedly higher in ROSC-treated cultures than in untreated controls. Moreover, leukemic cells were approximately eightfold more sensitive to ROSC than normal mononuclear cells, whereas the combined treatment (FM and CM) was cytotoxic also for normal peripheral blood mononuclear cells (PBMNCs). Furthermore, ROSC decreased the phosphorylation of survivin, CDK7, and RNA Pol II in CLL cells, resulting in reductions in cellular levels of highly expressed anti-apoptotic factors, thus facilitating the onset of apoptosis and suggesting that the multifactorial targeting may confer ROSC's strong therapeutic properties. Moreover, a comparison of the efficacy of in vivo and ex vivo therapy revealed a good correlation between the two, indicating that ex vivo results might be of predictive relevance. Thus, our results show that testing of CLL cells with different drugs in culture could facilitate the development of tailored therapy, and thus enhance its efficacy.

MATERIALS AND METHODS

PATIENTS

Studies were performed with CLL cells isolated from peripheral blood of 20 patients (Table I). Blood samples of CLL patients (9 men and 11 women) who had not received treatment prior to the onset of this study with a leukocytosis ranging from 79 to 541×10^9/L were studied. Diagnosis for these CLL patients was established according to standard clinical, cytological, and immunological criteria [Cheson et al., 1996]. The clinical staging of the disease was determined according to the Rai system [Rai et al., 1975]. The immunophenotypic characteristics of leukemic cells ($CD5^+/CD19^+/CD23^+$, presence on surface light immunoglobin κ or λ chains) were determined. We used monoclonal antibodies manufactured by DAKO AS (Glostrup, Denmark) and flow cytometry (Coulter,

TABLE I. Clinical Characteristics of CLL Patients

Patients number	Patients initials	Sex	Age	Stage of disease	Treatment/response to treatment
171	BR	W	51	IV	CC/CR
173	ES	W	53	II	CC/CR
175	TD	W	65	0	FC/PR
176	JR	M	57	I	FC/CR
177	HK	M	77	II	CC/PR
179	GS	W	49	II	CC/NR
182	SK	M	74	II	CC/CR
184	JW	M	71	0	FC/nd
185	IJ	W	68	0	CC/PR
187	HP	W	66	I	CC/CR
189	WP	M	54	IV	CC/PR
193	JG-S	W	77	IV	CC/CR
195	JK	M	73	I	CC/PR
196	JM	M	67	0	CC/CR
197	RR	W	69	I	CC/CR
199	HG	M	52	II	CC/NR
200	AP	W	57	IV	CC/NR
201	KH	W	56	IV	CC/NR
203	JJ	M	69	I	CC/CR
207	KK	W	80	IV	Chlorambucil/nd

Treatment protocol: CC, cladribine + cyclophosphamide; FC, fludarabine + cyclophosphamide.
The clinical staging of CLL was determined according to Rai et al.
CR, complete response; PR, partial response; NR, non-responder; nd, not determined.
In first experiments series six patients were studied. Results were shown in Figures 1 and 2. The main study was performed on 14 patients.

Hialeah, FL) (Table I). The present study was approved by the Local Ethics Committee of the Medical University of Lodz (No. RNN/237/03KE) and all the patients signed a declaration of consent.

These patients underwent next day therapy with CC (cladribine + cyclophosphamide) or FC (fludarabine + cyclophosphamide) or chlorambucil (one person) as was described previously [O'Brien et al., 2001; Robak et al., 2002].

Peripheral blood samples collected from four healthy volunteers were used as a control.

RESPONSE CRITERIA

Clinical response to the treatment was estimated by NCI Sponsored Working Group criteria [Cheson et al., 1996]. Complete response (CR) required the absence of symptoms and organomegaly, normal complete cell counts (absolute lymphocyte count $<4 \times 10^9$/L, absolute neutrofil count $>1.5 \times 10^9$/L, hemoglobin concentration >110 g/L, platelet count $>100 \times 10^9$/L) and bone marrow with $<30\%$ lymphocytes for at least 2 months and no lymphoid infiltrate in bone marrow biopsy performed 2 months after clinical evidence of CR. Partial response (PR) was considered in the case of lymph nodes, spleen, liver physical examination, and peripheral blood findings either identical to those of CR or improved over pretreatment values by at least 50%. Patients not included in these categories were considered as non-responders (NR).

CELL LINES

The human normal MRC-5 and F2000 fibroblasts obtained from American Type Culture Collection (ATCC), were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS). Cells were grown up to 60% confluence and then treated with indicated drugs.

ANTIBODIES

The following specific antibodies were used to detect the relevant proteins: the polyclonal anti-phospho-Thr161 CDK1, anti-phospho-Thr14/Tyr15 CDK1, anti-phospho-Thr160 CDK2, anti-phospho-Ser112 Bad, and corresponding antibodies against the total antigen (all from New England Biolabs (Beverly, MA)), polyclonal anti-phospho-Ser164/Thr170 CDK7 and anti-phospho-Thr34 survivin were from BioLegend (San Diego, CA), anti-caspase-3 (DAKO AS), monoclonal anti-CDK2 (Ab-4) antibodies (Lab Vision Co., Fremont, CA), monoclonal anti-PCNA (clone PC-10) and anti-MCM-7 antibodies (clone DCS-141), polyclonal anti-caspase-9 and anti-PARP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-CDK7 (clone MO-1.1, Sigma–Aldrich, St. Louis, MO), anti-RNA polymerase II (clone ARNA-3, Chemicon International); anti-RNA polymerase II phosphorylated on Ser-5 (clone H14) and anti-RNA polymerase II phosphorylated on Ser-2 (clone H5, both Abcam Ltd, Cambridge, England) were from Abcam Ltd; anti-actin antibodies were from ICN Biochemicals (clone C4) (ICN Biochemicals, Aurora, OH) and monoclonal anti-Ran antibodies were Becton and Dickinson.

Appropriate secondary antibodies linked to horseradish peroxidase (HRP) and to alkaline phosphatase (AP) were from R&D Systems (Minneapolis, MN) or from Sigma–Aldrich, respectively.

ISOLATION OF MONONUCLEAR CELLS

PBMNC samples obtained from healthy volunteers as well as from CLL patients before onset of therapy were collected to EDTA as anticoagulant. Mononuclear cells from blood samples were isolated in the Histopaque-1077 (Sigma–Aldrich) gradient density.

EX VIVO TREATMENT

Isolated PBMNCs were washed with phosphate-buffered saline (PBS) and resuspended at a final density of $2–5 \times 10^6$ cells/ml in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin. Leukemic cells were incubated with indicated drugs for 24 and 48 h at 37°C in an atmosphere of 5% CO_2. Mononuclear cells were treated with cladribine (C) ($C_E = 0.05$ μg/ml) or fludarabine (F) ($C_E = 7.3$ μg/ml) combined with mafosfamide (M) ($C_E = 1$ μg/ml) [Castejon et al., 1997; Bellosillo et al., 1999; Alvi et al., 2005; Kobylinska et al., 2006]. Additionally, the mononuclear cells from blood of 14 CLL patients were also incubated with ROSC (R), a tri-substituted purine derivative ($C_E = 20$ μM).

Cladribine (Biodrybin) was obtained from the Institute of Biotechnology and Antibiotics Bioton (Warsaw, Poland). Fludarabine was purchased from Schering AG (Berlin, Germany). The alkylating agent—mafosfamide was kindly donated by Baxter Oncology GmbH (Frankfurt, Germany). Roscovitine was kindly provided by Prof. Dr. M. Strnad (Palacky University, Olomouc, Czech Republic).

DETERMINATION OF THE NUMBER OF LIVING CELLS

The number of viable cells was quantified by a colorimetric MTT assay based on the cleavage of tetrazolium salt to formazan by mitochondrial dehydrogenases present in metabolically active cells. Isolated CLL cells were plated into 96-well microtiter plates and MTT

assay was performed as described previously [Cole, 1986; Rogalinska et al., 2009].

DETERMINATION OF THE RATIO OF LIVING CELLS

CLL cell samples were incubated in the absence (control) or in the presence of the drugs as indicated. The ratio of viable mononuclear cells after cultivation for 24 and 48 h was determined by flow cytometry using Vybrant Apoptosis Assay #4 according to manufacturer's procedure (Molecular Probes, Inc., Eugene, OR). Shortly, cells were simultaneously stained with green fluorescent Yo-Pro-1 and red fluorescent propidium iodide (PI) dyes and the cellular accumulation of the fluorochromes was measured at two channels and evaluated by a bivariate analysis. Yo-Pro-1 dye but not PI enters cells at early stages of apoptosis at which plasma membrane is slightly permeable, whereas living cells exclude the both dyes. Thus, using this assay the frequency of three distinct cell populations was determined: that of living (Q3), apoptotic (Q4), and dead (Q2—necrotic or late apoptotic) cells.

DETECTION OF CONDENSATION OF NUCLEI AND OF CHROMATIN FRAGMENTATION IN INDIVIDUAL CLL CELLS

In cultures morphology of cells and their density was evaluated microscopically under phase-contrast and Hoffman modulation contrast (HMC). For evaluation of nuclear morphology, cells were fixed in 3.7% paraformaldehyde in PBS and stained by Hoechst 33258 dissolved in PBS at a final concentration of 1.5 μg/ml. Preparations were then PBS washed four times and inspected under the fluorescence microscope (inverted microscope Eclipse TE300, Nikon Corporation, Tokyo).

DETERMINATION OF THE ACTIVITY OF CASPASES

The activity of caspase-9 was determined using the Homogenous Caspase-9 Glo Assay (Promega, Madison, WI). The assay provides a luminogenic caspase-9 substrate, which harbors the LEDH sequence. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the generation of light. Luminescence is directly proportional to the amount of the activated caspase-9.

The activity of caspase-3/7 was determined using the APO-ONE Homogenous Caspase-3/7 Assay (Promega) which uses the caspase-3/7 substrate rhodamine 110, *bis*-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R100) as described previously [Wesierska-Gadek et al., 2004, 2005b]. The culture medium samples of control and drug(s) treated leukemic cells were transferred into microtiter plate to separately determine the caspase activity in cells and in culture medium. Then an equal volume of caspase substrate was added and the probes were incubated at 37°C for different periods of time to assess the best signal-to-background ratio. The fluorescence was measured at 485 nm.

Moreover, the activity of both effector caspases was also determined using the Caspase-Glo 3/7 Assay (Promega). The assay provides a proluminescent caspase-3/7 substrate, which harbors the tetrapeptide DEVD sequence. Cleavage of the substrate by activated caspase-3/7 generates a "glow type" luminescent signal. Luminescence is proportional to the amount of the activated caspase. Luminescence and fluorescence were measured in the Wallac 1420 Victor, a multifunction microplate reader. Each point represents the mean ± SD (bars) of at least three replicates.

PREPARATION OF SUBCELLULAR FRACTIONS

The pelleted cells were rinsed with cold PBS and then suspended in isotonic sucrose solution containing 5 mM $MgCl_2$, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4) and protease inhibitors as previously described [Rogalinska et al., 2009]. Cell samples were homogenized in a Potter homogenizer for 3 min at 80 V as described previously by Blobel and Potter [1966]. The cell homogenates were centrifuged at 800*g* for 7 min resulting in the crude nuclear pellet and a supernatant representing a crude cytoplasmic fraction.

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Samples of the nuclear fraction isolated from leukemic mononuclear cells were incubated for 24 and 48 h without or with anti-cancer agents and prepared for calorimetric tests by the Almagor and Cole procedure [1989]. Samples were transferred into 300 μl steel pans and hermetically sealed. Calorimetric experiments were performed on a Setaram TG-DSC 111 calorimeter (Caluire, France) from 20 to 120°C at a scanning rate—R of 5 K/min (0.083 K/s) [Rogalinska et al., 2009].

The measured parameter—heat flow—HF [mW] allows to estimate the specific excess heat capacity—Cp_{ex} according to formula:

$$Cp_{ex} = \frac{HF}{R \times m}$$

where m is the DNA content [g] in the probe. The temperature calibration of the calorimeter was performed using the melting point of calibration materials gallium, indium, and tin. The temperature reproducibility was ±0.1 K. The measuring chambers were purged by dry nitrogen gas with a flow rate of 100 ml/min. For calibration of heat flow signal a sapphire standard was used.

PROTEIN SEPARATION AND IMMUNOBLOTTING

Protein concentration was determined by the method of Lowry [Lowry et al., 1951]. Protein samples loaded on 8%, 10%, or 15% SDS–polyacrylamide gels were separated by electrophoresis at 15 mA/slab according to the procedure of Laemmli [1970] and blotted onto Immobilon P membrane according to Towbin et al. [1979]. Equal protein loading and protein transfer were confirmed by Ponceau S staining. To avoid non-specific protein binding sites, the membranes were saturated with 5% non-fat dry milk in TBS (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) for at least 1 h at room temperature. After extensive washing in TBS containing 0.05% Tween-20 (TBST), blots were incubated with specific primary antibodies at the appropriate final dilution and the immune complexes were detected using appropriate HRP-conjugated secondary antibodies and the enhanced chemiluminescent detection reagent ECL+ (Amersham International, Little Chalfont, Buckinghamshire, England) [Wesierska-Gadek et al., 2004] or by development of color reaction for AP-coupled secondary antibodies after incubation with substrate solution (0.33 mg/ml of nitro blue tetrazolium, 0.17 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) prepared according to Leary et al. [1983]. Chemiluminescence was

analyzed and documented using the ChemiSmart 5100 equipped with a high resolution camera and image master software. Acquisition of chemiluminescence images using ChemiSmart 5100 offers unrivalled sensitivity and maximum dynamic range. Incubation with anti-actin or anti-Ran antibodies additionally confirmed equal protein loading.

STATISTICAL ANALYSIS

Results were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA). The statistical analyses were performed using SPSS Statistic Base 17.0 software (SPSS, Inc., Chicago, IL). Prior to the statistical analysis of the data, results of the measurement of the ratio of viable cells were normalized against control and the results of the determination of the activity of caspases were logarithmically transformed. The analysis was performed using One-way ANOVA with Dunett *t*-test, Turkey's test or Pearsońs correlation. The reported *P*-value is a result of a two-sided test. A *P*-value 5% to 1% is considered statistically significant (*), 1% to 0.1% statistically very significant (**) and <0.01% statistically extremely significant (***). The details of the statistical analysis are included in the legend to the corresponding figure.

RESULTS

PURINE ANALOGUES COMBINED WITH MAFOSFAMIDE HAVE A VERY WEAK APOPTOGENIC EFFECT ON CLL CELLS

Leukemic cells isolated from peripheral blood of six CLL patients before the onset of the therapy were treated ex vivo for 24 h with cladribine or fludarabine combined with mafosfamide, an active metabolite of cyclophosphamide (CM, FM). The viability of isolated leukemic CLL cells determined by MTT assays directly before plating averaged approximately 95% and apart from one case remained almost unaffected during cultivation for 24 h. The effects of the combined treatments on leukemic cells from five distinct patients are illustrated in Figure 1, which shows that these drug combinations had a negligible effect on the viability of the leukemic cells over a 1-day period. The addition of cladribine or fludarabine combined with mafosfamide only reduced the numbers of living cells by 11% and 13%, respectively, compared to the untreated controls. However, detailed analysis revealed that the susceptibility of leukemic cells to purine analogues combined with mafosfamide strongly varies. As shown in Figure 2, significantly stronger than average reductions in numbers of leukemic cells following FM and CM treatments (20% and 15%, respectively) were observed in cell culture from only one patient (No. 177). However, the viability of CLL cells during the 24 h cultivation of untreated controls was reduced by 40%, indicating that the CLL cells from patient No. 177 spontaneously died under ex vivo conditions (Fig. 2).

Using the Vybrant Apoptosis Assay #4 three distinct cell populations were distinguished: living cells, apoptotic cells, and dead cells. Remarkably, the frequency of apoptotic CLL cells after exposure to FM and CM for 24 h was very low (Fig. 2). Even the apoptosis-prone leukemic cells from patient No. 177 were only slightly affected; following exposure to CM and FM for 24 h the apoptotic rate of these cells increased by 8% or 9%, respectively (Fig. 2). These results imply that the low efficacy of the combined therapy might be attributable to its weak apoptogenic effect.

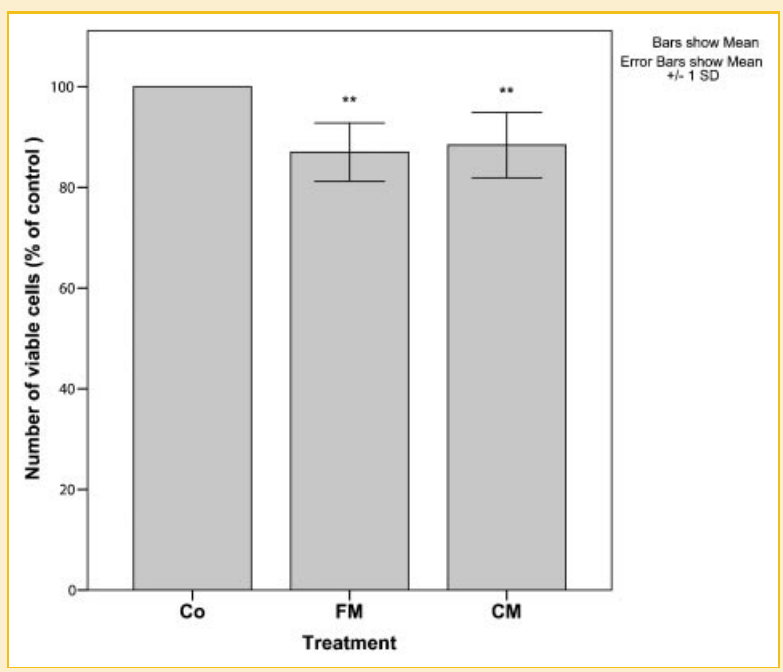

Fig. 1. Comparison of the effect of the ex vivo 24 h treatment with fludarabine and mafosfamide (FM), cladribine and mafosfamide (CM) on the number of viable leukemic mononuclear cells. Leukemic cells were seeded in 96-well microtiter plates immediately after isolation. The viability of isolated cells ranged from 95% to 98%. The measured values assessed by the MTT assay were normalized against those determined for untreated control (Co). The control cells without any drug were maintained in cell culture under the same conditions for 24 h. Bars represent mean values ± SD from five B-CLL patients. Results were analyzed using SPSS Statistic Base 17.0 software. Statistical significance was determined by Dunett *t*-test. Decrease of the number of viable cells was very significant.

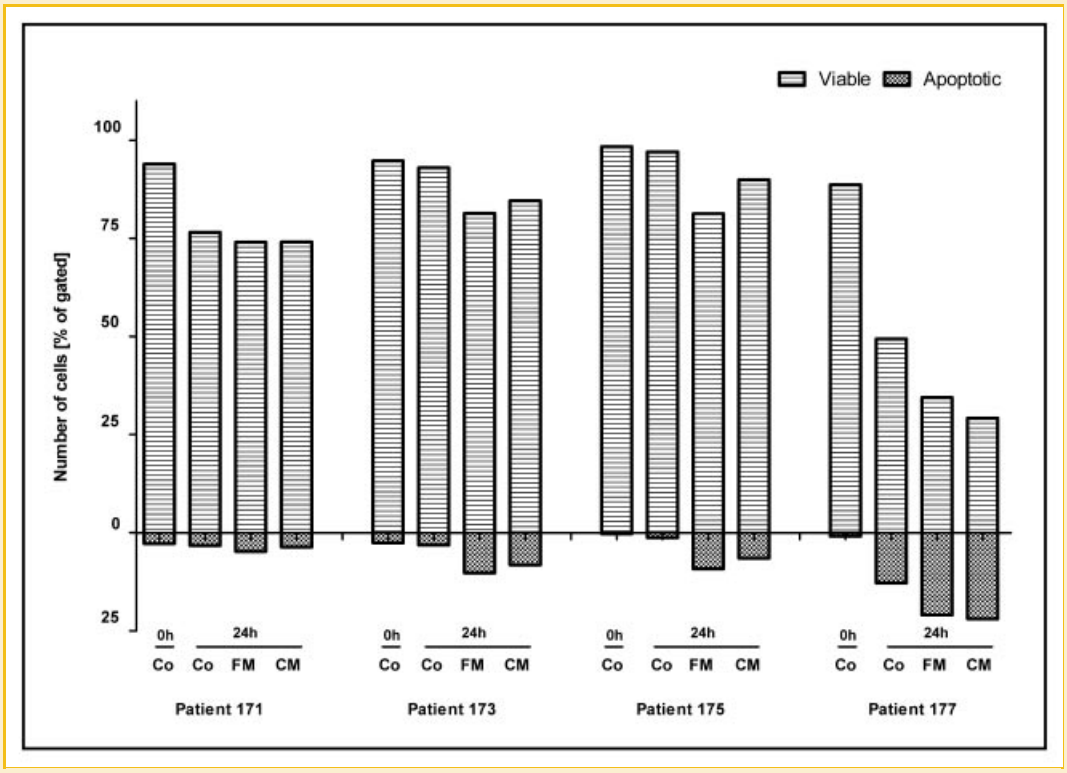

Fig. 2. Low pro-apoptotic effect of the combined FM and CM treatment of CLL cells for 24 h. Leukemic B-cells isolated from four patients were maintained in cell culture for 24 and for 48 h without drugs (Co) or in the presence of FM or CM. After treatment samples were collected and stained with the reagents provided in the Vybrant Apoptosis Assay #4. The Yo-Pro-1 and PI-stained cells were analyzed by flow cytometry after 488 nm excitation. Routinely 30,000 cells were measured by flow cytometry. On the basis of dyes accumulation three cell populations were distinguished. Viable (non-stained) and apoptotic (Yo-Pro-1-stained) cells in analyzed samples from four patients are shown. Results were evaluated using GraphPad Prism software.

ROSC HAS MUCH STRONGER EFFECTS ON THE VIABILITY OF CLL CELLS THAN OTHER PURINE ANALOGUES

In the next experimental series, leukemic cells isolated from 14 untreated CLL patients cultivated in a standard RPMI culture medium were exposed to ROSC alone, as well as to CM or FM for 24 and 48 h. The proportion of viable cells decreased following treatment in examined cell preparations from all CLL patients in a time-dependent manner (Fig. 3A). Of the three medications, ROSC affected the number of viable cells most potently (Fig. 3A), reducing the numbers of living cells by 50% after 24 h and 70% after a further 24 h. The reductions in the number of viable cells after short (24 h) and long (48 h) ROSC treatments were very highly statistically significant (Fig. 3A). However, the susceptibility of individual patients to the tested drugs markedly differed. Remarkably, the highest overall reduction in the number of living CLL cells in all tested patients was observed following treatment with ROSC (Fig. 4). Furthermore, the kinetics of the CLL cells' responses to the applied drugs significantly differed. In most cultures the CLL cells remained unaffected during the first 24 h of CM or FM treatment (Fig. 4, upper panels). Interestingly, 2 out of 14 CLL cell cultures were apoptosis-prone (those from patients Nos. 184 and 193) and an increase in the apoptosis rate was observed in control cells cultivated for 24 h without drugs. These samples were more susceptible to the action of CM and FM (Fig. 4). Unlike the combined treatment, ROSC noticeably eliminated living cells and increased the proportion of apoptotic cells (Fig. 4). Moreover, a considerable reduction in the number of viable cells was observed in 11 out of 14 CLL patients' cell samples after exposure to ROSC for 48 h (Fig. 4, lower panels). To exemplify the kinetics of the reduction in the number of living CLL cells and the progression of apoptosis, detailed results were compared for a selected patient (patient No. 200). The results (Fig. 4, Supplementary Data, Figs. S1 and S2) indicated that the combined treatments (CM and FM) did not significantly affect the proportion of viable CLL cells, even after 48 h, whereas ROSC alone reduced their numbers by ~30% after 24 h and 80% after 48 h.

In all except two cases, the spontaneous apoptosis rate in CLL patients was low. Exposure to ROSC markedly increased the proportion of apoptotic CLL cells, whereas standard therapies (CM and FM) led within the first 24 h primarily to the accumulation of dead cells (Supplementary Data, Fig. S1), then after a further 24 h the population of apoptotic cells increased (Supplementary Data, Fig. S2).

A WEAK EFFECT OF ROSC ON THE NUMBER OF LIVING NORMAL CELLS

PBMCs isolated from four healthy volunteers as well as normal human fibroblasts (F2000 and MRC-5) were used as healthy controls. The viability of isolated human mononuclear cells averaged 99%. Flow cytometric assays revealed that the human fibroblasts and isolated PBMCs were arrested in G_1 phase (more than 75% and 98%, respectively). Unlike CLL cells, normal PBMCs were barely affected by

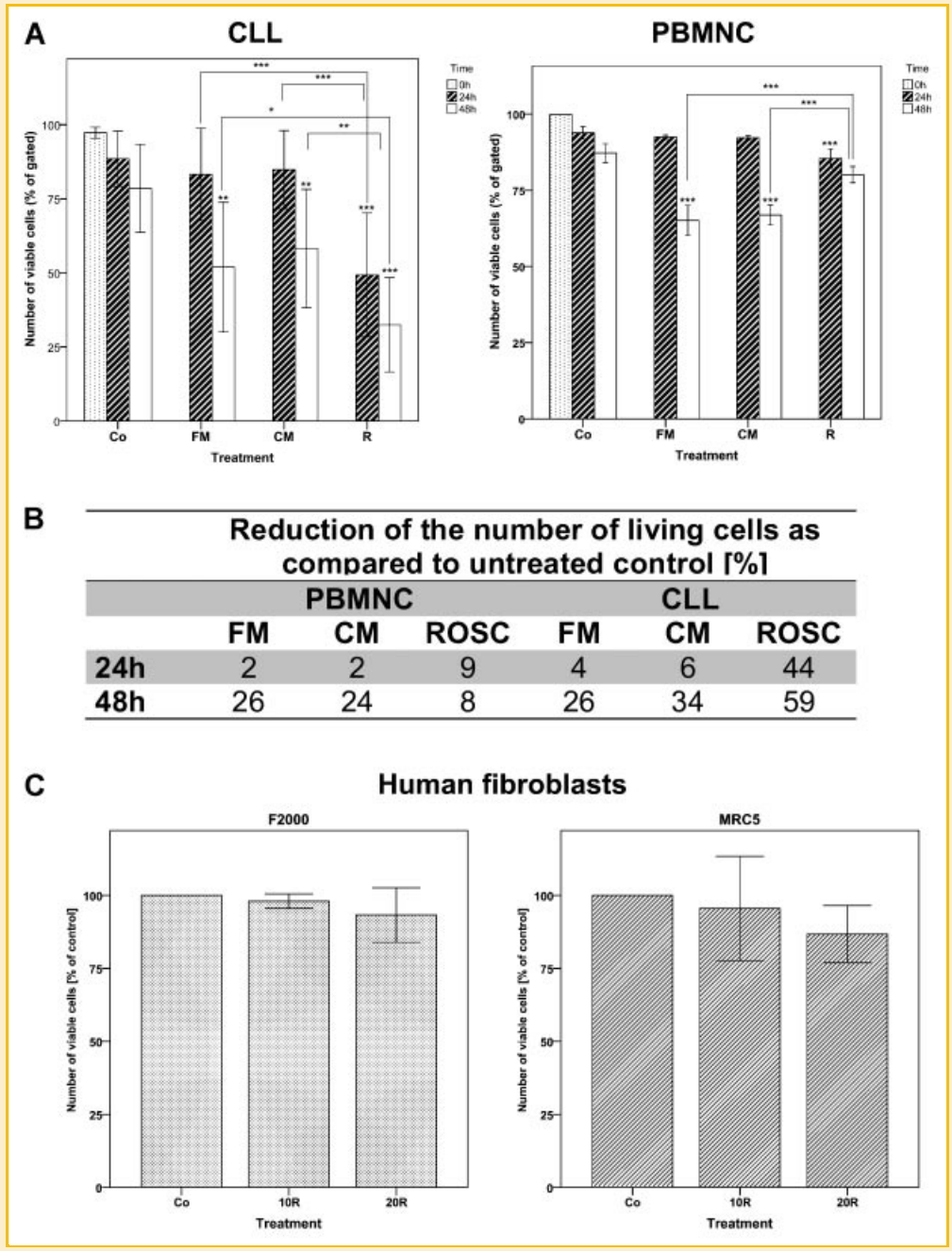

Fig. 3. Strong reduction of the number of living CLL cells after exposure to ROSC. A: Leukemic B-cells isolated from 14 patients (left panel) and normal PBMNC isolated from four healthy volunteers (right panel) were maintained in cell culture for 24 and for 48 h in the absence (Co), in the presence of FM, CM, or ROSC alone. After treatment samples (10^6 cells) were collected and stained with the reagents provided in the Vybrant Apoptosis Assay #4. Conditions of the test as described in detail in Figure 2. The Yo-Pro-1 and PI-stained cells were analyzed by flow cytometry after 488 nm excitation. Viable cells (non-stained) are shown. Bars represent mean values ± SD from 14 B-CLL patients. Data were analyzed by SPSS Statistic Base 17 statistic software. Statistic significance of the differences between treatment and control was assessed by Dunett *t*-test. Differences between distinct treatments were assessed by Turkey's test. The reduction of the number of living cells after treatment with ROSC for 24 and 48 h was statistically very highly significant ($P < 0.001$). B: Unlike ROSC, combined treatments reduce the number of viable normal PBMNCs. C: Low anti-proliferative effect of ROSC on normal human F2000 and MRC-5 cells. Normal human F2000 and MRC-5 cells were treated with ROSC as indicated for 24 h. Then the number of living cells was determined.

ROSC. After exposure to 20 μM ROSC for 48 h, the number of living mononuclear cells was reduced by 8% as compared to untreated controls (Fig. 3B). Remarkably, the number of living normal PBMCs were much stronger affected by the combined treatment (FM and CM) (Fig. 3A,B). The number of viable cells was reduced by ~25% but not before 48 h. The reduction rate and kinetics was similar to that of CLL cells and substantiates our observation on a delayed effect of the combined treatment (Fig. 3A,B). Furthermore, the effect of ROSC was also additionally tested on two human normal cells lines: F2000 and MRC-5. Human fibroblasts were only negligibly affected by 20 μM

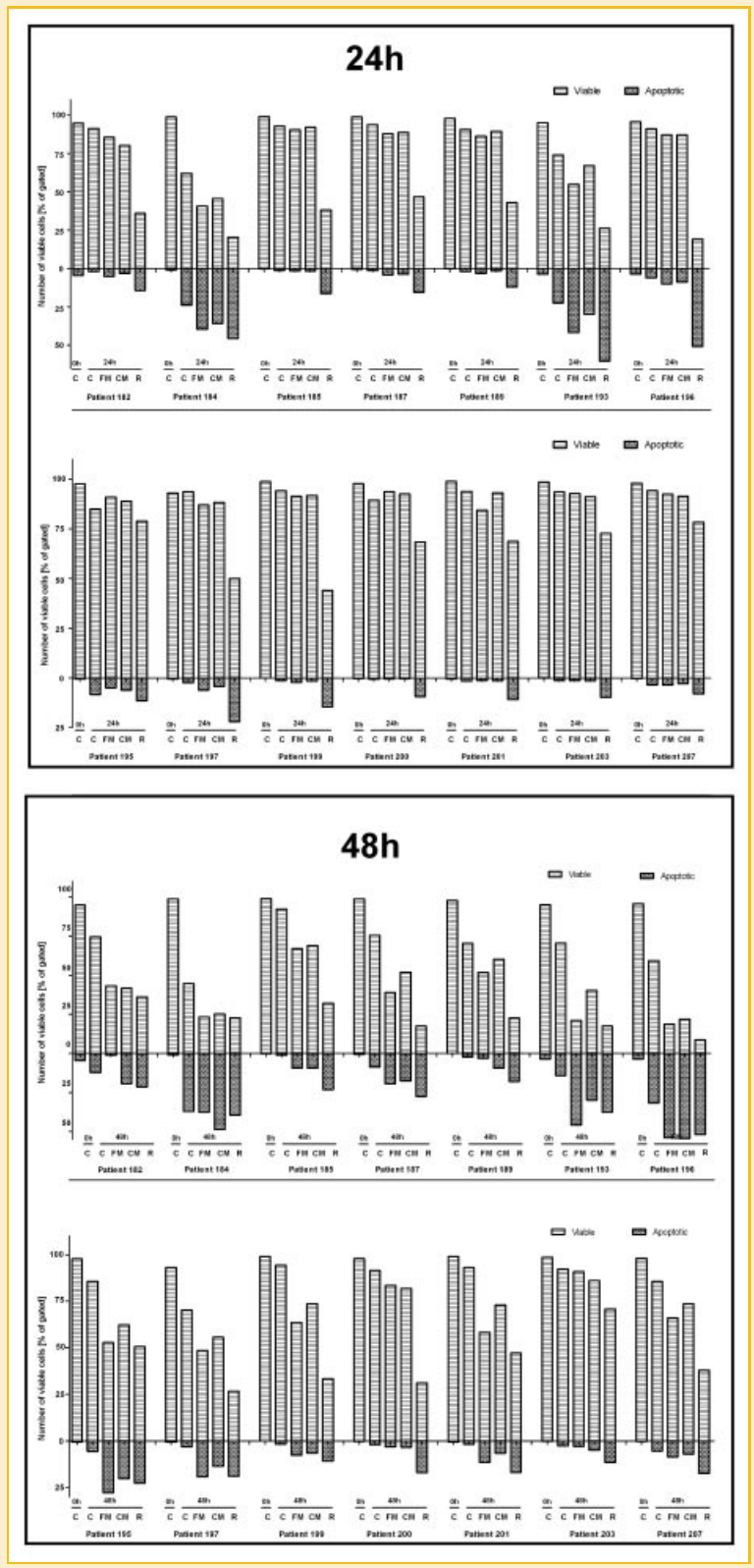

Fig. 4. ROSC-mediated decrease of the frequency of the population of viable cells after 24 h associates with the increase of the size of apoptotic population. Leukemic B-cells isolated from 14 patients were maintained in cell culture for indicated periods of time without drugs (Co), in the presence of FM, CM, or ROSC alone. After treatment samples (10^6 cells) were collected and stained with the reagents provided in the Vybrant Apoptosis Assay #4. Conditions of the assay as described in detail in Figure 2. The Yo-Pro-1 and PI-stained cells were analyzed by flow cytometry after 488 nm excitation. Viable cells (non-stained) and apoptotic cells (Yo-Pro-1-stained) were shown. Detailed results for each patient are depicted. Data were evaluated using GraphPad Prism software.

ROSC (Fig. 3C). The number of living F2000 and MRC-5 cells decreased by 8% and 15%, respectively, after treatment with ROSC for 24 h. Additional flow cytometric analyses (not shown) revealed that the sensitivity of normal cells to the CDK inhibitor strongly depends on their cell cycle status prior to the onset of treatment.

ROSC INDUCES MORPHOLOGICAL CHANGES CHARACTERISTIC OF APOPTOSIS IN CLL CELLS

Phase contrast or HMC microscopic inspection of cell cultures of untreated and ROSC-treated CLL samples revealed strong reductions in cell density following treatment with ROSC (Fig. 5, upper panel). Moreover, the drug-induced morphological changes (visualized by HMC) that are characteristic of apoptosis. These observations were further confirmed by examining fixed cells stained with Hoechst dye. Condensation of chromatin and fragmentation of the nuclei was detected in the majority of ROSC-treated CLL cells, even after treatment for 48 h (Fig. 5, lower panel). Remarkably, however, no cells with typical necrotic traits were detected in ROSC-treated cells. These observations substantiate the results of the Vybrant Apoptotis Assay and confirm the assumption that the population of Yo-Pro-1 + PI-positive cells after ROSC treatment for 48 h represented CLL cells in late apoptotic stage.

ANTI-CANCER AGENTS DIFFERENTIALLY AFFECT THE DSC PROFILES OF CLL CELLS

It has been previously reported that characteristic changes in the thermal transition at $95 \pm 3^{\circ}C$ in DSC analysis of samples from CLL patients following in vivo therapy reflect good responses to treatment [Rogalinska et al., 2009]. Therefore, we examined thermal profiles of nuclear preparations from untreated control leukemic cells and cells treated ex vivo with CM and FM, or ROSC. Significant reductions in the thermal transition at $95 \pm 3^{\circ}C$, accompanied by increases in the main thermal peak at $83 \pm 3^{\circ}C$, were observed in analyses of nuclear fractions of CLL cells from all patients after incubation with ROSC for 24 h. Remarkably, after incubating CLL cells with ROSC for a further 24 h the transition at $95 \pm 3^{\circ}C$ was strongly reduced or even lost. As shown for an illustrative patient (No. 200) in Supplementary Data (Fig. S3), a strong decrease in the thermal peak at $95 \pm 3^{\circ}C$ was accompanied by an increase in the main transition at $83 \pm 3^{\circ}C$ after incubating CLL cells with ROSC for 24 h. Further exposure of examined cells (48 h) to this agent was accompanied by almost complete loss of this transition in thermal scans. Interestingly, profiles of nuclear preparations of cell samples from the same patient exposed to CM and FM did not exhibit significant differences after 24 h incubation from controls, although incubation of the cells with FM for 48 h caused a slight decrease of a peak at $95 \pm 3^{\circ}C$, accompanied by an increase in the main transition peak at $83 \pm 3^{\circ}C$ (Fig. S3). Interestingly, this patient did not respond to the applied therapy (Table I). These data closely correlate with the results of the Vybrant Apoptosis Assay and reflect the higher efficacy of ROSC compared to both the CM and FM combinations.

ROSC MODULATES THE EXPRESSION AND ACTIVITY STATUS OF CELL CYCLE REGULATORS IN CLL CELLS

As expected, in CLL cells CDK2 is not active. Unlike in rapidly dividing human MCF-7 breast cells, used as positive controls, in leukemic lymphocytes the threonine at position 160 of CDK2 is

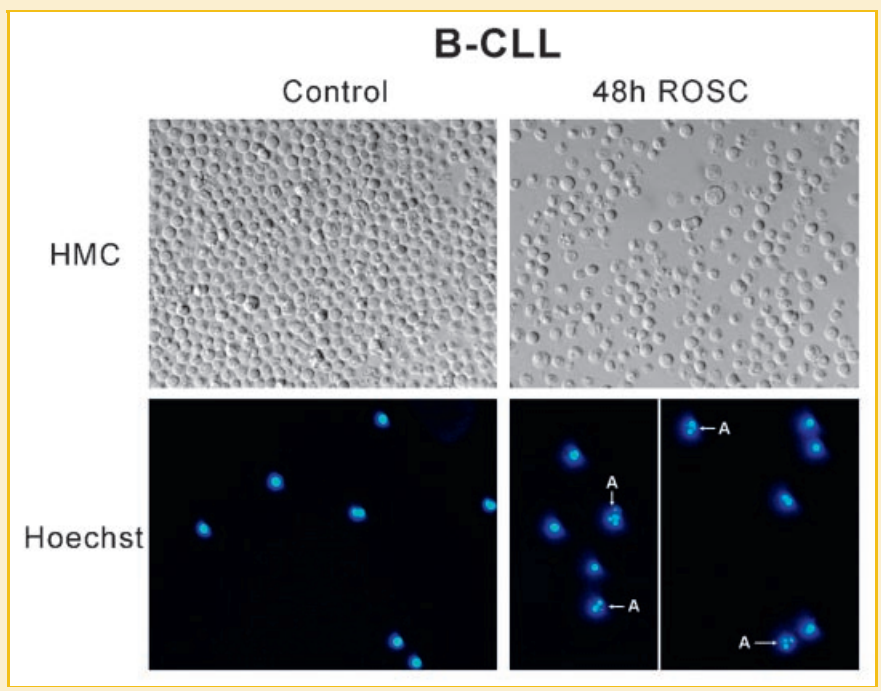

Fig. 5. Exposure of CLL cells to ROSC generates in a majority of cells chromatin changes characteristic for advanced stage of apoptosis. Untreated control and ROSC-treated CLL cells were inspected after 48 h under light microscopy (inverted microscope Eclipse TE300, Nikon Corporation, Tokyo) using Hoffman modulation contrast (HMC) (upper panel). Hoechst stained cells (lower panel) were inspected using to a longpass barrier filter (420 nm cut-on wavelength). The cell density upon treatment was markedly reduced as depicted in the (upper panel) and for these reasons Hoechst stained cells are shown in two distinct fields. Apoptotic cells are shown by arrows.

almost unmodified. This residue is localized within the T-loop and its phosphorylation is required for the change from the "closed" to the "open" conformation of CDK2. As shown in Figure 6, treatment of CLL cells with ROSC had no effect on the activity status of CDK2, but slightly reduced its cellular level. Interestingly, ROSC enhanced the phosphorylation of CDK1 at Thr14/Tyr15 in CLL cells from a few patients (Fig. 6). This indicates that ROSC is able to inhibit the activity of CDK1. Furthermore, in most CLL samples MCM-7 protein was undetectable. However, in a few cases a weak MCM-7 signal was detected by immunoblotting in untreated controls (Fig. 6). In these cases treatment with ROSC reduced MCM-7 to undetectable levels (Fig. 6). These results indicate that cells in the majority of CLL leukemic samples were G_0 arrested.

DETECTION OF CASPASE-9 AND CASPASE-3/7 ACTIVITY IN CULTURE MEDIA AFTER EXPOSING CLL CELLS TO ANTICANCER AGENT(S)

Activation of caspase-9 and caspase-3/7 upon treatment with the tested drugs was assessed by two independent approaches. First, the effects of the drugs on the intracellular processing that is required

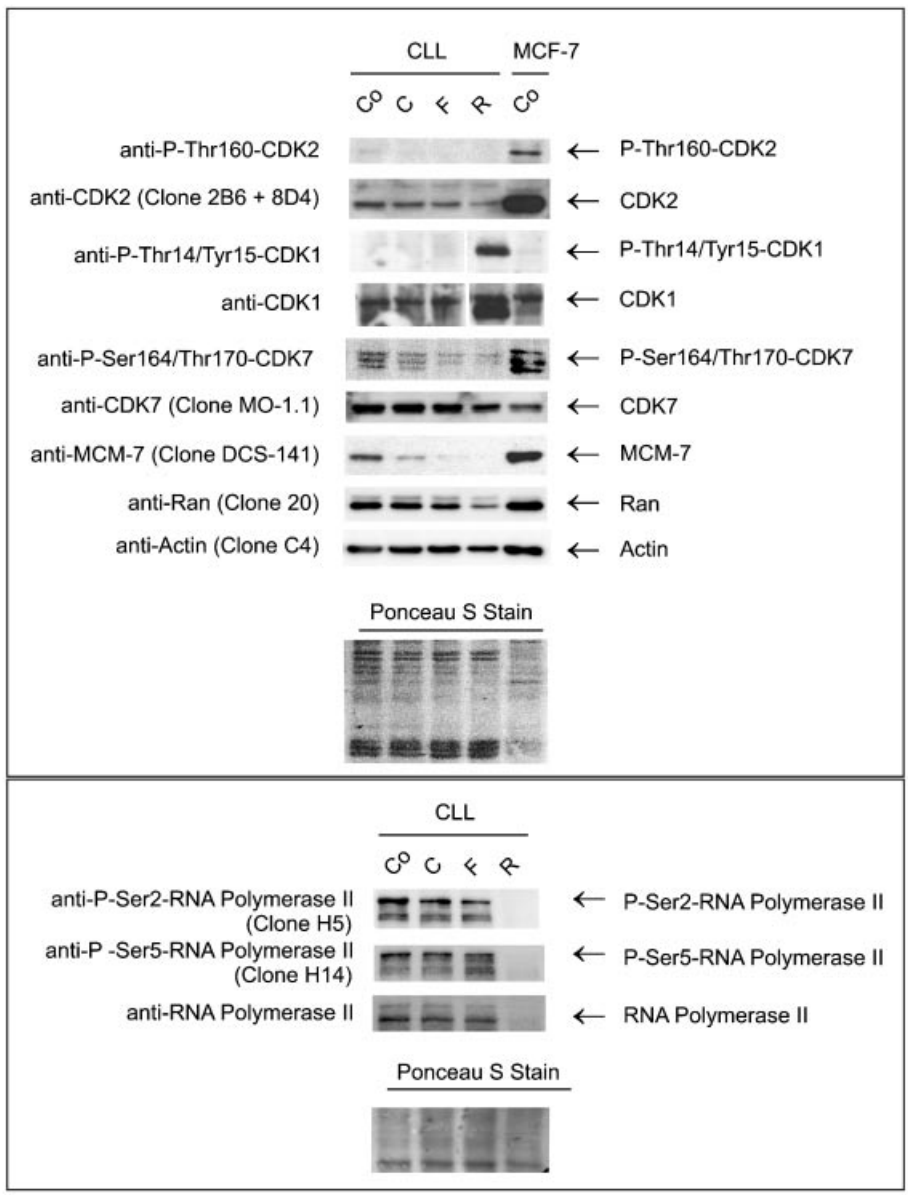

Fig. 6. Abolishing of the site-specific phosphorylation of CDK7 and RNA Pol II upon treatment with ROSC. WCLs from untreated control cells and CLL cells treated for 24 h as indicated were loaded on 12% SDS gels (30 μg/lane). After electrophoretic separation and transfer proteins immobilized on the membrane were incubated with specific primary antibodies as indicated. Immune complexes were detected after incubation with appropriate secondary antibodies coupled to HRP using ECL + chemiluminescence reagent. Acquisition of chemiluminescence images was performed using the ChemiSmart 5100 apparatus. WCL prepared from human MCF-7 breast cancer cells was loaded as a positive control. Proper transfer and equal protein loading was checked by Ponceau S staining and after incubation with anti-Ran and anti-actin antibodies.

for their activation was assessed by immunoblotting. For this purpose, proteins of whole cell lysates (WCLs) and cytoplasmic fractions, electrophoretically separated and membrane-immobilized, were incubated with antibodies recognizing pro-caspase-9 or pro-caspase-3 as well as their cleaved forms. As shown in Figure 7, in blots prepared from samples obtained from patient No. 200, the intensity of the protein bands representing intact pro-caspase-9 (Fig. 7A) and pro-caspase-3 (Fig. 7B) markedly decreased after treatment with ROSC for 24 h, whereas the combined treatments (CM and FM) for 24 h did not diminish the level of pro-caspase-9 (Fig. 7A). After 48 h treatment, processing of pro-caspase-9 was observed after administration of either FM or ROSC (Fig. 7A).

In the second approach, the activity of caspase-9 and caspase-3/7 released into the culture medium was quantified using caspase-9 and caspase-3/7 Glo assays. Both assays provide specific pro-luminescent substrates that, after cleavage by activated caspases, produce luminescence, the intensity of which is directly proportional to the amount of activated caspases present in the medium. The assays revealed that exposure of CLL cells to the tested anti-cancer drugs induced increases in the activity of caspase-9 and effector caspases-3/7 (Fig. 8). However, the caspase-inducing capability strongly varied both between the drugs and between individual patients. A weak (approximately twofold) increase in caspase-9 activity in the culture medium after exposing CLL cells from patient No. 200 to CM and FM for 24 h was observed (Fig. 8), fitting very well with the immunoblotting results (Fig. 7A). Unlike CM and FM combinations, ROSC alone very strongly activated both caspases (Fig. 8 and Table II). The activities of caspase-9 and caspase-3/7 increased 7- and 14-fold after exposing leukemic B-cells to the selective CDK inhibitor for 24 h, and increased 15- and 21-fold after 48 h exposure, respectively. Interestingly, the weak (twofold) increase of caspase-9 and caspase-3/7 activity observed after exposure of CLL cells from patient 200 to CM and FM for 48 h is statistically significant. Moreover, the ROSC-mediated enhancement of the activity of caspase-9 and caspase-3/7 is statistically significant relative to activities in both controls and samples treated with CM and FM for 24 or 48 h.

These results provide evidence that both Caspase-Glo assays are excellent tools to detect and measure the activity of caspases within very high activity ranges. In this context the more pertinent question appears to be whether the tests are suitable to determine weak enzyme stimulation. According to our experience the APO-One assay, a test similar to Caspase-3/7 Glo but based on the cleavage of pro-fluorescent caspase-3 substrate, seems to be more sensitive, however, there is no corresponding test for detecting caspase-9. Therefore, here we used the Caspase-Glo assays to assess the correlation of the activation of the two kinds of caspases in CLL cells.

The results (see plot in Supplementary Data, Fig. S4) demonstrate that there is a linear relationship between the activities of caspase-9 and effector caspase-3/7. Considering this close correlation, we decided to quantify the activity of effector caspase-3/7 in culture media of all 14 examined cell samples exposed to the tested drug combinations and to ROSC for 24 and 48 h using the Apo-One test. The results obtained by this assay provided additional data to that obtained using Caspase-3/7 Glo assays. While Caspase-3/7 Glo adequately measured the very high enzymatic activities induced by ROSC, the Apo-One assay was also able to quantify a weaker increase in caspase-3/7 activity. The results of the latter test for the 14 CLL patients are summarized in Table II and were statistically evaluated (Fig. 9). Exposure of CLL cells from the patients to standard therapy (CM and FM) for 48 h increased the activity of caspase-3 approximately threefold, whereas ROSC enhanced its activity sixfold. Detailed inspection of the results (Table II) revealed that ROSC stimulated caspases in leukemic cells from some patients (Nos. 187, 193, 196, and 208) very strongly and those in cells from other patients only moderately. Therefore, we divided the examined samples into two distinct groups, one of moderate ROSC responders (10 patients; Fig. 9B, left panel) and one of strong ROSC responders (4 patients; Fig. 9B, right panel), and compared the drug-induced activation of caspase-3/7 independently in the two groups. As depicted in Figure 9B, even in the group of moderate ROSC responders (left panel) the increases in caspase-3/7 activity after ROSC exceeded those observed after treatment with CM or FM. Taken together, our results show that of the three tested treatment regimens ROSC induces the strongest activation of caspases, after both short (24 h) and long (48 h) treatment.

EFFICACY OF THE TREATMENT OF CLL CELLS CORRELATES WITH THE ACTIVATION OF EFFECTOR CASPASES

Finally, we assessed whether the reductions in numbers of viable cells following the medications was linked to the release of the activated caspase-3 into the culture medium. The results (Fig. 10) show that the reduction in numbers of living cells correlates very strongly with the release of activated effector caspases into the culture medium (Pearson correlation coefficient = 0.754). This observation explains why the exposure of CLL cells to FM and CM combinations has a relatively weak and retarded effect on leukemic cells.

DIFFERENTIAL CELLULAR LEVELS OF APOPTOSIS-RELATED PROTEINS BETWEEN INDIVIDUAL PATIENTS

As mentioned in the previous section, the caspase-inducing capability strongly varied between individual patients. The observed variability may be attributable to differential expression of caspases and/or of inhibitors of apoptosis (IAPs). The analysis of the cellular levels of some regulators of apoptosis in leukemic cells from seven untreated CLL patients revealed marked differences (Fig. 7C) in the expression of Bcl-2, Mcl-1, pro-caspase-3 and 9 thereby substantiating the observation on varying apoptosis rate between leukemic cells from individual patients in response to medication.

ROSC REDUCES CELLULAR LEVELS OF INHIBITORS OF APOPTOSIS IN CLL CELLS

Determinations of the proportion of apoptotic cells (Fig. 4) and the activity of caspases (Figs. 8 and 9) revealed that, unlike CM and FM, ROSC primarily induces caspase-dependent apoptosis in CLL cells. In addition, immunoblot analyses revealed that cleavage of pro-caspase-9 and pro-caspase-3 (Fig. 7) in the ROSC-treated cells is associated with the decrease and subsequent loss of the intact poly(ADP-ribose) polymerase-1 (PARP-1), accompanied by the appearance of its truncated (89 kDa) form (Fig. 7). The shortened PARP-1 is a carboxy-terminal product generated by caspase-3

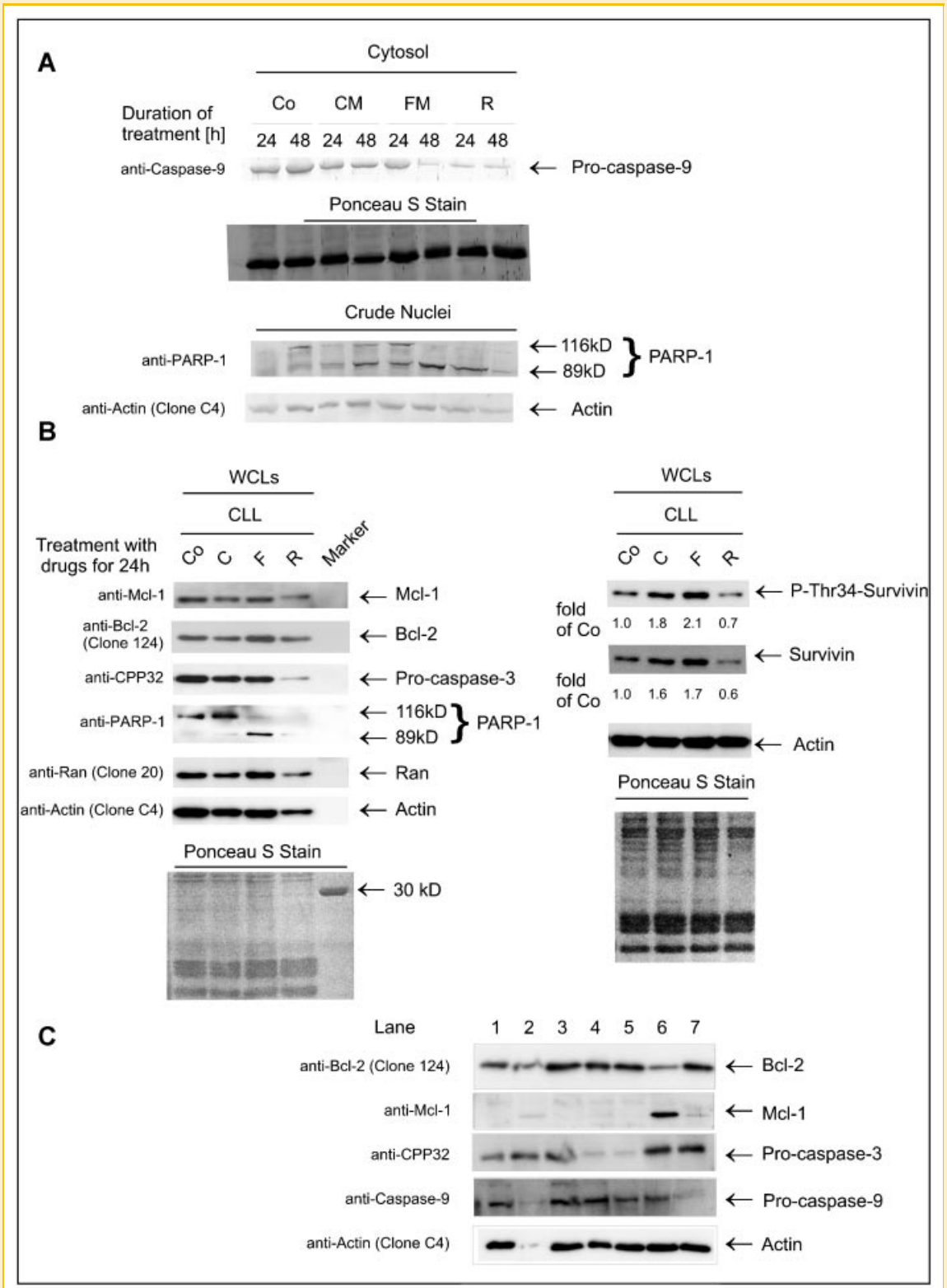

Fig. 7. ROSC reduces the cellular levels of inhibitors of apoptosis and results in cleavage of caspase-9. Subcellular fractions (A) or WCLs (B,C) were loaded. Samples isolated from patient No. 200 (A) and from a representative ex vivo late responder (B) were analyzed. Conditions of immunoblotting as described in detail in Figure 6. Antigens shown in upper part (A) were detected using AP-linked secondary antibodies and color reaction. Intensity of P-survivin and of total survivin was evaluated using software from Chemismart and was normalized against that of actin. C: Differential cellular levels of apoptosis-related proteins in leukemic cells. WCLs s from seven untreated CLL patients were loaded on 12% SDS gels, electroblotted and incubated with indicated antibodies. In the 2nd lane the actin signal is lacking due to incomplete electrotransfer (air buble).

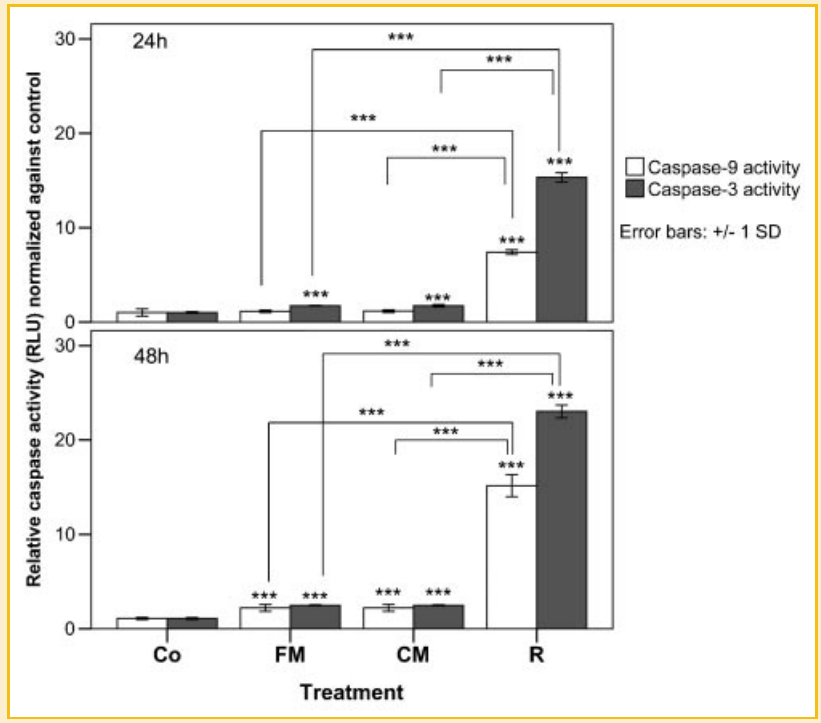

Fig. 8. A very strong accumulation of activated caspases in post-cultured media after exposure of CLL cells to ROSC. CLL cells isolated from patient 200 were exposed to indicated drugs for 24 h (upper panel) and 48 h (lower panel). Thereafter, post-cultured media of leukemic mononuclear cells were separated from cells, collected and aliquots were used for determination of the activity of caspases using Caspase-9 Glo and Caspase-3 Glo assays according to the protocol recommended by the manufacturer. Luminescence (relative luminescence units, RLU) was measured in the Wallac 1420 Victor, a multifunction microplate reader. The mean luminescence values of three replicates were normalized against the number of viable cells in corresponding cell culture. The data were analyzed using SPSS Statistic Base 17 software. Differences between treatment and control were assessed by Dunett *t*-test. Differences between different treatments were assessed by Turkey's test.

TABLE II. Relative Activity of Caspase-3 Released in Culture Medium After Treatment of CLL Cells for 48 h

Patient no.	Response to treatment		Relative caspase-3 activity			
	In vivo	Ex vivo[a]	Control	CM	FM	R
182	CC/CR	CM/late	1	6.02	2.73	3.38
184	FC/nd	FM/early	1	2.86	2.94	3.68
185	CC/PR	CM/late	1	2.51	1.24	4.86
187	CC/CR	CM/late	1	13.00	13.99	37.76
189	CC/PR	CM/late	1	0.95	1.69	2.70
193	CC/CR	CM/early	1	3.76	8.14	11.75
195	CC/PR	CM/late	1	3.80	5.46	6.30
196	CC/CR	CM/late	1	6.17	7.11	22.87
197	CC/CR	CM/late	1	2.24	2.82	5.91
199	CC/NR	CM/late	1	2.75	3.99	7.53
200	CC/NR	CM/MD	1	1.42	1.41	5.67
201	CC/NR	CM/late	1	4,28	3,95	7.16
203	CC/CR	CM/MD	1	1.01	0.91	1.36
207	Chlorambucil/nd	Late	1	5.77	7.49	15.52

CC, cladribine + cyclophosphamide; CM, cladribine + maphosphamide; FC, fludarabine + cyclophosphamide; FM, fludarabine + maphosphamide; R, roscovitine; CR, complete response; PR, partial response; NR, non-responder; nd, not determined.
Early, after 24 h; Late, after 48 h; MD, marginal decrease of the number of CLL cells.
[a]Ex vivo response was defined as a marked decrease of the number of CLL cells.

activity, and its accumulation further substantiates the results of other tests indicating the strong activation of caspases in ROSC-treated CLL cells.

Questions regarding the apoptotic trigger apparently stimulated by ROSC were addressed by monitoring cellular levels of anti-apoptotic proteins, such as Bcl-2, Mcl-1, and survivin, levels of all of which were found to decline markedly following treatment with ROSC (Fig. 7B). ROSC also abolished phosphorylation of the small inhibitor of apoptosis at threonine 34, which is required to maintain the stability of survivin. The quantification of protein bands revealed that intensity of threonine 34-phosphorylated form strongly correlates with the level of total survivin. After treatment with cladribine and fludarabine the intensity of both forms increased and after ROSC was diminished. Hence, following ROSC-mediated abrogation of the site-specific phosphorylation of survivin it became unstable and was degraded.

DIMINUTION OF PHOSPHORYLATION OF CDK7 AND RNA POL II IN ROSC-TREATED CLL CELLS

Finally, we addressed the mechanism whereby ROSC is able to reduce the cellular levels of other anti-apoptotic proteins, such as Mcl-1 or Bcl-2, by examining the phosphorylation status of CDK7 and RNA Pol II in ROSC-treated and control cells. ROSC abolished

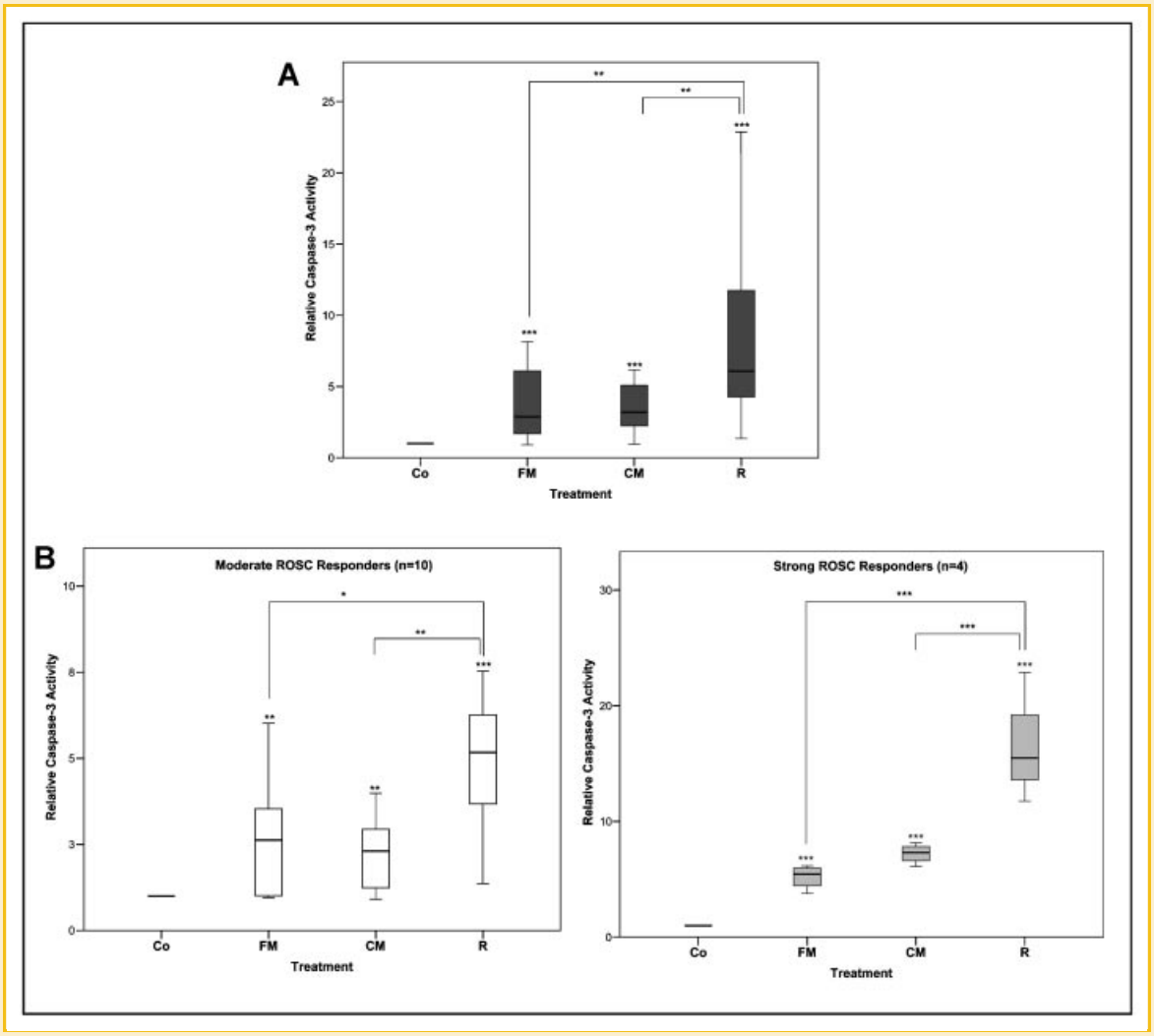

Fig. 9. A highest level of activated caspases detected in post-cultured media after exposure of CLL cells to ROSC. CLL cells isolated from 14 CLL patients were exposed to indicated drugs for 48 h. Thereafter, post-cultured media of leukemic mononuclear cells were separated from cells, collected and aliquots were used for determination of the activity of caspase-3 using APO-One assay according to the protocol recommended by manufacturer. Fluorescence (relative fluorescence units, RFU) was measured in the Wallac 1420 Victor, a multifunction microplate reader. The mean fluorescence values of three replicates were normalized against the number of viable cells in corresponding cell culture. The data were analyzed using SPSS Statistic Base 17 software. Differences between treatment and control were assessed by Dunett *t*-test. Differences between distinct treatments were assessed by Turkey's test. A: Analysis of the results of caspase-3 activity obtained for all patients. B: Depending on the susceptibility of leukemic cells to ROSC, the examined 14 patients were divided in two groups: moderate ROSC responders (9 patients) and strong ROSC responders (5 patients). As classification criterion of a strong ROSC response was used at least 10-fold induction of the caspase-3/7 activity cell number after exposure to ROSC for 48 h. The effect of three types of medication on the activity of effector caspases was evaluated in both groups. The data were analyzed using SPSS Statistic Base 17 software statistic software. Differences between treatment and control were assessed by Dunett *t*-test. Differences between distinct treatments were assessed by Turkey's test.

the modification of residues at positions 164 and 170 of CDK7, but did not affect its total level (Fig. 6B). The phosphorylation of the serine at position 164 and threonine at position 170 is critical for recognition of the substrate by CDK7 and phosphorylation of CTD of RNA Pol II. Hence, non-phosphorylated CDK7 is not able to induce phosphorylation of CTD, resulting in a transcriptional block. Accordingly, ROSC prevented the phosphorylation of CTD of RNA Pol II at two critical serine residues (Ser 2 and Ser5) within the repetitive heptad motif (Fig. 6). The total level of RNA Pol II was also diminished. Thus, ROSC diminishes cellular levels of IAPs by two independent mechanisms: inhibition of the phosphorylation of survivin and of CDK7. Whereas the first mechanism causes the destabilization of only one protein, the latter has a serious impact on global transcription in both dividing and non-dividing cells.

LEUKEMIC CELLS ARE MUCH MORE SENSITIVE TO ROSC THAN NORMAL MONONUCLEAR CELLS

Unlike FM and CM, ROSC only weakly decreased the number of living normal PBMNCs. The comparison of the impact of the tested medications on normal and leukemic cells revealed that the latter are approximately eightfold more sensitive to ROSC than the normal healthy cells (Fig. 3B). Furthermore, the doubling of the ROSC treatment time decreased only the number of leukemic cells but not of normal PBMNCs. Moreover, it became also evident that both

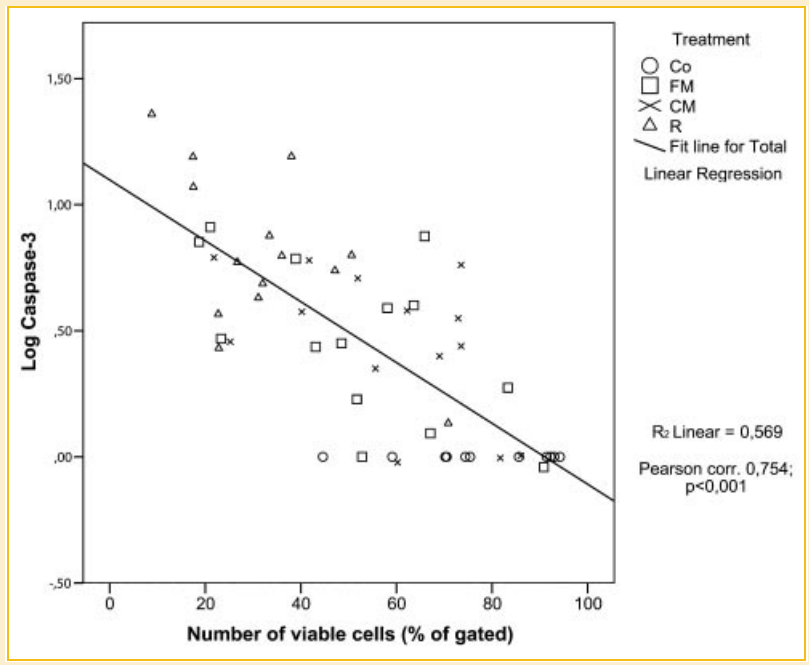

Fig. 10. Reduction of the number of living cells closely correlates with the enhancement of the effector caspases. SPSS Statistic Base 17 software (SPSS, Inc., Chicago, IL) was used to statistical calculations. Correlation between the number of viable cells and caspase-3 activity determined by APO-One assay was assessed by determination of Pearson's correlation index.

combinations: FM and CM display delayed effect not only on CLL cells but also on human healthy mononuclear cells. Surprisingly, treatment with FM for 48 h exerted the same cytotoxicity on normal and leukemic cells (reduction of the cell number by 26%). Leukemic cells were slightly more sensitive to CM (by 8%) than normal PBMNCs (Fig. 3B).

PREDICTIVE RELEVANCE OF EX VIVO TESTING

Finally, we compared the efficacy of ex vivo and in vivo therapy. Three (Nos. 199, 200, and 201) out of 14 CLL patients (21%) treated with cladribine combined with cyclophosphamide (CC) did not respond to the in vivo therapy (Table I). Furthermore, samples from these three patients did not respond under ex vivo conditions to either of the standard therapies (CC and FC) after 24 h, and displayed low susceptibility after 48 h (Fig. 4). However, ROSC efficiently triggered the leukemic cells of the non-responders into apoptosis (Fig. 4), reflected by the strong activation of effector caspases (Table II). In contrast, four (Nos. 182, 187, 193, and 196) out of the 14 CLL patients (29%) who provided samples that displayed marked reductions of numbers of leukemic cells and high activity of caspase-3 following ex vivo exposure to CC were good responders to the in vivo therapy and underwent complete remission. Hence, results of ex vivo testing of the sensitivity of leukemic cells to different therapy regimens showed very good correlations with the efficacy of the in vivo therapy in all except one case (No. 203) and to a lesser extent patient No. 197. How to explain the fact that in some cases there is no correlation between in vivo and ex vivo data? Ex vivo assays were performed on untreated CLL cells collected prior to the onset of first therapy cycle and the response was evaluated after treatment for 24 and 48 h. On the other hand, the treatment regimen for CLL patients encompasses 5–6 sequentially administered therapy cycles and the clinical response is evaluated after the last cycle. Considering the fact that patients respond to the treatment with different kinetics, it is conceivable that patient's No. 203 response to the in vivo therapy was long delayed and was therefore not registered under ex vivo conditions. In most cases the strongest reductions in the numbers of leukemic cells, accompanied by the highest rate of caspase-dependent apoptosis, were observed following treatment with ROSC. Taken together, our results show that testing of CLL cells with different drugs in culture should facilitate the choice of the optimal therapies for individual patients.

DISCUSSION

CLL is a highly heterogeneous, currently incurable disease occurring primarily in the elderly. Unlike most human malignancies, in CLL the appearance of cancer cells is attributable primarily to defects of the apoptotic pathway [Gale et al., 1994; Castejon et al., 1997; Reed, 1998; Bellosillo et al., 1999; Genini et al., 2000; Marzo et al., 2001; Reed and Pellecchia, 2005; Kobylinska et al., 2006; Reed, 2008]. Thus, leukemic lymphocytes in CLL patients consist of two distinct cell populations: dividing and non-dividing cells [Reed and Pellecchia, 2005]. Long-living quiescent cells constitute the vast majority of CLL cells in the peripheral circulation. Therefore, their susceptibility to conventional anti-cancer drugs that primarily interfere with DNA synthesis and cell division is relatively low, since

more than 90% of CLL cells are arrested in G_1 or G_0 phases of the cell cycle. The enhanced longevity of CLL cells results in their strong accumulation. The increased survival of leukemic B lymphocytes is attributable to the overexpression of anti-apoptotic factors and reduced levels of pro-apoptotic factors [Kitada et al., 1998]. Thus, drugs targeting apoptotic pathways should theoretically facilitate the initiation of apoptosis in CLL cells [Bentley and Pepper, 2000; Reed and Pellecchia, 2005; Reed, 2008]. Currently, standard therapies for CLL are based on the administration of cytotoxic drugs, which promote the initiation of apoptosis via activation of p53-mediated cellular responses to DNA damage. Activated p53 tumor suppressor protein is a potent inductor of cell death via multiple cellular targets. However, this therapeutic strategy can operate only when DNA damage signaling pathways and cellular p53 response are intact and operating properly in cancer cells. Unfortunately, in a number of CLL patients the DNA damage signaling machinery and/or p53 pathway is defective, rendering them insensitive to the standard therapy. Moreover, although satisfactory remission rates can be achieved with combined therapy, most CLL patients relapse after first therapy, and numerous CLL patients develop resistance to purine analogues such as fludarabine [Keating et al., 2002]. Surprisingly, patients with relapsed, and even with fludarabine-refractory, disease have responded well to ex vivo ROSC treatment [Weingrill et al., 2007]. Furthermore, ROSC can very efficiently induce apoptosis irrespective of the patients' pretreatment status, and can restore sensitivity to alemtuzumab in previously alemtuzumab-resistant leukemic cells [Weingrill et al., 2007].

However, apoptosis-based therapeutic strategies are unlikely to have high efficacy for treating CLL patients in isolation, since leukemic cells have varying proliferative status. However, pharmacological inhibitors of CDKs seem to be good candidates for treating CLL patients [Hahntow et al., 2004; Alvi et al., 2005; Fischer and Gianella-Borradori, 2005; Wesierska-Gadek and Schmid, 2006]. Furthermore, some of the selective CDK inhibitors, like ROSC, have dual activities and thus offer the possibility of simultaneously targeting cell proliferation and cell survival, since ROSC inhibits not only kinases involved in the regulation of the cell cycle like CDK2 and CDK1, but also affects the activity of CDK7.

In the present study the efficacy of the commonly used therapeutic regimens for treating CLL patients was compared under ex vivo conditions with that of ROSC. Interestingly, ROSC resulted in the highest reduction of the living CLL cells after both short (24 h) and long (48 h) treatments in all studied samples. Of 14 CLL patients nine (64%) were early (24 h) and five (36%) late responders (48 h) to ROSC. The strong reductions in the proportions of viable cells were accompanied by the accumulation of apoptotic cells, as evidenced by a selective uptake of permeant YO-PRO 1 dye and concomitant exclusion of PI. During the progression of apoptosis following ROSC treatment, the plasma membrane of leukemic lymphocytes became permeable and cells absorbed both fluorochromes. Simultaneously, cellular enzymes such as activated caspases and apoptotic protease (data not shown) were released into the culture medium. In addition, the most striking changes in DSC profiles occurred in the ROSC-treated nuclei and seem to reflect the chromatin condensation and/or fragmentation characteristic of cells undergoing apoptosis. Unlike other tested drugs, ROSC also triggered very strong activation of caspase-9 within 24 h, followed by strong increases in the activity of effector caspases, which were detected after a further 24 h. The strong activation of caspase-3 is also reflected in the appearance of the truncated form of the nuclear enzyme PARP-1, since the PARP-1 molecule harbors a DEVD motif in its amino-terminal domain that is recognized and cleaved by activated caspase-3 [Kaufmann et al., 1993; Lazebnik et al., 1994]. The loss of intact PARP-1 and generation of its truncated carboxy-terminal fragment was observed after exposure to ROSC for 24 h, and proteolytic processing of the truncated 89 kDa form of PARP-1 proceeded during the following 24 h of ROSC treatment, reflecting the strong activation of effector caspases detected in the measurements of caspase-3/7 activities.

Our results clearly show that the combined CM and FM treatments reduced populations of leukemic cells significantly less effectively than ROSC, possibly for the following reasons. The purine analogues cladribine and fludarabine, which are frequently used in the treatment of CLL in various combinations with alkylating agents (e.g., cyclophosphamide) mainly affect cycling cells [Robak, 2005; Van den Neste et al., 2005]. Cadribine inhibits the enzyme adenosine deaminase, thereby interfering with the processing of DNA, while fludarabine inhibits DNA replication by affecting the activity of ribonucleotide reductase and DNA polymerase [Genini et al., 2000]. Unlike these purines, ROSC prevents the activation of cellular CDKs; in dividing cells ROSC inhibits CDK2, and at higher concentrations CDK1 and CDK7. The latter kinase is a bifunctional enzyme that acts at the crossroads between cell cycling and transcription [Fisher, 2005]. It phosphorylates cell cycle kinases and thus its inactivation abolishes the activating phosphorylation of CDKs within the T-loop [Fisher, 2005; Lolli and Johnson, 2007]. CDK7 is also involved in regulation of the activity of RNA polymerase II, in which phosphorylation of the repetitive heptapeptide motif within its CTD is required for transcription [Komarnitsky et al., 2000; Buratowski, 2003; Gudipati et al., 2008]. The unphosphorylated CTD binds proteins of the preinitiation complex and is involved in the assembly of the inactive transcription machinery on the promoter DNA [Hirose and Ohkuma, 2007; Lolli 2009].

Promoter-proximal pausing of RNA Pol II after its recruitment and initiation at a gene promoter seems to be a universal rate-limiting step after transcription initiation [Krumm et al., 1995]. P-TEFb complexes facilitate the transition from abortive to productive elongation by sequential phosphorylating the CTD [Peterlin and Price, 2006]. There are three serine residues within the heptapeptide motif of CTD, two of which (at positions 2 and 5), might influence constellation of cellular factors associated with it and therefore are critical for its activity. In the first step CDK7 phosphorylates the Ser5 residues within tandemly repeated heptapeptides of CTD [Trigon et al., 1998] thereby priming it for further modification catalyzed by CDK9, a catalytic component of P-TEFb [Ahn et al., 2004]. Subsequent phosphorylation of both: CTD at the Ser2 residues within heptad repeats and of two negative elongation factors NELF and DSIF by active CDK9 is important for abrogation of the NELF- and DSIF-dependent block and promotes the processivity of elongation. It has been recently shown that ROSC prevents the phosphorylation of CDK7 at Ser164 and Thr170 [Wesierska-Gadek et al., 2009], resulting in abolition of the modification of Ser 5 of

CTD [Paprskarova et al., 2009]. These events lead to the inhibition of transcription and down-regulation of cellular anti-apoptotic proteins [Paprskarova et al., 2009] and the repression of virally encoded oncoproteins in human HeLa cervix carcinoma cells [Wesierska-Gadek et al., 2009].

In CLL cells the cell cycle kinases CDK2 and CDK1 are inactive, as evidenced by the lack of phosphorylation at their T-loops. Therefore, treatment with ROSC did not affect their activity status, but reduced their cellular levels. Remarkably, the pharmacological CDK inhibitor strongly enhanced the phosphorylation of CDK1 at inhibitory sites in leukemic B-cells, implying that it also has a marked impact on dividing B-cells. It is known that increased phosphorylation of CDK1 at Thr14/Tyr15 following ROSC treatment inhibits G_2/M transition, resulting in the accumulation of G_2 arrested cells [Wesierska-Gadek et al., 2005a]. However, ROSC abolished the site-specific phosphorylation of CDK7 and RNA Pol II, resulting in blockage of global transcription and (hence) reduction in the expression of some anti-apoptotic proteins such as Bcl-2 and Mcl-1. Moreover, ROSC prevented the phosphorylation of survivin at Thr34, the smallest member of the family of IAPs. This site-specific phosphorylation maintains the stability of survivin, a structurally unique IAP lacking a carboxy-terminal RING finger that is a bifunctional protein, acting not only as a suppressor of apoptosis, but also as a component of the chromosomal passenger complex; an essential regulator of cell division [Altieri, 2003]. Moreover, phosphorylation of survivin at Thr34 in addition to increasing its stability enhances its interaction with caspase-9 and SMAC/DIABLO [Song et al., 2003]. Thus, interference with the phosphorylation and expression of survivin stimulates caspase-dependent cell death [Wesierska-Gadek et al., 2009].

Our results are consistent with previously reported findings [Hahntow et al., 2004; Alvi et al., 2005], but also provide new insights. The cited authors subjected CLL cells to three different treatment protocols: irradiation (IR) plus fludarabine, IR combined with ROSC (CYC202) and fludarabine plus ROSC [Alvi et al., 2005]. They found that ROSC was the best therapeutic choice even as a single agent because it is able to eliminate leukemic cells by rapid induction of apoptosis [Alvi et al., 2005]. In our study the efficacies of the commonly used therapeutic regimens were compared for the first time with that of ROSC under ex vivo conditions. Our results demonstrate that ROSC rapidly sensitizes CLL cells from all tested patients to initiation of apoptosis and its efficiency exceeds the therapeutic effects of CM and FM combinations. In addition, we compared the efficacy of in vivo and ex vivo standard therapy for the first time. The results revealed a good correlation, showing that CLL patients that were resistant to the standard therapy in vivo and ex vivo efficiently responded to ROSC, indicating that ex vivo testing of leukemic cells is of predictive relevance.

Taken together, our results show that ROSC is the most efficacious of the tested drugs for ex vivo therapy of CLL cells. It enhances the rate of apoptosis in leukemic, but not healthy, cells by several independent mechanisms. It seems likely that application of ROSC in vivo would affect not only quiescent cells in the peripheral circulation, but also the dividing cells in proliferating centers (via other mechanisms, such as the inhibition of activating phosphorylation of CDK2 and induction of the inhibitory phosphorylation of CDK1). Moreover, our findings demonstrate that release of activated effector caspases into the culture medium upon ex vivo medication of CLL cells closely correlates with the efficacy of the therapy. The treatment with ROSC, a non-genotoxic CDK inhibitor, is advantageous because it induces caspase-dependent apoptosis in leukemic cells very rapidly, without a risk of a second cancer developing. This observation might be of clinical importance in the future.

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