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Action of resveratrol alone or in combination with roscovitine, a CDK inhibitor, on cell cycle progression in human HL-60 leukemia cells

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ABSTRACT

Results of a number of epidemiological and experimental studies indicate that polyphenols (e.g. resveratrol (RES), epicatechins etc.), antioxidant agents and abundant micronutrients in our food could have strong anti-mitotic as well as pro-apoptotic effects. In this study we raised the question whether roscovitine (ROSC), an inhibitor of cyclin-dependent kinases (CDKs) with increased selectivity towards CDK2, could be able to affect human leukemia HL-60 cells in which the *p53* gene is inactivated and whether ROSC-induced effects could be additionally modulated by compounds of natural origin, especially by polyphenols e.g. RES. Exposure of HL-60 cells to ROSC for 24 h inhibited their proliferation. Flow cytometric analyses revealed that unlike MCF-7 cells, HL-60 cells were arrested in G_1 upon ROSC treatment. Furthermore, ROSC also induced apoptosis in HL-60 cells. After treatment with 40 μM ROSC for 24 h the frequency of hypoploid cells representing cells undergoing apoptosis reached approximately 50%. In the next step the action of RES alone or in combination with ROSC was examined. Interestingly, synergistic effects were observed after combined treatment for 24 h and sequential post-incubation for 48 h in the presence of RES. Such combined treatment resulted in a marked reduction of the frequency of the S- and G_2/M-phase cells and simultaneously increased the G_1 cell population up to 80% at a fourfold lower ROSC dose. Further analyses revealed that the combined treatment strongly activated caspase-3. These results clearly evidence that RES strongly potentiates ROSC-induced apoptosis.

1. Introduction

In the last years great attention was paid to natural food constituents. Extensive investigations revealed that some dietary components have strong anti-proliferative and pro-apoptotic effects [1]. Resveratrol (RES) is a polyphenol produced in plants to prevent them from such environment stresses like fungal infection or UV radiation. Peanuts, berries, grapes and red wine are the natural sources of RES [2–5]. RES has been found to have a very wide spectrum of targets and has therapeutic and preventive potential (reviewed in [6,7]). The anti-cancer effect of RES was attested after application of RES as single agent or in combination with radiotherapy or conventional chemotherapy (reviewed in [8]). Apart from the

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Abbreviations: RES, resveratrol; ROSC, roscovitine; CDK, cyclin-dependent kinase.
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anti-proliferative and pro-apoptotic features [9–11] RES is also able to induce cell differentiation program in HL-60 promyelocytic leukemia cells [12–15].

Roscovitine (ROSC) is a small purine-like inhibitor of cyclin-dependent kinases (CDKs) with increased selectivity towards CDK2, CDK1, CDK5, CDK7 and CDK9 [16–19]. The anti-mitotic and apoptosis-promoting effects of ROSC have been shown in a number of cell lines and human tumor xenografts (reviewed in [20]). ROSC has been evaluated in several Phase I and is currently under investigation in several Phase II clinical trials for various clinical situations as monotherapy and in combination with conventional chemotherapeutic agents (reviewed in [20–22]). ROSC has been shown to have an activity in a number of haematological diseases [23,24], and HL-60 promyelocytic leukemia cells [25,26], or to potentiate the efficacy of other therapeutics [6,27].

Recently it has been described that combined treatment of human MCF-7 breast cancer cells with ROSC and RES enhanced the anti-proliferative effect of the drugs by activation of wild p53 protein and its downstream effects and additionally by negative regulation of oncogenic signal transduction. [28]. ROSC inhibits proliferation of human MCF-7 breast cancer cells by induction of cell cycle arrest [29–31]. ROSC blocks MCF-7 cells in G_2 phase in a time- and concentration-dependent manner. After exposure of MCF-7 cells to 20 μM ROSC for 8 h approximately 50% of cells were arrested in G_2 phase. Moreover, ROSC induces a p53-dependent apoptosis in human MCF-7 breast cancer cells that are relatively resistant to pro-apoptotic agents.

In this study we raised the question whether ROSC could be able to affect in a similar way human leukemia HL-60 cells in which the *p53* gene is inactivated and whether ROSC-induced effects could be additionally modulated by compounds of natural origin, especially by polyphenols e.g. RES.

2. Materials and methods

2.1. Cells and drugs

Human HL-60 promyelocytic leukemia cells were grown as suspension culture in RPMI medium supplemented with 10% FCS at 37 °C in an atmosphere of 5% CO_2. RES (Sigma–Aldrich, Inc., St. Luis, MO) and ROSC (a kind gift of Prof. Dr. M. Strnad, Medical Faculty, Palacky University, Olomouc, Czech Republic) were dissolved as a 50-mM stock solution in DMSO and stored at −20 °C until use.

2.2. Antibodies

The following antibodies were used in this study: rabbit polyclonal anti-CDK1, anti-phospho-CDK1 (Thr161), anti-phospho-CDK2 (Thr160), anti-phospho-cyclin B1 (Ser133) and anti-phospho-Ser139-H2A.X antibodies were from Cell Signalling Technology, Inc. (Beverly, MA), monoclonal anti-CDK2 (Ab-4) antibodies from Lab Vision Co. (Fremont, CA), rabbit polyclonal anti-phospho-CDK1 (Thr14/Tyr15), anti-cyclin B1 from Sigma–Aldrich, Inc. (St. Louis, MO), anti-cyclin E antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit polyclonal anti-human caspase 3 (CPP32) antibodies from DAKO AS (Glostrup, Denmark). Monoclonal anti-actin (clone C4) antibodies were from ICN (ICN Biomedicals, Aurora, OH). Appropriate secondary antibodies were obtained from Sigma–Aldrich, Inc. (St. Louis, MO).

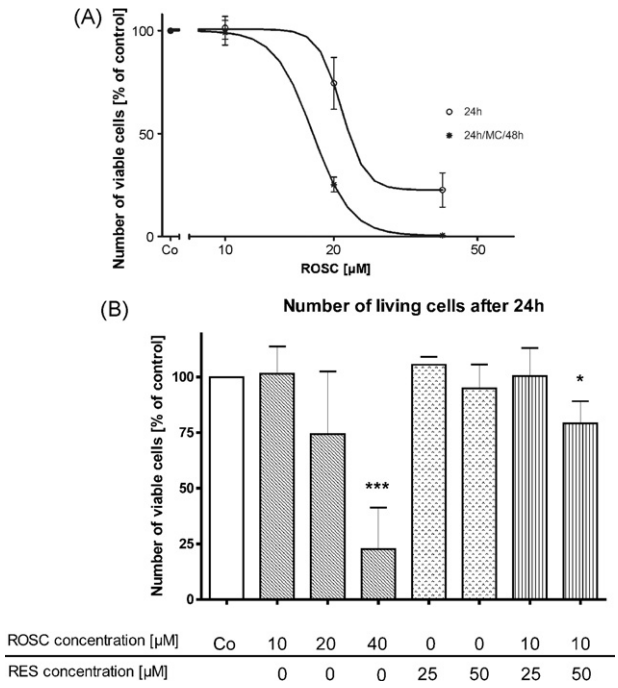
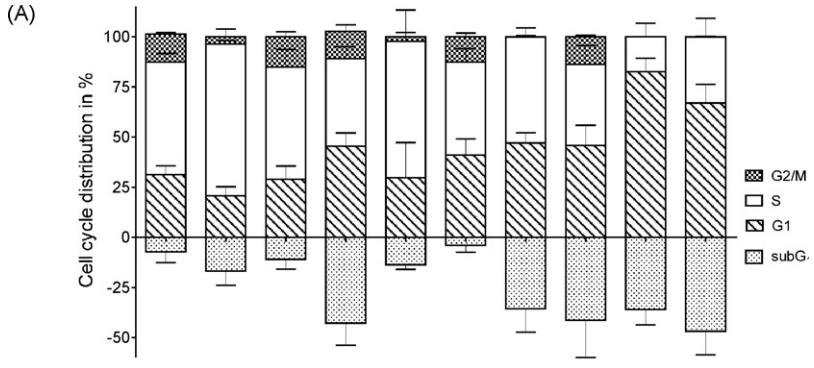


Fig. 1 – RES enhances ROSC-mediated reduction of the number of living HL-60 cells. (A) ROSC strongly reduces the number of viable human HL-60 cells. Exponentially growing HL-60 cells were plated in 96-well microtiter plates (5×10^3 cells/well). Twenty-four hours after plating cells were treated for 24 h with increasing concentrations of ROSC. The number of viable cells was determined directly after treatment (○ 24 h) and additionally, after post-incubation for 48 h in a drug-free medium (● 24 h/MC/48 h) by the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The graph represents mean values ± S.D. from three independent experiments, each performed at least in quadruplicate. IC_{50} value for ROSC = 21 μM after 24 h treatment; IC_{50} = 16 μM after treatment for 24 h, medium change (MC) and post-incubation for 48 h was evaluated by non-linear regression. The difference between IC_{50} values is statistically highly significant ($p < 0.001$). (B) RES potentiates the ROSC-mediated inhibition of cell proliferation. HL-60 cells plated in 96-well microtiter plates were exposed to ROSC or RES alone, or to a combination of both at indicated concentrations. The number of viable cells was determined after treatment for 24 h by the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The graph represents mean values ± S.D. from four independent experiments, each performed at least in quadruplicate. Statistical significance of treatment was determined by Bonferroni's multiple comparison test after comparison with a control. The effect of the treatment with 40 μM ROSC is statistically extremely significant ($p < 0.001$), the effect of the combined treatment is statistically significant ($p < 0.05$).



Control	+					+				
Roscovitine [μM]	-	-	10	40	10	-	50	40	10	10
Resveratrol [μM]	-	50	-	-	50	-	50	-	25	50
Duration of Treatment [h]	24h					72h	72h	24h		
Medium Change	-	-	-	-	-	-	-	+	+	+
Resveratrol [μM]	-	-	-	-	-	-	-	-	50	50
Post- incubation period [h]	-	-	-	-	-	-	-	48		

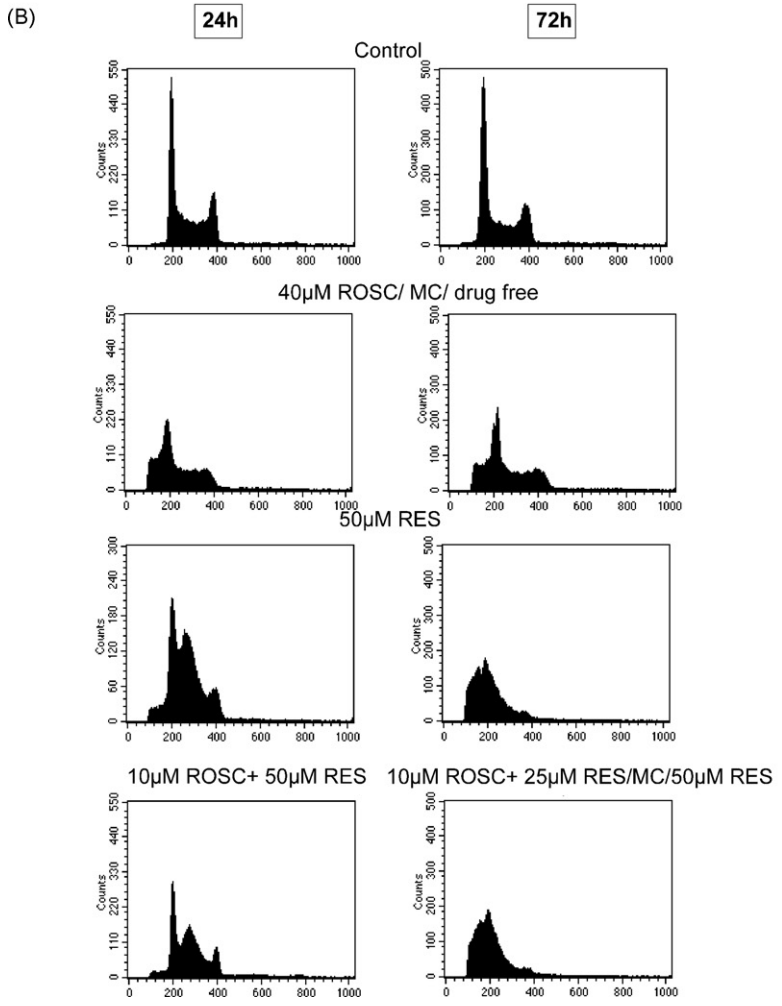


Fig. 2 – Accumulation of hypoploid HL-60 cells after treatment with ROSC alone or combined with RES. Exponentially growing HL-60 cells were exposed to ROSC and RES alone or to their combination for 24 h. In some cases samples were additionally post-incubated in a drug-free medium or and in the presence of RES. Cells were collected, stained with propidium iodide and DNA concentrations in single cells was measured by flow cytometry. (A) Effect of treatment with ROSC alone or in combination with RES on the distribution of HL-60 cells in distinct cell cycle phases. The graph represents mean values ± S.D. from at least three independent experiments. (B) Changes of DNA profiles after treatment. DNA histograms from a representative experiment.

2.3. Determination of the number of viable cells

Proliferation of HL-60 promyelocytic leukemia cells and their sensitivity to increasing concentrations of ROSC and/or RES was determined by the CellTiter-Glo™ Luminescent cell viability assay (Promega Corporation, Madison, WI). The CellTiter-Glo™ luminescent cell viability assay generating luminescent signals is based on quantification of the cellular ATP levels. Tests were performed at least in quadruplicate. Luminescence was measured in the Wallac 1420 Victor, a multilabel, multitask plate counter. Each point represents the mean ± S.D. (bars) of replicates from at least three representative experiments.

2.4. Measurement of caspase 3/7 activity

The activation of caspase 3/7 in human HL-60 promyelocytic leukemia cells in response to increasing concentrations of ROSC and/or RES was determined by the APO-ONE™ Fluorescence Assay (Promega Corporation, Madison, WI). Tests were performed at least in duplicate. To assess the enzymatic activity of caspase-3 released from dying cells into the culture supernatant, cells and medium were separately analyzed. Fluorescence was measured in the Wallac 1420 Victor, a multilabel, multitask plate counter. The relative values of the enzymatic activity were normalized to the number of living cells in corresponding wells. Each point represents the mean ± S.D. (bars) of replicates from at least two representative experiments.

2.5. Measurement of DNA of single cells by flow cytometry

The measurement of DNA content was performed by flow cytometric analyses based on a slightly modified method [32] described previously by Vindelov et al. [33]. The cells were harvested by centrifugation and washed in PBS. Aliquots of 1×10^6 cells were used for further analysis. Cells were stained with propidium iodide as described

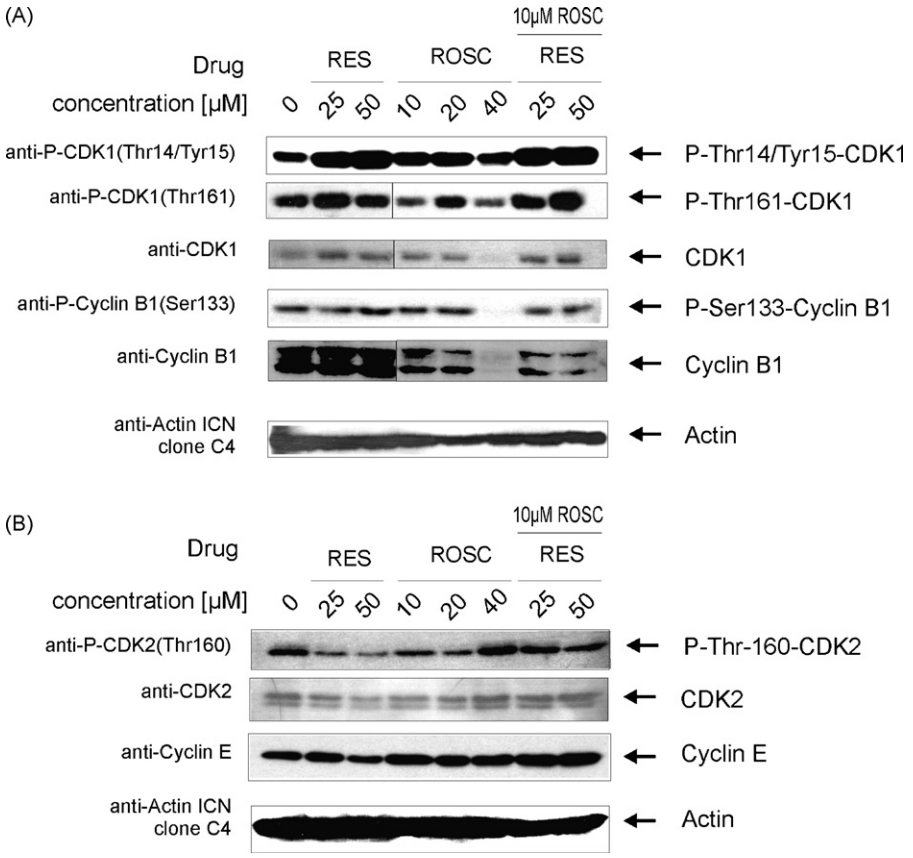


Fig. 3 – Effect of ROSC and RES alone and in combination on the functional status of the major cell cycle regulatory proteins. Exponentially growing cells were exposed for 24 h to ROSC and RES alone or to their combination, harvested and lysed in RIPA buffer. WCLs (30 μg protein/well) were electrophoretically separated on 10% or 12% SDS-polyacrylamide slab gels. Proteins immobilized on PVDF membranes were subjected to immunoblotting using different primary antibodies and appropriate secondary antibodies coupled to HRP. The immune complexes were detected by chemiluminescence using luminol (ECL+) as a substrate. To control the proper protein transfer and equal protein loading, membranes were stained with Ponceau S and incubated with anti-actin antibodies, respectively. (A) Inactivation of CDK1/cyclin B complexes by ROSC at high dose. Cellular proteins (WCLs) were separated on 12% (for detection of CDK1) and 10% and 12% (for detection of cyclin B1) SDS-polyacrylamide gels. Immunoblotting was sequentially performed with distinct antibodies as indicated. (B) Inhibition of the activating phosphorylation of CDK2 by lower ROSC dose. Cellular proteins (WCLs) were separated on 12% gels. The status of site-specific phosphorylation of CDK2 was determined using anti-P-Thr160-CDK2 antibodies.

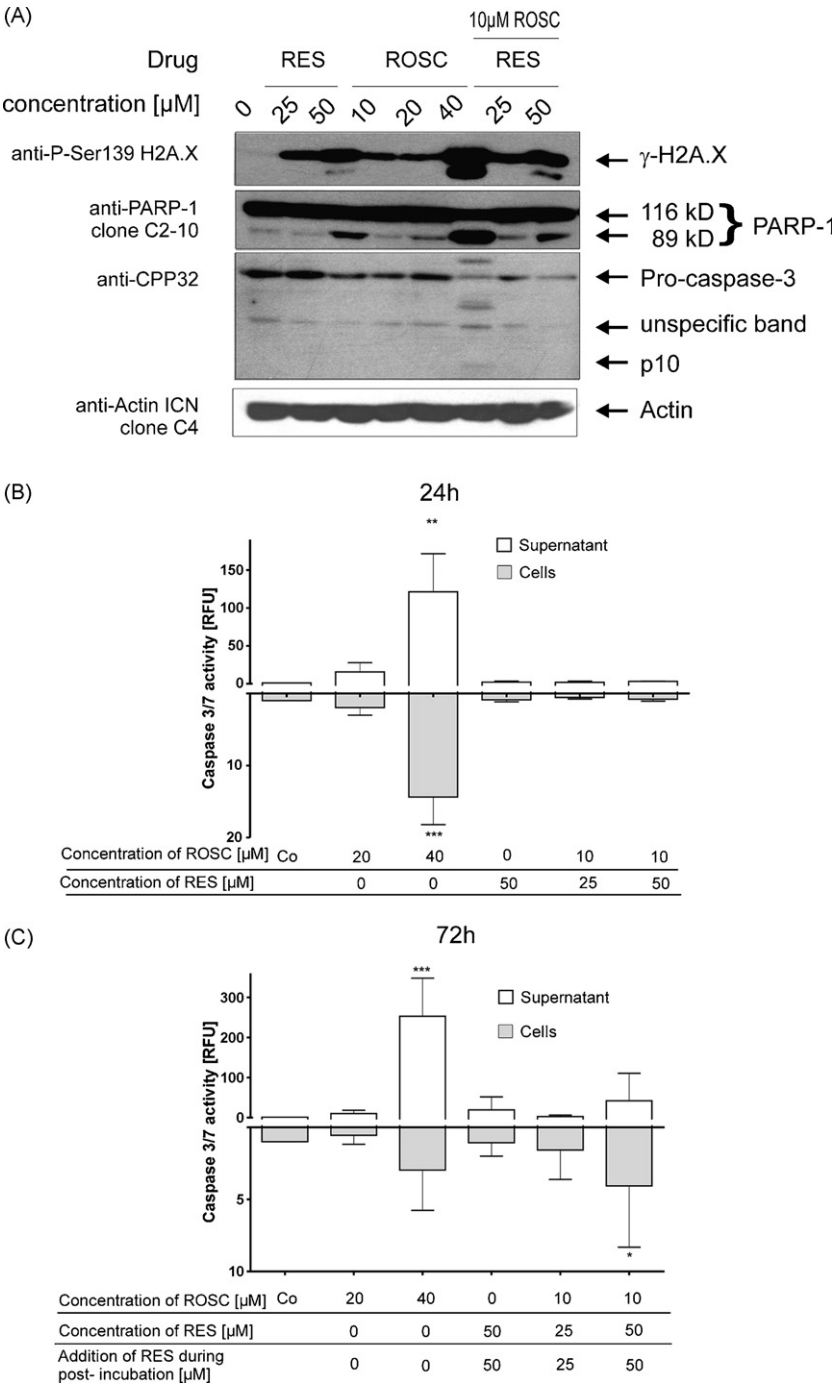


Fig. 4 – RES enhances the ROSC-induced apoptosis. (A) ROSC at high dose induces apoptotic changes driven by activated caspase-3. Exponentially growing HL-60 cells were exposed for 24 h to ROSC and RES alone or to their combinations, harvested and lysed in RIPA buffer. Conditions of electrophoresis and immunoblotting as described in detail in the legend to Fig. 3. Proteins were resolved on 10% (for detection of PARP-1) or 15% (for detection of γ-H2A.X and caspase-3) slab gels. Membranes were sequentially incubated with distinct primary antibodies as indicated. (B) and (C) HL-60 cells plated in 96-well microtiter plates were exposed to ROSC or RES alone, or to a combination of both at indicated concentrations. The activity of caspase 3/7 in cells and in culture supernatant was determined by the APO-ONE™ Homogeneous Fluorescence Assay (Promega Corporation, Madison, WI). For statistical significance the values after distinct treatments were compared to a control. The statistical significance was determined by Bonferroni's multiple comparison test. (B) Strong activation of caspase 3/7 after treatment for 24 h with ROSC at high dose. Enzymatic activity was separately determined in cells and in culture supernatant. The graph represents mean values ± S.D. from two independent experiments, each performed at least in duplicate. After the treatment with 40 μM ROSC the relative activity of the caspase 3/7 is statistically extremely significant ($p < 0.001$) in the cells and very significant in supernatant ($p < 0.01$). (C) Post-incubation of ROSC-treated HL-60 cells with RES potentiates the activation of effector caspase 3. The activity of caspase 3/7 was determined after treatment for

previously and the fluorescence was measured using the Becton Dickinson FACScan (Bedford, MA) after at least 2 h incubation at +4 °C in the dark. Distribution of cells in distinct cell cycle phases was determined using ModFIT cell cycle analysis software.

2.6. Protein separation and immunoblotting

Whole cell lysates (WCLs) were prepared in a modified RIPA buffer, mixed with 2× SDS sample buffer and separated on 10%, 12% or 15% SDS-polyacrylamide slab gels. Resolved proteins were transferred electrophoretically onto PVDF membranes (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire) and immunoblotted using Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire) as previously described [29,34].

2.7. Statistical analysis

Statistical analysis was performed using GraphPadPrism. Significance levels were evaluated using Bonferroni's multiple comparison test.

3. Results

3.1. Effect of ROSC and RES on proliferation of HL-60 cells

First, we investigated the short-time effect of ROSC on growth of HL-60 cells. In agreement with previously published data [26] exposure of exponentially growing HL-60 cells to ROSC for 24 h resulted in a marked reduction of the number of living cells (Fig. 1A). To detect the long-term effects of treatment, the drug was removed after 24 h and the cells were post-incubated in a drug-free medium for further 48 h. (Fig. 1A) The IC_{50} calculated from values measured directly after 24 h treatment with ROSC was 21 μM, and it decreased after post-incubation for 48 h to 16 μM ($p < 0.001$). This difference occurring between immediate and delayed effects of the inhibition of CDKs by ROSC was also detected in our previous studies performed on human MCF-7 breast cancer cells [28] and offers a window-time to test the ability of other agents to synergize the ROSC action in HL-60 cells. It implicates that also distinct dietary components such as polyphenols might be able to modulate the ROSC-induced inhibition of cell proliferation by promoting of apoptosis.

In next step we treated the cells with RES alone or in combination with ROSC. As shown in Fig. 1B, RES combined with 10 μM ROSC reduced the number of viable cells more strongly than treatment with the single agents (Fig. 1B). This anti-proliferative effect even increased after post-incubation of cells in the presence of RES (not shown). RES alone did not significantly affect the viability of cells within 24 h treatment (Fig. 1B) and its anti-proliferative effect became evident after continuous administration for 48 h (not shown).

3.2. Induction of G_1 arrest in HL-60 cells exposed to ROSC combined with RES

To evaluate the effect of ROSC on the cell cycle progression, untreated control HL-60 cells as well as cells exposed for 24 h to increasing concentration of ROSC were stained with propidium iodide and DNA concentration in single cells was determined by flow cytometry. Strong inhibition of the cell cycle became evident after treatment with ROSC at a final concentration of 40 μM (Fig. 2A and B). Unlike MCF-7 or HeLa cells, HL-60 cells were arrested in G_1 phase of the cell cycle. The increase of the frequency of G_1 cell population by approximately 20% coincided with the reduction of the S-phase-cells. Simultaneously, the ratio of hypoploid cells markedly increased and reached 50% at the highest dose of ROSC. In contrast, RES at high concentration led to the accumulation of the S-phase cell population that was accompanied by reduction of the ratio of G_1-cells. This displacement resulted in the appearance of a big S-phase peak that was associated with detraction of G_1 peak. Similar shift was observed in DNA histogram of HL-60 cells incubated for 24 h with 10 μM ROSC/50 μM RES. Interestingly, the most pronounced effect was observed after the combined treatment. The action of RES became evident in samples after combined treatment for 24 h and then after addition of RES to cells post-incubated for further 48 h (Fig. 2A and B). RES at a final concentration of 50 μM resulted in a strong G_1 arrest (up to approximately 90% depending on its concentration during the combined treatment). A strong increase of the ratio of hypoploid cells (approximately 30%) accompanied by the loss of G_2/M cells led to overlay of G_1 peak generating an untypical DNA histogram.

Analysis of the cellular levels and activity status of the key cell cycle regulators substantiates the results of the flow cytometry. The accumulation of G_1 cells after exposure to 40 μM ROSC coincided with inactivation of CDK1/cyclin B complexes evidenced by a dramatic decrease of the levels of the total cyclin B, of cyclin B1 phosphorylated at Ser133, as well as of the total CDK1 and of its activated form (Fig. 3A). As expected, ROSC inhibited the activating phosphorylation of CDK2 at lower doses. However, at the highest ROSC concentration the level of activated CDK2 form phosphorylated at Thr160 increased (Fig. 3B).

3.3. Induction of apoptosis upon treatment with ROSC and RES

The appearance of hypoploid cells indicates that in response to the treatment HL-60 cells undergo apoptosis. To substantiate this observation, two different approaches were per-

24 h, medium change (MC) and post-incubation for 48 h in a drug-free (0) medium or in the presence of RES. The graph represents mean values ± S.D. from four independent experiments, each performed at least in duplicate. After the treatment with 40 μM ROSC the relative activity of the caspase 3/7 determined in cells is not significant whereas in supernatant it is statistically extremely significant ($p < 0.001$). The activity of the caspase 3/7 in cells after combined treatment with 50 μM RES is statistically significant ($p < 0.05$).

formed. First, we monitored by immunoblotting the status of selected effectors and markers of apoptosis such as caspase-3 and PARP-1 and that of γ-H2A.X, a specific marker of DNA damage. In the second approach we determined the activity of effector caspases 3/7, both in the cells and the culture medium. Moreover, considering the fact that the drugs strongly reduced the cell number of living cells, the relative enzymatic activity was normalized to the cell numbers.

The Ser139 phosphorylated form of histone H2A, designated as γ-H2A.X was not detected in untreated controls but occurred after incubation with RES or ROSC alone and became even stronger after combined treatment. The appearance of a characteristic, caspase-cleaved form of PARP-1 was detected by immunoblotting in samples with high ratio of sub-G_1 cells and correlated with the decrease of the intensity of pro-caspase-3 protein band. Surprisingly, the intensity of the p10 subunit representing the cleaved fragment of caspase-3 did not coincide with the reduction of pro-caspase-3 band and with other markers of apoptosis. (Fig. 4A). In the second approach the enzymatic activity of the effector caspases 3/7 was measured. A strong activation of cellular caspase 3/7 was observed after 24 h treatment with 40 μM ROSC, and also after post-incubation for 48 h. The combined treatment enhanced caspase 3/7 activity after the post-incubation period (Fig. 4B and C). Moreover, the analysis of the culture supernatant revealed that a substantial portion of the activated caspase 3/7 was released from cells during apoptosis and was detectable in culture medium. These results strongly substantiate the assumption that hypoploid HL-60 cells represent cells undergoing apoptosis and evidence that ROSC induces in HL-60 cells caspase-dependent apoptotic pathway. Furthermore, the accumulation of activated caspase-3 in culture medium explains the lack of the reciprocal correlation between the intensity of pro-caspase band and p10 subunit. Taken together, our results clearly evidence that RES strongly potentiates ROSC-induced apoptosis and that it displays this action during post-incubation time.

4. Discussion

It has been found that a large number of chronic myeloid leukemias are associated with characteristic chromosomal translocation. The most frequent chromosomal rearrangement causing generation of Philadelphia chromosome leads to formation of BCR-ABL fusion protein with increased tyrosine kinase activity. On the other hand, some rearrangements in leukemias result in production of chimeric proteins such as TLS-ERG fusion protein [35] derived from the t(16;21) translocation and block differentiation of progenitor myeloid cells. Generally, constitutively activated kinases or transcription factors encoded by fusion proteins render them an advantage in proliferation and an arrest of differentiation at different stages of hematopoiesis [35]. Numerous studies evidence that the cell cycle progression is closely related to myeloid development and the proper cell cycle control regulation is necessary to regulate selfreneval of stems cells and rapid expansion of the hematopoietic progenitor cells. Human HL-60 promyelocytic leukemia cells represent myeloid progenitors that were transformed before terminal differentiation was completed. They are characterized by a number of changes e.g. loss of the p53 gene [36]. Inactivation of p53 gene connected with differentiation block confer them uncontrolled mitotic potential. In the light of above findings and considering the fact that restoration of terminal myeloid differentiation depends on cell cycle arrest, the combining of selective pharmacological inhibitors of CDKs with agents promoting differentiation may be useful in the search for new treatments of chronic myeloid leukemias.

RES, a polyphenol of natural origin might be a good candidate and seems to be of interest for a few reasons. It has been reported that RES is able to promote differentiation of HL-60 cells. Furthermore, RES is a component of our food and its intake as a dietary supplement can modulate the outcome of chemotherapy.

In this study primarily two issues were addressed. First, the question was raised whether the combined treatment of leukemic cells by agents that are known to block cell cycle progression and to promote myeloid differentiation might be an interesting therapeutic option. One would expect that increase of G_1 cell population would render cells more sensitive to pro-apoptotic stimuli and enhance the rate of apoptosis. Thus, the increased killing of leukemic cells would improve the efficacy of the therapy. Secondly, it was of interest to find out which schedule of the combined treatment would be optimal. The fact that ROSC has a retarded effect which becomes evident even in the absence of the drug during post-incubation time indicates that additional intervention during this post-incubation period could be especially effective.

Moreover, ROSC is known not only to arrest cell cycle progression but also to initiate apoptosis by inhibiting transcription that in turn leads to down-regulation of anti-apoptotic proteins [20]. In several previous studies ROSC was shown to induce cell death with slightly greater potency in p53 wild type cells compared with p53 mutant cells [16,17,37], and little or no difference between HCT-116 wild type p53 and isogenic $p53^{-/-}$ [26,37]. The strong induction and nuclear accumulation of p53 wild type protein with its downstream targets is a very important pathway activated by ROSC [31].

The anti-proliferative effect of ROSC in HL-60 cells has already been described [25,26]. In this study we showed that ROSC induces in HL-60 cells G_1 arrest and caspase-dependent apoptosis. However, the strongest effect was observed after treatment with high ROSC doses, which could lead in vivo to undesired side effects. Through administration of RES in combined settings the ROSC dose could be fourfold reduced without diminishing of therapeutic efficacy. A similar combined approach has been previously described in the case of MCF-7 cells. The anti-proliferative effect of the drugs was enhanced by activation of wild-type p53 protein and its downstream effects and additionally by negative regulation of oncogenic signal transduction. [28].

We decided to use 10 μM ROSC for combined treatment since it seems to be the upper tolerated level in human [38]; however, the newest reports about recent clinical studies are not available yet.

RES is a polyphenol which has been found out to have multiple targets in cells and their environment [8]. This provides the possibility to enhance the effectivity of the other compounds. RES is already previously described to induce

apoptosis in HL-60 cells [9–11]. However, the bioavailability of RES does not seem to be satisfying and has to be improved. Moreover, further studies are necessary to better understand the exact mechanisms of the action of RES [7]. It has also been advised that the main benefit of RES lies in using it as dietary supplement and combining it with other agents [3,7].

These results also indicate that the post-incubation time window offers an excellent opportunity for treatment with non-genotoxic agents to enhance the initial effect of ROSC. The comparison of the different treatment schedules implicate that the best results were obtained after treating the cells with a combination of ROSC and RES for 24 h, and then changing to a post-incubation of the cells with RES alone. When applying this schedule, the ratio of apoptotic cells increases nearly fivefold as compared to the ROSC-only treatment. The synergistic effect of both drugs and the post-treatment with RES should also open a much wider therapeutic window and dietary supplementation that should result in a clear benefit for patients receiving chemotherapy. Our promising findings suggest that a treatment regimen that makes use of a combination of the two drugs under an optimized schedule should be very effective.

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