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Antiproliferative Effect of 13-*cis*-Retinoic Acid is Associated with Granulocyte Differentiation and Decrease in Cyclin B1 and Bcl-2 Protein Levels in G0/G1 Arrested HL-60 Cells

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Abstract Retinoic acid (RA), similar to specific growth factors, can induce differentiation of proliferating promyelocytic precursors into terminally differentiated granulocytes, although little is known about effects of its 13-*cis* isomer on promyelocytic leukemia (PML). In this study we demonstrate that 13-*cis*-RA has a dose and time-dependant antiproliferative effect on HL-60 PML cell line, that it induces cell **accumulation** in resting G0/G1 phase of the cell cycle followed by an increase in CD11b granulocyte differentiation antigen expression. The obtained increase in the percentage of HL-60 cells in G0/G1 phase and complementary decrease in S phase of the cell cycle are accompanied by a decrease in the expression of cell cycle regulatory molecule cyclin B1. We also show the induction of interferon regulatory factor-1 (IRF-1) transcription that can, also, to some extent contribute to the antiproliferative effect of 13-*cis*-RA. Furthermore, down-regulation of Bcl-2 protein expression in 13-*cis*-RA treated HL-60 cells may contribute to sensitivity to apoptosis of growth arrested HL-60 promyelocytic cells.

Keywords Cell cycle · Promyelocytic leukemia · Proliferation · Retinoic acid · Differentiation

Abbreviations

RA	retinoic acid
ATRA	*all-trans* retinoic acid
PML	promyelocytic leukemia
IRF-1	interferon regulatory factor-1

Introduction

Chemotherapeutic agents that are presently used in the therapy of cancer often are cytotoxic and therefore harmful to healthy tissues. As imbalance of cell proliferation, apoptosis and differentiation leads to the development of malignant clones whose growth is often dependent on various growth factors or hormones, biological molecules that reduce proliferation and induce terminal differentiation or apoptosis of malignant cells represent potential alternatives for cancer treatment. In this sense, it has been known that retinoids, vitamin A and its functional and structural analogues, influence vertebrate cell differentiation, as well as proliferation [1]. Introduction of retinoids as differentiation therapeutical agents has shown significant impact on many malignancies, most notably on leukemias [2]. Pluoripotenet hematopoetic stem cells in the presence of external and internal stimuli undergo a genetically regulated program of maturation by acquiring functional and structural characteristics that result in the loss of capacity for self-renewal that distinguishes specific cell lineages [3].

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Antiproliferative and differentiating effects of 13-*cis*, unlike all trans isomer of retinoic acid (RA), have been studied on solid tumors and to a much lesser extent on leukemic cells [4]. Retinoic acid regulates proliferation, differentiation and susceptibility to apoptosis by binding to its nuclear receptors: retinoic acid (RAR) and retinoid acid X receptors (RXR) in target cells [5]. HL-60 promyelocytic leukemia (PML) cell line possesses characteristic t(15;17) translocation that fuses PML gene to retinoic acid receptor RARα gene, resulting in an aberrant PML-RARα protein which is dominantly negative to the normal RARα receptor. PML-RARα protein inhibits differentiation and promotes survival of immature myeloid precursor cells and therefore is associated with **leukemogenesis** [5]. PML-RARα competes with RARα for binding to retinoic acid response elements (RAREs) in target genes and causes transcriptional repression of RA responsive genes. PML-RARα forms complexes with large ubiquitous nuclear correpresors (CoR) and histone deacetylases (HDAC) which are, unlike the RARα-CoR complexes, more stable and resistant to treatment with physiological concentrations of RA [2]. Only the high pharmacological concentrations of RA could trigger a distinct conformational change of PML-RARα resulting in release of CoR complex and subsequent recruitment of coactivators (CoA) which convert PML-RARα from repressor to activator of transcription of the retinoid responsive genes [6]. RA treatment can also lead to the degradation of PML-RARα pathological product [6].

Promyeloid leukemic cells which fail to undergo the process of maturation and differentiation can upon treatment with retinoids, differentiate into mature granulocytes. HL-60 PML cells bearing PML-RARα translocation are a very illustrative model system for studying the molecular processes which underly the mechanisms of cell differentiation.

Materials and Methods

Cell Culture and Treatment

HL-60 cell line was cultured in suspension in RPMI 1640 (Sigma, St. Louis USA) culture media (CM) supplemented with 10% FCS (Sigma, St. Louis USA) at 37°C and 5% CO_2 in humid atmosphere. Cells growing in exponential phase in 6 well cell culture plates (Linbro, McLean, USA) in 2 ml CM were treated and for the period of 24, 48 and 72 h with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M concentrations of 13-*cis*-retinoic acid (Sigma, St. Louis USA) and with the same concentrations of *all-trans* retinoic (ATRA) acid. Both substances were dissolved in dymethyl sulfoxide (DMSO), while final concentration of DMSO in CM did not exceed 0.1%.

MTT Assay for Cell Growth

For the assessment of growth and proliferation 5000 HL-60 cells in 150 μl of CM per well, were cultured in 96 well microplates (Falcon, USA) in a incubator at 37°C and 5% CO_2 in humid atmosphere. MTT method is based on the ability of alive, metabolically active cells to reduce tetrazolium salt into formazan crystals. After 24, 48 and 72 h of incubation, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT) solution (5 μg/ml in PBS) was added to each well. After 4 h incubation the formazane crystals were dissolved by adding 100 μl of 10% SDS (sodium dodecylsulfate). Afterwards absorbance was measured at 570 nm on Microplate reader (Boerhing EL 311) and cell survival calculated using the following formula $A = (At - Ab)/(Ac - Am)$ (At* absorbance of treated cells, Ab* absorbance of the substance of applied concentration dissolved in cell culture medium, Ac* absorbance of untreated (control) cells and Am* absorbance of the cell culture medium)

Flow Citometry

For CD11b surface antigen expression 25×10^4 cells per sample resuspended in 50 μl RPMI 1640 CM were incubated for 30 min in the dark at 4°C with 5 μl of monoclonal mouse anti-human CD11b PE conjugated antibody (Becton Dickinson, San Jose, USA). The data was acquired on FACSCalibur argon laser flow cytometer (Becton Dickinson, San Jose, USA) and analyzed with CellQuest software. For cell cycle analysis$1x10^6$ cells per treatment were resuspended in polystyrene tubes in 400 μl of PBS. Fixation was preformed by incubating cell suspension with equal volume of 70% ice cold ethanol for 1 h on ice. The cells were then centrifuged, washed twice with PBS, resuspended in 500 μl of PBS and treated with 0.1 μg RNAse (type I-A, Sigma Chemicals, St. Louis USA) for 30 min at 37°C. Afterwards cells were stained with 40 μg/ml propidium iodide PI (Sigma Chemicals, St Louise USA) for 10 min in the dark at room temperature and PI fluorescence was measured on flow cytometer. The proportion of cells in G0, S and G2M phases of cell cycle were estimated using ModFit® software.

Western Blot

5×10^6cells were harvested and lysed for 30 min on ice in 200 μl RIPA buffer containing: Tris (ph 7.5 50 mM), NaCl (150 mM), NP-40 (1%), Na deoxycholate (0.5%), SDS (0.1%). Protein concentrations were determined by Lowry method [7]. 30 μg of proteins per sample was diluted in loading buffer (Tris pH 6.8, and then denaturated by cooking at 100°C for 5 min and afterwards kept on ice

for 10 min. The protein lysates were electrophoresed on 8% SDS-polyacrylamide (29:1 acrylamide/bisacrylamide) gel at 100 V and then electrotransfered (Trans Blot Cell; Bio-Rad, Hercules, CA USA) to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, USA) using voltage of 100 V for 1 h. The nitrocellulose membrane was blocked for 1 h with 5% non-fat dried milk in 1×TBST buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20).

Primary antibodies were dissolved in TBST and the blots were incubated overnight at 4°C temperature. Rabbit anti-human cyclin B1, rabbit anti-human MEK1/2 and mouse anti-human Bcl-2 antibodies (Sigma, St. Louise, USA) were diluted 1:200, 1:1,000 and 1:1,000 respectively. The blots were washed 3 times in 1×TBST buffer and incubated with peroxidase conjugated secondary antibodies (Amersham, Arlington Heights, IL USA) for 1 h at room temperature, washed 3 times in 1×TBST buffer and protein bands were detected using enhanced chemiluminescence (ELC) detection system (Amersham, Arlington Heights, IL USA). The blots were analyzed with Gel Doc BioRad system using Multi-Analyst 1.1 software.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated form HL-60 cells using TRIZOL (Sigma, St. Louise, USA). RNA was reverse transcribed by random priming while incubated with 1 μl reverse transcriptase MuLV, 10 mM deoxynucleotide triphosphate mix (dNTP), RNAse inhibitor and 2 μl 0.1 M dithiothreitol (DTT) for 1 h at 37°C and then 5 min at 99°C (Fermentas). Total cDNA was then subjected to PCR program 5 min at 95°C, and 38 cycles of amplification (15 s at 95°C, 30 s at 57°C, 45 s at 72°C) and 7 min at 72°C. Sequence of primers used for amplification of 414 bp (base pair) fragment of IRF-1 was: GACCAGAG CAGGAACAAG corresponding to bp 483–501 and the antisense was TAACTTCCCTTCCTCATCC corresponding to bp 881–889. β actin primer selected to amplify 685 bp fragment was: TGGGTCAGAAGGATTCCTAT corresponding to 181–200 and the antisense AAGGAAGGCTGGA AGAGT corresponding to 821–838 from the published β actin sequence. The results were quantified using Image Scion program.

Statistical Analysis

Significance of the differences in cell proliferation, percentage of the cells in S and G0/G1 phases of cell cycle and CD11b antigen expression was determined by one-way ANOVA. Values of $p<0.05$ were considered significant. Significance of the differences in these parameters between concentrations of 13-*cis*-RA and with increase in time of treatment was determined by Friedman test.

Results

Proliferation of treated HL-60 cells was assed by MTT test and calculated as the percentage ratio of absorbance of treated cells versus the absorbance of untreated control cells. Higher concentrations (10^{-6} and 10^{-5} M) of 13-*cis*-RA significantly (ANOVA, $p<0.05$) decreased proliferation of HL-60 cells after the shortest follow up time of 24 h. After 48 h of treatment all applied RA concentrations, except the lowest (10^{-8} M), significantly (ANOVA, $p<0.05$) decreased cell proliferation and such trend is also sustained after 72 h of treatment (Fig. 1). Proliferation of treated cells decreased significantly (ANOVA, $p<0.05$) with the increase of RA concentration. Friedman test showed statistically significant decrease of HL-60 cells proliferation ($p<0.05$) with increase of period of treatment for each 13-*cis*-RA concentration, except for the lowest concentration. The obtained results imply strong and prompt time and dose dependant antiproliferative action of 13-cis-RA.

Agents with antiproliferative action may also affect the cell cycle phase distribution of treated cells. Cell cycle analysis shows that consistent to MTT results, after 24 h treatment, the percentage of HL-60 cells in S phase of cell cycle decreased significantly (ANOVA, $p<0.05$) only after treatment with the highest 13-*cis*-RA concentration (10^{-5} M) compared to the control treated with CM alone (Fig. 2d), while the percentage of cells in G0/G1 phase of cell cycle at that time point did not change. After longer, 48 h treatments, with 13-*cis*-RA or ATRA simultaneously, 10^{-7}, 10^{-6} and 10^{-5} M concentrations of 13-*cis*-RA and also 10^{-6} and 10^{-5} M concentrations of ATRA induced significant (ANOVA, $p<0.05$) increase in the percentage of HL-60 cells in G0/G1 phase compared to the control. The obtained accumulation of cells in resting G0/G1 phase of cell cycle was accompanied with subsequent significant (ANOVA, $p<0.05$) decrease in the percentage of the cells in S phase of cell cycle (Fig. 2b, e). The similar

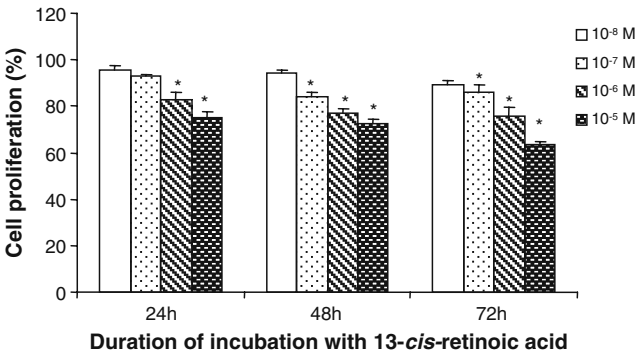

Fig. 1 Proliferation of HL-60 cells treated with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M 13-*cis*-RA with respect to untreated control cultures determined by MTT test. Results are presented as mean ± SER of 6 representative experiments performed in triplicates, * $p<0.05$ of treated vs. control cultures (one-way ANOVA)

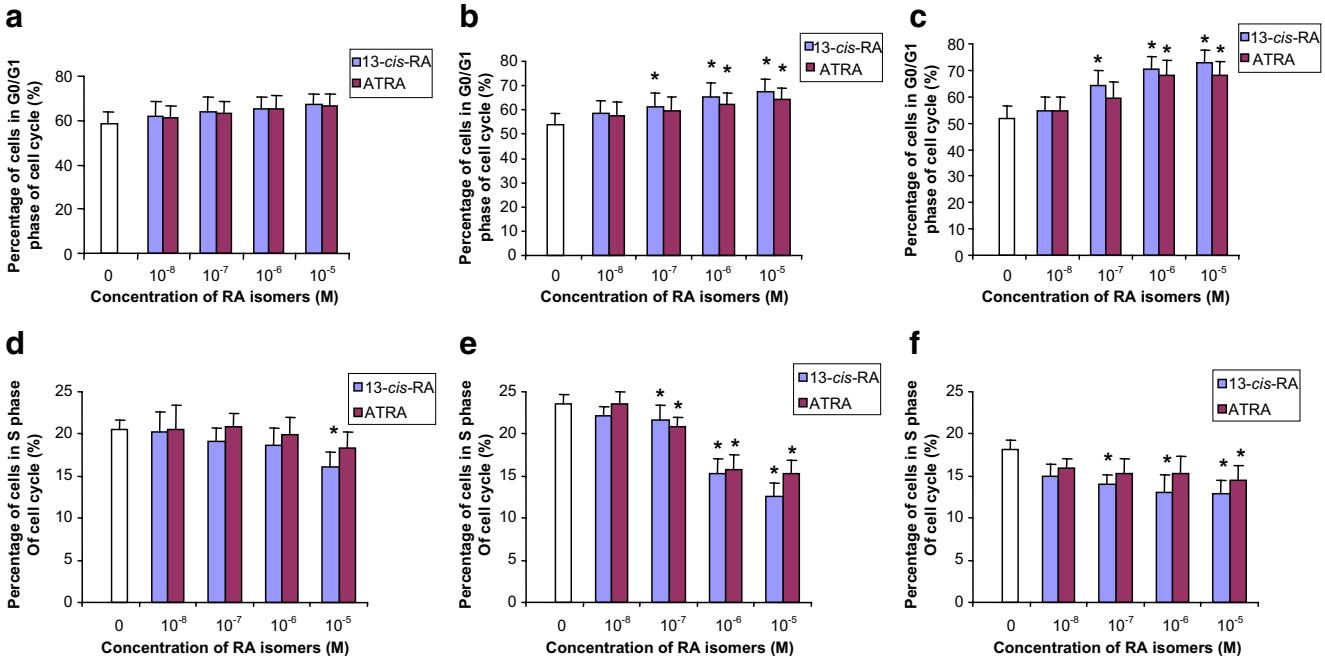

Fig. 2 Percentage of HL-60 cells in G0/G1 and S phase of cell cycle after 24 h **a**,**d**, 48 h **b**, **e** and 72 h **c**, **f** of treatment with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M concentrations of 13-*cis*-RA and ATRA. Data shown here represent mean values ± SER of four representative experiments, * $p<0.05$ of treated vs. control cultures (one-way ANOVA)

increase in the percentage of G0/G1 and decrease in S phase cell population was induced after 72 h treatment with either 13-*cis*-RA or ATRA (Fig. 2c, f). Our results show that treatments with 13-*cis*-RA induced change in cell cycle phase distribution of greater magnitude than ATRA (Fig. 3). Evaluation of the change in cell cycle distribution after all applied concentrations of 13-*cis*-RA with time using Friedman statistical test showed a time dependant effect ($p<0.5$, Friedman test) of 13-*cis*-RA on G0/G1 accumulation and S **phase** decrease.

Flow cytometric evaluation of CD11b granulocyte differentiation antigen expression shows no change after 24 h treatment with 13-*cis*-RA, while after 48 h the expression of CD11b differentiation antigen increased significantly($p<0.05$, ANOVA) with the increase of concentration of applied 13-*cis*-RA, compared to its expression on control treated with CM only. The similar increase in CD11b expression was observed after 72 h treatment (Table 1). CD11b expression increased significantly with the increase of concentration 13-*cis*-RA ($p<0.05$, ANOVA) as well as with the extension of period of incubation ($p<0.05$, Friedman test). These findings indicate strong dose and time dependant influence of 13-*cis*-RA on CD11b differentiation antigen expression. Simultaneous 72 h treatments with either ATRA or 13-*cis*-RA, show that ATRA causes greater increase in CD11b differentiation antigen expression that 13-*cis*-RA (Fig. 4).

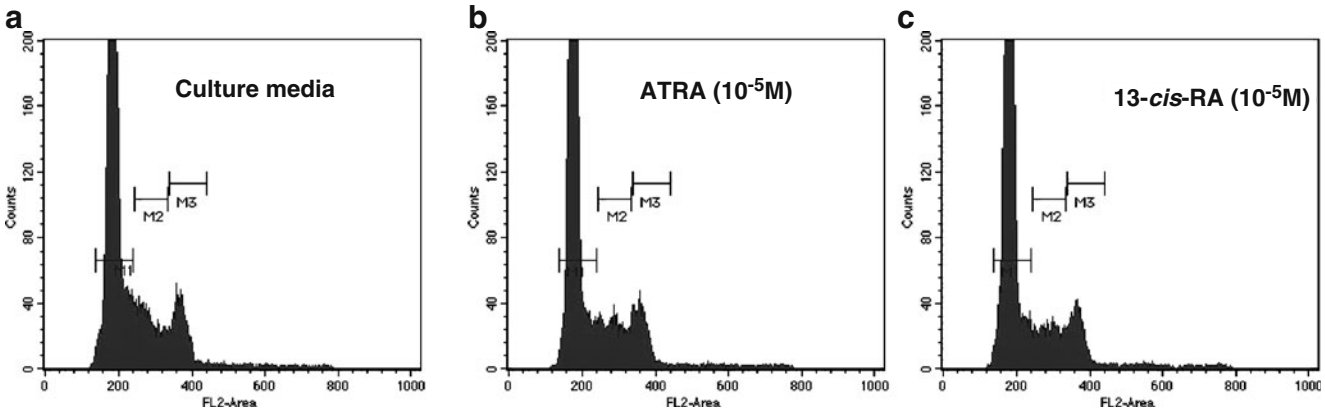

Fig. 3 Representative flow cytometric histograms showing cell cycle distribution after 72 h of treatment with culture media **a**, 10^{-5} M 13-*cis*-RA **b** and 10^{-5} M ATRA **c**. Obtained increase in G0/G1 phase and S phase decrease are greater after 13-*cis*-RA than after ATRA treatment. Marker positions M1, M2 and M3 designate G0/G1, S and G2M phase of cell cycle, respectively

Table 1 Percentage of $CD11b^+$ HL-60 cells after treatment with13-*cis*-retinoic acid

CD11b antigen expression (%)					
	Concentration of 13-*cis*-retinoic acid				
	0 M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
24 h	8.79±0.62	7.83±0.21	6.23±0.67	6.94±0.39	8.14±0.25
48 h	9.37±0.44	19.79±0.52**	24.00±0.39**	42.54±0.47**	59.73±0.48**
72 h	8.6±0.28	17.09±0.11**	28.01±0.23**	42.14±0.79**	62.98±0.42**

**$p<0.01$ treatment vs. control culture (one-way ANOVA)

Obtained increase in CD11b differentiation antigen expression after 48 and 72 h treatment with 13-*cis*-RA is directly proportional to G0/G1 cell cycle phase accumulation and inversely proportional to proliferation of HL-60 cells.

Western blot results show that compared to the controls treated with CM alone, cyclin B1 protein expression in HL-60 cells decreased after 24 h treatment only with higher (10^{-6}, 10^{-5} M) 13-*cis*-RA concentrations, while after 48 and 72 h treatment all applied RA concentrations induced cyclin B1 protein level decrease. We also, show that cyclin B1 protein expression has reached its lowest level after 48 h concordant with G0/G1 arrest (Fig. 5). Western blot evaluation of the anti-apoptotic Bcl-2 protein expression shows after 24 h of 13-*cis*-RA treatment a decrease of Bcl-2 expression compared to the untreated control that persisted also after 48 and 72 h of treatment (Fig. 5). We also show that the expression of MEK1/2 signaling molecule compared to the control increased after 24 h treatment with 13-*cis*-RA (Fig. 6), which is prior to the onset of granulocyte differentiation i.e. G0/G1 cell cycle arrest and increased expression of CD11b granulocyte differentiation antigen on HL-60 cells.

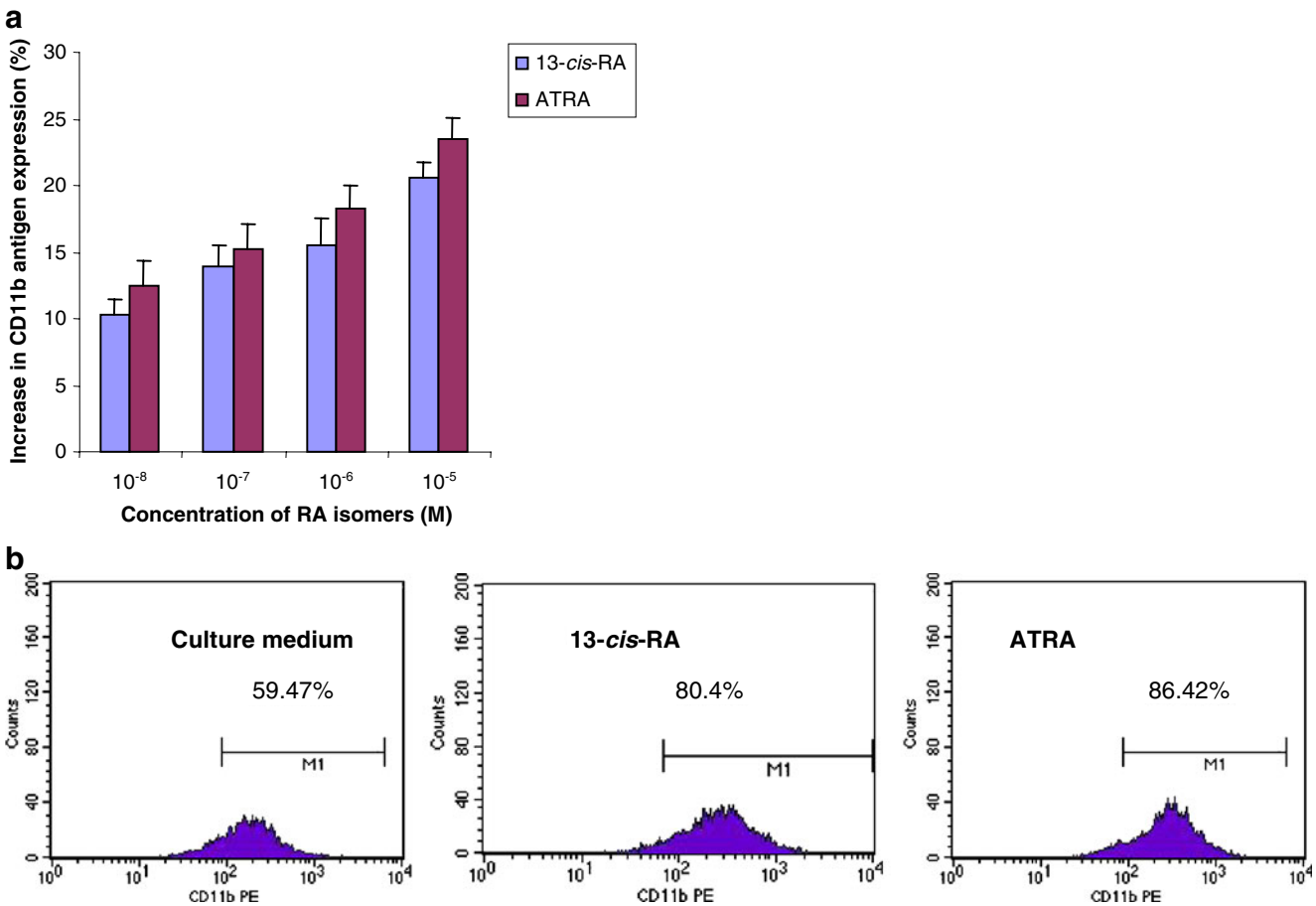

Fig. 4 Increase in CD11b differentiation marker expression after 72 h of treatment with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M concentrations of 13-*cis*-RA and ATRA. Data shown here represent mean values mean ± SER of four representative experiments **a**. Representative flow cytometric histograms showing greater increase in CD11b differentiation marker expression compared to the untreated control after treatment with 10^{-5} M ATRA, than with the same concentration of 13-*cis*-RA **b**

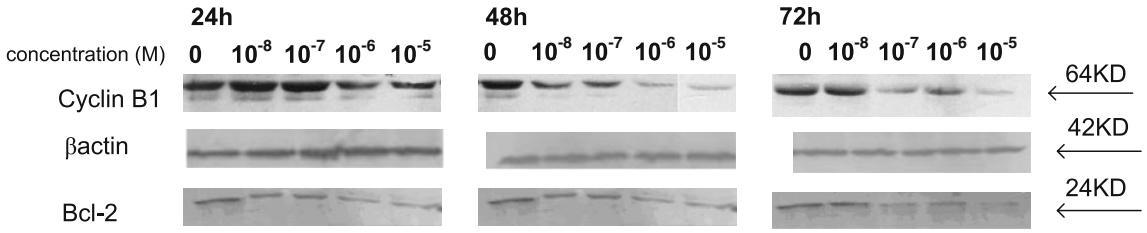

Fig. 5 Decrease in cyclin B1 **a** and Bcl-2 **b** protein expression after 24, 48 and 72 h of treatment with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M concentrations of 13-*cis*-RA (representative Western blot of four independent experiments)

RT-PCR results show compared to the control treated with CM only, the induction of IRF-1 iRNA in 13-*cis*-RA treated HL-60 cells, relative to the level of β-actin house keeping gene, after the first follow up time of 24 h. Our results show the highest level of IRF-1 transcription after 48 h of treatment (up to17- 19 fold) which remained higher than control after 72 h, although at lower than in previous 48 h interval (Fig. 7).

Discussion

The specific mechanisms of action of biological compounds on malignant cells may be important for the development of cancer therapeutics with lower toxicity. Different transcription factors, including activated retinoic acid receptors and specific cytokines can induce differentiation of immature myeloid cells. As HL-60 is a human promyelocytic leukemia cell line that is capable of differentiating into granulocytes, macrophages and basophiles by various chemical inducers and cell culture conditions [6], we have extensively investigated some aspects of this phenomenon in this study.

We show after the shortest 24-hours treatment a dose dependant antiproliferative effect of 13-*cis*-RA isomer on HL-60 cells concomitantly with the reduction of DNA replication, i.e. decrease in percentage of cells in S phase of cell cycle. Antiproliferative effect of RA has been previously reported on promyelocytic cells only for the all trans isomer [8, 9] but little is known about the effect of 13-*cis* isomer, that has been studied more on cutaneous cell lines. Published data indicate that 13-*cis* has been proven more effective than all trans RA isomer in inhibiting DNA synthesis and proliferation of SEB-1 sebocyte cell line [4]. It has been shown that retinoic acid in cell culture medium undergoes a thiol- radical mediated isomerization resulting in mixture of four isomers (13-*cis*-, 9-*cis*-, *all trans*-RA and 9,13-*di-cis*-) that have been also detected in treated HL-60 cells, with all trans-RA, compared to other RA isomers, reaching about four times higher concentration in HL-60 cells [10]. The smaller antiproliferative effect of 13-*cis*-RA obtained in this study compared to the existing published data for *all trans* isomer, could be due to shorter incubation periods [8, 9].

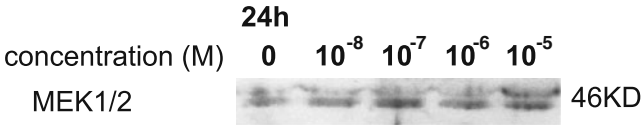

Fig. 6 Increase in MEK1/2 protein expression in HL-60 cells after 24 h of treatment with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M concentrations of 13-*cis*-RA (representative Western blot of four independent experiments)

To our knowledge, there are no existing data regarding the effect of 13-*cis*-RA on cell cycle distribution and differentiation of promyelocytic leukemia cell lines. Our results obtained after simultaneous treatments of HL-60 cells with 13-*cis*-RA and ATRA show that compared to ATRA, lower 13-*cis*-RA concentrations induced significant accumulation of cells in G0/G1 phase, as well as a decrease in the percentage of cells in S phase of cell cycle. As the obtained decrease in HL-60 cell proliferation and changes in cell cycle distribution are in agreement with previous findings for the more investigated *all trans* RA obtained on several PML-RARα positive cell lines [11, 12], the new findings in this study indicate that 13-*cis*-RA could be more effective than ATRA in inducing favorable changes in cell cycle distribution of HL-60 cells. Effects of RA on cell cycle and proliferation are mediated thorough the expression of target genes that suppress proliferation and cell cycle progression, such as p21, p19, GADD153 and due to this they down-regulate the expression of genes favoring DNA synthesis and/or repair and G1-S and G2-M cell cycle transition, such as c-myc, c-myb, GATA2, XECC1, P55CDC, as well as the expression of cell cycle regulatory molecules, cyclines A and B1 [13].

CD11b surface molecule is considered to be a granulocyte differentiation antigen since it is expressed mostly on mature granulocytes and to a much lesser extent on monocytes and NK cells. It has been first shown by Brietman *et al.* that RA in vitro treatment of PML-RARα positive cell lines, as well as PML cells isolated from leukemia patients, induces granulocyte differentiation [14]. Our results indicate that expression of CD11b differentiation antigen started to increase after 48 h of 13-*cis*-RA treatment, simultaneously with increase in the percentage of cells accumulated in G0/G1 phase of cell cycle, and

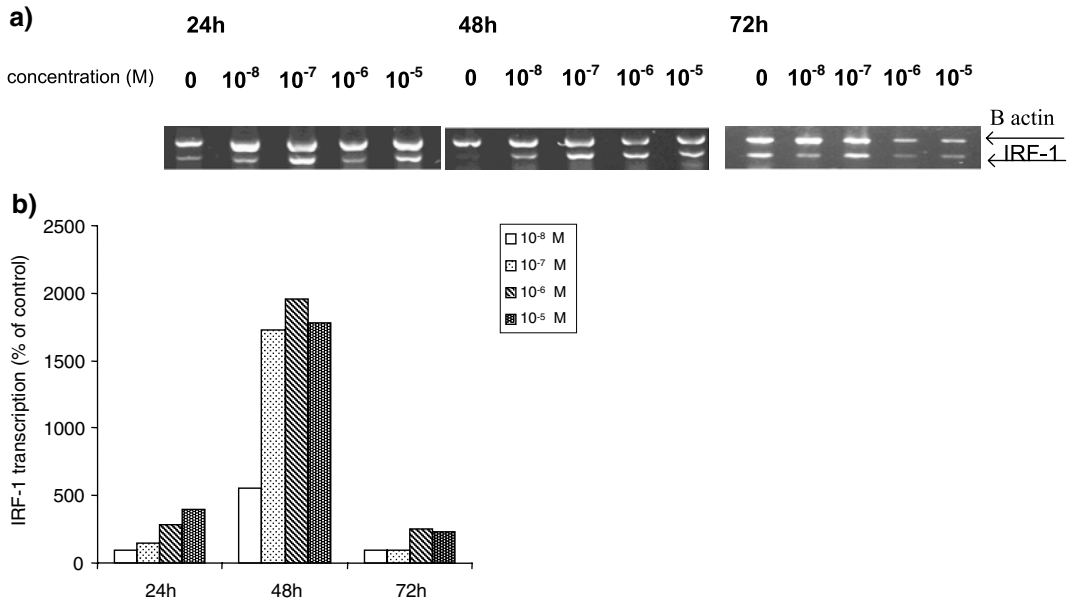

Fig. 7 Increase in IRF-1 transcription in HL-60 cells after 24, 48 and 72 h of treatment with 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M concentrations of 13-*cis*-RA RT-PCR of a representative experiment **a**. Level of IRF-1 normalized for β-actin level in 13-*cis*-RA treated cells is expressed as percentage of the control treated with CM **b**

persisted after 72 h of treatment showing not only time but also a significant dose-dependant effect. Our results obtained after simultaneous treatments with either 13-*cis* or *all trans* retinoic acid isomers also show that despite the greater beneficial effect of 13-*cis*-RA on cell cycle distribution in terms of G0/G1 phase accumulation and complementary decrease of S phase, *all trans* retinoic acid induces a greater increase in CD11b differentiation antigen expression. This finding of greater CD11b antigen expression is in accord with existing reports on *all trans* RA in vitro treatments [15-17], including lower concentrations (10^{-9} M) [18] and longer incubation time [8].

Differentiation of PML-RARα positive promylocytes is induced by activation of retinoid responsive genes and caspase-mediated or proteasome degradation of PML-RARα pathological product [19]. RA up-regulates the ubiquitin-activated E1 (UAEE1) and ubiquitin-conjugated (UCE) enzymes which catalyze the binding of the ubiquitin polymer to target protein [20] in proteasomes and also by inducing the SUMO-1 ubiquitin-like protein expression [21] involved in PML relocalization and regaining of its role as tumor suppressor. RARE sequences are present in the promoters of CDK (cycline dependant kinase) inhibitors, $p21^{waf/cip}$, p19 genes, and cause accumulation of HL-60 cells in resting G0/G1 phase of cell cycle and their differentiation. RA induced granulocyte differentiation is also associated with up-regulation of the CEBPε transcription factor which is important for granulocyte differentiation of myeloid cells [22].

Level of cell cycle regulatory molecules CDKs and cyclines is dependent on the phase of cell cycle [23] and every alteration in cell cycle distribution of proliferating cells is usually associated with the change in the level of cell cycle regulatory molecules. Transcription of cyclin B1 is the lowest in G0/G1 phase while it increases during S phase and reaches its highest level in the beginning of G2M phase and again declines from the mid-G2M until the beginning of G1 phase of the cell cycle [24]. We show decrease in cyclin B1 protein level after 24 h treatment only with higher concentrations of 13-*cis*-RA. It is interesting that the lowest level of cyclin B1, obtained in this study, is reached after 48 h of 13-*cis*-RA treatment at the same time when HL-60 cells begin to accumulate in G0/G1 phase of cell cycle and simultaneously with a significant increase in CD11b differentiation antigen expression. A report on another PML-RARα positive cell line [11] treated with other differentiation agents also shows that G0/G1 arrest could be accompanied with reduction of cyclin B1 protein level as found in this study.

Mitogen activated kinase (MAPK) transduction pathway has a significant role in survival of haematopoetic cells and it has been shown that MAP kinase may be constitutively active in some human malignancies including promyelocytic cells such as HL-60 cell line, with constitutively active MEK1/2 [25]. In this study we show that overall level of MEK1/2 signaling molecule is elevated in HL-60 cells after 24 h treatment with 13-*cis*-RA, contrary to another report [26] that demonstrates an increase in phosphorylated MEK1/2, with no change in the overall level of this protein after treatment of HL-60 cells with all trans RA. Since MEK1/2 is phosphorylated and active in G0/G1, as well as in G2M phase of cell cycle [25], it appears that MAP kinase signaling

pathway may also be involved in the process of differentiation of 13-*cis*-RA-treated HL-60 cells as we show in this study their accumulatin in G0/G1 phase of cell cycle. Therefore, as the increase of the overall level of MEK1/2 component of MAP kinase signal transduction pathway in 13-*cis*-RA treated HL-60 cells appears before granulocytic differentiation its increase may be one of the preconditions that enable differentiation. Moreover, it has also been shown on HL-60 cell line that antiproliferative activity of *all trans* RA is mediated through phosophorylation of ERK2 form of MAP kinase [27].

Various chemotherapeutical agents often display their therapeutic benefit by inducing apoptotic death of malignant cells. Level of antiapoptotic Bcl-2 protein and its functional homologue Bcl-X_l has been proven to be important for sensitivity of malignant cells to chemotherapy [27, 28]. High level of Bcl-2 positive blasts has been shown to correlate with the lower probability for promyelocytic leukemia patients to achieve chronical remission and is also associated with shorter overall survival [29]. Decrease in Bcl-2 protein expression in HL-60 cells shown in this study after 13-*cis*-RA treatment is consistent with the data that have been previously published for *all trans* RA stereoisomer [28, 30]. Down regulation of Bcl-2 protein expression could be due to serine phosphorylation of Bcl-2 which has been shown in PML blasts [31] after in vitro treated with all trans RA that can make Bcl-2 susceptible to degradation. There are also data that PML-RARα pathological product could also facilitate degradation of Bcl-2 in promyelocytic cells [32] and therefore decrease Bcl-2 protein expression.

Since antiproliferative effect of RA is very similar to interferon's and both interferon-α and RA share a similar signaling pathway [33], in this paper we investigated the influence of 13-*cis*-RA isomer on interferon regulatory factor 1 (IRF-1) transcription factor. In this study we show induction of IRF-1 transcription in 13-*cis*-RA treated HL-60 cells that reached highest level after 48 h, the time when HL-60 cells have been shown to arrest in G0/G1 phase of cell cycle, along side with detected decreased proliferation. This is in accord with the finding that IRF-1 level is dependent on the phase of cell cycle and is highest in G0/G1 phase [34].The shown increase in IRF-1 transcription has also previously been demonstrated on HL-60 and other PML-RARα positive cell lines (NB4, THP-1, U937) after treatment with all trans RA [35, 36]. It has been shown that RA promptly induces IRF-1 transcription, after 1 h of treatment [33] and that maximal level of IRF-1 protein product itself is correlated with maximal growth inhibitory effect of RA [37]. In case of damaged DNA, which is present in almost all tumor cells, IRF-1 together with p53 induces transcription of p21 CDK inhibitor and in that way inhibits progression to the S phase of cell cycle and proliferation [38]. Induction of IRF-1 transcription shown in this study on HL-60 cells may be another mechanism that also underlies 13-*cis*-RA antiproliferative activity.

Our data are in accord with a clinical study showing that patients with refractory APL treated with oral 13-*cis*-RA showed dramatic rise in peripheral blood maturing myeloid cells. These maturing cells were morphologically similar to those observed during in vitro culture of bone marrow promyelocytic cells with 13-*cis*-RA. These cultured cells showed most characteristics of differentiated cells with increased capacity for superoxide production, increase of leukocyte alkaline phosphatase activity, as well as much more effective phagocytosis, although they never matched morphologically normal granulocytes [39, 40].

In this study we show two major dose and time dependent effects of 13-*cis*-RA on HL-60 promylocytic cell line. The antiproliferative effect was the first to appear, after only 24 h of RA treatment, which is followed, after prolonged in vitro incubation, by a prodifferenting effect comprised of accumulation of HL-60 cells in the resting G0/G1 phase of cell cycle and simultaneous increase in CD11b granulocyte differentiation antigen expression. We also show that both, the decrease in the expression of cell cycle regulatory protein cyclin B1 and the increase in IRF-1 transcription factor are most intense when the highest percentage of HL-60 cells becomes arrested in the quiescent G0/G1 phase of cell cycle. Another aspect of 13-*cis*-RA action, shown in this study, deals with sensitivity of tumor cells to apoptosis, associated with down-regulation of Bcl-2 expression, a finding that is associated with acquiring sensitivity to chemotherapeutical agents. The new presented aspects of the effect of 13-*cis*-RA isomer on PML cell line may further facilitate the development of new pharmacological strategies i**n** the treatment of PML.

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