Growth Inhibition and Proapoptotic Activity Induction by IIF and Valproic Acid on RA-resistant Leukemia Cells

G. BARTOLINI[1], M. ORLANDI[1], A. PAPI[1], K. AMMAR[1], R. TONELLI[3], M. FRANZONI[3], A. PESSION[3], P. ROCCHI[2] and A.M. FERRERI[2]

Departments of [1]Experimental Evolutive Biology and [2]Experimental Pathology, and [3]Laboratory of Pediatric Hematology-Oncology, "Lalla Seragnoli" University of Bologna, Bologna, Italy

Abstract. *All-trans retinoic acid (RA) induces complete remission in a high proportion of patients with acute promyelocytic leukemia (APL). Nevertheless, most of these patients develop RA resistance and relapse. In an attemp to mimic clinical conditions for the treatment of leukemia, a stably RA-resistant subclone of the human promyelocytic leukemia cell line HL60 (HL60-R) was developed to study the antiproliferative and proapoptotic effect of the retinoid IIF (6-OH-11-O-hydroxyphenantrene) in comparison with RA. Moreover whether the inhibitor of histone deacetylase (HDAC) activity, valproic acid (VPA), could enhance sensitivity to retinoids in HL60-R cells was evaluated. Finally, the effect of IIF on the expression of multidrug resistance-associated protein 1 (MRP1) and P-glycoprotein (P-gp) was evaluated. It was found that IIF strongly suppressed cell proliferation (as measured by growth curves) and induced apoptosis (as measured by DNA fragmentation and Annexin V detection assays), while RA was practically ineffective. The addition of VPA to IIF accentuated the antiproliferative effect of IIF alone and increased apoptosis; the combination of VPA with RA allowed growth arrest. Moreover IIF caused a reduction of transmembrane transporter expression, particularly of P-gp, as shown by Western blotting. Our results suggest that IIF may be useful in controlling the proliferation of RA-resistant leukemia cells, especially in combination with an HDAC inhibitor, such as VPA.*

The approach to the treatment of acute promyelocytic leukemia (APL) has changed drammatically over the past decade; as a result, the long-term event-free survival for patients has improved significantly. The addition of the vitamin A derivative all-*trans* retinoic acid (RA) to treatment regimens has been found to be responsible for this improvement in survival (1-4). However, the therapeutic use of this compound is limited by a number of problems, which include serious systemic toxicity (5, 6) and induction of RA resistance (7-12). For this reason, major efforts are underway to identify new agents or strategies capable to overcome some or all of these problems.

In a previous study, we described the strong antiproliferative and prodifferentiating effect of the retinoid IIF (6-OH-11-*O*-hydroxyphenantrene) in comparison with RA in different tumor cell lines, including HL60 leukemic cells (13-16). In this study we investigated if IIF was also effective on RA-resistant leukemic cells. For this purpose we developed an RA-resistant subclone of the human promyelocytic leukemic cell line HL60 (HL60-R); we investigated the antiproliferative and proapoptotic effect of IIF on this derivative cell line. Strategies to overcome this resistance may include combination therapy of retinoids with other differentiation-inducing or chromatin-remodeling agents, such as histone deacetylase (HDAC) inhibitors (17-23). HDAC inhibitors are emerging as a promising class of antineoplastic agents for the treatment of solid and hematological malignancies; many studies suggest that a combination of retinoids plus HDAC inhibitors may have a greater therapeutic action than treatment with either drug alone (24-27). In addition, valproic acid (VPA) has been shown to inhibit HDAC activity and to synergise with RA as a differentiation inducer of leukemic cells both *in vitro* and *in vivo* (28-33). For these reasons, in the present work we tested whether the addition of VPA to IIF leads to an increase in growth inhibition and apoptosis in HL60-R cells with VPA plus RA with respect to IIF alone. In certain cases, pharmacoresistance can be overcome if the administered drug acts also as inhibitor of transmembrane transporters (34-37); on this basis, we investigated the effect of IIF on P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) expressions.

Correspondence to: Prof. Anna Maria Ferreri, Dipartimento di Patologia Sperimentale, Sezione Cancerologia, viale Filopanti 22, 40126 Bologna, Italy. Fax: +39 051242169, e-mail: annamaria.ferreri@unibo.it

Key Words: Leukemia cells, retinoids, valproic acid, RA-resistance.

Materials and Methods

Cell culture and treatment. HL60 cells, a human promyelocytic leukemia cell line, were cultured in RPMI 1640 (Sigma, Milan, Italy) supplemented with 10% fetal bovine serum (Sigma), 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml) solution and grown at 37˚C in a humidified atmosphere of 5% CO_2. Retinoic acid-resistant HL60 cells (HL60-R) were generated by exposing HL60 cells to a stepwise increase from $1x10^{-8}$ to $5x10^{-6}$ M RA (Sigma) for 3 months. The HL60-R cells were then propagated for 1 month in RA-free media and retested for resistance in $5x10^{-6}$ M RA. These cells, cultured in RA-free medium, under the same conditions as the HL60 parental cells, remain RA resistant. IIF (pat.WIPO WO 00/17143) and RA were dissolved immediately before use in propylene glycol and ethanol, respectively, and the final concentrations were obtained using the culture medium. The concentration of the solvents in the highest dose of the drugs did not affect cell proliferation of either cell lines. VPA (Sigma) was dissolved in the culture medium just before use.

Assessment of cell growth. A classic growth curve analysis was used to determine the effect of the compounds on cellular proliferation. A total of $0.5x10^5$ cells were placed in 25 cm^2 flasks and exposed to IIF or RA (20 and 40 μM), or to VPA (2 mM) alone or in combination with IIF or RA 20 μM. A cell count of replicate cultures was performed every 24 hours for 4 days. Cell viability was determined using trypan blue dye-exclusion test, which distinguishes viable and non-viable cells. Additional studies were conducted in order to evaluate the growth inhibitory effect of the drugs, using an MTT colorimetric assay, following an established protocol (38).

DNA fragmentation assay. Exponentially growing cultures were treated with IIF or RA (20 μM for 5 days). The cells ($4x10^6$/sample) were collected by centrifugation (220 x g) for 10 min at 25˚C. Cell pellets were resuspended in 500 μl of TE (10 mM Tris-HCL, pH 8; 1 mM EDTA, pH 8) and lysed for 1 h on ice by the addition of 500 μl of lysis buffer (5 mM Tris-HCl, pH 8; 20 mM EDTA, pH 8; 2% Triton X-100). The samples were then centrifuged for 12 min at 1000 x g; supernatants were collected in a clean microcentrifuge tube. For a qualitative evaluation of DNA laddering, 66 μl Na-acetate 0.2 M and 1 ml of absolute ethanol were then added to the samples. After overnight incubation at –20˚C, samples were centrifuged for 30 min at 1000 x g. Pellets, resuspended in 30 μl of TE, were subjected to RNAse (0.5 μg/ml at 37˚C for 1 h) and then to proteinase-K (1 μl/ml at 37˚C for 1 h) treatment. After addition of 6x DNA loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol in water), samples were separated in a 1% agarose gel containing 0.5 μg of ethidium bromide per ml and visualized under UV light. The DNA ladder 100 pb (Gibco, Milan, Italy) was used as a molecular marker.

Annexin V/propidium iodide apoptosis detection assay. Apoptosis was evaluated using the Annexin-V-FLOUS Staining Kit (Roche Diagnostic, Basel, Switzerland) according to the manufacturer's standard procedures. Analysis of apoptosis was performed by a FACSCalibur flow cytometer (Becton Dickinson, Mansfield, MA, USA). Data were analyzed by the Cell Quest (Becton Dickinson) software.

Evaluation of transmembrane transporters. Expression of P-gp and MRP1 was determined by Western blotting on wild-type HL60 and HL60-R cells with the use of the monoclonal antibody anti-P-gp clone JSB1 (Signet Laboratories, Dedham, MA, USA) and antibody anti-MRP1 (Biodesign International, Saco, MA, USA). The cells ($4x10^6$/ sample) were collected by centrifugation at 220 x g for 10 min and pellets were resuspended in phosphate-buffered saline (PBS) and sonicated on ice in the presence of protease inhibitors. Protein concentration was determined by the method of Lowry *et al.* (39). Cell lysates (50 μg of protein per lane) were size fractioned in sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gels prior to transfer to Hybond TM-C Extra membranes (Amersham, Italy) by standard protocols. Membranes were blocked overnight with 3% milk in TBS buffer (Tris-HCl, 2.42%, NaCl, 8%, Tween 20, 0.1%, pH 7.4) at 4˚C. The anti-P-gp and the anti-MRP1 antibodies were diluted 1:200 and 1:50 respectively; the antimouse peroxidase conjugated antibody was diluted 1:1000 with 5% milk in TBS. The P-gp and MRP1 proteins were detected by Chemiluminescent Peroxidase Substrate (Sigma) for Western blotting. The amount of protein in each lane was the same, as confirmed by Western blotting of actin (Sigma).

Results

Growth inhibition. To assess the antiproliferative effect of IIF in comparison with RA, cells were exposed to equimolar doses of the retinoids for four days. The effect of IIF and RA on HL60-R cells, as shown by growth curves, is illustrated in Figure 1. Interestingly, IIF caused a strong growth inhibition, increasing with time, at both concentrations: the percentage of inhibition in 40 μM treated cells was approximately 70% as compared to the control ones after 4 days treatment. The effectiveness of RA on cell proliferation at equimolar doses was far weaker, reaching a maximum of 20% inhibition. These results were confirmed by the MTT assay (data not shown). Subsequently, combination studies using 20 μM IIF or RA with 2 mM VPA were performed: the antiproliferative effect of VPA alone or in combination with IIF or RA on HL60-R cells as shown by growth curves is given in Figure 2. The results showed that the combination of IIF or RA with VPA produced a greater inhibition of growth than each agent alone, increasing with incubation time. After four days exposure, the effects of the combinations on cell viability were similar, however the antiproliferative effect of IIF combined with VPA was affected far earlier (60% loss of viability after 1 day exposure). VPA alone induced growth inhibition increasing with time and reaching 60% loss in viability after four days exposure.

Apoptotic activity. In order to evaluate whether the growth inhibition effect was accompanied by apoptosis, DNA fragmentation assay and Annexin V/propidium iodide staining analysis were performed. DNA fragmentation assay on HL60-R cells resulted in the formation of a DNA ladder

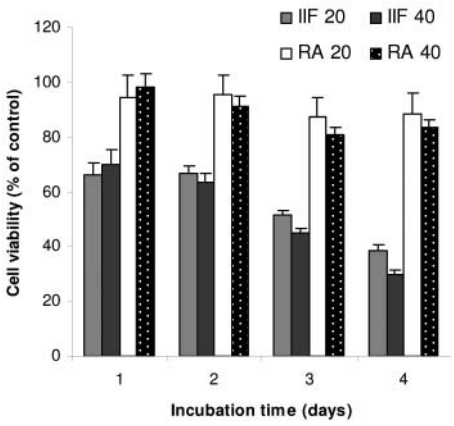

Figure 1. *Growth curves of HL60-R cells treated with 20 or 40 μM IIF or RA assessed by viable cell counts. Each bar represents the mean (±SE) of replicate cultures from three independent experiments.*

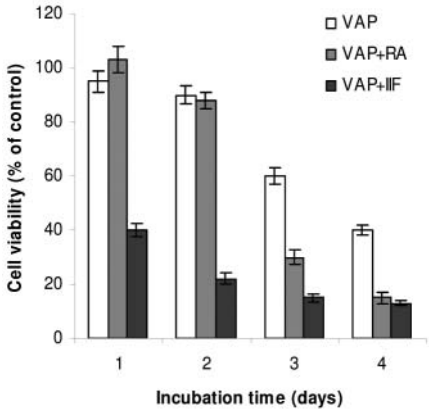

Figure 2. *Growth curves of HL60-R cells treated with 2 μM VPA alone or in combination with 20 μM IIF or with 20 μM RA assessed by viable cell counts. Each bar represents the mean (±SE) of replicate cultures from three independent experiments.*

after 5 days treatment with 20 μM IIF, while DNA fragmentation was quite negligible after equimolar RA treatment (Figure 3). As demonstrated by Annexin V/propidium iodide staining analysis, 24 h exposure to VPA and RA, alone or in combination, did not increase the percentage of Annexin V/propidium iodide-positive cells, while treatment with IIF increased the percentage to 14% and IIF plus VPA to 18% (Figure 4).

Transmembrane transporters expression. Experiments were performed to assess P-gp and MRP1 expression in HL60 parental cells. The effect of IIF as a possible modifier of P-gp and MRP1 expression in HL60-R cells was investigated. As shown in Figure 5, treatment with 20 μM IIF caused a remarkable reduction of P-gp expression (approximately 60% after 3 days treatment). The same treatment produced a weak effect on MRP1 expression (Figure 6).

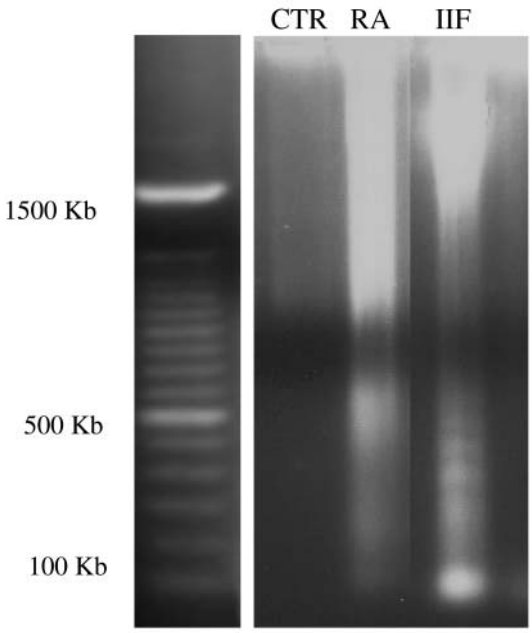

Figure 3. *Analysis of DNA fragmentation in HL60-R cells after a five-day exposure to 20 μM IIF or RA. The molecular marker (200 bp) is shown on the left of the figure. CTR: untreated cells. The results are representative of three independent experiments.*

Discussion

Despite an excellent initial response, APL cells develop resistance to RA and relapse occurs in APL patients treated with RA alone (7-12). Major efforts are being made to develop pharmaceuticals that could overcome the problem of RA resistance. IIF is a new retinoid of particular interest as an anticancer agent, being active in different cell lines, including HL60 leukemic cells, as we reported in previous studies (13-16). The present work was undertaken in order to investigate the potential of IIF as an antitumoral agent on the HL60-R cell line, based on the hypothesis that IIF could bypass RA resistance. Treatment with IIF caused a marked growth inhibition: the antiproliferative effect in RA-resistant cells was early and time-dependent, even at a low dose, while, as expected, RA treatment at equimolar doses displayed a very weak effect. Since a number of synthetic retinoids cause growth suppression through induction of apoptosis (40-46), we analysed whether IIF affected HL60-R cell growth by triggering programmed cell death. As demonstrated by the DNA fragmentation assay and Annexin V analysis, IIF was a strong inducer of apoptosis. These data indicate that IIF, unlike RA, has a very significant activity as an antiproliferative and proapoptotic agent in HL60-R cells:

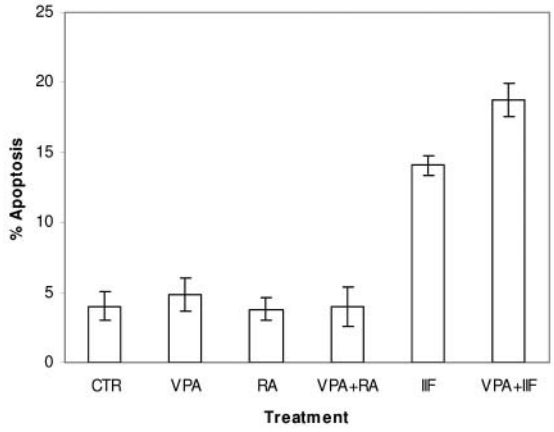

Figure 4. *Apoptosis induction by 20 μM IIF and 20 μM RA alone or in combination with 2 mM VPA after 1 day exposure, in HL60-R cells. Apoptosis was assessed by Annexin V/FITC binding. Each bar represents the mean±SE of three experiments. CTR: Untreated cells.*

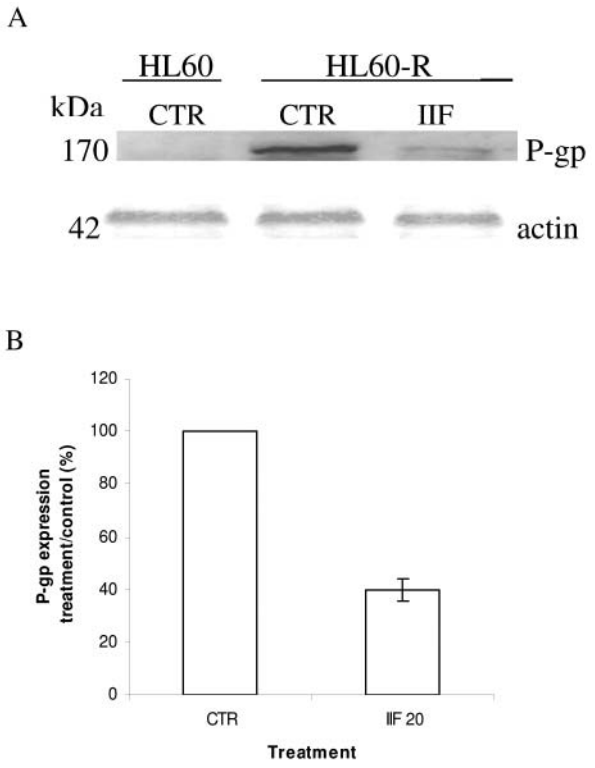

Figure 5. *A) Expression of P-gp in HL60-R cells, after a three day exposure to 20 μM IIF, evaluated by Western blot. CTR: untreated cells. B) Densitometric data, normalized by actin, are expressed as treatment/CTR% and are the mean±SE of three independent experiments.*

this effect can be obtained with low doses and short exposure to the drug and could be due – at least in part – to the remarkable reduction observed in P-gp expression.

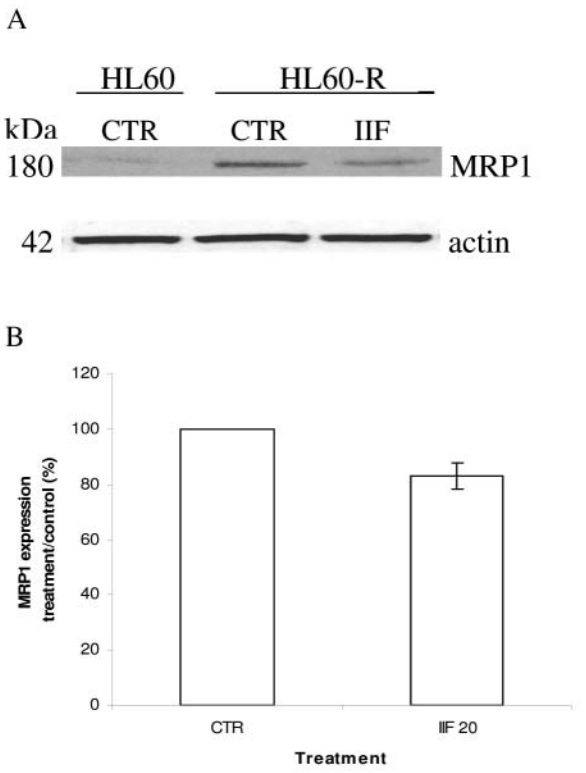

Figure 6. *A) Expression of MRP1 in HL60-R cells, after a three-day exposure to 20 μM IIF, evaluated by Western blot. CTR: Untreated cells. B) Densitometric data, normalized by actin, are expressed as treatment/CTR% and are the mean±SE of three independent experiments.*

Several studies indicate that the combination of RA with HDAC inhibitors, such as VPA, causes synergistic antitumor effects on transformed cells from APL patients (31-33, 47-48). On this basis, cell proliferation and apoptosis of HL60-R cells were assessed in the presence of VPA with IIF or RA. The addition of VPA to RA allowed growth inhibition; the combination of VPA with IIF accentuated the antiproliferative and proapoptotic activity of IIF alone. Collectively our results demonstrated that IIF by itself is a strong antiproliferative and proapoptotic inducer, capable of relieving resistance of HL60-R cells. These effects were enhanced when IIF was associated with the HDAC inhibitor VPA. In a previous study we demonstrated the remarkable antitumoral activity of IIF in different cancer cells (13-16); the results of this study support the possibility that IIF may be an effective compound for anticancer treatment, including of drug-resistant human cancer.

Acknowledgements

This work was supported by a grant from MURST, Italy. We thank Dr. A. Lucchetti for his support in the DNA fragmentation assay.

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Received July 24, 2007
Revised November 16, 2007
Accepted December 10, 2007