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Retinoids increase idarubicin cytotoxicity in human myeloid leukemia cell lines

Patrizia Tosi, Giuseppe Visani, Emanuela Ottaviani, Annalisa Pellacani, Silvia Manfroi and Sante Tura

Institute of Hematology and Medical Oncology 'Seràgnoli', University of Bologna, Via Massarenti 9, 40138 Bologna, Italy. Tel: (+39) 51390413; Fax: (+39) 51398973.

All-*trans*-retinoic acid (ATRA) has proven useful in acute promyelocytic leukemia (APL). In order to reduce the side effects and to improve the efficacy of this compound, conventional chemotherapy, and anthracyclines in particular, are frequently added either during remission induction or in consolidation therapy. In this study we aimed at investigating the rationale of the combination of ATRA plus idarubicin in two human leukemic cell lines, HL-60 and K562, that display a different sensitivity to ATRA treatment. The effects of ATRA were compared with those of two clinically active retinoids, 13-*cis*-retinoic acid (13-*cis*-RA) and 9-*cis*-retinoic acid (9-*cis*-RA). Both in HL-60 and in K562 cells, the majority of the combinations of ATRA and idarubicin were synergistic, while the combinations with 9-*cis*-RA and 13-*cis*-RA were more effective in HL-60 and K562 cells, respectively. A 72 h pre-incubation with retinoids was able to further increase the cytotoxicity of ATRA plus idarubicin in the two cell lines. Intracellular idarubicin accumulation was enhanced by retinoids, as demonstrated by a cytofluorimetric method. Our results could contribute to provide a rationale for ATRA plus idarubicin combinations not only in APL but also in acute leukemia of other cytotypes.

Key words: Acute myeloid leukemia, idarubicin, retinoids.

Introduction

All-*trans*-retinoic acid (ATRA) has proven to be a useful therapeutic approach for acute promyelocytic leukemia (APL). The administration of ATRA at the onset of the disease has several advantages over chemotherapy, as this compound is able both to induce complete hematological remission without bone marrow aplasia[1] and to improve coagulopathy.[2] Several problems, however, are related to ATRA therapy: first, the occurrance of hyperleucocytosis,[3] that could contribute to the onset of ATRA syndrome that is a potentially fatal complication.[4] Second, and most important, ATRA alone is not able to guarantee long-lasting complete remissions, as the majority of the patients relapse within a year if conventional consolidation chemotherapy is not administered.[3,5] For these reasons, several groups have proposed ATRA plus chemotherapy combination schedules both for induction of remission and for consolidation. Fenaux *et al.*[6] have demonstrated that the addition of chemotherapy after achievement of complete remission with ATRA was more effective than conventional therapy in prolonging disease-free survival. Alternative treatments were also designed, including simultaneous administration of ATRA plus idarubicin. The choice of this latter drug was based upon the evidence of a peculiar sensitivity of APL cells to antracyclines[7] and to idarubicin in particular,[8] and to superiority of idarubicin over daunorubicin on MDR models[9] and in terms of reduced cardiotoxicity.[10] Preliminary results obtained in 185 patients[11] indicate that this drug combination is able to induce a high frequency of early molecular remissions.

At present, few studies have been reported concerning the interactions between antracyclines and ATRA *in vitro*.[12] The aim of the present study was to evaluate the cytotoxic relationship occurring between ATRA and idarubicin in two leukemic cell lines that show a different pattern of sensitivity to the activity of retinoids. The effects of ATRA were also compared with those of two clinically active retinoids, 9-*cis*-retinoic acid (9-*cis*-RA) and 13-*cis*-retinoic acid (13-*cis*-RA).

Materials and methods

Drugs and chemicals

ATRA and 9-*cis*-RA were kindly provided by Hoffman-La Roche (Basel, Switzerland). 13-*cis*-RA was purchased from Sigma (St Louis, MO). All the

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Correspondence to P Tosi

retinoids were dissolved in absolute ethanol to a concentration of 10^{-2} M. Further dilutions were performed in RPMI 1640 medium (Gibco, Paisley, UK). Idarubicin was purchased from Pharmacia-Farmitalia Carlo Erba (Milan, Italy). The drug was initially dissolved in sterile water; final dilutions were performed in RPMI 1640.

Cell lines

Exponentially growing HL-60[13] and K562 cell lines[14] were used throughout the study. The cells were cultured in 25 mm sterile plastic flasks containing RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Gibco). The flasks were kept at 37°C in a humidified incubator with 5% CO_2 and passed three times weekly. Under these conditions, the doubling time was approximately 24 h for both the cell lines.

Plasma clot clonogenic assay

The cytotoxicity of idarubicin plus retinoids was evaluated in two different schedules.

(a) Co-administration: 1×10^3 HL-60 or K562 cells were resuspended in 1 ml of RPMI 1640 medium plus 10% FCS, 10% citrated bovine plasma, 3.4 μg/ml of $CaCl_2$, with or without retinoids at 10^{-7} M and with or without idarubicin at concentrations ranging from 1 to 50 ng/ml. Samples were plated in 35 mm plastic Petri dishes and kept at 37°C in a humidified incubator with 5% CO_2.

(b) 72 h retinoid pre-incubation: 2×10^6 HL-60 or K562 cells were resuspended in 10 ml of RPMI with or without retinoids at 10^{-7} M. After 72 h of incubation the cells were washed twice, scored for viability and plated as described above.

Colonies (aggregates of > 50 cells) were scored after 7 days, using an inverted microscope. Each experiment was performed in duplicate and repeated at least three times. Colony growth inhibition was calculated as percent of control culture colony growth.

The activity of the drug combination was evaluated as follows: the ratio between the product of the survival fraction for each agent alone (expected survival) and the survival fraction observed with the drug combination was calculated.[15] Values greater than 1 were considered to show synergism, those less than 1, antagonism and those of about 1, an additive effect.

Intracellular idarubicin accumulation

In order to evaluate whether incubation with retinoids could affect intracellular Idarubicin accumulation, 4×10^6 HL-60 or K562 cells were resuspended in 20 ml of RPMI 1640 with 10% FCS. Idarubicin was added at the following concentrations: 0, 25, 50, 100, 250, 500 and 1000 ng/ml, either together with retinoids at 10^{-7} M (schedule *a*) or after 72 h pre-exposure to retinoids (schedule *b*). After 2 h of incubation, cells were washed twice and intracellular idarubicin concentration was measured according to a previously reported cytofluorimetric method.[16] Briefly, a FACScan (Becton Dickinson, San Jose, CA) was used, with a laser excitation of 488 nm; forward-angle light scatter was determined in order to evaluate cell volume; relative intracellular idarubicin accumulation was finally measured as normalized median fluorescence intensity (NMFI).

Statistical analysis

Data were analyzed by Student's *t*-test and differences were considered significant when $p < 0.05$ (two-tailed test).

Results

Effects of retinoids and idarubicin on clonogenic growth of HL-60 and K562 cells

The effects of various retinoids on HL-60 and K562 clonogenic growth are shown in Table 1. As reported elsewhere,[17] ATRA and 9-*cis*-RA at 10^{-7} M displayed a similar activity on HL-60 cell line, with an average 35 and 41% growth inhibition after 7 days

Table 1. Activity of retinoids on HL-60 and K562 colony growth

	HL-60		K562	
	A	B	A	B
ATRA	35 ± 6.9[a]	51 ± 5.5[a]	26 ± 6.3	37 ± 3.9
9-*cis*-RA	41 ± 4.7[b]	53 ± 5.8[b]	23 ± 8.4	31 ± 4.6
13-*cis*-Ra	31 ± 6.3	46 ± 8.2	28 ± 2.7	37 ± 5.3

Data are expressed as percent growth inhibition compared to control (no drug). The average number of colonies in control samples was 123 ± 17 (HL-60) and 145 ± 33 (K562). A, plating in retinoid-containing media. B, 72 h pre-treatment before plating in retinoid-containing media.
[a] $p = 0.03$.
[b] $p = 0.04$.

of incubation, while 13-*cis*-RA was less effective. K562 cell line colony growth was less affected by retinoids, 13-*cis*-RA appeared to be the most active agent (28% growth inhibition) followed by ATRA (26% growth inhibition). Culture in retinoid-containing media 72 h prior to plating increased overall cytotoxicity, both in HL-60 cells and, to a minor extent, in K562 cells.

The data concerning the cytotoxicity of co-administered idarubicin plus retinoids on clonogenic growth of HL-60 and K562 cells are reported in Figure 1(A and B). The IC_{50} of idarubicin, after 7 days of continuous exposure, was 9.5 ng/ml in HL-60 cells, this was decreased by 71, 52 and 23% by ATRA, 9-*cis*-RA and 13-*cis*-RA, respectively. The effects of ATRA plus idarubicin appeared to be synergistic in the majority of the combinations tested. On K562 cells the IC_{50} of idarubicin was 9 ng/ml, the most effective combinations were those with ATRA (45% decrease in the IC_{50} of idarubicin) while 9-*cis*-RA and 13-*cis*-RA exerted a reduced activity. A 72 h pre-incubation with retinoids was able to further increase the cytotoxicity of the combination with idarubicin; with regard to the HL-60 cell line (Figure 2A), a synergistic effect was observed in the samples where ATRA and 9-*cis*-RA were added to idarubicin while in the K562 cell line (Figure 2B) the most active combinations were those with ATRA and 13-*cis*-RA.

Intracellular idarubicin accumulation

In order to evaluate whether an increased activity of idarubicin plus retinoids was the result of the summation of two different cytotoxic pathways or if retinoids could instead interfere with the cellular uptake of idarubicin, we measured the intracellular concentration of the anthracycline by a cytofluorimetric method. In the HL-60 cell line, both

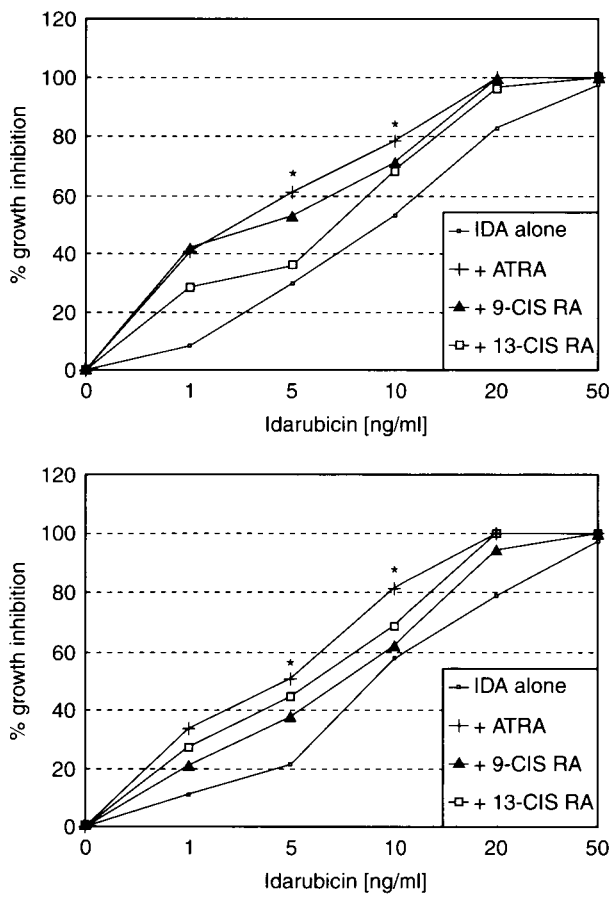

Figure 1. Clonogenic growth inhibition of HL-60 (A) and K562 (B) after co-administration of retinoids plus idarubicin. Asterisks indicate a synergistic effect.

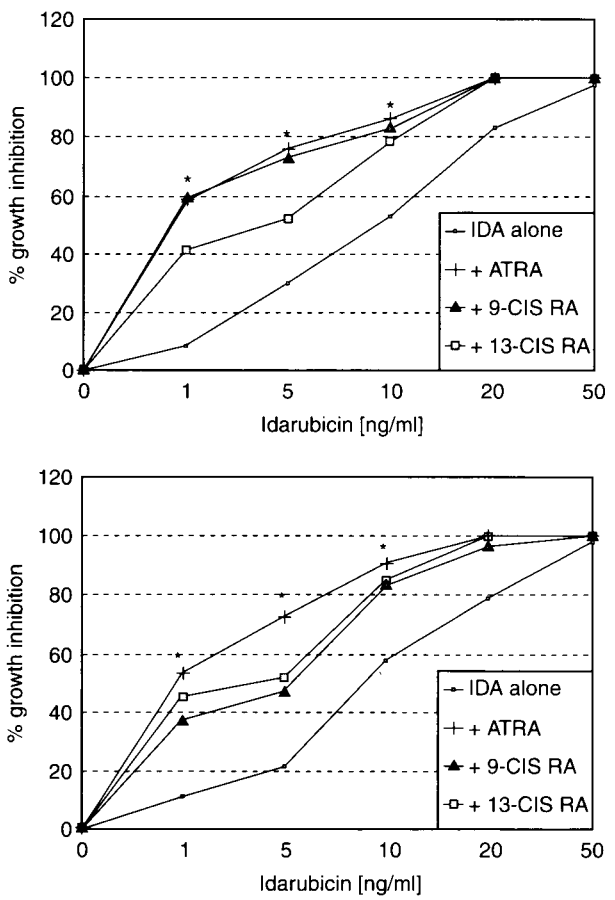

Figure 2. Clonogenic growth inhibition of HI-60 (A) and K562 (B): 72 h pre-incubation with retinoids. Asterisks indicate a synergistic effect.

co-incubation and pre-treatment with retinoids determined an increase in intracellular idarubicin concentration (Figure 3A and B), that always reached statistical significance in ATRA-treated samples. In K562 cells the same phenomenon occurred, even though, in this cell line, co-incubation with retinoids caused a less marked increase in intracellular idarubicin compared to control (Figure 3C and D).

Discussion

The clinical activity of retinoids has been demonstrated in several neoplastic models, including skin cancer[18] and hematological malignancies, such as myelodysplastic syndromes,[19,20] juvenile chronic myeloid leukemia[21] and, above all, APL, where ATRA represents a well established therapeutic approach,[1,3] while 9-*cis*-RA has shown promising activity.[22] In order to improve the efficacy and to reduce the side effects, several retinoid-based combination schedules have been proposed, either with biologic agents or with conventional drugs. In particular interferon (IFN)-α plus 13-*cis*-RA have proven effective in cutaneous T cell lymphoma[23] and in squamous cell carcinoma of the skin;[24] furthermore, the addition of IFN-α seems to be able to restore the sensitivity to ATRA in APL.[25] The combinations of ATRA with chemotherapy have been recently evaluated; specifically it has been demonstrated that the combination with low-dose Ara-C is useful in poor prognosis acute myeloid leukemia (AML),[26] while several groups have reported that high-dose conventional consolidation therapy is mandatory after ATRA treatment, in order to prolong disease-free survival in APL.[6] The biochemical interaction between retinoids and chemotherapy has not been thoroughly investigated yet. *In vitro*, ATRA is able to potentiate Ara-C and daunorubicin-induced DNA damage, probably by inhibiting

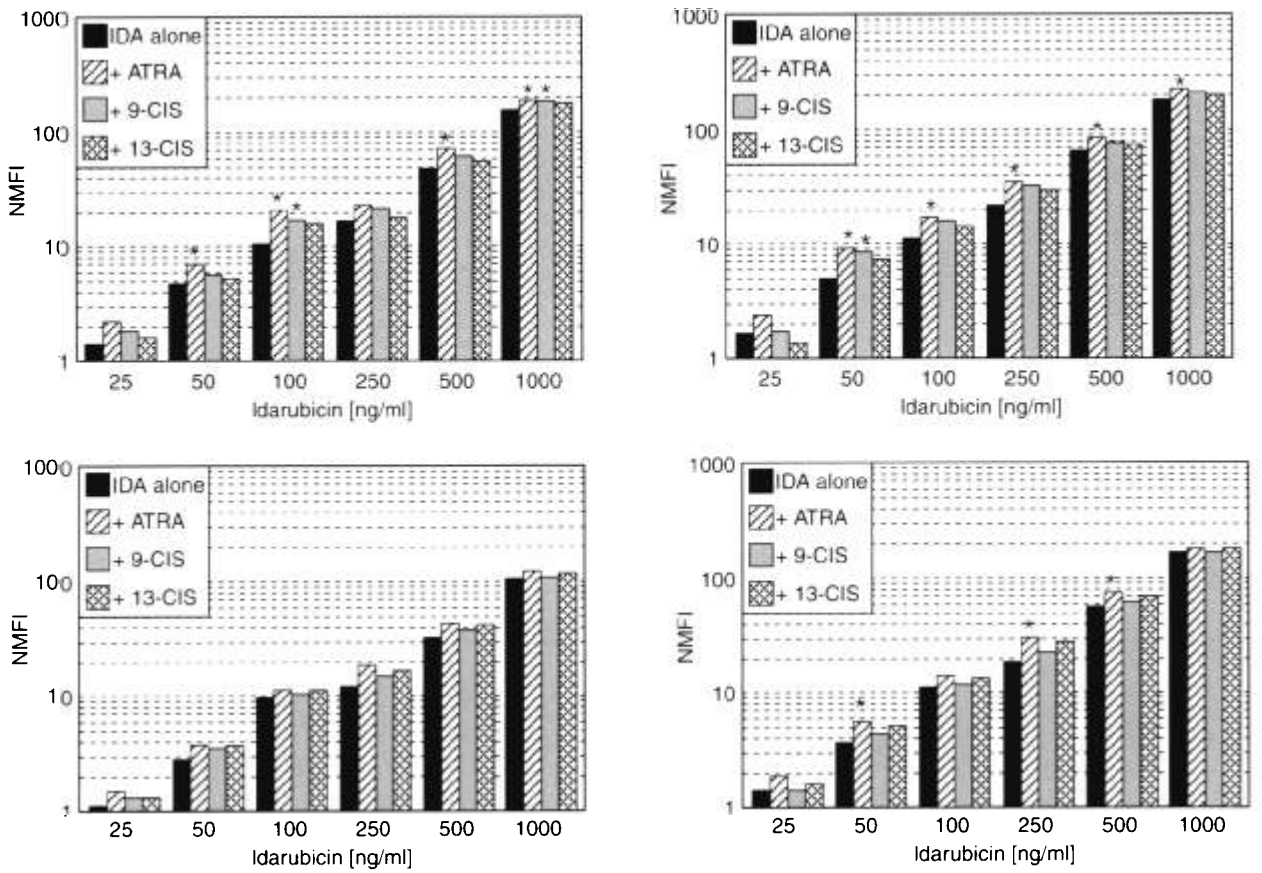

Figure 3. Intracellular concentration of idarubicin in HL-60 and K562 co-incubated or pre-incubated with retinoids. (A) Hl-60, co-incubated; (B) HL-60, pre-incubated; (C) K562 co-incubated; (D) K562 pre-incubated. Asterisks indicate a significant increase in NMFI compared to control.

the mechanisms of DNA repair;[12,27] the relationship between differentiation and drug-induced cytotoxicity, however, appears to be more controversial. It has been reported that DNA fragmentation induced by topoisomerase II inhibitors could actually be reduced by *in vitro* treatment with differentiation promoting agents such as TPA[28,29] while ATRA has been described to increase etoposide cytotoxicity.[28–30]

In the present paper we have demonstrated that retinoids, and ATRA in particular, are able to synergistically increase idarubicin-induced cytotoxicity, both in HL-60 cells, that are known to undergo granulocytic differentiation upon treatment with retinoids,[31] and in K562 cells, that are relatively insensitive to ATRA treatment, thus suggesting a mechanism not related to differentiation. Evaluation of intracellular Idarubicin concentration showed that, in both the cell lines, the accumulation of the drug was increased in retinoid-containing samples compared to controls. The reason for this phenomenon has not been clarified yet. It could be postulated that the compounds display some kind of interaction at the cellular membrane level and this could be indirectly confirmed by the reported observation that an epirubicin-resistant cell line was also resistant to ATRA-induced differentiation.[32]

Whatever the exact mechanism of the retinoid-induced increase in idarubicin sensitivity could be, our findings may help to provide a rationale for ATRA plus chemotherapy combinations, not only in APL, but also in differentiation insensitive settings, such as AML of other cytotypes.

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