A Search for Multidrug Resistance Modulators: The Effects of Retinoids in Human Colon Carcinoma Cells

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Abstract. The development of multidrug resistance (MDR) is one of the major causes of failure in cancer therapy. The use of cell lines with acquired resistance to anticancer agents represents a very good tool for investigation into the possibility of reversal of MDR. In this study the ability of all-trans-retinoic acid (RA) and its derivative 6-OH-11-O-hydroxyphenanthrene (IIF; pat. WIPO W000 /17143) as antitumor agents was investigated in the human colon carcinoma cell line LoVo and in the counterpart resistant derivative LoVo/MDR cell line. Cell proliferation was measured by MTT assay, apoptosis was evaluated using DNA fragmentation and Annexin V detection assay. Retinoids suppressed cell proliferation in a time- and dose-dependent manner. Interestingly, IIF was significantly more effective than RA, particularly on LoVo/MDR cells. RA and IIF induced apoptosis in both cell lines, with IIF effect significantly higher than that of RA. Furthermore, on the basis that MDR phenotype is often caused by drug efflux due to overexpression of the membrane P-glycoprotein (P-gp), it was demonstrated that RA and IIF reduced P-gp synthesis in LoVo/MDR cells. Our results suggest that IIF could be a powerful tool in the development of colon carcinoma treatments, even when tumor cells present an MDR phenotype.

In a previous study, we have demonstrated the remarkable antitumor activity of the retinoids all-trans-retinoic acid (RA) and 6-OH-11-O-hydroxyphenanthrene (IIF) in the colon cancer cell lines CaCo-2 and HT29 (13). In this study, we investigated if RA and IIF can also induce growth inhibition in colon carcinoma cells that present an MDR phenotype. The human colon carcinoma cell line LoVo and a LoVo/MDR cell line, selected after doxorubicin exposure, were used. As the MDR phenotype is, in most cases due to an increased drug efflux, mediated by P-glycoprotein (P-gp) (14-17), the effect of RA and IIF as possible modifiers of the expression of P-gp protein in LoVo/MDR cells was analyzed.

Materials and Methods

Cell line. The LoVo cell line was derived from a metastatic human colon carcinoma and LoVo/MDR was the derivative MDR clone. LoVo and LoVo/MDR cell lines were maintained in RPMI 1640 (Sigma-Aldrich, Milano, Italy) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 Ìg/ml) and grown at 37°C in a humidified atmosphere of 5% CO2 in air.

Cell treatment. IIF (pat. WIPO W0 00/17143 ) and RA (Sigma-Aldrich) were dissolved in propylene glycol and ethanol, respectively, immediately before use and the final concentrations in the range 10 ÌM - 40 ÌM were obtained using the culture medium. The concentration of the solvent in the highest dose of drugs did not affect cell proliferation of either cell lines.

Growth inhibition. The effect of IIF and RA on cell proliferation was evaluated by MTT assay (18) based on the reduction of the number of metabolically active cells, and the results were expressed as percentage of the controls. This method eliminates the problems due to the tendency of cells to form aggregates after trypan blue staining. In order to examine the ability of cells treated with IIF and RA to form colonies, LoVo and LoVo/MDR cells were plated in Petri dishes in culture medium: one day after plating, the cells were exposed to the drugs. After seven days of incubation, the plates were fixed, stained with Giemsa and colonies consisting of more than 20 cells were counted.

DNA fragmentation assay. Exponentially growing cells (2x10⁴/cm² in T-25 flasks) were treated with IIF or RA (10 and 20 ÌM). The cells (3x10⁶/sample) were detached by tripinsization and collected by
centrifugation at 220 xg 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 xg; 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 xg. Pellets, resuspended in 30 μl of TE, pH 8, 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 BRL) was used as a molecular marker.

**Annexin V apoptosis detection assay.** Experiments were performed on cells seeded in 96-well plates at a density of 6x10^4/well, treated for two days with IIF or RA 20 μM. Samples were prepared according to the protocol of kit sc-4252 AK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using 0.5 μg of Annexin V FITC and 10 μl of propidium iodide per 100 μl assay buffer. After 15 min incubation in the dark, the buffer was substituted by 100 μl of RPMI supplemented with FCS, and samples were observed under reversed fluorescence microscopy using a dual filter set for FITC and rhodamine. Apoptosis was evaluated by counting FITC-labeled cells in at least five random fields, and was expressed as % of total cells.

**Evaluation of P-gp.** Expression of P-gp protein was determined by Western blotting on LoVo/MDR cells with the use of the monoclonal antibody anti-P-gp clone JSB1 (Signet Laboratories, USA). The cells (3x10^6/sample) were collected by centrifugation at 220 xg for 10 min and pellets were resuspended in PBS and sonicated on ice in the presence of protease inhibitors. Protein concentration was determined by the method of Lowry et al. (19). 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% BSA in transfer buffer saline (TBS) (Tris-HCl, 2.42%, NaCl, 8%, Tween 20, 0.1%, pH 7.4) at 4°C. The anti-P-gp and the antimouse peroxidase conjugated antibodies were diluted 1:200 and 1:1000 respectively with 3% BSA in TBS. The P-gp protein was detected by Chemiluminescent Peroxidase Substrate for Western blotting (Sigma). The amount of protein in each lane was the same, as confirmed by Western blotting of actin.

**Results**

**Growth inhibition.** Treatment with both retinoids in LoVo cells resulted in a remarkable dose- and time-dependent decrease in cell viability (Figure 1). Interestingly, this effect was also obtained in LoVo/MDR cells (Figure 2). The effectiveness of IIF, as compared with RA, was shown in both cell lines, particularly in LoVo/MDR cells. Further studies on growth inhibition induced by IIF and RA were performed by clonogenic assay in liquid medium. The ability to form colonies was suppressed by both retinoids in both cell lines: only in LoVo/MDR cells treated with 20 μM RA, clonogenic efficiency was 10% with respect to controls (14.25±1.6 versus 140.45±6.8).

**Apoptotic activity.** In order to evaluate whether retinoid induction of growth inhibition was accompanied by apoptosis, Annexin V/propidium iodide staining assay and DNA fragmentation assay were performed. As demonstrated by Annexin V analysis, both retinoids displayed apoptotic activity in both cell lines; furthermore, apoptotic activity induced by IIF was significantly higher than that induced by RA in LoVo/MDR cells (Figure 3). DNA fragmentation assay performed on LoVo/MDR cells resulted in the formation of DNA ladder after treatment with 20 μM IIF, while DNA fragmentation was not detected after RA treatment (Figure 4).

**P-gp expression.** The effect of IIF and RA as possible modifiers of P-gp expression was investigated in LoVo/MDR cells. As expected, P-gp was absent in LoVo cells, while it was detectable in LoVo/MDR cells (Figure 5A); treatment with RA or IIF caused a remarkable reduction of P-gp expression (Figure 5B).
Chemotherapy of colorectal cancer is unsatisfactory and mortality remains very high (20, 21). One of the major causes of the failure or even discontinuation of cancer treatment is the development of pleiotropic drug resistance (22, 23).

Retinoids have long been investigated in preclinical models, and clinical data have already supported the potential of these compounds as anticancer preventive and therapeutic agents (7-12, 24, 25).

In previous studies, we described a potent antitumor activity of the retinoid IIF, in comparison with RA, in different cancer cell lines (13, 26, 27). The present study was performed to explore the ability of RA and its derivative IIF to induce growth inhibition and apoptosis in the human colon carcinoma cell line LoVo and in the counterpart resistant derivative LoVo/MDR cell line.

Our results, besides confirming the antitumor effect of retinoids on colorectal carcinoma cells (12, 13, 28-30), clearly demonstrated the effectiveness of IIF and RA on doxorubicin resistant colon carcinoma cells. In LoVo/MDR cells, RA and IIF reduced the expression of P-gp, the protein responsible for increased drug efflux in cells with MDR phenotype (14-17).

The data obtained on cell growth indicated that both retinoids exhibited a potent antitumor activity against LoVo and LoVo/MDR cells. The activity of RA on LoVo/MDR cells was far weaker than that obtained in LoVo parental cells. Treatment with IIF exerted severe growth inhibition, especially on LoVo/MDR cells: the antiproliferative effect of IIF on these resistant cells was earlier and higher (even at lower doses) than on LoVo cells. When the effects on cell growth induced by RA and IIF were compared, it was apparent that in both cell lines the inhibition by IIF is always higher.

A progressive inhibition of apoptosis may cause cancer transformation which further progresses to malignancy on the colon cells (31-33). Therefore, the factors that control the balance between cellular proliferation and apoptosis in colonic epithelium are of great importance (33). In general, the protective/therapeutic potential of retinoids against human cancer has been considered to be primarily due to restoration of the differentiation potential of their target cells. Some studies, however, have demonstrated that retinoids cause tumor cell growth suppression through induction of apoptosis in certain types of cancer cells (34-36). Unlike natural

![Figure 2. Effect of doses (nM) of RA (top) and IIF (bottom) on LoVo/MDR cell growth as assessed by MTT assay. Each bar represents the mean ±SE of six replicate cultures from three independent experiments.](image)

![Figure 3. Apoptosis induction by 20 µM RA and 20 µM IIF in LoVo cells (top) and in LoVo/MDR cells (bottom) after a 2-day exposure. Apoptosis was assessed by Annexin V-FITC binding. Each bar represents the mean±SE of four experiments. CTR: untreated cells.](image)
retinoids that often induce differentiation, a number of synthetic retinoids cause growth suppression through induction of apoptosis (37). Therefore, in this study we assayed whether or not IIF and RA induced apoptosis in colon cancer cells. As shown by Annexin V analysis, RA and IIF confirmed their ability to trigger this physiological pathway of cell death, that seems to play an important role in the response to anticancer treatment (38, 39). Apoptotic activity displayed by IIF on LoVo/MDR cells was significantly higher than that induced by RA; IIF also confirmed its ability as an apoptotic agent when the DNA fragmentation assay was performed in LoVo/MDR cells; these results are in agreement with others indicating that synthetic retinoids cause growth suppression through induction of apoptosis (12, 37).

Our data underline the fact that the antimitotic activity induced by IIF, in particular on LoVo/MDR cells was more pronounced than that exerted by RA; thus, using IIF, better effects were obtained at lower doses and on shorter exposure to the drug. Our results suggest that IIF may be a promising drug for anticancer treatment, particularly on drug resistant human solid tumors.

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References