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Research Paper

Inhibition of P-Glycoprotein-Mediated Multidrug Resistance by Unfractionated Heparin

A New Potential Chemosensitizer for Cancer Therapy

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KEY WORDS

UFH, heparin, Verapamil, MDR, Pgp, calcein-AM, MDA-MB321, doxorubicin, MTT

ABBREVIATIONS

UFH	unfractionated heparin
Pgp	P-glycoprotein
MDR	multidrug resistance
MDA-MB231	breast tumor cell line
Calcein-AM	calcein acetoxymethylester
doxo	doxorubicin
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Ver	Verapamil
DMSO	dimethyl sulfoxide
D-MEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
PBS	phosphate buffered saline

ABSTRACT

Anticoagulant treatment with heparins is frequently used to prevent venous thromboembolism in cancer patients. In the present study, we investigated the ability of unfractionated heparin (UFH) to inhibit P-glycoprotein (Pgp)-mediated multidrug resistance (MDR) on human breast cancer cell line (MDA-MB231) and its doxo-resistant subline. Results were a compared to the classic reversing agent, Verapamil (Ver), used, as reference at 50 μM concentration. We analysed the Pgp function by calcein acetoxymethylester (calcein-AM) uptake, a fluorescent marker substrate, before and after in vitro exposure to UFH at clinically achievable dose of 20 U/ml. The mean percentage of calcein-AM retained into cancer cells after 3 and 12 h were 32 ± 10.9 and 45 ± 12.3, respectively, for UFH pretreated cells and 25.3 ± 8.7 and 29.4 ± 10.4, respectively, for Ver pretreated cells when compared to control cells, receiving only medium. Pgp activity was studied by measuring intracellular drug accumulation in doxo-resistant subline, treated (2 h) with either UFH or Ver, prior exposure (2 h) at different doxo concentrations (2, 4 and 8 μM). The mean percentage of remaining intracellular doxo were 55.4 ± 4.5 , 51.4 ± 3.9 and 50 ± 1.8 percent, respectively for UFH treated cells, and 44.1 ± 5.8, 39.3 ± 4.4 and 19.4 ± 8.6%, respectively, for Ver treated cells as compared with control cells, receiving only doxo. These results were consistent with the increase of sensitivity to doxo of the same doxo-resistant subline resulting in a 2.2, 2.6 and 2.2-fold increase, respectively, for UFH-doxo combination and 2.2, 2.5 and 2.0-fold respectively, for Ver-doxo combination respect to cells receiving doxo alone, as assessed by MTT test. In conclusion, these findings demonstrate the potentiating effect in vitro of UFH on doxo accumulation and cytotoxicity in the MDA-231 cell line and its doxo-resistant subline and suggest that UFH could to be used as an potential chemosensitizer in clinical chemotherapy for increasing in vivo, the efficacy of anticancer treatment.

INTRODUCTION

Cancer patients are frequently treated with heparins for the prevention and treatment of venous thromboembolism due to the impact of cancer cells and chemotherapy on the blood coagulation cascade. However, heparins other that anticoagulant properties have several biological activities binding with to wide range of proteins and molecules.[1-3] In this context, we sought to examine whether the unfractionated heparin (UFH) interacts with the 170-kD plasma membrane P-glycoprotein (Pgp), the product of the multidrug resistence (MDR) gene, which functions as an energy dependent efflux pump which decreases intracellular drug accumulation and cytotoxicity.[4-6] As a results of the interaction of the UFH to this multidrug transporter protein, alterations of the activities of the transmembrane pump system would be induced and as consequence to influence the drugs response. Since MDR mediated by Pgp has been documented in a variety of human cancer cells and in same case, such as in some carcinomas including breast, ovarian, and renal, has been shown to be associated with a poor response to cancer chemotherapy,[7-16] UFH could be used as chemosensitizer in the clinical setting to inhibit the mechanism of pleiotropic chemoresistance Pgp-mediated and to potentiate chemotherapy.[17,18] In this study, we evaluated the in vitro effects of UFH on the Pgp function and activity using a human breast cancer cell line, such as MDA-MB-231[19] and assessed whether these effects increased the sensitivity of this carcinoma cell type to antineoplastic drugs. The functional ability of Pgp was tested by measuring intracellular retention of the MDR Pgp-mediated marker, calcein acetoxymethylester (calcein-AM).[20-22] This hydrophobic fluorescent molecule is actively transported by transmembrane efflux pump, thereby its intracellular signal levels

give a measure of the level of expression of MDR-Pgp in cancer cells and an indication of the degree to which the pump is or is not blocked by Pgp inhibitors. The modifying effects of UFH on Pgp activity were studied by cytoplasmatic doxorubicin (doxo) accumulation assay, a known Pgp substrate, which is frequently used for the antineoplastic treatment of human breast cancer in combination with other chemotherapeutic agents. To investigate relationship between UFH effects on Pgp function and activity and the drug sensitivity of the tumor cells to anticancer drugs, we measured the in vitro cytotoxic activity of clinically achievable doses of doxo in the presence or absence of UFH, utilizing a 4 day MTT colorimetric assay.[23,24] We have used UFH at a concentration which can be achieved in the blood of patients undergone at anticancer treatment. The results of this analysis were then compared with those obtained by a well known Pgp specific modulator such as the calcium channel blocking agent, Verapamil (Ver) that blocks binding of drugs to Pgp competing against anticancer drug for binding site on Pgp.[25]

MATERIALS AND METHODS

Chemical and drugs. Doxo (doxorubicin hydrochloride), Ver (verapamil hydrochloride) and heparin sodium salt were purchased from ICN Biomedicals Inc. (Aurora, Ohio, MA); calcein acetoxymethylester (calcein-AM) and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Doxo was stored in a stock solution of 1 mg/ml at 4°C in the dark. Calcein-AM was stored in a stock solution of 1 mM in DMSO at -20°C. MTT was prepared as a solution at 5 mg/ml in PBS filtered through a 0.22-μm filter, and stored at 4°C for periods of up to 30 days. All drug dilutions were made in D-MEM and freshly prepared for each experiment.

Cell and culture conditions. Adenocarcinoma cell line (MDA-MB-231) was purchased by Cancer Research Institute (Genova, Italy). Monolayer of cell line were grown in 75 cm^2 tissue culture flask (Nunc, Denmark) containing 15 ml of Dulbecco's modified Eagle's minimal essential medium (DMEM) pH 7.4 (EuroClone, UK) supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 1% nonessential aminoacids, 1% penicillin-streptomycin. Cells were maintained at 37°C under air/5% CO_2 and were subcultured every 3-4 days by treatment with 0.05% trypsin and 0.02% EDTA in Ca^{++} and Mg^{++} free PBS.

Calcein-AM assay. Cells harvested by trypsinization were washed in growth medium and resuspended to 1 x 10^6 cells/ml. One hundred μl of this cell suspension (1 x 10^5 cells) were dispensed per tube in polystyrene round-bottom tubes (12 x 75 mm style tubes; BD Falcon, Bedford, MA), containing 90 μl of DMEM and incubated at 37°C in 5% CO_2 for 24 h. After this period cells were pretreated with either UFH 20 U/ml or Ver 50 μM for 3 and 12 h at 37°C in 5% CO_2. Following pretreatment with the individual compounds, the cells were further incubated at 37°C with calcein-AM at concentration of 0.25 μM for 15 min. After centrifugation (12000 rpm/min) the supernatants were removed and pellets were sonicated (75 W, 20 KHz, 30s) and frozen till the day of measurements. The intracellular fluorescence was then measured using a spectrofluorophotometer (SFM 25, Kontron Inst.) at an excitation wavelenght of 488 nm and emission wavelength of 525 nm. Cells incubate with medium alone (without inhibitors) were used as control. Each measurement was performed in triplicate. The ratios of the mean fluorescence intensities of treated cells (F treated) relative to those of the control wells (F control) were expressed as inhibitory capacity of the transport activity of the Pgp pump and calculated as follows:

Pgp inhibition (%) = 100 x (F treated - F control) / F treated.

Doxorubicin accumulation assay. For drug accumulation studies breast cancer cells were rended doxo-resistant to increase Pgp activity. For the development of a doxo-resistant subline, MDA-MB231 cells were maintained with exposure to doxo until their growth rate approached that of the untreated parental cells. Doxo resistant cells were selected by exposure to drug at initial concentration of 10 ng/ml. The doxo concentration was then increased 2 to 3 times. A period of 9–10 weeks were required to establish adequate growth at each concentration. All cultures were free of Mycoplasma. Cellular doxorubicin accumulation was determined using a standard procedure by incubating doxo-resistant tumor cells at concentration of 1 x 10^6 cells/ml, in a polystyrene round-bottom tubes series (12 x 75 mm style tubes; BD Falcon) at 37°C in 5% CO_2 for 24 h. After this period, the cells were incubated for 2 h at 37°C with either UFH 20 U/ml or Ver 50 μM. At the end intracellular doxo accumulation was determined by exposing cells to varying concentration of doxo (2 μM, 4 μM and 8 μM) at 37°C for 2 h. Each measurement was performed in triplicate. Following drug exposure, cells were washed twice in ice-cold (0°C) PBS (pH 7.4) and the final pellets were sonicated and frozen until analysis. Three ml butanol were thus added and allowed to extract the doxo during 2 h at 37°C. After extraction 1 ml water was added and the phases were mixed vigorously on a vortex mixer and separed by centrifugation (4000 rpm/30 min) on Eppendorf centrifuge. The fluorescence of doxo in the lysate was determined in the butanol phase by fluorescence spectrometry using 475 and 580 nm as excitation and emission wavelenght, respectively. For comparative purposes, the more amount of doxo remaining in cells pretreated with either UFH or Ver was expressed as a percentage of the doxo remaining in control cells (doxo alone) at the selected concentration and calculated from the following formula:

Doxo accumulation (%) = 100 x (F treated – F control) / F treated

Where F treated = mean fluorescence of tubes treated with UFH or Ver prior doxo treatment; F control = mean fluorescence of tubes treated with doxo alone.

Drug sensitivity assay. Cultured cells prepared as above were adjusted at a concentration of 1 x 10^6 cells/ml in growth medium. One hundred μl of this cell suspension were plated into individual well of 96-well round bottom microtiter tissue culture plate (BD Falcon, Bedford, MA) and incubated at 37°C in a 5% CO_2 95% air atmosphere for 24 h. In order to evaluate the ability of UFH and Ver to increase the sensitivity to doxo, we measured in triplicate the tumor growth reduction by incubating cells in the presence of UFH (20 U/ml) or Ver 50 μM in combination with different doxo concentrations (2 μM, 4 μM and 8 μM). The plates were incubated at 37°C and examined daily for evidence of nutrient depletion at each doxo concentration. The medium was removed after 72 h of incubation. Other medium containing 0.5 mg/ml MTT was then added to each culture well in a volume of 100 μl and incubated at 37°C for a further 4 h. The tetrazolium salt MTT is reduced to a coloured formazan by living cells but not by dead cells. After incubation, the supernatants were removed and formazan crystals produced were solubilized by the addition of 100 μl of 0.04 N HCl-isopropyl alcohol. Thereafter, the well contents were mixed thoroughly with a multichannel pipet and the absorbance of each well was measured at 550 nm wavelenght and 660 nm reference wavelenght. with a microplate reader (Spectra Max 190, Molecular Devices). Untreated control wells were set up in six wells. The optical density (OD) is linearly related to cell number. The percentage of growth inhibition was calculated as follow:

Growth inhibition (%) = (1 - OD treated well / OD control well) x 100

Cancer cell survival, a given doxorubicin concentration, was indeed expressed as a percentage of the control wells and calculated as follow:

Cell survival (%): 100 (OD treated well/OD control well).

Statistical analysis. Data were expressed as mean ± SD and compared with chi-square and Student's t-test. All statistical results were reported as significant when $P<0.05$.

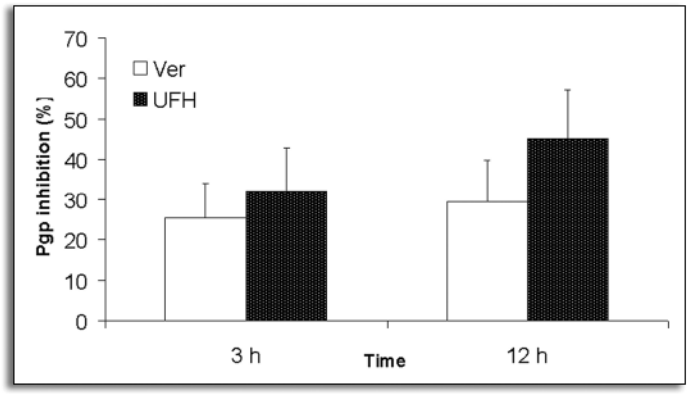

Figure 1. Effect of either UFH 20 U/ml or Ver 50 μM on intracellular calcein-AM retention measured in breast cancer cell line. Results were expressed as a percentage of inhibition of Pgp, determined at the indicated time periods. The mean values of triplicate experiments ± SD are shown.

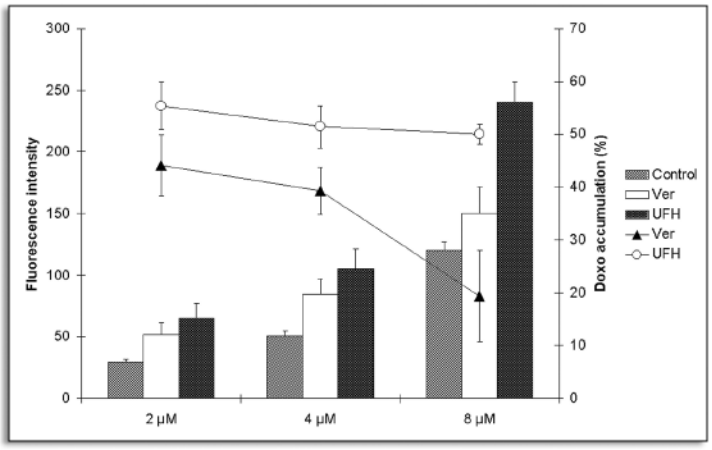

Figure 2. Mean fluorescence intensities retained in doxo-resistant subline pretreated with either UFH 20 U/ml or Ver 50 μM after incubation with the indicated doxo concentrations for 2 h. Mean percentage of increase of intracellular doxo accumulation in both UFH and Ver cells, relative to untreated control, were also reported. Each value represents mean ± SD of three experiments.

RESULTS

Calcein-AM assay. After a preincubation step of 3 and 12 h with either UFH (20 U/ml) or Ver 50 μM, the intracytoplasmatic calcein-AM fluorescence level in breast cancer cells, was markedly increased when compared to that of cells incubated with medium alone ($P<0.05$). In particular, the mean percent value of calcein-AM retained after 3 and 12 h were 32 ± 10.9 and 45 ± 12.3, respectively, for UFH pretreated cells and 25.3 ± 8.7 and 29.4 ± 10.4, respectively, for Ver pretreated cells, respect to untreated control. In Figure 1 is shown the cellular retention of calcein-AM fluorescence, at the times above indicated, expressed as percentage of the inhibitory capacity of the transport-Pgp mediated. These data demonstrate that tumor cells pretreated with UFH have a significantly higher degree of Pgp inhibition following both incubation times, when compared to that of the cells preincubated with Ver ($P<0.05$). Again, when the duration of the preincubation was lengthened to 12 h, cells previously treated with UFH showed a further increase of intracellular calcein-AM as compared with Ver pretreated cells. These data indicate that UFH was able to inhibit the function of membrane Pgp, expressed on this type of cancer cells in a dose dependent manner and that a enhancement of intracellular calcein-AM were obtained by a longer preincubation times.

Intracellular doxorubicin accumulation. Figure 2, illustrates that a preincubation step of 2 h with either UFH 20U/ml or Ver 50 μM, greatly increased intracellular doxo accumulation in doxo-resistant breast cancer cells after incubation for 2 hours at 37°C with increasing concentrations of doxo respect to untreated cells (without inhibitors). In particular, the remaining intracellular fluorescence intensities, after exposure at 2, 4 and 8 μM doxo concentrations were 55.4 ± 4.5 , 51.4 ± 3.9 and 50 ± 1.8 %, respectively for UFH treated cells, and 44.1 ± 5.8, 39.3 ± 4.4 and 19.4 ± 8.6%, respectively, for Ver treated cells. Thus, cellular doxo accumulation capacity into human malignant cells was significantly higher for the UFH pretreated cells than for the cells previously exposed to Ver, at all doxo concentrations, when compared to untreated control cells that exhibit reduced drug uptake typical of MDR cells ($P<0.05$). Again, while for the Ver treated cells the doxo percentage decreased progressively, reaching only 19% of the control cells after exposure to 8 μM doxo concentration, the UFH treated cells were able to maintain intracellular doxo to levels superior to 50% of control cells for all doxo concentrations (Fig. 2). The reason for these different patterns remains still to be established. It is possible that above a critical concentration, resistant cells actively extrude doxo or limit the interaction of Ver to Pgp sites, leading to reduced intracellular doxo accumulation.

MTT assay. Table 1 summarizes the results of the cytotoxic activity of doxo on tumor cells at different doses (2 μM, 4 μM and 8 μM) incubated in continuous presence or absence of either UFH or Ver for 4 days at 37°C. As shown, the UFH at 20 U/ml resulted not cytotoxic by itself for tumor cell. While the Ver at a concentration of 50 μM exhibit a growth inhibition around 7-8 % whereas at 100 μM produced a high cytotoxic effect reducing tumor cell growth from 20 to 30% (data not shown). The results shown that the combination of either UFH or Ver together with doxo greatly increased the sensitivity of the breast carcinoma cells to the cytotoxic effects of anticancer drug respect to cells treated with doxo alone ($P<0.05$). In particular, the cytotoxic effects by using 2 μM, 4 μM and 8 μM doxo concentrations following 72 h resulted in a 2.5, 2.8 and 2.3-fold increase, respectively, for the cells incubated with UFH-doxo combination and in a 3.2, 3.0 and 2.1-fold increase, respectively, for the cells incubated with Ver-doxo combination. However, cytotoxic effect of Ver by itself on tumor cells alone was about 3-fold higher than UFH as shown in Table 1. In fact, whether the percentage of cell survival was calculated subtracting the cytotoxic activity of both UFH and Ver alone from the respective values obtained in combination with different doxo concentrations, it is possible to evidence similar cytotoxic effects of two compounds when combinated with doxo (Fig. 3). As shown in Table 1, the combination UFH 20U plus doxo 2 μM determined an inhibition of tumor cell growth lower than Ver 50 μM plus doxo 2 μM combination and resulted similar to that of 4 μM doxorubicin alone. Furthermore, at 8 μM doxo concentration, while the growth inhibition percentage decreased rudely in Ver treated cells, at the same doxo concentration, the effects of UFH resulted to be significantly more efficacy that those observed in presence of either Ver 50 μM plus doxo or doxo alone ($P<0.05$). It is of interest, to note the dramatic enhancement of cytotoxicity (>50%) caused of the combination UFH 20/ml plus doxo 8 μM, respect to doxo treatment alone.

DISCUSSION

One of major problems in anticancer chemotherapy is the ability of malignant cells to survive exposure to different cytotoxic agents than is frequently associated with Pgp expression.[26] In the last years, various agents have been experimented in vitro to modulate Pgp activity but the low specificity and/or the high toxicity of these compounds when coadministered with anticancer drugs limit their use in the clinical chemotherapy.[27,28] In this study we have demonstrated that exposure of Pgp expressing human breast cancer cells at non toxic concentration of UFH resulted in the inhibition of Pgp function and activity with an increase in sensitivity to doxo respect to untreated cancer cells (without UFH). We showed that breast

Table 1 **Cytotoxic effect of doxo alone and in combination with either UFH or Ver in doxo-resistant breast cancer cells**

Treatment	Absorbance (570 nm)	Growth inhibition (%)*
Control	0.913	——
Doxo 2 μM	0.85 ± 0.04	7.2 ± 0.2
Doxo 4 μM	0.78 ± 0.03	15.2 ± 0.4
Doxo 8 μM	0.64 ± 0.02	30.4 ± 0.3
UFH 20 U/ml	0.9 ± 0.02	1.8 ± 0.2
UFH 20 U + doxo 2 μM	0.75 ± 0.03	17.8 ± 0.8
UFH 20 U + doxo 4 μM	0.52 ± 0.03	42.7 ± 1.1
UFH 20 U + doxo 8 μM	0.27 ± 0.02	70.4 ± 1.8
Ver 50 μM	0.85 ± 0.04	6.8 ± 1.1
Ver 50 μM + doxo 2 μM	0.71 ± 0.04	22.8 ± 2.3
Ver 50 μM + doxo 4 μM	0.50 ± 0.03	45.4 ± 2.5
Ver 50 μM + doxo 8 μM	0.31 ± 0.04	66.1 ± 3.1

*Each value represent the average of three experiments in which each drug combination was examined in triplicate.

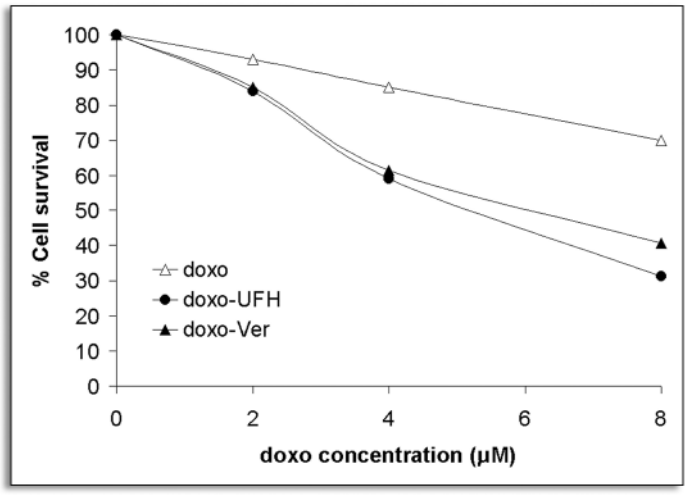

Figure 3. Mean percentage of cell survival determined in doxo resistant breast cancer cells after treatment with doxo alone (white triangle) and in combination with either UFH 20U/ml or Ver 50 μM at the indicated doxo concentrations. The results are corrected for the toxicity of both UFH and Ver on resistant tumor cells as described in Materials and Methods. Each data point is a mean of three experiments.

malignant cells, when preincubated with UFH, produced an increase in calcein-AM fluorescence intensity in a dose dependent fashion. Since, calcein-AM is an excellent fluorescent indicators for Pgp function, measuring directly the transport function Pgp mediated, our results indicate that UFH is certainly involved in the transmembrane transport and thus can act as inhibitor of the drug efflux pump function. These observations were also consistent with the intracellular doxo accumulation assay. In fact, we have evidenced that doxo-resistant breast cancer cells following the UFH pretreatment periods (3 and 12 h), enhanced the cellular doxo retention of a percentage of up to 50%, respect to cells treated with doxo alone, and that this increase was apparently not dependent upon the extracellular doxo concentration. Figure 1 shown that the percentage of the intracellular doxo levels achieved by UFH pretreated cells resulted significantly higher than that observed in presence of Ver in all doxo concentration ($P<0.05$). This latter seems to be less able to inhibit Pgp activity, retaining less than 20% of doxo at more high extracellular doxo concentration, thus clearly demonstrating the enhanced efflux Pgp mediated of the anticancer drug. In contrast, the increase of doxo accumulation in UFH pretreated cells confirms the decreased of the efflux protein activity. Taken together, these results strongly suggest that there is a reasonable relation between UFH and P-gp function and activity, however, the exact molecular mechanism by which UFH leads to the intracellular doxo accumulation in these tumor cells remain unclear. One explanation for the results described here is that UFH by their strong negative charges, might bind to cancer cells, accumulates within the cells, and thus to act as substrate for the Pgp transporter, resulting in inhibition of drug efflux function. Therefore, UFH might work as a competitive inhibitor to the transport of chemotherapeutic agent via Pgp in a way which is similar to first and second generation of Pgp inhibitors such as Ver, cyclosporine A or analogues. Another explanation is that UFH is not transported by Pgp, but induces, by oneself or in cooperation with other cellular factors, a conformational change in the protein such as third generation Pgp inhibitors (anthranilamide derivative), resulting in a decreased efficiency of the transport of chemotherapeutic drug.[29,30] The effects of UFH on cellular doxo accumulation were supported by an increased of the cancer cells sensitivity to the doxo treatment. In fact, using the MTT test to measure the degree of cell growth inhibition, we found that UFH potentiated (up to 2.5-fold) the antiproliferative effect of doxo on resistant cells, at a level similar to that produced by the known MDR modulator Ver which, however, is more toxic. This latter compound, seemed also to lose its activity in time in tissue culture environment, while UFH appeared to possess a prolonged activity. This result is, also, consistent with calcein-AM assay, in that P-gp pump efflux appeared to be better blocked when cells are exposed to UFH for prolonged periods (12 h). Furthermore, Ver levels of 50 μM are not clinically achievable, while levels of 20 U/ml of UFH are considered therapeutic. As shown in Table 1, the effect of UFH is very interesting in that it can potentiate the antiproliferative effect of doxo on cancer cells also when this agent is used a lower cytotoxic concentration (1.1 μg/ml) that is less of the maximal serum concentration when standard dose is used clinically (8–10 μg/ml). This results is clinically relevant because by the combined therapy of UFH and doxo, could be possible reduce doxo concentration and its cumulative myelotoxicity and cardiotoxicity, when administered systemically. In conclusion, our results demonstrate that UFH inhibits Pgp function and activity in MDA-231 cell line and its doxo-resistant subline enhancing doxo uptake and indicate that a combined approach of UFH with doxo results in a more effective form for potentiating the in vitro cytotoxic effects. Therefore, UFH although structurally and functionally different from Ver, appears to be a good chemosensitizer of MDA-231 doxo-resistant cells and could thus play an important role in the chemotherapy of human breast cancer in drug resistant cases. Moreover, UFH at a concentration of 20 U/ml is very less toxic and better tolerated respect to other reversing agents such as Ver that is highly toxic with significant arrhythmia and hypotension as the most prominent of its side effects at serum levels below those that are the optimal for reversing MDR in our tissue culture (50 μM). These findings, suggest that UFH in cancer patients might have a dual function: on the one hand, UFH as anticoagulant to protect from

the thromboembolic complications during antineoplastic treatment and on other hand, UFH as adjuvant to anticancer therapy to overcome Pgp-mediated drug resistance and make tumoral cells more sensitive to the cytotoxic effects of chemotherapeutic agents. Again, UFH or analogues of these substances with diminished anticoagulant activity, could be selected and used at higher dosages concomitantly with lower dosages of doxo (side effect free) for the treatment of intrinsically drug-resistant tumors, Pgp expressing, without reducing its antitumor activity and without increasing the bleeding risk. Further in vitro studies are in progress in our laboratories in order to investigate in various human tumors derived cell lines, the effectiveness of UFH as chemosensitizing agent, whose combination with cytostatics in clinical therapy could lead to a synergistic overcome of drug resistance Pgp-mediated and open new perspectives in cancer treatment.

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