Modulatory Effects of Heparin on Cellular Accumulation and Cytotoxicity of Doxorubicin in MRP1-overexpressing HL60/doxo Cells

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Abstract. Background: The overexpression of multidrug resistance protein (MRP1), associated with high levels of intracellular glutathione (GSH), is a well characterized mechanism of multidrug resistance (MDR) in several malignancies. Various chemosensitizers have been used in vitro to modulate the MRP1 activity, but the high toxicity limits their clinical application. Unfractionated heparin (UFH), is frequently used to prevent thrombo-embolic complications in cancer patients. This in vitro study aimed to elucidate the potential role of UFH as a sensitizer in anticancer clinical chemotherapy. Materials and Methods: The human leukemic doxorubicin-resistant cell line (HL60/doxo), which overexpresses the MRP1 protein was treated with UFH alone or in combination with three different concentrations of doxo. The intracellular accumulation and cytotoxicity of doxo and the cellular GSH content were measured in comparison with the leukotriene LTD4 receptor antagonist, MK571, a specific MRP1 inhibitor. Results: UFH increased doxo accumulation and cytotoxicity in the HL60/doxo cell line with respect to cells treated with doxo alone. UFH also decreased the cellular GSH content in the HL60/doxo cells with respect to the control, suggesting a potential involvement of UFH in doxo co-transport with GSH. Conclusion: Our results demonstrate that UFH modulates MRP1-mediated MDR in HL60/doxo cells expressing high MRP1 levels. These findings suggest a potential clinical application of heparin as an adjuvant to overcome MRP1-mediated drug resistance in cancer patients.

The development of multidrug resistance (MDR) to chemotherapeutic agents is one of the major obstacles to anticancer therapy (1-3). The MDR phenomenon consists of an intrinsic or acquired cross-resistance to many structurally and functionally unrelated chemotherapeutic agents used in the treatment of different tumors (4). Several molecular investigations have characterized different genes codifying various proteins associated with MDR (5). A well known MDR-associated protein is the multidrug resistance protein (MRP1) a member of the ATP-binding cassette transporter superfamily, present in several human tumors (6-9). The MRP1 is a transmembrane glycoprotein of 190 kDa which acts as an efflux pump that transports several antineoplastic drugs, as well as conjugated metabolites and other organic anions through the plasma membrane out of the tumor cells. Although the exact mechanism(s) of MRP1-mediated MDR has not been clarified yet, reduced glutathione (GSH), a cellular non-protein thiol present in almost all mammalian cells, seems to play an important role in MRP1-mediated transport (10-12). The MRP1 is an active transporter of important natural product-derived anticancer drugs, such as doxorubicin (doxo), an anthracycline antibiotic frequently utilized in association with other cytostatic agents for the chemotherapy of various human tumors (13). Although the molecular mechanism(s) by which MRP1 excretes doxo through the cell membrane is not fully understood, it was shown that an unmodified lipophilic form of doxorubicin was not transported by MRP1 (14). It was suggested that extrusion from cell of doxo is effected by a co-transport mechanism with GSH, whose intracellular concentration was considerably increased in association with the overexpression of MRP1 protein, cancer cells (15). In the last years, several in vitro studies and clinical trials evidenced that this form of drug resistance could be overcome by the combined use of chemotherapies and MRP1-modulators, known as chemosensitizers. Recently, many natural and synthetic compounds have been tested for their ability to modulate MRP1-mediated MDR and to improve the anticancer therapy, but currently none are in clinical use (16-21). Treatment with anticoagulants, such as heparins, is frequently used to prevent thromboembolic complications in cancer patients, due to the
impact of tumor cells and chemotherapy on hemostatic function (22-24). Heparins, besides their anticoagulant effects, they can also bind to a wide variety of molecules and proteins thereby interfering with their biological activities. The aim of this study was to investigate whether unfractionated heparin (UFH) interferes with MRP1 activity to influence tumor cell sensitivity to doxo. The modulating effects of UFH on MRP1-mediated MDR were analyzed in a human promyelocytic leukemia cell line selected for resistance to doxo (HL60/doxo), which expresses high levels of MRP1 (25). HL60/doxo cells were treated with three different doxo concentrations in the presence or absence of UFH and intracellular doxo accumulation, doxo cytotoxicity and cellular GSH content were measured. The results were then compared to those obtained with the leukotriene D4 antagonist MK571, a specific MRP1 inhibitor, used as a positive control. UFH and doxo concentrations, which can be achieved in the blood of patients undergoing anticancer treatment, were utilized.

Materials and Methods

Chemicals and drugs. Doxorubicin (doxorubicin hydrochloride), DL-buthionine-S-R-sulfoximine (BSO) and heparin sodium salt were obtained from ICN Biochemicals Inc. (Aurora, OH, USA); glutathione (GSH), diethionitrobenzoic acid (DTNB), NADPH, glutathione reductase, dimethyl sulfoxide (DMSO) and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); metaphosphoric acid was from Aldrich Chemical Co. (Milwaukee, WI, USA); MK571 sodium salt was from Calbiochem (San Diego, CA, USA). All chemicals and drugs were freshly dissolved before use, except for doxo, which was stored in a stock solution of 1 mM in PBS at ~20°C in the dark.

Cell culture. The human promyelocytic leukemia cell line HL60 was obtained from Interlab Cell Line Collection (Cancer Research Institute, Genova, Italy) and was cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 2 mM of L-glutamine, at 37°C under a humidified atmosphere of 5% of CO2 in air. The doxo-resistant HL60 cell subline was selected by exposure to increasing concentrations of doxo over 2 h at 37°C. After extraction, 1 ml water was added and the phases were mixed vigorously on a vortex mixer and separated by centrifugation. The intrinsic fluorescence of doxo was measured in the butanol phase by a spectrofluorimeter (SFM 25, Kontron Inst.; excitation wavelength of 465 nm and emission wavelength of 580 nm). Accumulated doxo in the cells pre-treated with either UFH or MK571 was expressed as the percentage of doxo remaining in the untreated control cells (doxo alone) at the selected concentration and determined using the formula:

\[ \text{Doxo accumulation (\%) = } 100 \times \frac{F_{\text{treated}} - F_{\text{control}}}{F_{\text{treated}}} \]

where \( F_{\text{treated}} \) was the mean fluorescence of HL60/doxo cells treated with UFH or MK571 prior doxo treatment, while \( F_{\text{control}} \) was the mean fluorescence values of HL60/doxo cells treated with doxo alone.

Cytotoxicity assay. HL60/doxo cells were seeded in a 96-well microtiter plates (Nunc, Denmark) at 1x10^5 cells/well in RPMI-1640 supplemented with 10% FBS and with three doxo concentrations (2 µM, 4 µM and 8 µM) for 72 h in the presence of UFH (250 µg/ml) or MK571 (30 µM). Subsequently, the cells were washed and resuspended in 100 µl of medium containing 0.5 mg/ml MTT and incubated at 37°C for 4 h (26). The supernatants were then removed and the formazan crystals solubilized with 100 µl of 0.04 N HCl-isopropyl alcohol. Untreated HL60/doxo wells containing medium alone were used as control. All measurements were performed in triplicate. The reduction of MTT by viable cells to a colored (blue) formazan product was measured spectrophotometrically at 550 nm wavelength and 660 nm reference wavelength, using a 96-well plate reader (Spectra Max 190, Molecular Devices) and expressed as percentage of the control cells and calculated as follows:

\[ \% \text{ Cytotoxic Index (CI): } (1-OD_{\text{treated well}} / OD_{\text{control well}}) \times 100 \]

where \( OD_{\text{treated well}} \) were the mean absorbance values of HL60/doxo cells treated with either UFH or MK571, while \( OD_{\text{control well}} \) were the mean absorbance values of the untreated HL60/doxo cells, respectively. Cancer cell survival, a given doxo concentration, was indeed expressed as a percentage of the control wells and was estimated as follows:

\[ \text{Cell survival (\%): } 100 (OD_{\text{treated well}} / OD_{\text{control well}}) \]

Measurement of intracellular glutathione. The total intracellular GSH levels of HL-60/doxo cells was measured according to the enzyme recycling procedure by Tietze (27). Briefly, the HL60/doxo cells prepared as above were washed and resuspended in 2 ml tubes at a density of 1x10^6 cells/ml in RPMI-1640 complete medium and were exposed for 3 h at 37°C to either UFH (250 µg/ml) or MK571 (30 µM). As positive control, tumor cells were exposed to BSO, a specific inhibitor of GSH biosynthesis to deplete intracellular GSH concentration (28). BSO was used at a final concentration of 30 µM, which by itself did not disturb cellular proliferation. Cells following incubation were pelleted, washed in PBS and resuspended in 500 µl of 5% metaphosphoric acid. Then cells were sonicated and frozen until analysis. For total GSH determinations, cells were thawed, centrifuged and 100 µl aliquot of supernatant were added at 800 µl of phosphate/EDTA buffer (0.2 mM potassium phosphate, 0.01 M EDTA, pH 7.4) containing GSH reductase (0.5 U/ml) and diethionitrobenzoic acid (DTNB, 0.2 mg/ml). After 2 min incubation at 25°C, 100 µl of a NADPH solution (5 mg/ml) were added to the samples to start the reaction and the
increase in absorbance at 412 nm (TNB formation) was monitored with a Cary 3C UV-visible Spectrophotometer for 15 min against an appropriate blank and quantified using molar adsorption coefficient $\varepsilon=13600$ M$^{-1}$ cm$^{-1}$. Untreated HL60/doxo wells containing medium only were used as negative control. The assay was carried out in triplicate. Results were expressed as nmoles of GSH for 1x10$^6$ cells.

Statistical analysis. Results are expressed as mean±SD and compared with the Chi-square and the Student's t-test. All results were considered to be statistically significant at $p<0.05$.

**Results**

**Cellular doxo accumulation.** The doxo accumulation analysis revealed a significant increase of doxo fluorescence in those HL60/doxo cells pre-incubated with either UFH or MK571 before treatment with doxo, with respect to the corresponding untreated HL60/doxo cells ($p<0.05$) (Figure 1). The accumulation capacity at the 2, 4 and 8 $\mu$M concentrations of doxo, were 41.8±3, 39.7±3.6 and 39.4±4%, respectively, for the UFH-pretreated cells and 35.8±3.2, 34.6±3.6 and 33.4±3.0%, respectively, for the MK571-treated cells. These data demonstrate that the intracellular doxo accumulation capacity was significantly greater for the UFH-pretreated cells than for those cells incubated with MK571 at all doxo concentrations, when compared with untreated control cells ($p<0.05$).

**Cytotoxicity assay.** As reported in Table I, the co-exposure of HL60/doxo cells to different concentrations of doxo in presence of either UFH (250 $\mu$g/ml) or MK571 (30 $\mu$M) greatly increased the sensitivity of the HL60/doxo cells to doxo alone. In particular, the growth inhibition using 2 $\mu$M, 4 $\mu$M and 8 $\mu$M doxo concentrations resulted in an increase of 2.5-, 2.3- and 2.2-fold, respectively, for the cells incubated with UFH-doxo combination and in a 2.4-, 2.0- and 2.0-fold increase, respectively, for the cells incubated with the MK571-doxo combination. These results demonstrate that simultaneous association of UFH and doxo significantly potentiated the cytotoxic action of doxo, compared to cells treated with doxo alone ($p<0.05$), revealing a synergistic effect in these resistant cancer cells. The percentage of cell survival calculated by subtracting the corresponding cytotoxicity values of both UFH and MK571 alone from the respective values obtained in combination with different doxo concentrations is reported in Figure 2.

**GSH assay.** The effect of either UFH (250 $\mu$g/ml) or MK571 (30 $\mu$M) on intracellular GSH levels in HL60/doxo cells is shown in Figure 3. In cells treated with UFH, MK571 and BSO, the percentage of reduction for GSH intracellular level was 30%, 5%, and 50%, respectively, compared to the control. These results indicate that UFH is more able than MK571 to modulate intracellular GSH concentrations.

**Discussion**

The overexpression of MRP1 confers resistance to numerous anticancer drugs in the treatment of several human tumors (29-31). The modulation of MRP1 activity could be a valid approach to increasing the therapeutic efficacy of anticancer
drugs in cancer patients. However, the introduction of the chemosensitizers into clinical practice is slow because of the high toxicity of these compounds, as well as of the changes associated with the pharmacokinetics of antineoplastic agents that could significantly alter their efficacy. In this study, it was demonstrated that UFH at non-toxic concentrations produced an increase in the cellular doxo accumulation (up to 40%) higher than that obtained with MK571 (30%), in MRP1 overexpressing HL60-doxo cells, exposed to increasing concentrations of doxo. These results suggest that UFH was more able than MK571 to reduce the MRP1-mediated doxo efflux activity in this human cancer cell line. However, the exact molecular mechanism by which the UFH produced an increase in the intracellular accumulation of doxo remains to be established.

One potential mechanism involves the accumulation of UFH within the cells, which then interacts competitively or non-competitively with doxo-binding sites or with the ATP-binding sites of MRP1. Alternatively, UFH might affect MRP1 activity by altering cellular GSH levels. This second mechanism seems to be consistent with the reduction of the intracellular levels of GSH obtained in resistant HL60/doxo cells treated with UFH. Indeed, as shown in Figure 3, the pre-incubation of HL60/doxo cells with UFH resulted in decreased levels of cellular GSH (30% loss), compared to the GSH control level, while MK571 had no significant effect. This observation suggests that UFH might exert, at least in part, a modulatory effect on MRP1 activity by reducing the co-transport of doxo with GSH. All these possibilities require further examination. It is reasonable to hypothesize that a relationship exists between UFH and MRP1 activity in this leukemic cell line.

The effect of UFH on cellular doxo accumulation was also consistent with the increase of sensitivity of resistant cells to doxo when treated concomitantly with UFH. Indeed, UFH combined with doxo, significantly potentiated (p<0.05) the cytotoxic action (up to 2.5-fold) of the anticancer drug in cultured HL60/doxo cells at all doxo concentrations, with respect to doxo alone. Furthermore, UFH treatment alone produced minimal toxicity in HL60/doxo cells, less than that measured with MK571.

In conclusion, it was shown that UFH is an effective chemosensitizer of MRP1-associated MDR in HL60 cells enhancing the cytotoxicity at a low doxo concentration (1.1 µg/ml). The standard dose which is used clinically is 8-10 µg/ml. Therefore, in addition to its anticoagulant effect, UFH could have potential antitumor activity in vivo, potentiating the pharmacological action of antiblastic agents, such as doxo in chemotherapeutically-treated patients with MRP1-overexpressing tumor cells without adverse side-effects. However, the administration of high concentrations of UFH carries the risk of causing hemorrhaging from systemic anticoagulation. This disadvantage can be compensated by a new generation of chemically modified heparins with lower anticoagulant activities and still preserving their modulatory properties on MRP1-mediated MDR. These synthetic heparin derivatives, might be used in vivo at higher dosages in combination with lower dosages of anticancer drugs like doxorubicin (side-effect free) for treatment of MRP1-overexpressing tumor cells without reducing its cytotoxic activity and without increasing the risk of hemorrhaging (32).

Further studies assessing the value of UFH and its derivatives as chemosensitizing agents in several human cancer cell lines in combination with various chemotherapeutic agents, could provide the basis for the
potential clinical application of heparin as an adjuvant to antineoplastic therapy to overcome MRP1-mediated drug resistance in cancer patients.

References
