Are low molecular weight heparins able to sensitize chemoresistant tumor cells?

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Introduction

Venous thromboembolic complications are often associated with malignant diseases. The treatment and prevention of these events in cancer patients is frequently carried out using low-molecular-weight heparin (LMWH). However, heparin has other pharmacological effects in addition to the antithrombotic activities [1], e.g., antimetastatic and antiangiogenic activities and these are currently subjects of intensive research [2]. In clinical trials with cancer patients it has been shown that treatment with LMWH is associated with an increase in survival rate and this effect of LMWH is not only attributable to its prevention of lethal thromboembolic complications [3].

Recent additional findings show that unfractioned heparin (UFH) can alter the chemoresistance of tumor cells by interference with P-glycoprotein (P-gp)-mediated multidrug resistance [4]. Therefore, it appears worthwhile to investigate whether LMWH acts as a potential synergist or modulator of chemotherapy. However, published data on this topic are rare.

The aim of this in vitro study was to obtain an insight into the possible interaction between LMWH and P-gp in multidrug resistant cells and to investigate whether this can be considered as a plausible mechanism explaining the improvement in survival of cancer patients receiving anticoagulant treatment. In this study the influence of the LMWH tinzaparin on P-gp-independent chemoresistance phenomenon such as that associated with cisplatin resistance was also investigated.

Materials and Methods

Cell lines

The human ovarian carcinoma cell line A2780, its subline A2780adr overexpressing P-gp and the cisplatin-resistant subline A2780cis, were obtained from the European Collection of Cell Cultures. They were cultured in RPMI1640 medium with a 10% FCS, 1.5% L-glutamine and 1% penicillin/streptomycin supplement (PAN Biotech).

P-gp activity

The effect of the LMWH tinzaparin and the pentasaccharide fondaparinux on P-gp-mediated transport in A2780adr ovarian carcinoma cells was studied using calceinAM and rhodamine123 assays [5].

Tinzaparin was obtained from Leo Pharma GmbH and fondaparinux from gsk. Concentrations in sterile 96-well plates for tinzaparin and fondaparinux were $5 \times 10^{-1}$ mg/ml to $5 \times 10^{-6.5}$ mg/ml corresponding 7.7 x $10^{-2}$ mM to 2.4 x $10^{-7}$ mm and 2.9 x $10^{-1}$ mM to 9.2 x $10^{-7}$ mm respectively. The established P-gp inhibitors X-24 and verapamil ($10^{-3}$ mM to $10^{-7}$ mM) were used as controls [6].

For the calceinAM assay A2780adr cells were seeded in 200 µl (27,000/well) and incubated (37 °C, 5% CO₂) with tinzaparin, fondaparinux or inhibitor for 3 h (using medium) or 1 h (using Krebs-HEPES buffer). After addition of 0.3 µM calceinAM, fluorescence was measured in the POLARstar™ OPTIMA multiwell reader (BMG Labtech).

Flow cytometric detection using BD FACSCalibur (BD Biosciences) was conducted with both calceinAM and rhodamine123. Cells were seeded in 200 µl, 100,000/well and incubated for 30 min (37 °C, 5% CO₂). Rhodamine123, 0.3 µM was added for an incubation period of 2 h and calceinAM 0.3 µM for an incubation period of 15 min and then fluorescence was determined by FACS.
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Determination of cisplatin sensitivity

The sensitivity to cisplatin of A2780 and A2780cis cells pre- or coincubated with tinzaparin was detected by the MTT assay and compared to incubation with sole cisplatin. Here, the UV absorption of the formazan emerged from MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) correlates directly with cell vitality. Resistance was characterized as resistance factors (Rf) meaning the IC50 ratio of the A2780cis subline to A2780 cells.

Cisplatin was used in concentrations from $10^{-3}$ M to $10^{-7}$ M and a tinzaparin concentration of 50 µg/ml matchable with average innohep® (tinzaparin) dosage.

Cells were seeded in 100 µl 40.000/well in 96-well plates, while one sample was already preincubated with tinzaparin, and after 20 – 24-h incubation (37 °C, 5% CO2) cisplatin was added together with or without tinzaparin. The absorption of each sample was measured 24, 48 and 72 hours after cisplatin addition.

Cellular platinum accumulation

The platinum uptake after LMWH incubation compared to sole cisplatin by A2780 and A2780cis cells was analyzed by flameless atomic absorption spectrometry (FAAS). The cells were seeded in 1 ml and incubated for 20 – 24 h (37 °C, 5% CO2) before addition of 20 µM cisplatin. After multiple washing steps with PBS and cell lysis, platinum concentrations were measured. Samples were taken after 0.5, 1, 2, 4, 8, 12 and 24 hours and related to total protein amount.

Transporter blotting

The expression of the influx/efflux transporters CTR-1 and ATP7A/B participating in the origination of cellular platinum equilibrium was examined by SDS-PAGE and Western Blotting.

Cell lysis was prepared from A2780 and A2780cis cells incubated with 2 µM cisplatin over 72 h (37 °C, 5% CO2). Some samples were preincubated with 50 µg/ml tinzaparin for 20 h or coincubated with the same amount.

For detection we used a goat anti-CTR-1 antibody (Santa Cruz Biotechnologies), which was bound to a complementing HRP-conjugated anti-goat secondary antibody. Amplification was conducted via the Lumionol reaction in the ChemiDoc XRS+ System (Bio-Rad Laboratories). ECL solution, Precision Plus Standard and Streptactin were also purchased from Bio-Rad.

Results

P-gp activity

Recent data referred to the fact that unfractionated heparin may affect P-gp activity [4]. To obtain further information on this activity and to cover a wider range of structural parameters, we selected the LMWH tinzaparin, representing the largest size fractions among the LMWH products with an average of 18 subunits compared to the synthetic pentasaccharide fondaparinux.

As shown in Figure 1 neither tinzaparin nor fondaparinux caused a significant rise in calcien fluorescence indicating that they are not able to block P-gp. In contrast the potent P-gp inhibitor X-24 caused a considerable increase demonstrating the activity of P-gp inhibitors.

![Figure 1. P-gp inhibition measured by the CalceinAM assay with fluorometric detection of the highly active inhibitor X24 ($10^{-3}$ mM to $10^{-7}$ mM) in comparison to the LMWH tinzaparin ($7.7 \times 10^{-2}$ mM to $2.4 \times 10^{-7}$ mM) and the pentasaccharide fondaparinux ($2.9 \times 10^{-1}$ mM to $9.2 \times 10^{-7}$ mM).]
in the A2780adr cell line in transporting the fluorescence marker. Although we used a wide range of concentrations, IC\textsubscript{50}-values could not be calculated for tinzaparin or fondaparinux. A slight increase in fluorescence indicates some type of MDR-related inhibition due to unspecific and indirect heparin effects. Nevertheless, in comparison to the effects of the established P-gp inhibitor this minor rise in fluorescence appears to be an artefact.

To consolidate these findings we modified our assay using rhodamine123 as an alternative P-gp substrate and again calcine-AM as P-gp activity markers in combination with flow cytometric detection. Even those modifications led to the same results showing no P-gp interaction of both saccharides (data not shown).

**Influence of tinzaparin on cisplatin chemoresistance**

In order to investigate whether the LMWH tinzaparin can affect the P-gp-independent cisplatin resistance of A2780cis cells, MTT assays were performed comparing the sensitive and resistant cells after treatment with cisplatin in addition to a cellular tinzaparin preincubation. Cisplatin-resistant ovarian cancer cells tolerate 2 – 3 fold higher concentrations of cisplatin compared to the sensitive cells. While tinzaparin did not affect the parental A2780 cells, the LMWH obviously sensitized the A2780cis cells.

Already after 24 hours cisplatin showed similar toxicity for A2780 and cisplatin-resistant A2780cis cell lines pretreated with tinzaparin. After 72 hours the resistance factor was decreased by tinzaparin to 1.46 in comparison to 2.79 without the heparin. The simultaneous application of the LMWH seemed to have less impact (data not shown).

**Effects of tinzaparin on cellular platinum accumulation**

Cisplatin resistance in A2780cis cells is associated with lower intracellular platinum levels [7]. To investigate whether the sensitization of A2780cis cells by tinzaparin is in line with increased intracellular platinum accumulation, platinum levels were determined by AAS as described previously (data not shown).

However, tinzaparin did not affect platinum levels, which does not explain the higher cisplatin sensitivity already after 24 hours in presence of the heparin.

**Alterations in transporter expression**

A Western blot analysis was performed to investigate whether the sensitization of A2780cis cells by tinzaparin is related to changes in the transporter expression profile.

In a first step without tinzaparin incubation we reconfirmed the fact that in the native in vitro situation the sensitive cells show a much higher expression of the influx transporter CTR-1 than cisplatin resistant cells underscoring its role in chemoresistance. Subsequently, using a cellular preincubation with tinzaparin for 16 hours, the expression level of CTR-1 in resistant cells was raised significantly while sensitive cells were much less affected. It is notable that the effect seems to be cisplatin-independent. As in the cytotoxicity experiments, simultaneous tinzaparin/cisplatin incubation had much less impact. On the other hand we observed no change in ATP7A/B efflux transporter expression (data not shown).

**Conclusion**

In our experiments using two different assays, we found no P-gp blocking activity for LMWH or the anticoagulant pentasaccharide fondaparinux. These findings are in contrast to recent publications. Although these contradictions might be a matter of different experimental parameters, referring to our data in comparison to well known and established P-gp inhibitors, a heparin interaction affecting P-gp appears unlikely.

Nevertheless, the effect of tinzaparin to sensitize cisplatin-resistant A2780 cells appears highly interesting, although we presently cannot provide a molecular explanation. Since the sensitization of A2780cis cells to cisplatin treatment by tinzaparin preincubation is not reflected by higher intracellular
platinum levels, a more complex effect of heparin pretreatment on signaling pathways might be involved but this needs further investigation. Ongoing research is directed to explaining whether deregulated transporter expression after tinzaparin-preincubation may be responsible for heparin acting as a chemosensitizer in resistant cells.

References


