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Original Article

Inhibition of protein glycosylation reverses the MDR phenotype of cancer cell lines

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ABSTRACT

Background: Multidrug resistance proteins are one of the most important factors that cause chemotherapy resistance, which in turn reduces therapeutic efficacy and survival for cancer patients. Tunicamycin is one of the most well-known inhibitors of *N*-glycosylation and is considered a powerful adjunct that can increase the effectiveness of many drugs. Tunicamycin blocks the first step of P-gp (glycoprotein P) and BCRP (breast cancer resistance protein) *N*-glycosylation, which is a very important modification for the activity and cellular localisation of these proteins.
Methods: The effects of tunicamycin on ovarian and colorectal cancer cells were examined in multiple cell lines. The primary ovarian cancer cell line W1 and the established ovarian cancer cell line A2780 were compared against their drug-resistant derivatives W1TR/W1PR (TR: topotecan resistant; PR: paclitaxel resistant) and A2780T1 (topotecan resistant), respectively. We also compared the colorectal cancer cell line LoVo against its doxorubicin-resistant derivative LoVo/Dx. Cell viability was determined by the MTT assay. The glycopeptides were subjected to deglycosylation using the endoglycosidase PNGase F. A2780T1, LoVo/Dx and W1PR cells were treated with the protein degradation inhibitors MG132 and BMA. Protein expression was detected by western blot and immunocytochemistry.
Results: In this study, we showed via the MTT assay that tunicamycin significantly decreased the viability of cancer cell lines that were co-treated with a chemotherapeutic drug. Western blot analysis showed that, in LoVo/Dx and W1PR cells, tunicamycin treatment resulted in the expression of a 70 kDa P-gp protein instead of the mature 170 kDa P-gp. Treatment with MG132 or BMA fully suppressed the effect of tunicamycin in the case of W1PR cells only. In tunicamycin-treated W1TR cells, the size of the BCRP protein did not differ from that of its native unglycosylated form. In tunicamycin-treated A2780T1 cells, BCRP expression was completely inhibited, but pre-treatment with MG132 or BMA suppressed the effect of tunicamycin. Immunocytochemistry analysis indicated that tunicamycin only affected the translocation of P-gp but not that of BCRP. After treatment, we observed higher P-gp expression in the cytoplasm than at the cell membrane.
Conclusions: Our results indicated that tunicamycin may enhance the effect of chemotherapy by interfering with the localisation and function of transporter proteins that are responsible for multidrug resistance.

1. Introduction

Cancer is one of the major health crises and causes of death in industrialised countries. Only 30% of adult cancer patients can be completely cured [1]. Therefore, we must improve current therapeutic options to reduce cancer mortality rate. At present, the most effective cancer treatment methods are surgery and chemotherapy. Surgery often allows for complete recovery without further complications or recurrences for early stage

Abbreviations: ABC, ATP binding cassette superfamily; BCRP/ABCG2, breast cancer resistance protein; BSA, bovine serum albumin; FBS, foetal bovine serum; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; MDR, multidrug resistance; MEME, minimum essential medium eagle; PBS, phosphate buffered saline; P-gp, glycoprotein P; PNGase F, *N*-glycosidase F; TBS, tris buffered saline; Tun, tunicamycin.

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diseases. By contrast, chemotherapy is more effective against late-stage, disseminated, or non-operable diseases. Thus, increasing the efficacy of chemotherapy is one of the most sought-after goals in cancer research. A major obstacle to chemotherapy efficacy is the development of multidrug resistance (MDR). MDR is defined as cross-resistance to a broad spectrum of structurally unrelated cytotoxic drugs. The causes of MDR are diverse, but they are mostly connected with the overexpression of drug transporters of the ABC family, which can remove drugs from the cells [2,3].

The most well-studied ABC protein is glycoprotein P (P-gp), which is encoded by the *MDR1* (*ABCB1*) gene. It was the first protein that was identified to be connected with multidrug resistance. Today we know that many more proteins are associated with MDR, one of which being breast cancer resistance protein (BCRP), which is encoded by the *ABCG2* gene. The MDR proteins are mainly localised to the cell membrane, but P-gp and BCRP have also been detected at the nuclear envelope [4,5]. Therefore, it is likely that there exist additional mechanisms of resistance specifically associated with the cell nucleus.

P-gp comprises two homologous and symmetrical domains, each containing six transmembrane (TM) segments, where the N-terminal is located at the cytoplasmic side of cell membrane [3,4]. P-gp is *N*-glycosylated at amino acid (aa) positions 91, 94 and 99 in the first extracellular loop [4,6]. The molecular weight of unglycosylated P-gp is 140 kDa based on the aa structure, and glycosylation increases the molecular weight to 170 kDa [7]. BCRP (ABCG2) belongs to the G family of ABC transporters. It is considered a "half-transporter", and it is composed of six transmembrane domains. Homo-dimerisation is necessary to create a functional BCRP efflux pump. BCRP is glycosylated at Asn596 in the third extracellular loop [8–10]. The molecular weight of unglycosylated BCRP is 60 kDa and it is increased to 70 kDa after glycosylation [11].

Glycosylation is one of the major post-translational protein modifications. Sugar residues are covalently attached to BCRP and P-gp via *N*-type binding (*N*-linked glycosylation) to asparagine residues. The entire process of sugar synthesis and protein binding occurs at the endoplasmic reticulum (ER). The first stage of *N*-glycosylation involves the synthesis of a sugar core on ER membrane-anchored dolichol phosphate at the cytoplasmic side of ER. When a premature sugar core is formed, the entire glycan chain is translocated to the luminal side of ER. An activated sugar core is then created and translocated to an asparagine residue on the target protein. The protein with the sugar core attached is translocated to the Golgi apparatus, where terminal glycosylation occurs. The mature protein is then directed to its final destination [6,12].

For many proteins, including BCRP and P-gp [4,13], glycosylation plays a very important role in protein folding and intracellular translocation. The significance of glycosylation can perhaps be reflected from the functions of glycoproteins in a cell [6,14,15]. For proteins such as BCRP and P-gp, one of the questions of interest is whether the accompanying sugar residues have any effect on the cellular localisation and/or activity of these proteins. In addition, among the past studies on the importance of glycosylation for ABC proteins and the potential application of glycosylation inhibitors as chemotherapy adjuncts, most of which focused on P-gp, and tunicamycin (tun) was the most often used glycosylation inhibitor [16,17]. Recently, several studies focused directly on the effect of glycosylation inhibitors on BCRP activity. The BCRP transporter has not been extensively investigated in the past. Therefore, valuable information may be gained from an in-depth look at BCRP activity [8,9]. Furthermore, the specific role of *N*-glycosylation inhibitors in improving chemotherapy response has not been clearly elucidated.

This paper discusses the effect of tun on the cellular localisation of P-gp and BCRP in human ovarian and colorectal cancer cell lines. We also assessed the effects of glycosylation inhibition on the protein expression of P-gp and BCRP and the sensitivity of the cell lines to cytostatic drugs.

2. Materials and methods

2.1. Reagents and antibodies

Doxorubicin, topotecan, paclitaxel, tunicamycin, RIPA Lysis Buffer, BMA, and MG132 were obtained from Sigma (St. Louis, MO). RPMI-1640 and MEME medium, foetal bovine serum (FBS), antibiotic-antimycotic solution, and L-glutamine were also purchased from Sigma (St. Louis, MO). PNGase F was obtained from New England Biolabs (Hitchin, UK). Bradford Dye Reagent was obtained from Bio-Rad Laboratories (Hemel Hempstead, UK). Nitrocellulose membrane was obtained from GE Healthcare (Buckinghamshire, UK). Cell Proliferation Kit I (MTT) and protease inhibitor cocktail were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Rabbit anti-ABCG2 polyclonal Ab (H-70), rabbit anti-GADPH polyclonal Ab (FL-335), goat anti-mouse HRP-conjugated Ab, and goat anti-rabbit HRP-conjugated Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-P-glycoprotein Ab (C219) was obtained from Alexis Biochemicals (Lörrach, Germany). The MFP488 fluorescent secondary antibodies were obtained from MoBiTec. Mounting medium with DAPI was obtained from Santa Cruz Biotechnology.

2.2. Cell lines and cell culture

Two ovarian cancer cell lines were used for this study: (1) W1, which is a primary ovarian cancer cell line, and (2) A2780, which is a commercially available established human ovarian carcinoma cell line. The W1 cell line was established from ovarian cancer tissues that were obtained from an untreated patient in December 2009. From the W1 cell line, we generated the W1TR subline, which is resistant to topotecan (W1 topotecan resistant), and the W1PR subline, which is resistant to paclitaxel (W1 paclitaxel resistant). These cell lines were derived in our laboratory as described previously by Januchowski et al. [18]. From the A2780 cell line (obtained from ATCC, Poland), we generated the topotecan-resistant subline A2780T1. The drug-resistant sublines were generated by exposure of the drug-sensitive cells to incrementally higher concentrations of each drug. The final concentration of top was 24 ng/ml, and that of pac was 1100 ng/ml. We also used the human colon adenocarcinoma cell line LoVo and its commercially available, doxorubicin-resistant subline LoVo/Dx (obtained from ATCC, Poland), which was cultured in the presence of 200 ng/ml dox to retain its resistant phenotype. The W1TR and A2780TR1 cells were shown to overexpress BCRP, while the W1PR and LoVo/Dx cells were shown to overexpress P-gp [5,18].

The LoVo, LoVo/Dx, A2780, and A2780T1 cell lines were cultured in MEME medium, and the W1, W1PR, and W1TR cell lines were cultured in RPMI-1640 medium. Media were supplemented with 10% FBS, 2 mM L-glutamine and 1% antibiotic-antimycotic solution. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 (v/v).

2.3. MTT assay

The MTT survival assay was performed to estimate the extent of cell resistance to chemotherapeutic agents and tunicamycin. Briefly, cells were seeded at 4×10^3 cells per well into 96-well culture plates and pre-incubated for 48 h. To examine the effect of tun or chemotherapeutic drugs on cell survival, the cells were treated with increasing concentrations of tun or different drugs for