New approaches in the treatment of multidrug resistant tumors

Theses of Ph.D dissertation

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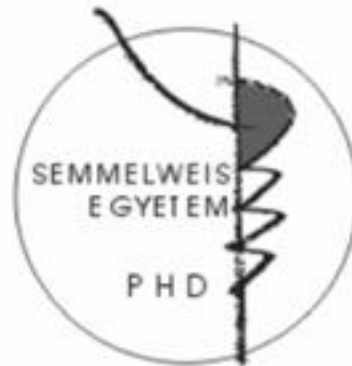

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Introduction

The mortality of malignant diseases is increasing worldwide. While in 2008 12.7 million new cancer cases were diagnosed and 8.2 million cancer-related deaths occurred, in 2012 the number of new diagnoses were up by 11% and the mortality increased by 8%. Although newly developed targeted therapies can significantly prolong the survival of patients with different tumors, clinical success often proves to be temporary because of the emergence of drug resistance.

Drug resistance mechanisms can limit the potency of a wide variety of structurally unrelated anticancer drugs (multidrug resistance (MDR)). Mechanisms ensuring MDR may involve the downregulation of target proteins, modification of apoptotic pathways, enzymatic inactivation of the toxic substances or the increase of DNA-repair. The most common and most efficient mechanism of cellular resistance encountered in the laboratory relies on the active efflux of chemotherapeutical agents from the intracellular compartment. In malignant cancers this mechanism is often linked to the function of P-glycoprotein (ABCB1, MDR1, Pgp), a member of the ATP Binding Cassette (ABC) transporter superfamily, which reduces intracellular drug levels below a cell killing threshold.

Although Pgp-mediated drug resistance has been known for decades, clinically viable solutions to overcome this form of MDR are still lacking. Selective inhibition of the transporter offered a promising approach, however due to the unexpected pharmacological side effects, inhibitors failed in clinical trials. In the present work we describe two alternative approaches, based on the exploitation of collateral sensitivity linked to the resistance phenotype, or the by-passing of the transporter-mediated efflux.

Biological changes that are responsible for cancer cell survival may at the same time also result in pharmacologically exploitable weaknesses. The concept of collateral sensitivity was established by Szybalski and Bryson in 1952, based on the observation showing that antibiotic-resistance in bacteria may be accompanied by an increased sensitivity to other types of antibiotic treatments. Our laboratory has identified molecules that can selectively target Pgp-expressing tumor cells through exploiting the collateral sensitivity of MDR cells. Based on these results, it was suggested that MDR-selective compounds may be suitable for the treatment of resistant tumors, by selectively eliminating MDR cancer cells during chemotherapy.

Another possible strategy to evade MDR relies on the improved delivery of commonly used drugs. It has been suggested that “stealth” formulations may increase the efficacy of treatment by shielding commonly used chemotherapeutics from Pgp-mediated efflux. Pegylated

liposomal doxorubicin (PLD) was developed to overcome the severe side effects of doxorubicin. Although reducing doxorubicin's (DOX) side effects was a major improvement proving the benefit of advanced drug delivery systems, the effect of PLD on MDR tumors or the evolution of drug resistance was never fully investigated.

Aims

Because success of chemotherapy is often hampered by the resistance of cancer cells, our goal was to establish efficient strategies against multidrug resistant tumors. In order to achieve this objective, the specific aims were:

1. Identification and in vitro characterization of new MDR-selective compounds
2. Assessing the impact of the MDR-selective compounds on Pgp expression in cell lines, primary cells isolated from tumors and in a cellular model of the Blood-Brain Barrier (BBB)
3. Testing the efficiency of PLD *in vitro* and *in vivo* using drug-sensitive and drug resistant models
4. Examining the therapeutic activity of PLD in a clinically relevant mouse model of mammary carcinoma

Materials and methods

Drug sensitive and resistant cell line pairs. The MES-SA (sensitive), MES-SA/Dx5 (resistant), MDCKII (sensitive), MDCKII-ABCB1 (resistant), A431 (sensitive), A431-ABCB1 (resistant), KB-3-1 (sensitive), KB-V1 (resistant), P388 (sensitive), P388/ADR (resistant) cell line pairs and the human breast cancer cell panel (MCF7, T47D, MDA-MB-231, MDA-MB-468, Hs578T, BT-549) were obtained either from the American Type Culture Collection (ATCC) or from the National Cancer Institute Developmental Therapeutics Program (NCI DTP) cell repository. Cells were cultured in DMEM or RPMI media (Life Technologies), supplemented with 10% fetal bovine serum, 5 mmol/L glutamine, and 50 units/mL penicillin and streptomycin (Life Technologies). All cell lines were cultivated at 37 °C, 5% CO2.

Blood-Brain Barrier endothelial cell line. The immortalized hCMEC/D3 BBB endothelial cell line was cultured in EndoGRO™-MV media supplemented with 1 ng/ml FGF-2 growth factor. Plastic surfaces used for cell culturing were coated with 20-fold diluted collagen type I to ensure the appropriate environment for cell growth.

Cytotoxicity assay. Viability was assessed by the PrestoBlue® assay (Life Technologies), according to the manufacturer's instructions. Briefly, cells were cultured in 96-well plates for 24h and then treated with the indicated drugs for 72 or 120h. After treatment 5% PrestoBlue® reagent was applied and the viability was measured using an EnSpire plate reader (Perkin Elmer). Curves were fitted by the Prism software using the sigmoidal dose–response model. Curve fit statistics were used to determine IC_{50} values.

Immuncytochemistry. 5000 MES-SA or MES-SA/Dx5 were plated in 8-well chambers (Thermo Scientific). 24 hours later the cells were fixed with 4% paraformaldehyde. After fixation, cells were permeabilized with a solution containing 2 mg/mL BSA, 1% fish gelatin, 5% goat serum, and 0.1% Triton-X 100 for 1 h at room temperature. The samples were stained for 1h with anti-P-glycoprotein antibody (1:500 MRK16, Kamiya Biomedical) and then with 250-fold diluted Alexa488-conjugated goat anti-mouse IgG antibody (Life Technologies) for 1h. Nuclei were stained with DAPI (Dojindo Molecular Technologies). The stained samples were examined by a Zeiss LSM 710 confocal laser scanning microscope.

RNA isolation and RT-PCR. Snap-frozen tumor samples were pulverized under liquid nitrogen and were homogenized in TRIzol™ Reagent (Life Technologies). Total RNA was isolated from tissue samples using Direct-zol® MiniPrep kit (Zymo Research) according to the manufacturer's guidelines. cDNA samples were prepared from 300 ng total RNA using the Promega Reverse Transcription System Kit. The Pre-Developed TaqMan® assay Actin ß (Actß) (Life Technologies) was used as endogenous control in real-time qPCR experiments; for quantifying Abcb1a and Abcb1b mRNA levels the respective TaqMan® primers were used. Real time PCR analyses were carried out using the StepOne™ Real-Time PCR System (Life Technologies).

Flow cytometry. The calcein assay was used to assess P-glycoprotein function. MES-SA and MES-SA/Dx5 cells were detached with 0.1% trypsin solution and 250.000 cells were incubated with 0.25 µM calcein AM (Dojindo Molecular Technologies) with or without 10 µM verapamil at 37°C. Calcein accumulation was measured with a FACSCANTO II flow cytometer (BD Biosciences).

Allograft tumors. P388 and P388/ADR cells (1×10^6/animal) were injected into the intraperitoneal cavity of 6-8 week old male BDF1 mice and 48h later a single dose of saline, doxorubicin (3 mg/kg) or PLD (3 or 5 mg/kg) were administered intraperitoneally. The animals were weighted 3 times per week and monitored multiple times per day for any sign of pain.

Spontaneous, transplantable Brca1$^{-/-}$;p53$^{-/-}$ mouse mammary tumors. Tissue pieces (1–2 mm in diameter) obtained from Brca1$^{-/-}$;p53$^{-/-}$ FVB mouse mammary tumors (a kind gift from Sven Rottenberg, NKI) were transplanted orthotopically under anesthesia. Treatment was initiated when the volume of the tumors reached ~200 mm^3. The maximum tolerable dose (MTD) of doxorubicin (5 mg/kg) and PLD (8 mg/kg) was applied through the tail vein. Treatments using the MTD was repeated every 10 days unless the size of the tumors decreased to 50% of its original volume. In that case treatment was repeated when the tumor relapsed to its original size. Animals were sacrificed when the tumor volume reached ~2000 mm^3.

Isolation of primary cancer cells from the spontaneously occuring mouse mammary tumors. The freshly removed tumor tissue was cut into ~1mm^3 pieces. The pieces were transferred to the dissociation media containing 200 U/ml type IV collagenase and 0,6 U/ml dispase (Life Technologies) in completed DMEM, and digested for 2 hours. The cell suspension was supplemented with completed media.

Measurement of serum doxorubicin levels after DOX and PLD treatment. FVB mice were treated with a single dose of intravenous doxorubicin (5 mg/kg) or PLD (8 mg/kg). Blood samples were taken before administration and after 5, 15, 30, 60, 180, 360, 1440, 2880 minutes by cardiac puncture of euthanized animals. Blood serum was separated by centrifugation at 4000 rpm for 15 minutes at 4°C. Samples were stored at -20°C until mass spectrometry analysis.

Results

1. Identification and in vitro characterization of new MDR-selective compounds

Through correlating Pgp mRNA expression data with publicly available DTP drug toxicity profiles we have identified Pgp substrates and MDR-selective compounds. MDR cancer cells expressing Pgp showed hypersensitivity to MDR-selective compounds unless P-glycoprotein was silenced or inhibited. To identify further MDR-selective compounds we correlated the cytotoxicity pattern of 49,169 compounds to Pgp expression levels measured across the NCI60 cell line panel. The analysis resulted 21 new MDR-selective candidates. We characterized three compounds (NSC57969, NSC297366, NSC608465) using three different cell line pairs. Two molecules (NSC57969, NSC297366) showed significantly higher toxicity and selectivity than the formerly discovered MDR-selective agents (the new structures are 6-7-fold more toxic to Pgp-expressing cells, and their IC_{50} values are lower by an order of magnitude). MDR-selectivity was proved by combining the new compounds with Pgp inhibitor Tariquidar (TQ).

Inhibition of the transporter abolished toxicity in Pgp-expressing cells, but did not influence the sensitivity of the parental cell lines.

2. Assessing the impact of the MDR-selective compounds on Pgp expression in cell lines, primary cells isolated from tumors and in a cellular model of the Blood-Brain Barrier

Continuous exposure of cells to MDR selective compounds was described to result in a gradual loss of Pgp expression, supporting the causal link between toxicity and Pgp function. To characterize the ability of the compounds analyzed in this study to modulate the expression of Pgp, we incubated MES-SA/Dx5 cells with IC_{20} concentrations of the compounds and followed the expression of Pgp in the surviving cell population. Surprisingly, a single, high-dose treatment with the newly identified molecules completely eradicated Pgp-expressing cells. Loss of Pgp-mediated drug efflux immediately re-sensitized cells to Pgp substrate drugs such as doxorubicin. In parallel, the cells acquired cross-resistance to MDR-selective compounds, providing yet another evidence supporting the causal role of Pgp in the initial collateral sensitivity of MES-SA-Dx5 cells

To test if the MDR-selective toxicity of the compounds identified in this study is maintained in a more realistic model of clinical drug resistance, we used a genetically engineered model of resistant murine mammary cancer. Pgp expression associated with the resistance of the doxorubicin resistant Brca1$^{-/-}$;p53$^{-/-}$ spontaneous mouse mammary carcinoma cells could be eliminated by a single treatment with NSC57969, suggesting that that MDR-selective compounds can effectively revert the MDR phenotype of cells that express Pgp at clinically relevant levels.

In contrast to tumor cells, despite high Pgp levels, human brain microvascular endothelium cells (hCMEC/D3) do not show collateral sensitivity to NSC57969, and treatment with NSC57969 does not induce loss of Pgp. These results suggest that normal tissues expressing Pgp are not differentially sensitive to MDR-selective agents, raising the possibility that it is the combination of the malignantly transformed state with the expression of Pgp that leads to increased toxicity.

3. Investigating efficiency of PLD in sensitive and resistant in vitro and in vivo systems

To evaluate the effect of pegylated liposomal formulation on the *in vitro* toxicity of doxorubicin, we compared the toxicity of DOX, PLD and cisplatin in the NCI-60 breast cancer cell lines. Whereas DOX showed strong toxicity in all six cell lines (BT-549, Hs578T, MDA-MB-231, MDA-MB-468, MCF-7, T47D), the IC_{50} values of PLD were on average 45-fold

higher. We also evaluated the susceptibility of DOX and PLD to Pgp-mediated MDR by using pairs of drug-sensitive and multidrug resistant cell lines. A431-B1, MESS-SA/Dx5 and P388/ADR cells expressing Pgp were resistant to DOX as compared to their sensitive counterparts. Pgp expression also conferred resistance to PLD, which was virtually nontoxic to MDR cells. Addition of the Pgp-inhibitor tariquidar restored sensitivity, proving that P-glycoprotein can protect cells against doxorubicin despite its stealth formulation.

In further experiments we monitored the effect of DOX or PLD treatment on the survival of BDF1 mice bearing drug-sensitive (P388) or drug-resistant (P388/ADR) ascites tumors. P388 tumors responded well to DOX and PLD, which was reflected in a significant increase in the median survival (DOX: 29 days; PLD (3 mg/kg): 28 days; PLD (5 mg/kg): >63 days as compared to saline (15.5 days)), while the same treatment failed to prolong survival of mice inoculated with resistant P388/ADR cells (DOX: 12.5 days; PLD (3 mg/kg): 13 days; PLD (5 mg/kg): 16 days). Thus, in line with the *in vitro* data, this particular *in vivo* model indicated that, despite the stealth formulation, PLD cannot be considered as an effective solution for the treatment of multidrug resistant tumors.

4. Investigating efficiency of PLD in a clinically relevant mouse model of mammary carcinoma

To test the therapeutic value of PLD in a more relevant model, we treated mice bearing orthotopically transplanted mammary tumors obtained from Brca1$^{-/-}$;p53$^{-/-}$ mice. These animals develop invasive ductal carcinoma (IDC) that share similar molecular, pathological and immunohistochemical characteristics with their human counterparts. Treatment with the MTD of DOX induces resistance, which is in most cases mediated by Pgp. Whereas saline-treated mice had to be sacrificed within 12 days, treatment with the MTD of doxorubicin increased the median survival of mice to 49.5 days. As compared to doxorubicin, treatment with the MTD of PLD resulted in a 6-fold increase in median relapse-free survival, and a 3-fold increase in median overall survival (9 vs 56 days and 49.5 vs 151.5 days, respectively). Growth kinetics of individual tumors revealed fundamental differences between the DOX and PLD groups. In some tumors, treatment with the MTD of DOX resulted in an initial response, but eventually all tumors relapsed and became resistant to therapy within 60 days. In contrast, 8 of the 10 tumors treated with the MTD of PLD were efficiently contained and only two tumors became refractory to treatment. Moreover, with the re-transplantation of doxorubicin-resistant tumors into the mammary fat pad of wild-type mice we proved PLD treatment is still successful while DOX remained ineffective.

Analysis of excised tumors revealed that Pgp expression levels associated with PLD resistance are in average 100-fold higher than those observed in DOX-resistant tumors. Favorable pharmacokinetics could explain the superior efficacy of PLD over DOX. Liposomal formulation of doxorubicin allowed a 60% increase of the MTD (8 mg/kg vs 5 mg/kg for PLD and DOX, respectively), which resulted in a 35-fold increase in the maximum peak doxorubicin concentration (31600 ± 6023 ng/µl vs 885.67 ± 240, measured 5 min after administration). Following intravenous injection of DOX, doxorubicin plasma levels decayed rapidly, whereas 7 days after treatment with a single dose of PLD, doxorubicin concentrations were still comparable to maximum levels observed in DOX-treated mice. Consequently, the AUC value was ~2600-fold higher for PLD as compared to DOX (4.47×107 vs 1.7×104 ng×h/ml). Despite sustained high doxorubicin plasma concentrations, treatment with PLD was not limited by signs of pain, serious side effects or weight loss.

Conclusions

1. Using *in silico* correlations and *in vitro* screening we identified 3 new MDR-selective compounds showing improved toxicity and selectivity.
2. A single high-dose treatment of MDR cells with the newly identified MDR-selective compounds resulted in the loss of Pgp expression and the resensitization of cells against conventional chemotherapy agents.
3. The new MDR-selective agents were active against primary cancer cells isolated from the brca1$^{-/-}$;p53$^{-/-}$ mouse mammary carcinoma model, expressing clinically relevant levels of P-glycoprotein.
4. Using a cellular model of the BBB, we found that MDR-selective compounds may not alter Pgp expression in pharmacological blood-tissue barriers.
5. In vitro comparison of doxorubicin (DOX) and pegylated liposomal doxorubicin (PLD) demonstrated that DOX is more toxic than PLD, and both compounds are subject to Pgp-mediated MDR.
6. Additionally, using the P388 mouse leukemia tumor model we showed that DOX and PLD are effective only against drug-sensitive tumors devoid of P-glycoprotein expression.
7. PLD treatment significantly increased the relapse-free and the overall survival of genetically engineered, brca1$^{-/-}$;p53$^{-/-}$ mouse mammary tumor bearing animals compared to DOX due to the delayed onset of resistance.

8. PLD-resistance of brca1$^{-/-}$;p53$^{-/-}$ mouse mammary tumors was due to Pgp overexpression; the mRNA levels of P-glycoprotein were in average 100-fold higher than those in DOX-resistant tumors.
9. Pgp expression levels associated with DOX-resistance cannot protect tumors against PLD treatment, suggesting that re-challenge of DOX-resistant tumors with PLD is a viable clinical strategy.

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