Statins and their effect on ABCB1 in neuroblastoma

Doctoral thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Philosophy
(PhD)

Submitted by

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Vienna, 10/2015
Declaration

This work was carried out in the Institute of Pharmacology – Medical University of Vienna in the laboratory of Prof. Dr. Martin Hohenegger. FACS analysis for detection of rhodamine 123 efflux was performed in the Institute of Medical Chemistry – Medical University of Vienna in the laboratory of Prof. Peter Chiba. YFP-ABCB1 plasmid construct used in transfection experiments was kindly provided by Prof. Peter Chiba and Oliver Kudlacek (Institute of Pharmacology – Medical University of Vienna). Lipid extractions and HPLC analysis were partly performed in the Institute of Vascular Biology – Medical University of Vienna in the laboratory of Dr. Valery Bochkov. Confocal microscopy experiments were carried out in the Institute of Physiologie – Medical University of Vienna.
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Neuroblastoma is a rare childhood cancer with poor outcome. Though investigations in identification of biologic and genetic markers, neuroblastoma is still a therapeutic challenge, especially because of chemoresistance. Notably, chemoresistance is mainly based on the upregulation of ABC transporters. Our previous studies have shown that HMG-CoA reductase inhibitors statins are able to downregulate and alter the glycosylation of the most prominent ABC transporter ABCB1 (Sieczkowski et al, 2010; Werner et al, 2013). In this study, we wanted to investigate the underlying mechanism for the downregulation as well as altered glycosylation of ABCB1 in more detail. Data presented here were performed with neuroblastoma cell line SH-SY5Y in vitro as well in vivo using xenograft murine model. In both studies we found that simvastatin induces apoptosis in these cells which could be prevented by addition of dolichol. Moreover, simvastatin inhibits efflux activity of ABCB1 and depletes cells from endogenous dolichol which can partly explain the downregulation and altered glycosylation of ABCB1.

In conclusion, these data provide novel evidence to use statins in anticancer strategies.

Diese Ergebnisse liefern somit Evidenz für eine neue Ansatzmöglichkeit von Statinen wie Simvastatin in der Krebsbehandlung.
Publications arising from this thesis


In vitro and in vivo downregulation of the ATP binding cassette transporter B1 by the HMG-CoA reductase inhibitor simvastatin

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Received: 11 February 2015 / Accepted: 18 August 2015
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Abstract Extrusion of chemotherapeutics by ATP-binding cassette (ABC) transporters like ABCB1 (P-glycoprotein) represents a crucial mechanism of multidrug resistance in cancer therapy. We have previously shown that the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor simvastatin directly inhibits ABCB1, alters the glycosylation of the transporter, and enhances the intracellular accumulation of doxorubicin with subsequent anti-cancer action. Here, we show that simvastatin reduces endogenous dolichol levels and ABCB1 in human neuroblastoma SH-SY5Y cells. Coapplication with dolichol prevents the downregulation of the ABCB1 transporter. Importantly, dolichol also attenuated simvastatin-induced apoptosis, unmasking involvement of unfolded protein response. Direct monitoring of the fluorescent fusion protein YFP-ABCB1 further confirms concentration-dependent reduction of ABCB1 in HEK293 cells by simvastatin. In simvastatin-treated murine xenografts, ABCB1 was also reduced in the liver and rhabdomyosarcoma but did not reach significance in neuroblastoma. Nevertheless, the in vivo anti-cancer effects of simvastatin are corroborated by increased apoptosis in tumor tissues. These findings provide experimental evidence for usage of simvastatin in novel chemotherapeutic regimens and link dolichol depletion to simvastatin-induced anti-cancer activity.

Keywords ABC-transporter · Apoptosis · Dolichol · Glycosylation · HMG-CoA reductase inhibitors · Neuroblastoma · Rhabdomyosarcoma

Abbreviations
ABC transporter · ATP-binding cassette transporter
ABCB1 · ABC transporter B1, P-glycoprotein
AFC · 7-Amino-4-trifluoro-methyl coumarin
ANOVA · Analysis of variance
CHOP · CCAAT/enhancer-binding protein (C/EBP)-homologous protein
DMEM · Dulbecco’s modified Eagle’s medium
ECL · Enhanced chemiluminescence
ER · Endoplasmic reticulum
FACS · Fluorescence-activated cell sorting
FPF · Farnesyl pyrophosphate
GGPP · Geranylgeranyl pyrophosphate
HEK cells · Human embryonic kidney cells
HMG-CoA reductase · 3-Hydroxy-3-methylglutaryl coenzyme A reductase
HPLC · High-performance liquid chromatography
HRP · Horseradish peroxidase
MDR · Multidrug resistance
PI · Propidium iodide
RIPA buffer · Radioimmunoprecipitation assay buffer
TLC · Thin-layer chromatography

Published online: 30 August 2015
Introduction

The ATP-binding cassette (ABC) transporters are one of the largest families of transmembrane proteins and dispose xenobiotics, lipids, and metabolite products across the plasma membrane, mainly in an ATP-dependent manner (Dean and Annino 2005; Fletcher et al. 2010). The overexpression of ABC transporters is generally associated with resistance to chemotherapy, which is prominently mediated by transporters like ABCB1 (P-glycoprotein; MDR1), ABCBC1, and ABCG2 (Gottesman et al. 2002; Cascorbi 2006; Robey et al. 2007; Fletcher et al. 2010). ABCB1 is heavily glycosylated at asparagine residues 91, 94, and 99, which are not affecting transport activity (Schinkel et al. 1993; Grills et al. 2000; Kvakajova-Kisecka et al. 2001; Dean and Annino 2005; Sere et al. 2011). Several studies have shown that glycosylation is important for protein quality control in the endoplasmic reticulum (ER) and trafficking to the plasma membrane (Schinkel et al. 1993; Loo and Clarke 1998). Noteworthy, inhibition of glycosylation of ABCB1 induced increased sensitivity to different drugs (Hiss et al. 2007).

Three generations of ABCB1 inhibitors were developed to counteract transporter-driven multidrug resistance in tumors (Gottesman et al. 2002; Fletcher et al. 2010). At last, third-generation compounds tamuridar, elacridar, and others are specific and potent inhibitors of ABCB1, but due to severe side effects and limited efficacy, clinical trials were declined (Fox and Bates 2007; Abraham et al. 2009; Cripe et al. 2010). Thus, it has been postulated that down-regulation of ABCB1 or inhibition of compensatory upregulation may represent a novel pharmacological access to transporter-mediated multidrug resistance (MDR) (Tamaki et al. 2011; Amiri-Kordestani et al. 2012).

Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and thereby block the mevalonate pathway, which results in reduced levels of cholesterol, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) (Goldstein and Brown 1990). Based on this mechanism of action, statins are widely used in the treatment of cardiovascular diseases accompanied by hypercholesterolemia (Demirre et al. 2005; Gazzero et al. 2012; Taylor et al. 2013). Beside this cholesterol-lowering effects, so-called pleiotropic effects have emerged, which are in part explained by reduction of intermediates of the mevalonate pathway (Goldstein and Brown 1990; Takemoto and Liao 2001; Gazzero et al. 2012). The isoprenoids FPP and GGPP are involved in the posttranslational modifications of several proteins, such as small G proteins, which play a critical role in maintenance of cell shape, motility, differentiation, and proliferation (Demirre et al. 2005; Gazzero et al. 2012). Moreover, dolichol, a side product of the mevalonate pathway, is an important lipid carrier for the glycogen precursor in N-linked glycosylation of proteins in the ER (Behrens and Leloir 1970; Cantagrel and Lefebvre 2011).

Statins’ anti-tumor effects have been investigated in many cellular systems (Farmer 2000; Werner et al. 2004; Demirre et al. 2005; Minichsdorfer and Hohenegger 2009; Sieczkowski et al. 2010; Gazzero et al. 2012; Chang et al. 2013). Previous studies indicate that human melanoma, neuroblastoma, and rhabdomyosarcoma cells are susceptible to statin-induced apoptosis via the mitochondrial pathway (Werner et al. 2004, 2013; Minichsdorfer and Hohenegger 2009; Sieczkowski et al. 2010). Indeed, statins also directly inhibit ABCB1 in many cellular systems, e.g., with an IC_{50} of 9 μM for simvastatin or 26 μM for lovastatin (Wang et al. 2001; Goard et al. 2010; Martirosyan et al. 2010; Sieczkowski et al. 2010; Werner et al. 2013). The co-application of simvastatin or lovastatin with doxorubicin, a well-known ABCB1 substrate, increased the accumulation of the anthracycline in many cellular systems and resulted in enhanced nuclear accumulation, potentiated DNA damage, and apoptosis (Goard et al. 2010; Martirosyan et al. 2010; Sieczkowski et al. 2010; Werner et al. 2013). Beside this immediate blockade of ABCB1, we also observed reduced levels of ABCB1 in membranes from simvastatin-treated rhabdomyosarcoma and neuroblastoma cells (Sieczkowski et al. 2010; Werner et al. 2013). The downregulation of ABCB1 affected mainly the fully glycosylated 170-kDa band compared to the core-glycosylated 140-kDa band. This is a clear indication for an involvement of statins in the glycosylation of the ABCB1 transporter (Sieczkowski et al. 2010). Finally, downregulation of ABCB1 by a preincubation with simvastatin was sufficient to result in reduced calcine efflux rates in rhabdomyosarcoma (RD) cells (Werner et al. 2013).

Hence, the aim of this study was to show whether statin exposure to human neuroblastoma cells has an impact on endogenous dolichol levels and whether coadministration of dolichol could prevent ABCB1 downregulation and apoptosis. Moreover, induction of apoptosis and reduction of ABCB1 by simvastatin was evaluated in vivo by murine xenograft models with rhabdomyosarcoma and neuroblastoma cells.

Materials and methods

Chemicals and reagents

Simvastatin was purchased from Merck (Darmstadt, Germany), and all other reagents and chemicals from Sigma-Aldrich (St. Louis, MO, USA) or Carl Roth (Karlsruhe, Germany), if not otherwise stated.

Cell culture

Experiments were performed with human neuroblastoma (SH-SY5Y), rhabdomyosarcoma (RD), and human embryonic kidney (HEK)-293 cells (ATCC–LGC Standards, Wesel,
Germany). SH-SY5Y cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 medium, HEK-293, and RD cells in DMEM high glucose medium, all supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and kept in humidified atmosphere of 5% CO₂ at 37 °C.

Lipid extraction and chromatography

SH-SY5Y cells (1 × 10⁶) were treated according to the figure legend and lysed in methanol plus 3% acetic acid (v/v), supplemented with the same volume of hexane and vortexed vigorously. Lipid extraction into the upper phase was repeated and collected, and the organic solvent evaporated. The collected lipids were dissolved in CHCl₃ and separated by thin-layer chromatography (TLC) using TLC Silica gel 60 plates (Merck; Darmstadt, Germany) and hexane including 20% ethyl acetate as mobile phase. The separated lipids were visualized by CuSO₄ and heating of the plate. C₂₀:0 dolichol, isolated from bovine heart, and C₁₄;0 dolichol were used as standards. Samples corresponding to the relative motility of the dolichol standards were scratched and transferred to a glass column retaining the matrix of the TLC. Dolichol elution from the silica gel was accomplished by 500 μl CHCl₃ and repeated four times. The elute was collected, evaporated, dissolved in 20 μl 2-propanol/methanol/n-hexane (45:45:10), and applied to high-performance liquid chromatography (HPLC). Dolichol fractions were separated with the running buffer (propanol/methanol/n-hexane, 45:45:10) at 1.7 ml/min (Hitachi pump L-2130 and UV detector L-2400; Tokyo, Japan) using C₁₄;0 dolichol or cholesterol as an internal standard. Peaks were analyzed with Elite LaChrom software (Hitachi; Tokyo, Japan).

Cell lysis and membrane extraction

Untreated and simvastatin-exposed SH-SY5Y cells were washed with phosphate-buffered saline (PBS), shock-frozen with liquid nitrogen, and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 10 mM glycerophosphate; 0.1% SDS; 1% NP-40) containing protease inhibitors aprotinin (2 μg/ml), leupeptin (10 μg/ml), and peafblock (1 mM). Phosphatase inhibitors NaF (1 mM) and Na₃VO₄ (1 mM) were supplemented to detect phosphorylated protein species. After 10 min on ice, cells were again shock-frozen in liquid nitrogen and centrifuged at 3000×g for 30 min at 4 °C. The supernatant fractions were used for Western blots. Protein concentrations were determined with Bradford protein assay using bovine serum albumin as a protein standard (Bradford 1976).

Caspase 3 activity

The fluorescence-based caspase 3 assay was carried out as previously described (Sacher et al. 2005; Werner et al. 2013).

PCR

Following drug treatment, total RNA from SH-SY5Y cells was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). After reverse transcription (RevertAid First Strand cDNA Synthesis Kit; Thermo Scientific; Waltham, MA, USA), the cDNA was used for quantitative (real-time) PCR using SensiMix SYBR and Fluorescein (GenXpress, Vienna, Austria) and the specific primers given in Table 1. Quantitative PCR was initiated by a step of denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 30 s, and DNA synthesis at 72 °C for 30 s. The final melting step included denaturation at 95 °C for 15 s, 60 °C for 15 s, a linear temperature gradient to 95 °C in 20 min, and 95 °C for 15 s. Cₚ values were normalized to the four control genes (B2M, RPLP0, RPS14, and GAPDH), and quantification was performed using the comparative Cₚ method. The PCR for ER stress markers (BiP and CHOP) was run under identical conditions except for 30 cycles, an annealing temperature of 58 °C, and normalization to GAPDH.

Table 1

<table>
<thead>
<tr>
<th>Primer sequences</th>
<th>ABCB1</th>
<th>ABCC1</th>
<th>ABCC4</th>
<th>ABCC6</th>
<th>ABCG2</th>
<th>BIP</th>
<th>CHOP</th>
<th>B2M</th>
<th>RPLP0</th>
<th>RPS14</th>
<th>GAPDH</th>
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<tr>
<td>Forward</td>
<td>GCCGCCGCGTCAATGGAGATCTTGGAGGGGACC-3'</td>
<td>GATCAGCAAGGAGTCAGGAT-3'</td>
<td>GCCGCCGCCGCAGCAGAC-3'</td>
<td>GCCATCGGTCATCCTGAG-3'</td>
<td>GCCGCCGCCGCTCCATCTC-3'</td>
<td>GCCGCCGCGTCAATGGAGATCTTGGAGGGGACC-3'</td>
<td>GCCGCCGCCGCAGCAGAC-3'</td>
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<td>GCCGCCGCCGCAGCAGAC-3'</td>
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<td>Reverse</td>
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FACS analysis

SH-SY5Y cells (5 × 10⁵) were treated with simvastatin as indicated in figures, and surface ABCB1 transporter was mapped with MRK16 antibody (1:100 dilution in PBS, 30 min at room temperature; Kamiya Biomedical Company, Seattle, WA, USA) and visualized with the corresponding Alexa Fluor® 488-conjugated goat anti-mouse antibody (1:100 in PBS, 30 min at 4 °C; Invitrogen, CA, USA). Alternatively, C219 (Abcam, Cambridge, UK) or p170 (Neomarkers, Fremont, CA, USA) antibodies (1:50 in 10% FCS with 1% NaN₃ in PBS, 2 h at room temperature) were used for ABCB1 staining in fixed cells and gave similar results. In all FACS experiments, unstained cells and/or cells mapped with mouse IgG₂a (Becton Dickinson, Heidelberg, Germany) were used as negative controls to correct for background. The data were processed off-line with Flowing software (www.flossingsoftware.com).

Apoptosis was determined by bivariate FACS analysis using FITC-conjugated annexin V (Ebioscience, San Diego, CA, USA) and propidium iodide (PI) as previously described (Minichsdorfer and Hohenegger 2009).

Rhodamine 123 efflux

Rhodamine 123 efflux was performed as previously described by Donmez Cakil et al. (Donmez Cakil et al. 2014). Briefly, SH-SY5Y cells (1 × 10⁵) were exposed to increasing concentrations of simvastatin for 48 h, washed, and incubated with 0.53 μM rhodamine123 for 30 min at 37 °C. Fluorescence (excitation at 488 nm and emission wavelength at 534 nm) was continuously monitored (5 min) with a FACScalibur. Surface expression of ABCB1 was controlled by FACS with MRK16 staining, and off-line analyses were done with CellQuest software (Becton Dickinson, Heidelberg, Germany), as previously described (Chiba et al. 1996; Donmez Cakil et al. 2014).

Protein turnover of YFP-ABCB1 fusion protein

HEK-293 cells (5 × 10⁴) were transfected with 0.4 μg YFP-ABCB1-pcDNA3 plasmid using TurboFect following the manufacturer’s protocol (Thermo Scientific; Waltham, MA, USA). The NH₂-terminal-tagged YFP-ABCB1 construct was kindly provided by Prof. Peter Chiba (Institute of Medical Chemistry) and Dr. Oliver Kudlacek (Institute of Pharmacology, Medical University of Vienna). After 48 h of recovery, cells were treated for another 48 h in the absence or presence of cycloheximide (10 μg/ml), doxorubicin (0.1 μM), simvastatin (1 or 3 μM), or vehicle (empty pcDNA3 plasmid). Thereafter, cells were shock-frozen and resuspended in PBS, and the YFP fluorescence (excitation 515 nm, emission 530 nm) was measured with a fluorescence spectrophotometer (FL-4500 Hitachi; Tokyo, Japan). Signals were corrected for protein concentration and background signal of the vehicle (FL-Solutions 2.0 software; Hitachi; Tokyo, Japan).

Murine xenograft experiments

Two xenograft experiments were performed and approved by the Animal Welfare Committee of the Medical University of Vienna and the Austrian Science Ministry (GZ66.009/0271-BrtG/2005 and GZ66.009/0274-II/3b/2010). Female CD-1 Nu/Nu mice (6 weeks old; Charles River; Sulzfeld, Germany) were subcutaneously inoculated with SH-SY5Y neuroblastoma cells (1 × 10⁶ in PBS) into the left and right flank. Twelve days after inoculation, groups of four mice were assigned to control group or simvastatin (4.25 mg/kg/day; oral) group. In both groups, one inoculum did not turn into a tumor. A day–night rhythm was emulated by light every 12 h, and the welfare of the animals was checked every day. The animals were killed by neck dislocation after 2 months or earlier due to critical tumor size. Organs and tumors were excised and weighted, and aliquots were rapidly frozen in liquid nitrogen and stored at −80 °C for further analyses.

Under similar conditions, female CD-1 Nu/Nu mice were inoculated with rhabdomyosarcoma (RD) cells (1.5 × 10⁶ cells in PBS) into the right flank. One week after inoculation, animals received water (control; n=10), simvastatin (1.15 mg/kg/day; n=6), cyclophosphamide (2 mg/kg/day; n=6), or a combination of simvastatin and cyclophosphamide (n=6). The animals were killed after 54 days or earlier due to critical tumor size. Tumors and livers were excised, weighted, and fixed in 4% paraformaldehyde for staining and immunohistochemistry. Small aliquots of organs and tumors were also rapidly frozen in liquid nitrogen and stored at −80 °C for further analyses.

For tissue analysis, liver and tumor samples (25–50 mg) were homogenized in solution A (10 mM HEPES, pH 7.5; 10% sucrose; 5 mM EDTA; 1 mM DTT; 1 mM pefabloc; 100 μM aprotinin; 100 μM leupeptin; 10 μM calpain inhibitors I and II). Lysates were centrifuged at 100,000 × g for 5 min, and the supernatant was centrifuged again with 600 × g for 10 min. The supernatant was again centrifuged at 11,600 × g for 20 min. At last, the supernatant was centrifuged again at 100,000 × g for 45 min. Pellet corresponding to the membrane fraction was resuspended in 100–200 μl solution B (10 mM HEPES, pH 7.5; 10% sucrose; 2 mM EDTA; 1 mM DTT) supplemented with the above protease inhibitors and stored at −80 °C. All steps were carried out at 4 °C.

Immunohistochemical analysis of tumors

Tumors were embedded in optimal cutting temperature compound (OCT Tissue-Tek, Sanova, Vienna, Austria) prior to frozen sectioning on a microtome cryostat (Microm HM-
500-OM, Walldorf, Germany). Alternatively, samples were fixed with 5% formalin buffered in PBS. Sections were cut (3–5 μm) and formalin was removed by increasing concentrations of ethanol. If necessary, sections were dehydrated by methanol and again hydrated in water. Fixed slices were incubated with 4% parafformaldehyde and then blocked with 4% bovine serum albumin in PBS. Nuclei were stained with Hoechst 33258 (15 min) and activated caspase 3 with an antibody selective for cleaved caspase 3 (1:200, overnight; Cell Signaling, Millipore, Vienna, Austria). Immunoreactive species were detected with a corresponding Cy3-conjugated antibody (1:200; excitation 543 nm, emission 570 nm; PA43004, GE Healthcare, Vienna, Austria), and images were taken with a Zeiss fluorescence microscope (Axioimager Z1, Jena, Germany).

Western blot analysis

Proteins (15–30 μg) were separated on a 7 or 10% SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% bovine serum albumin, and incubated overnight at 4°C with the following primary antibodies: ABCB1 (C219, 1:300; Merck; Darmstadt, Germany), cleaved poly-ADP ribose polymerase (cleaved PARP, 1:1000; Cell Signaling Millipore, Vienna, Austria), extracellular-signal-regulated protein kinases 1 and 2 (ERK1/2) and phospho-ERK1/2 (1:2000; Cell Signaling Millipore, Vienna, Austria), actin (AC-40, 1:20, 000), or α-tubulin (anti-α tubulin, 1:40,000). Proteins of interest were visualized by enhanced chemiluminescence (ECL) system (ECL Plus, GE Healthcare, Vienna, Austria) using a species-corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000 in 2% BSA; Cell Signaling Cell Signaling Millipore, Vienna, Austria) for 1 h at room temperature. Actin and α-tubulin were alternatively used as loading controls depending on compatibility with species of the antibody and molecular mass range of the protein of interest. Three independent experiments were carried out for Western blots, and samples were used up to three times to repeat and optimize X-ray exposure times for ECL detection. Bands of interest were quantified and analyzed using ImageJ software (http://rsweb.nig.go.jp/ij/).

Statistical analysis

Typically, three independent experiments were carried out in duplicates (n=3) and the data are presented as mean ± standard deviation (SD) if not otherwise stated. Statistical analyses were performed using SigmaPlot software (Jandel, Erkrath, Germany) with either unpaired Student’s t test or for multiple comparisons ANOVA and Holm–Sidak test or post hoc Tukey test. A p value <0.05 is considered statistically significant.

Results

ABCB1 downregulation in simvastatin-treated SH-SY5Y cells

Simvastatin reduces the fully glycosylated form of the ABCB1 transporter in rhabdomyosarcoma (RD) and SH-SY5Y neuroblastoma cells shown by Western blots (Sieczkowski et al. 2010; Werner et al. 2013). In order to quantify this effect, we here confirm significant reduction of cell surface ABCB1 protein expression in simvastatin-treated SH-SY5Y neuroblastoma cells by FACS analysis (Fig. 1a–c). The kinetics of cell surface reduction of ABCB1 is detected with MRK16 antibody in non-permeabilized SH-SY5Y neuroblastoma cells. ABCB1 reduction is hardly observed after 24 h simvastatin exposure (Fig. 1b), indicating an early compensatory mechanism. However, a significant reduction of ABCB1 surface expression is observed after 48 h with already 1 μM simvastatin (Fig. 1c).

In order to further confirm ABCB1 downregulation by simvastatin in another experimental setting including also intracellular ABCB1, the fluorescent fusion protein YFP-ABCB1 was heterologously expressed in HEK-293 cells (Fig. 1d). This experimental setup was validated by doxorubicin as a positive control for upregulation of the ABC transporter and cycloheximide as a negative control mirroring transcriptional inhibition. A concentration of 3 μM simvastatin was sufficient to reduce the transporter within 48 h significantly. Simvastatin had no effect on the fluorescence signal of YFP-ABCB1 in the presence of cycloheximide, a blocker of translational elongation. Accordingly, inhibition of transcription is considered to be responsible for the simvastatin-dependent reduction of ABCB1 protein. These findings together with previous reports confirm that simvastatin in the low micromolar concentration range downregulates ABCB1 on protein level, which accounts for a 20–40% reduction, depending on the experimental conditions (Fig. 1) (Sieczkowski et al. 2010; Werner et al. 2013).

Accordingly, one would expect a decrease of ABCB1 messenger RNA (mRNA) levels in the presence of simvastatin. The tissue distribution, spectrum of substrates and regulators, and the role in multidrug resistance are overlapping for ABCC1 and ABCG2 with ABCB1 (Sharam 2008). In contrast, little is known about ABCC6 in regard to multidrug resistance and compensation of ABCB1 downregulation (Vanakk er et al. 2013). A significant reduction of ABCB1, ABCC1, and ABCG2 mRNA is already seen after 6 h of simvastatin exposure, which is further enhanced upon longer incubation times (Fig. 1e, f). The amount of ABCC6 mRNA is not affected and documents specificity of the simvastatin effect. Most prominently, mRNA of ABCB1 and ABCC1 is already downregulated by 1 μM simvastatin. Noteworthy, these
findings are not indicative for a compensatory upregulation of alternative transporters like ABCC1 and ABCG2.

Transporter activity of ABCB1 is affected by simvastatin

Functionally, statins directly inhibit ABCB1 (Bogman et al. 2001; Wang et al. 2001; Goard et al. 2010; Martirosyan et al. 2010; Sieczkowski et al. 2010; Werner et al. 2013). In order to confirm previous findings using calcein-AM, we here determined first-order rate constants of rhodamine 123 efflux which were plotted against the corresponding expression of ABCB1. This approach allowed correcting for different expression levels of the transporter, which was reduced in the presence of simvastatin (Fig. 2a). The slopes of such linear regressions yield rhodamine 123 transportation rates (Fig. 2b), which were significantly reduced by simvastatin in a concentration-dependent manner. However, a significant difference between simvastatin concentrations was not obtained.
Simvastatin depletes cells from endogenous dolichol

In support to our conjecture, that glycosylation of proteins is impaired by simvastatin exposure, we have determined endogenous dolichol levels in SH-SY5Y cells (Fig. 3) (Sieczkowski et al. 2010). Expectedly, simvastatin exposure reduced endogenous dolichol levels in a concentration-dependent manner (Fig. 3b). Noteworthy, a significant reduction of dolichol was observed already at 0.1 µM simvastatin compared to untreated cells (Fig. 3c, d).

Dolichol phosphate is a downstream product of the HMG-CoA reductase and critically required for glycoprotein biosynthesis (Behrens and Leloir 1970; Goldstein and Brown 1990). One would now postulate that dolichol C50 165 coapplication prevents downregulation of ABCB1 by simvastatin (Fig. 4a–d). Interestingly, the addition of dolichol alone, but also in the presence of simvastatin, strongly intensified the ABCB1 bands, indicating full saturation of the glycosylation machinery. Conversely, with simvastatin alone, a significant downregulation of ABCB1 is detectable at 24 h and longer incubation times (120 h, Fig. 4c, d). Importantly, the addition of dolichol significantly protected from simvastatin-induced downregulation of ABCB1 (Fig. 4c, d). The protein bands for ABCB1 are fuzzy and broad, as this is assumed to be due to different levels of glycosylation, in particular in the presence of exogenous dolichol. Similar findings have been described by others previously (Loo and Clarke 1999; Gribar et al. 2000; Zhang et al. 2004; Seres et al. 2011). However, the reduction of ABCB1 is specific since protein levels of α-tubulin and ERK1/2 were not affected by simvastatin or dolichol (Fig. 4f). Moreover, the well-described inhibitory effect of statins on ERK1/2 phosphorylation is seen in simvastatin-exposed probes and significantly reversed by coadministration with dolichol (Fig. 4g) (Campbell et al. 2006). Noteworthy, dolichol per se significantly reduced ERK1/2 phosphorylation, which is currently not understood.

The dolichol dependency of simvastatin effects was also observed on the level of apoptosis. The cleaved PARP fragment significantly accumulates in simvastatin exposed cell extracts and is reduced by coadministration of dolichol (Fig. 4b, e). This observation is supported by simvastatin-dependent activation of caspase 3 and positivity for annexin V/PI staining, which were again significantly reduced by coadministration with dolichol (Fig. 5). Thus, impairment of glycosylation in the endoplasmatic reticulum (ER) leads to reduction of heavily glycosylated proteins but also to cellular stress resulting in apoptosis. Mechanistically, this can be explained by simvastatin-induced ER stress, which is detected by the upregulation of the specific genes like BPG/GRP78 and the transcription factor CCAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP) (Table 2) (Haataja et al. 2008; van Schadewijk et al. 2012). Already at 0.1 µM simvastatin, BiP and CHOP were significantly induced, while at 10 µM simvastatin, no induction of the ER chaperone BiP was observable. An explanation for this latter observation is currently not available.
**In vivo effects of simvastatin on tumor growth and ABCB1 expression**

In order to confirm in vivo relevance of our findings, CD-1 Nu/Nu mice were inoculated with either SH-SY5Y neuroblastoma cells or RD rhabdomyosarcoma cells, and simvastatin was applied orally, 4.25 or 1.15 mg/kg/day, respectively. These doses of simvastatin translate into a human equivalent dose of 7 and 20 mg, which is a typical starting dose for simvastatin (Reagan-Shaw et al. 2008; Gazzarro et al. 2012).

There was no significant difference between the body mass of control and simvastatin-treated mice in both models (Table 3 and Fig. 6a). In the neuroblastoma xenograft model, there was also no significant difference in organ weights (Table 3), indicating no gross metastatic activity. Conversely, tumor weight in simvastatin-treated mice was diminished in comparison to control animals; however, this decrease was only significant in the RD xenograft model (Table 3 and Fig. 6b).

Following oral administration, simvastatin reaches the highest concentrations in the liver (Gazzarro et al. 2012). Accordingly, ABCB1 was significantly reduced in tissue homogenates from liver of simvastatin-treated animals (Figs. 6 and 7). Moreover, a similar trend was observed in rhabdomyosarcoma and neuroblastoma, which was...
Fig. 5 Dolichol restores simvastatin-induced apoptosis. Caspase 3 activation is monitored in lysates from SH-SY5Y cells treated with simvastatin (Sim) in the absence (CTL) and presence of dolichol C20 for 48 h (a). Similar treated cells were analyzed for apoptosis by FACS analysis of annexin V-PI-positive cells (b). The symbols represent mean ± SD (n=3). Asterisks denote significance versus control (*p<0.05; **p<0.01; ***p<0.005)

only significant in rhabdomyosarcoma (cf., Figs. 6 and 7). Taken together, these in vitro findings confirm that simvastatin is able to downregulate ABCB1 also in vivo at pharmacological relevant dosages.

Discussion

Statin treatment alters the glycosylation pattern of ABCB1 in neuroblastoma and rhabdomyosarcoma cells, similar to tunicamycin or PNGase F treatment (Siekzowski et al. 2010; Werner et al. 2013). The ABCB1 transporter exists in a full glycosylated 170-kDa form which upon deglycosylation shifts to the core glycosylated 140-kDa species (Loo and Clarke 1999). Although impaired glycosylation leads to a reduction in cell surface expression, the protein is still functional as this has been shown in various cellular systems (Germann et al. 1990; Kuchler and Thorner 1992; Loo and Clarke 1999).

The detection of ABCB1 by Western blot results in diffuse and fuzzy bands surrounding the 170- and 140-kDa region, due to the different levels of glycosylation (Loo and Clarke 1999; Greer and Ivey 2007; Seres et al. 2011). We also observed diffuse bands in particular in the presence of dolichol, which supports the conjecture that the level of glycosylation of the transporter is responsible for the overlap of the full and

**Table 2** Simvastatin-induced unfolded protein response

<table>
<thead>
<tr>
<th>Simvastatin (µM)</th>
<th>BiP (a.u.)</th>
<th>CHOP (a.u.)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.1</td>
<td>1.37±0.10 (**)</td>
<td>1.64±0.12 (**)</td>
</tr>
<tr>
<td>1</td>
<td>1.28±0.04 (**)</td>
<td>1.39±0.13 (*)</td>
</tr>
<tr>
<td>10</td>
<td>1.06±0.05 (n.s.)</td>
<td>2.35±0.27 (**)</td>
</tr>
</tbody>
</table>

Markers for ER stress, BiP and CHOP, were detected on mRNA level by PCR and normalized to GAPDH values from probes treated with increasing simvastatin concentrations for 48 h (mean±SD, n=3)

(*p<0.02; **p<0.005; ***p<0.001; n.s. denotes not significant; statistical significance is calculated with one-way ANOVA and Holm-Sidak method)

as this has been postulated previously (Demierre et al. 2005; Gazzzaro et al. 2012).

**Table 3** Analysis of the neuroblastoma xenograft experiment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Simvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (n=4)</td>
<td>1.49±0.14</td>
<td>1.59±0.24 (n.s.)</td>
</tr>
<tr>
<td>Heart (n=4)</td>
<td>0.14±0.02</td>
<td>0.13±0.01 (n.s.)</td>
</tr>
<tr>
<td>Spleen (n=4)</td>
<td>0.19±0.06</td>
<td>0.18±0.05 (n.s.)</td>
</tr>
<tr>
<td>Kidney (n=8)</td>
<td>0.53±0.20</td>
<td>0.52±0.10 (n.s.)</td>
</tr>
<tr>
<td>Lung (n=4)</td>
<td>0.21±0.05</td>
<td>0.20±0.02 (n.s.)</td>
</tr>
<tr>
<td>Tumor (SH-SY5Y cells; n=7)</td>
<td>2.77±1.87</td>
<td>2.25±1.61 (n.s.)</td>
</tr>
<tr>
<td>Body weight (n=4)</td>
<td>34.48±4.54</td>
<td>36.06±5.51 (n.s.)</td>
</tr>
</tbody>
</table>

Postmortem, the organs of four mice in each group were weighted (g) and presented as mean±SD. In both groups, one tumor was not detectable. Comparison of the two groups with Student’s t test revealed no statistical significance (n.s.).
core glycosylated species (Fig. 4). In the presence of simvastatin, the overall amount of ABCB1 is reduced and thereby also the intensities of the 140-kDa core glycosylated band, which may explain that, in some cases, the shift from the 170 to the 140-kDa form is hardly pronounced. Another explanation for the heterogeneity of ABCB1 glycosylation is depending on cellular systems, in particular transformed versus untransformed cells (Ichikawa et al. 1991; Fakla et al. 1998). For example, the different glycosylation branches have been found to be alternatively bisected at β(1-4)-N-acetyl-D-glucosamine residues (GlcNAc) in transformed cells. Nevertheless, we can exclude a methodical issue since other proteins are readily detected in these samples, like ERK1/2, cleaved PARP, or α-tubulin (Fig. 4).

The cell surface expression of ABCB1 in SH-SY5Y neuroblastoma cells is significantly reduced for longer incubation times in the presence of simvastatin (Fig. 1). This decline in ABCB1 expression is supported by Western blots of whole cell lysates probed for total ABCB1, which showed only a slight reduction after 24-h simvastatin exposure compared to 120-h incubations (Fig. 4a–d). This reduction of ABCB1 is considered to be not due to enhanced protein degradation, since the expression of the fusion protein YFP-ABCB1 is not further reduced in the presence of cycloheximide plus simvastatin compared to cycloheximide alone. Conversely, in the absence of transcriptional inhibition, simvastatin reduced YFP-ABCB1 within 48 h to the level obtained with cycloheximide alone. Moreover, this assumption is further
corroborated by qPCR and a significant downregulation of mRNA for ABCB1, ABCC1, and ABCG2 over time (Fig. 1e, f). A compensatory upregulation of ABCC1 or ABCG2 is not observed, although these transporters share overlapping drug specificity and importance in multidrug resistance (Sharom 2008). In contrast, the mRNA for ABCC6 is hardly affected by simvastatin, indicating the specificity of the drug effect. A significant reduction of mRNA and functional activity for ABCB1 has been previously described in human hepatoma and blood cells (Rodrigues et al. 2006). Interestingly, in these cellular systems, 10 to 20 µM of atorvastatin caused an increase of ABCB1 on protein level.

Importantly, our findings have been verified by various methods (heterologous expression of YFP-ABCB1, FACS, and Western blots) and different antibodies against ABCB1 (C219, MRK16) in order to control for unspecific reactivity. In particular, heterologous expression of YFP-ABCB1 enabled direct monitoring in simvastatin-treated HEK-293 cells and corroborated downregulation in a concentration-dependent manner (Fig. 1d), while the anthracycline doxorubicin, a known inducer of ABCB1, significantly augmented YFP-ABCB1.

Besides plasma membrane, ABCB1 has been allocated to the endoplasmic reticulum, various endosomes, Golgi, and lysosomes, but not mitochondria (for review see (Fu and Arias 2012)). This intracellular organelle pattern reflects the protein synthesis, maturation and posttranslational modification, the translocation to the target membrane, and the degradation, respectively. Posttranslational modification of ABC transporters by N-glycosylation has been investigated intensively and plays a critical role in protein folding, protein export, and maintenance of protein stability (Richert et al. 1988; Asano et al. 1993; Schinkel et al. 1993; Lee et al. 2003; Zhang et al. 2004; Unquhart et al. 2005). Indeed, simvastatin reduced endogenous dolichol levels (Fig. 3) and thereby the prerequisite for proper
protein glycosylation in the ER (Behrens and Lebœuf 1970). Others have shown that inhibition of glycosylation augments ubiquitination and degradation of ABCB1 (Kramer et al. 1993; Seres et al. 2011). Importantly, altered glycosylation does not affect the transport activity of ABCB1 (Germann et al. 1990; Kuchler and Thormer 1992; Schinkel et al. 1993; Loo and Clarke 1999; Seres et al. 2011), whereas ER trafficking is impaired (Loo and Clarke 1998). In non-polarized cells, approximately 30% of the ABCB1 is found in EAA1- and Rab5-positive endosomes which shuttle between the plasma membrane and thereby control the cell surface expression of ABCB1 (Kim et al. 1997). It is known that Rab proteins control vesicle transport by interaction with microtubules and the actin cytoskeleton. Statins reduce isoprenylation of small G proteins, in particular, those interacting with the cytoskeleton (Kato et al. 2004; Gazzarro et al. 2012). This has been shown for Rab5 and Rab7 in lovastatin-treated FRTL-5 thyroid cells, which then accumulate in the cytosol (Laezza et al. 1998). This translocation from membrane fractions to the cytosol is explained by a decrease of geranylgeranylation and farnesylation, which serve as lipid anchor for membrane insertion. Coapplication of mevalonate, the product of the statin-inhibited HMG-CoA reductase, was sufficient to prevent cytosolic accumulation of Rab proteins (Laezza et al. 1998).

A link between reduced glycosylation and apoptosis is provided by the induction of BiP and CHOP, two sensitive markers for ER stress (Table 2). Thus, in a scenario of dolichol depletion, ER stress and reduced functional vesicular transport would explain pro-apoptotic events and less cell surface expression of ABCB1. However, reserve granules and intact transporters might be present in these endosomes which may serve as a compensatory reservoir within the first 24 h after simvastatin application. Consequently, in add-back assays, dolichol not only overcomes deglycosylation and downregulation of ABCB1 but also reduces simvastatin-induced apoptosis (Fig. 4).

Simvastatin-induced apoptosis is also shown in our in vivo findings. Two xenograft models confirm anti-tumor activity of simvastatin as well as ABCB1 downregulation. CD-1 Nu/Nu mice inoculated with rhabdomyosarcoma or neuroblastoma cells received clinically relevant simvastatin concentrations and showed a remarkable induction of apoptosis in both tumor tissues indicated by PARP and caspase 3 cleavages (Figs. 7 and 8). Most importantly, ABCB1 downregulation is found in the liver and tumor tissues but did not reach significance in neuroblastoma. Importantly, simvastatin is capable to trigger apoptosis in neuroblastoma and rhabdomyosarcoma. Moreover, the extent of apoptosis is comparable to cyclophosphamide and further amplified by the combination of the two drugs, although cyclophosphamide is not a substrate for ABCB1 (Fig. 8). This latter issue highlights the potential of simvastatin to act as an anti-tumor drug.

Fig. 8 Activation of caspase 3 in rhabdomyosarcoma of simvastatin-treated animals. Rhabdomyosarcomas from animals described in Fig. 5 were used for immunohistochemistry to detect cleaved caspase 3 (a) in controls (CTL), simvastatin (Sim)-, and/or cyclophosphamide (Cyc)-treated groups. Nuclei were stained with Hoechst dye (green), and cleaved caspase 3 (cl. casp 3; red). The merged pictures allow identification of the caspase 3 translocation into the nucleus equivalent to ongoing apoptosis (magnification ×63; white scale bars, 20 μm). Nuclei positive for cleaved caspase 3 were counted, and normalized data of 20 pictures from each group are depicted (b). Statistical significance for multiple comparisons was calculated with one-way ANOVA and post hoc Turkey test (* p<0.05; ** p<0.005)
Statins are well established and safely used compounds in the treatment of hypercholesterolemia (Guzziero et al. 2012). However, anti-tumor activities caught most attention in clinical trials and meta-analyses (Demierre et al. 2005; Guzziero et al. 2012; Nielsen et al. 2012). Although available data are still controversial and conflicting, recent studies showed that statin use before a cancer diagnosis might be associated with lower risk of cancer incidence and cancer-related mortality (Guzziero et al. 2012; Nielsen et al. 2012). Notably, all-cause mortality by statin users with cancer was reduced by 15% (Nielsen et al. 2012).

Statins are also known to inhibit directly ABCB1, and for the various statins, the IC_{50} values match congruently the inhibition of ATP hydrolysis (Bogman et al. 2001; Wang et al. 2001; Goard et al. 2010; Sieczkowski et al. 2010; Werner et al. 2013). Consequently, statins enhance the intracellular accumulation of ABCB1 substrates like doxorubicin, which augments cytotoxicity in neuroblastoma and rhabdomyosarcoma cells and may provide the classical argument for usage in an anti-cancer therapy (Tamplin et al. 1954; Werner et al. 2004, 2013; Demierre et al. 2005; Goard et al. 2010; Sieczkowski et al. 2010; Tanaka et al. 2010).

In summary, simvastatin has a profound anti-tumor activity per se, which is now further highlighted by the fact that ABCB1 is downregulated in vitro and in vivo. Although simvastatin is well tolerated, these data also implicate that drug–drug interactions not only may take place on the level of CYP3A4 but also have the potential to interfere with drug extrusion via ABCB1. In fact, there is a strong overlap of compounds, which are substrates for both proteins (Sharam 2008; International Transporter C et al. 2010). Nevertheless, this study shows that it is feasible to downregulate ABCB1 in vitro and in vivo by simvastatin, which may act as a lead compound in novel anti-cancer therapies.

Acknowledgments The authors thank Prof. Dr. Valery Bochkov (Institute of Physiology, Medical University of Vienna) for instructions and help with dolichol measurements, Prof. Peter Chiba, (Institute of Medical Chemistry) and Dr. Oliver Kudlacek (Institute of Pharmacology, Medical University of Vienna) for providing the NH$_2$-terminal tagged YFP-ABCB1 construct, and Mag. Martin Künzl for advice in qPCR. The authors are grateful for adequate help in animal housing by Sandra Bolzer, Jasminka Kanlic and Parvani, Tajjama. This study was supported by Herzfelder’sche Familienstiftung and FWF grant P-22385 from the Austrian Science Founds.

Authors’ contribution Bhiter Atil, Evelyn Berger- Sieczkowski, Johanna Bardy, and Martin Werner performed experimental work. Bihter Atil and Martin Hohenegger analyzed data, performed conceptual work, and wrote the manuscript. All authors critically revised the manuscript and gave final approval for submission.

Conflict of interest The authors confirm that they have no conflict of interest.

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Simvastatin-induced compartmentalisation of doxorubicin sharpens up nuclear topoisomerase II inhibition in human rhabdomyosarcoma cells

Martin Werner · Bihter Atli · Evelyn Sieczkowski · Peter Chiba · Martin Hohenegger

Received: 10 December 2012 / Accepted: 21 March 2013 / Published online: 7 April 2013 © The Author(s) 2013. This article is published with open access at Springerlink.com

Abstract Tumours, which are initially sensitive to cytotoxic agents, often develop resistance to a broad spectrum of structurally unrelated drugs. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have been shown to inhibit ATP-binding cassette (ABC) transporters but have also impact on glycosylation of such proteins. Doxorubicin is a substrate for ABC transporters like P-glycoprotein (ABCB1) which is present in human RD rhabdomyosarcoma cells. It was therefore the aim of this study to identify the compartmentalisation and action of doxorubicin in simvastatin-treated RD cells. Due to autophorescence of doxorubicin, intracellular distribution was monitored by confocal microscopy. The biological effects were traced on the level of colony formation, caspase activation and DNA injury. Here we show that simvastatin treatment leads to ABCB1 inhibition and down-regulation of the transporter. Consequently, these cells accumulate significant amounts of doxorubicin, predominantly in the nucleus and lysosomes. While clearance of the anthracycline into lysosomes is not altered by simvastatin treatment, it significantly enhanced nuclear accumulation in a HMG-CoA reductase-independent manner. Thus, in such treated cells, topoisomerase II activity is significantly inhibited, which is further corroborated by augmented double-strand DNA breaks. Moreover, colony formation was synergistically inhibited by the combination of simvastatin and doxorubicin. Given the fact that ABCB1 expression correlates with an adverse prognosis in many tumours, adjuvant chemotherapy including statins might represent a novel therapeutic concept to overcome ABCB1-mediated multidrug resistance by direct inhibition and down-regulation.

Keywords ATP-binding cassette transporters · HMG-CoA reductase inhibitors · Apoptosis · Topoisomerase II · Rhabdomyosarcoma

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ABC transporter</td>
<td>ATP-binding cassette transporters</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ABC transporter B1, P-glycoprotein</td>
</tr>
<tr>
<td>ACF</td>
<td>7-Amino-4-trifluoro-methylcoumarin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>3-Hydroxy-3-methylglutaryl coenzyme A reductase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>Radio immunoprecipitation assay buffer</td>
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Introduction

Rhabdomyosarcoma is the most common soft tissue sarcoma of childhood, accounting for more than half of such cases in paediatric patients (Raney et al., 1993). Initial treatment is often successful, but relapses due to multidrug resistance (MDR) remain a major obstacle to survival. Thus, the overall outcome in
paediatric rhabdomyosarcoma is therefore still a therapeutic challenge. In part, MDR may be caused by changes in the expression level or activity of ATP-binding cassette (ABC) transporters (Endicott and Ling 1989; Liscovitch and Lavie 2002; Szakacs et al. 2006).

Ways of reversing MDR via ABC transporters have been investigated extensively in recent years, and three generations of inhibitors have been developed (Liscovitch and Lavie 2002; Szakacs et al. 2006). Among these, verapamil, cyclosporine A and tarzicipar have emerged as lead compounds for characterisation in vitro. Nevertheless, due to severe side effects, such as nephrotoxicity for cyclosporin A or cardiac toxicity for verapamil, these MDR modulators failed to convince in clinical trials (Cocker et al. 2000; Szakacs et al. 2006). Although there are many new MDR-modulating agents available for in vitro usage, no effective and clinically applicable therapy has yet evolved (Liscovitch and Lavie 2002; Szakacs et al. 2006; Tamaki et al. 2011).

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, are widely used in hypercholesterolaemia and prevent cardiovascular events (Lennernas and Fager 1997; Corsini et al. 1999; Group 2000). In recent years, evidence emerged that anti-inflammatory and anti-proliferative actions of statins also contribute to beneficial cardiovascular outcomes. These effects have led to a series of in vitro studies which came to the conclusion that statins also exert anti-tumour activity which has been targeted to cell cycle arrest, depletion of cholesterol precursors like isoprenoids and oxidative stress (Demierre et al. 2005; Sleijfer et al. 2005). However, in clinical studies, the role of statins in oncology is still controversial and seems to be strongly dependent on the molecular identity of the tumour (Graaf et al. 2004; Poynter et al. 2005; Nielsen et al. 2012).

Statins are capable to inhibit the rhodamine 123 efflux from tumour cells via their lactone form but not in the hydrolysed acidic form (Bogman et al. 2001). Such inhibition of efflux activity could be attributed to ABCB1 interaction with statins (Wang et al. 2001; Sakaeda et al. 2002; Chen et al. 2005). In a previous work, we made similar observations and could show that glycosylation of ABCB1 is reduced in simvastatin-treated human neuroblastoma cells (Sieczkowski et al. 2010). The mutual enhancement of cytotoxicity between statins and doxorubicin has been shown in neuroblastoma cells and RD rhabdomyosarcoma cells to trigger the mitochondrial apoptotic machinery (Werner et al. 2004; Sieczkowski et al. 2010). Since statins are capable to trigger caspase activity per se, it is difficult to estimate the apoptotic potential of doxorubicin in drug combinations. Moreover, it is unclear whether enhanced intracellular doxorubicin accumulation is targeted to individual organelles and whether this translates into specific doxorubicin-mediated cytotoxicity. However, one would postulate that enhanced intracellular doxorubicin concentrations might translate into augmented topoisomerase II inhibition.

Thus, it was the aim of the present work to confirm synergism between simvastatin and doxorubicin. Moreover, intracellular compartmentalisation of doxorubicin and functional implications thereof were investigated.

Materials and methods

Materials

Dulbecco’s modified Eagle medium (DMEM) was purchased from Gibco® (Invitrogen®, Paisley, Scotland, UK). Doxorubicin, daunorubicin, rhodamine 123, protease inhibitors (pefabloc, leupeptin and aprotinin), soft agar and other common chemicals were from Sigma Chemical Co.® (St. Louis, MO, USA). Simvastatin and verapamil were purchased from Merck® Research Laboratories (Rahway, NJ, USA). Concanamycin A and fluorescent substrates for caspases 3 and 8 were from Alexis Biochemicals® (San Diego, CA, USA). Lysotracker® Red was obtained from Molecular Probes® (Eugene, OR, USA).

Cell culture

Human rhabdomyosarcoma cells (RD cells) of the spindle cell type were obtained from ATCC® (Manassas, VA, USA) and kept in growth medium (DMEM, 10% foetal calf serum, 50 U/ml penicillin G and 50 μg/ml streptomycin) at 37 °C under 5% CO₂ for 2–5 days prior to experiments. The CCRFvcer1000 cells were cultured as previously described (Chiba et al. 1996; Hiessbock et al. 1999) and used to confirm simvastatin as inhibitor of ABCB1.

Caspase assays

RD cells were incubated with simvastatin, doxorubicin and/or mevalonic acid for the indicated times and concentrations. Thereafter, the cells were lysed (lysis buffer—25 mM HEPES (pH 7.4), 5 mM EDTA, 1 mM EGTA, 5 mM MgCl₂, 5 mM DTT, supplemented with protease inhibitors 1.4 μg/ml aprotinin, 10 μg/ml leupeptin and 100 μM pefabloc) and centrifuged (35,000 g, 30 min), and aliquots of the supernatant were used for a fluorescence-based caspase assay (reaction buffer—25 mM HEPES pH 7.4, 10% sucrose, 1.4 mM CHAPS and 5 mM DTT and 50 μM AFC-conjugated caspase substrates) (Werner et al. 2004). The corresponding pellet is termed “membrane fraction”, resuspended in radio immunoprecipitation assay (RIPA) buffer and further used for Western blotting.
Topoisomerase II decatenation assay and histone H2AX phosphorylation

RD cells (5 × 10^7 cells) were treated with drugs according to the figure legends for 24 h. The cells were lysed and processed for nuclear extraction according to Dansk et al. (1988). Nuclear extracts (20 µg) were assayed for topoisomerase II activity (30 min at 37 °C) using a topoisomerase II decatenation kit (Inspiral™, Norwich, UK). The reaction was terminated with 2.5 % SDS and the DNA separated on a 1 % agarose gel. Densitometric analysis was carried out using the ImageJ™ software (http://rsweb.nih.gov/ij/index.html).

Alternatively, the phosphorylation of histone H2AX was used as a sensitive measure for double-strand DNA breaks. A flow cytometry based histone H2AX phosphorylation kit was used (Millipore™, Temecula, CA, USA). RD cells were treated as depicted in the figure legend and further processed and stained with an FITC-conjugated antibody specific for serine139 phosphorylation of histone H2AX according to the instructions of the manual. The positive stained cells were quantified by flow cytometry. The fluorescence signal in the absence of the antibody was used as a negative control and RD cells treated with 10 µM etoposide as a positive control.

Western blot analysis

Aliquots of membrane fractions (15 µg) from RD cells resuspended in RIPA buffer were applied to an 8 % sodium dodecyl sulphate polyacrylamide gel. The separated proteins were transferred to nitrocellulose membranes (Whatman™, Dassel, Germany) and exposed to an ABCB1-specific primary antibody (C219 mouse monoclonal P-gp antibody, Abcam™, Cambridge, UK; diluted at 1:300) for 90 min at room temperature. Specific bands were visualised with an appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG, horseradish peroxidase-linked antibody, GE Healthcare™, Bucks, UK; diluted 1:15,000, 60 min at room temperature) using an ECL Plus detection system (GE Healthcare™, Bucks, UK). Immunodetection of α-tubulin (monoclonal anti-α-tubulin; Sigma™, St. Louis, USA; dilution of 1:7,500) was used to control protein loading.

Colony forming assay

Trypsinised RD cells were seeded in 6- and 12-well plates at a density of 500 cells per well, precoated with 0.5 % soft agar. After 24 h, cells were incubated in the absence and presence of doxorubicin, simvastatin or a combination of both at the given concentrations. After 15 days, the cells were fixed with 50 % ethanol and stained with 0.5 % methylene blue. Cell growth was evaluated by counting colonies with more than 50 cells.

Fluorescence-activated cell sorting (FACS) analysis

RD cells were treated with simvastatin or verapamil for 10 min and subsequently with doxorubicin for further 60 min. Cells were analysed for doxorubicin accumulation on a FACSScan (Beckton Dickinson™, New York, USA) with an excitation of 488 nm and an emission filter of 600 nm.

For histone H2AX phosphorylation, RD cells were exposed to the indicated compounds for 18 h and treated according to the kit instructions. The FITC-conjugated antibody against histone H2AX phosphorylation was detected with an excitation at 488 nm and an emission at 515 nm. A total of 10^6 cells were counted per sample and the data were processed using WinMDI software™ (http://www.cyto.purdue.edu/flowcyt/software/Winmdि.htm) or FACS Diva™ (BD Biosciences™, San Jose, CA, USA).

Confocal fluorescence microscopy

RD cells were grown on glass cover slides and incubated for 18 h in the absence or presence of simvastatin, verapamil or concanamycin A for 1 h. Such treated cells were subsequently exposed to 1 µM doxorubicin for an additional hour. Cells were washed with PBS and supplied with fresh culture medium. Images were collected using a confocal microscope (Zeiss® LSM 410; Jena, Germany), an excitation of 488 nm and an emission of 543 nm for LysoTracker® Red (Molecular Probes®, Leiden, Netherlands) or 570 nm for doxorubicin. The digitized pictures from a single focal plane containing two to six cells were stored. The doxorubicin intensity was measured in the nucleus (55–69 cells) and lysosomes (91–296 cells) using MetaMorph® software (Universal Imaging Corporation, Downingtown, PA, USA) and ImageJ™ software (http://rsweb.nih.gov/ij).

For the doxorubicin co-localisation in the lysosomes, cells were pre-incubated in the culture medium with 10 µM simvastatin and 15 µM verapamil for 1 h and another hour together with 1 µM doxorubicin. Pictures of doxorubicin autofluorescence were obtained as described above. Subsequently, 20 nM LysoTracker® Red was added and the fluorescence signal collected.

The kinetic analysis of nuclear accumulation of doxorubicin was carried out under conditions described above with minor changes. RD cells were incubated for 20 min in the absence and presence of simvastatin or the combination of simvastatin plus mevalonic acid. Immediately after the doxorubicin addition, a series of pictures was taken for the indicated period with the settings described above.
Daunorubicin, rhodamine 123 and calcine flux measurements

CCRFcrl000 cells were pre-treated with various concentrations of simvastatin or 15 μM verapamil for 20 min. Daunorubicin and rhodamine 123 were used as transporter substrates as previously reported (Chiba et al. 1996; Hiessbock et al. 1999). The IC_{50} values for simvastatin were determined in zero trans efflux and steady-state protocols. In the steady-state protocol, apparent IC_{50} values were corrected for differences in ABCB1 expression considering a pump-leak situation.

For calcine efflux measurements, RD cells were pre-treated with various concentrations of simvastatin or 15 μM verapamil for 20 min or 48 h. Cells were gently detached and 5×10^5 cells were resuspended in HEPES/Na-puffer (20 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose) supplemented with the respective drugs from the 20-min pre-incubation. No drugs were supplemented to cell suspensions from the 48-h pre-treatment. The experiment was started with 1 μM calcine-AM uptake and reaching a plateau. After 30 min, the extracellular calcine was quenched by 1 mM of CoCl₂. The fluorescence was monitored at an excitation wavelength at 485 nm and an emission at 535 nm using a fluorescence plate reader (VICTOR-3, Perkin-Elmer, MA, USA) with sampling rates of 10 and 30 s or 3 min, depending on the phase of the experiment (calcine efflux, calcine-AM uptake or plateau phase, respectively).

Miscellaneous procedures

Experiments were carried out at least three times in duplicates and presented as mean ± SD, if not otherwise stated. The curves were subjected to non-linear least squares regression and fitted to the according equations (Hill equation) using the SigmaPlot software (Jandel, Erkrath, Germany). Statistical analysis was carried out by Student’s t test or for multiple comparisons by ANOVA and post hoc Dunnett’s test (when compared to control) or Tukey’s test (for pairwise comparison). A value of p<0.05 was considered statistically significant.

**Results**

Simvastatin and doxorubicin synergistically activate the mitochondrial pathway of apoptosis

In human RD cells, HMG-CoA reductase inhibitors like simvastatin readily activate the effector caspase 3 in a concentration-dependent manner (Fig. 1a). The EC_{50} of simvastatin-induced caspase 3 activation shifted by more

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**Fig. 1** Potentiation of caspase 3 activity by simvastatin plus doxorubicin. RD cells were grown in the absence and presence of simvastatin (sim), doxorubicin (dox) and mevalonic acid (ma, 1 mM) at the given concentrations for 24 h. Aliquots of the supernatant of lysed cells were used for caspase 3 (a, c) and caspase 8 activity (b). The data are given as mean values ± SD (n=4). Statistical significance was determined with ANOVA and post hoc Tukey’s test (asterisk denotes significance versus control *p<0.039, **p<0.001; number sign denotes significance versus sim #p<0.001; plus sign denotes significance versus sim+dox ++p<0.001; n.s. not significant)
than one order of magnitude from 9.9±1.2 to 0.69±0.2 μM (mean ± SD, n=4; p<0.001) upon co-application with doxorubicin at a clinically relevant concentration (Lee et al. 1980). Under these conditions, caspase 8 was not significantly activated, neither by simvastatin alone nor by a combination with doxorubicin (Fig. 1b). The simvastatin-induced caspase activation is strongly dependent on HMG-CoA reductase inhibition and thus prevented by co-application with mevalonic acid (Werner et al. 2004; Minichsdorfer and Hohenegger 2009; Sieczkowski et al. 2010). In contrast, doxorubicin-induced caspase 3 activation is insensitive to co-administration of mevalonic acid (Fig. 1c). However, a more than additive stimulation of caspase 3 is seen with the co-administration of doxorubicin and simvastatin. This is significantly reduced by mevalonic acid to the level of doxorubicin alone. Thus, the mutual sensitisation for apoptosis is only seen for the intrinsic pathway of apoptosis, which has been previously depicted for statins in more detail (Werner et al. 2004). These results confirm that simvastatin potentiates doxorubicin toxicity in RD cells at concentrations that have been well tolerated in patients receiving simvastatin (Lennernas and Fager 1997; Larner et al. 1998).

Synergistic inhibition of colony formation by simvastatin and doxorubicin

HMG-CoA reductase inhibitors have been shown to synchronise tumour cells by blocking the transition of G1–S in the cell cycle, which explains their anti-proliferative effects in many cell types (Rao et al. 1999; Dimitroulakos et al. 2001; Ukomadu and Dutta 2003). We have therefore investigated colony formation of RD cells in the presence of increasing concentrations of simvastatin and doxorubicin, exemplified in Fig. 2a. In fact, RD cells are highly susceptible to inhibition of colony formation with an IC50 of 3.42±0.7 nM doxorubicin (mean ± SD; n=3) (data not shown). Consistent with our previous observations on caspase 3 and 9 activation, the efficacy to suppress colony formation is more pronounced in the combination of simvastatin and doxorubicin as compared to the individual compounds (Fig. 2a) (Werner et al. 2004). The potency of the individual compounds to prevent colony formation is not influenced by the presence of the second compound as depicted in Fig. 2a (IC50 for simvastatin 3.0±1.3 μM versus simvastatin plus 5 nM doxorubicin 2.8±2.1 μM; mean ± SD, n=3). The isobologram analysis revealed a clear synergistic interaction for the drug combination of simvastatin with doxorubicin (Fig. 2b).

Inhibition and down-regulation of ABCB1 by simvastatin

Taking advantage of the fluorescence properties of doxorubicin, a short pre-treatment of RD cells with simvastatin resulted in a significant increase in doxorubicin fluorescence as monitored by FACS analysis (Fig. 3a, b). Similarly, the first generation ABCB1 inhibitor verapamil also enhanced the intracellular accumulation of doxorubicin. In a control experiment without doxorubicin, autofluorescence of the cells in the absence or presence of simvastatin was not observed (data not shown).

There are already reports that statins are capable of inhibiting ABC transporters (Bogman et al. 2001; Wang et
approach, first-order rate constants of daunorubicin and rhodamine 123 efflux show a clear inhibition by simvastatin with $IC_{50}$ values of 4.6±1.6 and 49.3±4.7 µM (mean ± SD; n=3), respectively (Fig. 4). The Hill coefficient between 1.1 and 1.2 in both cases is indicative for a non-cooperative interaction. Comparable results were obtained when verapamil was used as a P-glycoprotein inhibitor which gave $IC_{50}$ values in the same concentration range (Chiba et al. 1996; Hiesbock et al. 1999). In order to further corroborate this assumption, calcine efflux was determined in RD cells.

![Graph showing efflux rates](image)

Fig. 3 Enhanced intracellular doxorubicin content in the presence of simvastatin or verapamil. Doxorubicin accumulation was assessed with flow cytometry in RD cells treated for 1 h with 1 µM doxorubicin (ctl) (a). Preincubation of the cells for 10 min with 3 µM (sim3), 10 µM (sim10) simvastatin or 15 µM verapamil (ver) enhanced the doxorubicin fluorescence intensity. The experiment was repeated twice with similar results. The mean increase in doxorubicin fluorescence under conditions given in a is depicted in the bar diagram (b). The data were processed with WinMDI software and presented as geometrical mean ± SD (n=3).

![Graph showing simvastatin effects](image)

Fig. 4 Simvastatin inhibits ABCB1 in CCRFver1000 cells. The ABCB1 expressing CCRFver1000 cells were loaded with daunorubicin (a) or rhodamine 123 (b) and the kinetics of the decrease in fluorescence was determined in the presence of increasing concentrations of simvastatin. The calculated first-order rate constants ($V_{max}/K_{m}$) were plotted in these concentration–response relationships. The symbols represent the mean ± SD (n=3).

al. 2001; Sakaeda et al. 2002; Sieczkowski et al. 2010). We have therefore investigated the effects of simvastatin on ABCB1 in greater detail in CCRFver1000 cells, which express a defined number of transporter molecules (Chiba et al. 1996; Hiesbock et al. 1999). Such an approach enables to normalise the transporter activity to the actual number of transporters in the cells. Using this experimental
The calcine efflux is best described by fitting the data points to a biexponential decay. Thus, the two half lives of calcine efflux were significantly prolonged already in the presence of 1 μM simvastatin (Fig. 5b, c).

ABCB1 is present in RD cells and, importantly, is downregulated upon incubation for 48 h with increasing concentrations of simvastatin (Fig. 6a, b). Thus, these observations corroborate and expand earlier observations of our laboratory in human neuroblastoma cells that deglycosylation might impair not only the function of ABCB1 but also protein expression (Sieczykowski et al. 2010). To confirm a functional consequence, such treated RD cells were washed and subjected to calcine efflux measurements, and again, a significant prolongation of the half lives was observed. Hence, a direct inhibition of ABCB1 by simvastatin is in accordance to earlier observations and is now expanded by a significant down-regulation of the transporter (Bogman et al. 2001; Wang et al. 2001; Sakaeda et al. 2002; Sieczkowski et al. 2010).

Mining intracellular doxorubicin accumulation under simvastatin treatment

In order to further trace intracellular doxorubicin accumulation, RD cells were scanned by confocal microscopy. Doxorubicin accumulates in the nuclei, but also in punctate-patterned organelles, which co-localise with lysosomes (Fig. 7a). A lysosomal sequestration of doxorubicin represents a possible clearance mechanism of RD cells. However, no changes were observed in lysosomal doxorubicin fluorescence intensity or lysosomal radius (Fig. 7b, c).

The nucleus is the primary target organelle for doxorubicin. The anthracycline binds to DNA and inhibits topoisomerase II, which results in blockade of DNA replication and transcription (Larsen et al. 2000). Consequently, one can postulate that cytotoxicity of doxorubicin depends on the concentration in the nucleus. Under control conditions, doxorubicin accumulates readily in the nuclei (Fig. 8). Upon treatment with simvastatin or verapamil for 18 h, the nuclear doxorubicin fluorescence increased significantly (Fig. 8b). As a weak base, doxorubicin is sequestered in acidic compartments such as lysosomes as already shown in Fig. 7a. This sequestration is thought to represent one mechanism of multidrug resistance.

Fig. 5 Simvastatin inhibits ABC transporter-mediated calcine efflux in RD cells. RD cells were incubated in the absence (ctl) or presence of 3 μM simvastatin (sim) 20 min prior to 1 μM calcine-AM exposure (a). The fluorescence (excitation 485 nm, emission 535 nm) was monitored over time until a plateau was reached. Calcine efflux wasvisualised by quenching extracellular calcine by the addition of 1 mM CoCl₂. The symbols and bars represent the mean ± SEM (n=6). The calcine efflux was fitted to bi-exponential decays and the half lives are depicted from applications with 1 (1 sim) or 10 (10 sim) μM simvastatin or 15 μM verapamil (15 ver) (b, c). The bars and errors represent the mean ± SD (n=8).
Fig. 6 Down-regulation of ABCB1 by simvastatin. The presence of ABCB1 was confirmed by Western blot analysis in untreated and simvastatin-treated (sim) RD cells, incubated for 48 h (a). Protein loading is controlled with α-tubulin. The intensity of the 140- and 170-kDa ABCB1 protein band was corrected for protein loading and the normalised intensities are depicted as mean ± SD (n=3) in b. The asterisk denotes statistical significance versus control (p<0.05). Similar to the experiments depicted in Fig. 5, calcine efflux was determined in RD cells after 48 h of incubation with 3 and 30 μM simvastatin (sim) versus control (ctl). Simvastatin was removed before calcine-AM loading. The half-lives of the first (c) and second calcine release phase (d) were presented as mean ± SD (n=6).

that may exist separately from the ABC transporters (Schindler et al. 1996). Treatment of the cells with concanamycin A, an inhibitor of the lysosomal proton pump, destroys these organelles and still leads to accumulation and nuclear deposition of doxorubicin (Fig. 7a, b). Importantly, 50 nM concanamycin A is below the concentration range which has been described to interfere with ABCB1 function (0.1–10 μM) (Sharon et al. 1995).

To analyse the kinetics of nuclear doxorubicin uptake, time series were taken from doxorubicin-exposed cells using confocal microscopy. Simvastatin immediately accelerated the nuclear compartmentalisation of doxorubicin, which was not prevented by co-incubation with mevalonic acid (Fig. 8c). This is of utmost importance and indicates that an HMG-CoA reductase-independent mechanism is responsible for the nuclear doxorubicin accumulation, which is already observed within 1 h of co-administration.

Enhanced topoisomerase II inhibition and DNA double-strand breaks by simvastatin plus doxorubicin

Simvastatin-induced inhibition of ABCB1 results in enhanced nuclear deposition of doxorubicin. Therefore, nuclear extracts from RD cells were probed for decatenation of kinetoplast DNA.

This assay is specific for topoisomerase II activity, the target enzyme of anthracyclines. Expectedly, the topoisomerase II activity is inhibited to a greater extent by the drug combination of simvastatin plus doxorubicin (Fig. 9a, b). Complementary, topoisomerase II inhibition may result in less DNA repair and this should facilitate the detection of DNA double-strand breaks. Thus, the increment in histone H2AX phosphorylation on serine139 was used as a sensitive measure for DNA double-strand breaks (Waters et al. 2009). Again, a combination of simvastatin and doxorubicin significantly enhanced DNA injury compared to the individual compounds alone (Fig. 9c, d). Interestingly, simvastatin alone or in a combination with mevalonic acid failed to increase DNA double-strand breaks. This indicates that the induction of apoptosis by simvastatin alone has not reached DNA laddering within the experimental time frame of 18 h. Moreover, these findings corroborate the initial hypotheses that simvastatin-induced nuclear compartmentalisation of doxorubicin significantly enhances efficacy of the anthracycline.

Discussion

Statins, like simvastatin, are used for the treatment of hypercholesterolaemia and prevention of cardiovascular
events (Corsini et al. 1999; Group 2000; Nielsen et al. 2012). They belong to the most widely prescribed drugs and are well tolerated, besides occasionally occurring skeletal muscle side effects, which are now understood as apoptotic events (Corsini et al. 1999; Sacher et al. 2005). In recent years, evidence has accumulated that statins also exert an anti-proliferative activity including cell cycle arrest, inhibition of angiogenesis, stimulation of anti-tumour immunity and impairment of metastatic potential (Sleijfer et al. 2005). In different tumour cell lines, it was shown that statins induce growth inhibition by blocking the transition of G1-S phase in the cell cycle (Lee et al. 1998; Rao et al. 1999; Dimitroulakos et al. 2001; Wachtershauser et al. 2001; Ukomadu and Dutta 2003) and by induction of apoptosis via the mitochondrial pathway in human RD cells (Werner et al. 2004), melanoma cells (Minichsdorfer and Hohenegger 2009), human lymphocytes, myeloma cells (Caffiro et al. 2005) and others (Dimitroulakos et al. 2001; Siecikowski et al. 2010). However, apoptosis is seen in tumour cells only above 1–3 μM of the individual statins which represent a more than 10-fold higher concentration compared to pharmacological statin treatment (Corsini et al. 1999). This is hampered by co-application of the anthracycline doxorubicin which is capable to shift the concentration-response curve for simvastatin to the left by a factor of 10 (Fig. 1a).

In our study, we show that 100 nM doxorubicin is capable of shifting the EC_{50} of simvastatin-induced caspase 3 activation from 9.9 to 0.7 μM. This anthracycline concentration is found in the plasma of tumour patients treated with doxorubicin (Giaccone et al. 1997; Swenson et al. 2003). In contrast, the peak plasma concentrations reached in patients after oral simvastatin administration are approximately 0.14 μM (Lennernas and Fager 1997). Thus, induction of apoptosis with an EC_{50} of 0.7 μM simvastatin is clearly above this value. However, we speculate that lower doses of these drugs may also be effective in prolonged incubations, since the apoptotic effects of statins are time and dose dependent (Werner et al. 2004; Minichsdorfer and Hohenegger 2009). Indeed, low-dose statins given continuously seem to exhibit some anti-tumour activity (Sleijfer et al. 2005; Nielsen et al. 2012), which might explain the
protective effect observed in case-control studies (Boudreau et al. 2004; Graef et al. 2004; Poynter et al. 2005) and in a study performed in hepatocellular carcinoma (Kawata et al. 2001). The latter observation is of particular interest because the concentrations which statins might reach in the liver are significantly higher than those in the serum. The liver/plasma ratio of 10:2 after subcutaneous application of atorvastatin, simvastatin, lovastatin or pravastatin confirms this assumption (Chen et al. 2007). Importantly, liver injury is a rare statin side effect and often linked to concomitant drug administration or pre-existing liver diseases (Corsini et al. 1999).

Although doxorubicin represents a hallmark in many anti-cancer drug regimens, its role in children soft tissue sarcoma has been controversially discussed (Bisogno et al. 2005). However, our in vitro results are indicative of a rational with a potential role of statins as an emerging candidate for combinatory chemotherapy. Synergistic interactions between statins and various cytotoxic drugs were found in vitro and in vivo: (1) simvastatin was found to act synergistically with carbamustine in vitro and in vivo (Soma et al. 1995). (2) Lovastatin potentiated the anti-tumour effects of cisplatin in MmB16 melanoma cells (Feleszko et al. 1998), of 5-fluorouracil and cisplatin in colon cancer cells (Agarwal et al. 1999), of doxorubicin in Colon-26 cells, v-Ha-ras-transformed NIH-3T3 sarcoma cells and Lewis lung carcinoma cells in vitro and in vivo (Feleszko et al. 2000). (3) Cervastatin synergistically enhanced cytotoxicity of 5-fluorouracil in colorectal cancer cells (Wang et al. 2002) and of doxorubicin and cisplatin in human breast cancer cells (Kozar et al. 2004). Interestingly, with the exception of doxorubicin and in part carbamustine, these chemotherapeutics are no substrates for ABCB1 (Szakacs et al. 2006). Cisplatin is a substrate for ABCC2, ABCC5 and ABCC6 and the thiopurine 5-fluorouracil for ABCC5 and ABCC11 (for review, see Szakacs et al. 2006). Accordingly, statins may impact also other ABC transporters, which has been confirmed for ABC1 and ABCC2 (Wang et al. 2001; Sakaeda et al. 2002; Chen et al. 2005). One should have in

Fig. 8 Enhanced nuclear deposition of doxorubicin by simvastatin, verapamil or concanamycin A treatment. RD cells were grown on glass cover slides and incubated for 18 h in the absence (ctl) or presence of 10 µM simvastatin (sim), 15 µM verapamil (ver) or 50 nM concanamycin A (con) for 1 h. Such treated cells were subsequently exposed to 1 µM doxorubicin for an additional hour (a). Representative experiments are depicted and the bar indicates 10 µm. The intensity of the nuclear doxorubicin accumulation is plotted for the conditions described under (b). The bars represent the mean ± SD (n=55–69 cells). To resolve the kinetics of the nuclear doxorubicin accumulation, RD cells were preincubated for 20 min in the absence (filled circles) or presence of 10 µM simvastatin (open triangle) or a combination of 10 µM simvastatin plus 1 mM mevalonic acid (filled square) (c). The experiment was started by the addition of doxorubicin (1 µM) and pictures were taken every 2 min. Images were analysed with Metaflour® imaging software. The symbols represent the mean ± SEM (n=14–38)
mind that CYP3A4 substrates in many cases share also substrate specificity for ABCB1 (Gottesman et al. 2002; Szakacs et al. 2006). However, in the clinics, side effects due to interactions via ABC transporters are not described for statins (Corsini et al. 1999; Szakacs et al. 2006; Tamaki et al. 2011), although a polymorphism in the gene of the organic anion-transporting polypeptide (OATP1B1) has been attributable for elevated statin plasma levels and increased risk for myotoxicity (Link et al. 2008). Given the fact that tumour cells replicate faster than normal tissue, one can speculate that statins might have a greater impact on the expression of ABC transporters in tumour cells compared to normal cells.

In RD cells, simvastatin induced nuclear accumulation of doxorubicin, which is not related to inhibition of HMG-CoA reductase (Fig. 8). Thus, one has to postulate a novel drug target for simvastatin. In accordance with previous findings (Bogman et al. 2001; Wang et al. 2001; Sakaeda et al. 2002; Sieczkowski et al. 2010), we confirm that simvastatin is a potent inhibitor of ABCB1. This is corroborated by the following findings: (1) verapamil, a prototypical first generation ABCB1 inhibitor, also led to enhanced nuclear accumulation of doxorubicin. (2) Doxorubicin is a substrate of ABCB1 and confers doxorubicin resistance which is therefore of clinical relevance (Endicott and Ling 1989; Gottesman et al. 2002; Szakacs et al. 2006). Moreover, ABCB1 is expressed in RD cells and calcine efflux is inhibited by simvastatin (Fig. 6a). (3) Simvastatin is also efficient in another cellular system. In CCRFver1000 cells which stably express a defined number of ABCB1 transporters, daunorubicin and rhodamine123 fluxes were inhibited by simvastatin. (4) An alternative explanation might be provided by our recent observations on the glycosylation level of ABCB1 in the presence of simvastatin in human neuroblastoma cells (Sieczkowski et al. 2010). Simvastatin reduced the 170-kDa fully glycosylated species and thereby might impair the plasma membrane export of the transporter. A similar down-regulation is observed in RD cells upon simvastatin exposure times up to 48 h (Fig. 6a, b). Western blot analyses confirm a strong decline
of the 170-kDa ABCB1 band, but also of the 140-kDa core-
glycosylated species.

Interestingly, the lactone forms of statins are more potent to
inhibit P-glycoprotein, compared to their corresponding acid
forms (Bogman et al. 2001). For comparison, lovastatin in the
acid form with the open lactone ring exerts a greater potency in
pro-apoptotic action in cancer cell lines (Dimitroulakos et al.
2001). Pravastatin already exists in its acidic form and cannot
inhibit ABCB1 (Bogman et al. 2001; Wang et al. 2001).

Indirectly, these results imply that HMG-CoA reductase inhi-
bition is not a prerequisite for ABCB1 inhibition. This is
supported by our finding that the enhanced nuclear accumula-
tion of doxorubicin is not abrogated by the co-application of a
saturating concentration of mevalonic acid (Fig. 8c). Thus,
ABCB1 and other ABC transporters like ABCC1 and ABCC2
are confirmed to be new targets for statins (Bogman et al. 2001;
Wang et al. 2001; Chen et al. 2005; Szieczkowski et al. 2010).

Besides the efflux mechanism of ABC transporter, cellular
drug clearance may also involve sequestration to acidic com-
partments such as lysosomes, recycling endosomes, the trans-
Golgi network or secretory vesicles (Schindler et al. 1996;
Larsen et al. 2000). Indeed, a punctuated staining of doxorubi-
cin was detected in RD cells, which was attributed to lysosomes.
In contrast to nuclear deposition, simvastatin did not modulate the
sequestration of the weak base doxorubicin to this acidic com-
partment (Figs. 7 and 8). Conversely, destruction of lysosomes
by the V-type ATPase inhibitor concanamycin A resulted in a
significant elevation of nuclear doxorubicin (Fig. 7b). Remark-
ably, this finding highlights the importance of the lysosomal
drug clearance machinery of tumour cells. Concanamycin A was
also found to inhibit ABCB1, although in a concentration range
between 0.1 and 10 µM (Shalom et al. 1995). Thus, in our
experimental settings, 50 nM concanamycin A is assumed to
have no effect on the ABC transporter.

Nevertheless, the combination of simvastatin and doxorubi-
cin shows a significant increment in topoisomerase II
inhibition and DNA double-strand breaks (Fig. 9). Impor-
tantly, incubation with simvastatin alone had no effect on
topoisomerase II activity or the occurrence of DNA double-
strand breaks. This is again indicative for a synergistic
action of the two drugs and highlights the importance of
doxorubicin guidance into the nucleus by simvastatin.

Taken together, these data lead to the conclusion that
simvastatin enhances the nuclear deposition of doxorubicin
by direct inhibition of ABCB1 and on long term by down-
regulation of the transporter. Consequently, this effect trans-
lates into further inhibition of topoisomerase II and enhanced
DNA double-strand breaks. Interestingly, ABCB1 expression
assessed by immunohistochemistry was an adverse prognostic
factor in paediatric rhabdomyosarcoma patients (Chan et al.
1990). It remains to be elucidated whether statins can partially
overcome chemotherapy resistance in vivo, prevent relapses
and improve the overall outcome in rhabdomyosarcoma.

Acknowledgments The authors are indebted to Dr. Peter Ambros
(St. Anna Children’s Cancer Research Institute, Vienna, Austria) for
helpful discussion and critical reading of the manuscript and to Anton
Karel for technical assistance. The work was supported by grants from
the Herzfeldsche Familienstiftung and the Austrian Science
Foundation, FWF-Project P22385.

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Refractory Neuroblastoma Cells: Statins Target ATP Binding Cassette-Transporters

Evelyn Sieczkowski, Bihter Atıl, and Martin Hohenegger

Abstract
Statins lower plasma cholesterol and also its intermediates which play important roles in membrane integrity, cell signalling, protein synthesis and cell cycle progression. This pharmacological profile displays adjuvant chemotherapeutic potential and is currently a matter of debate and investigations. The overall success of statins in preventing cardiovascular events is only in part attributable to a decrease in low density lipoprotein (LDL)-cholesterol. Other effects like anti-proliferative and anti-inflammatory actions have been summarized as so called pleiotropic effects. The latter effects might only in part be related to inhibition of the HMG-(3-hydroxy-3-methylglutaryl) CoA reductase, thus other pleiotropic targets have to exist. Accumulating evidence exists that human tumor cells, in particular neuroblastoma cells are susceptible to statin induced apoptosis. Moreover, statins are capable to inhibit ATP binding cassette (ABC)-transporters which participate in multidrug resistance and chemotherapy resistance. Statins also have an impact on glycosylation of ABC-transporters and reduce the overall expression of prototypical transporters like P-glycoprotein (ABCB1). Based on these observations the hypothesis is elaborated, that statins may have adjuvant chemotherapeutic potential in cancer and in particular neuroblastoma.

Keywords • Statins • Cancer • ABC transporters • Neuroblastoma cells • Apoptosis • P-glycoprotein

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Introduction

Action of HMG-CoA Reductase Inhibitors

The 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors, commonly referred to as statins, are used successfully in the prevention and treatment of cardiovascular diseases (Corsini et al., 1999). They inhibit HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway (Goldstein and Brown, 1990). Thereby, statins deplete cells of mevalonate and subsequent products like ubiquinone, dolichol, farnesyl- and geranylgeranylpyrophosphate and cholesterol (Sinensky, 2000) (Fig. 17.1). Recent studies have shown that statins induce apoptosis in a wide range of different cell types, including skeletal muscle, leukemia, lymphoma, rhabdomyosarcoma and others (Dimitroulakos et al., 2001; Sacher et al., 2005; Werner et al., 2004). It is noteworthy to mention that farnesyl and geranylgeranyl transferase inhibitors have been tested as anti-cancer drugs in clinical trials, but results have not (yet) met the criteria for market approval (Caraglia et al., 2005). In contrast, statins are widely used in people and their human pharmacology is accordingly well understood and safe (Corsini et al., 1999). Similarly, their potential beneficial action in human cancer is starting to be widely appreciated (Brown, 2007; Demierre et al., 2005).

Pleiotropic Effects of Statins

The pleiotropic effects of statins may be divided into HMG-CoA-reductase-dependent and independent actions. The successive depletion of farnesyl pyrophosphate (15-carbon isoprenoid; FPP) or geranylgeranylpyrophosphate (20-carbon isoprenoid; GGPP) is clearly related to HMG-CoA-reductase inhibition (Fig. 17.1). Prenylation with GGPP is crucial for small G protein family members responsible for cell motility, in particular Rho, Rac and Cdc42. Rho A and C (but not Rho B) are implicated in transformation and metastasis (Caraglia et al., 2005; Zhong et al., 2005). Statins, like cerivastatin, lovastatin and simvastatin have been shown to reduce Rho A, for example in a breast cancer cell line and anaplastic thyroid cancer cells (Zhong et al., 2005).

An HMG-CoA-reductase independent target for statins has also been identified. The lymphocyte-function-associated antigen 1 (LFA1) which is important in migration and T cell activation is a direct binding partner for lovastatin (Weitz-Schmidt, 2003). Novel lovastatin analogues have been identified, which are more selective for the integrin LFA1. Not surprisingly, these derivatives do not inhibit the HMG-CoA-reductase. These immunomodulatory effects of statins have been recently summarized and expanded by beneficial clinical impacts on inflammation, nitric oxide synthesis and the coagulation cascade (Smaldone et al., 2009).

Taken together, these pleiotropic effects have led to a panel of diseases not related to elevation of LDL-cholesterol, but benefit from susceptibility to statin induced pleiotropic action. In particular diseases of the central nervous system, like ischemic and hemorrhagic stroke, Alzheimer disease, Parkinson disease, and multiple sclerosis have been shown to improve from statin application although the molecular mechanisms behind these observations are not fully understood (for review see Willey and Elkind, 2010).
Statins and Cancer

The efficacy of statins was evaluated in several hematological neoplasms, solid tumors of the prostate, advanced gastric cancer, hepatocellular cancer and central nervous system (Brown, 2007; Demierre et al., 2005; Kawata et al., 2001). In advanced hepatocellular cancer: pravastatin significantly increased the median survival to 18 months compared to 9 months in the control (Kawata et al., 2001). Importantly, pravastatin was combined with standard chemotherapy, i.e. with 5-fluorouracil. Similar results were recently obtained for pravastatin in acute myeloid leukemia and with simvastatin in relapsed myeloma patients. Again, in both cases statins were combined with conventional chemotherapy (for review see Jakobisiak and Golab, 2010). But also in the prevention of colon cancer statins have been discussed, in particular in combination with cyclooxygenase inhibitors. While evidence for a preventive role of statins in colon cancer is questionable, evaluation of statins is promising in adjuvant and neo-adjuvant chemotherapy as indicated by a recent phase II study (Brown, 2007; Jakobisiak and Golab, 2010).

Statins in Neuroblastoma Cells

The first description of efficacy of statins in neuroblastoma is provided by Maltese et al. (1985). In a murine neuroblastoma model lovastatin showed efficacy in vivo to suppressed tumor growth and on histological analysis tumors revealed marked cellular degeneration. However, this analysis was performed with very high lovastatin doses, i.e., 5 mg/kg/h, which translates into a human daily dose of more than 8 g. Obviously, this drug application exceeds the recommended human dosage 100-fold.

On cellular level anti-cancer efficacy was corroborated in neuroblastoma cells by the finding that clonogenic colony formation in soft agar is inhibited by lovastatin and reversed by co-administration mevalonate (Girgert et al., 1999). The human SH-SY5Y neuroblastoma cell line is successfully used in models investigating neuroprotective compounds, Parkinson’s disease, neuronal signal transduction and neuronal cell differentiation. When exposed to micromolar concentrations of simvastatin (Fig. 17.2a) or atorvastatin (data not shown) SH-SY5Y cells clearly change their morphology. The cells shrink and the nucleus is prominent compared to cytosol. The rounded cells have a tendency to detach from the surface especially at concentrations above 30 µM and incubation times of at least 24 h. These changes are indicative of apoptosis. This is further corroborated by a significant augmentation of annexin V staining in statin treated cells (Sieczkowski et al., 2010). We could confirm that simvastatin and atorvastatin trigger apoptosis by caspase 3 and 9 activation in time and concentration dependent manner (Sieczkowski et al., 2010).

Interestingly, there is a synergism between the combination of simvastatin with the anthracycline doxorubicin, which is used in the clinical treatment of neuroblastoma, but also rhabdomyosarcoma. Caspase 3 activation was significantly enhanced with a combination of simvastatin plus doxorubicin, compared to caspase activation by simvastatin or doxorubicin alone (Fig. 17.2b). Moreover, in RD rhabdomyosarcoma cells colony formation was also synergistically suppressed by the combination of simvastatin and doxorubicin (Sieczkowski et al., 2010; Werner et al., 2011). Clearly, the combinatorial effects of statins with the anthracycline doxorubicin are more than additively.

Statins and ATP-Binding Cassette (ABC) – Transporters

The development of multidrug resistance is a major problem throughout cancer chemotherapy. Drug efflux via ATP-binding cassette (ABC) transporters is the main mechanism responsible for resistance to chemotherapeutics. There is accumulating evidence from recent studies that HMG-CoA reductase inhibitors directly inhibit the ABC transporter, P-glycoprotein (ABCB1) (Bogman et al., 2001; Sieczkowski
Fig. 17.2 Statins effect on human SH-SY5Y neuroblastoma cells morphology and caspase 3 activation. Panel a: Morphological changes of undifferentiated SH-SY5Y cells upon simvastatin treatment for 24 h. Phase contrast images at a 100 magnification. Panel b: SH-SY5Y neuroblastoma cells were treated in the absence (control) and presence of 0.1 µM doxorubicin (Dox) or 1 µM simvastatin (Sim) for 24 and 48 h. Cytosolic fractions from cell lysates were used for a fluorescence based caspase 3 assay (Werner et al., 2004). Bars represent mean ± S.D. (n = 3). This over-additive effect has been found to be related to inhibition of ABC transporter proteins, but exceeds simple inhibition as observed with verapamil, a first generation inhibitor of ABC transporters (Sieczkowski et al., 2010). Moreover, the cytotoxic effect of statins has been previously linked to drug resistant P-glycoprotein expressing neuroblastoma cells (Dimitroulakos and Yeger, 1996).

Therefore, an interaction via this class of proteins is likely, because doxorubicin is a well known substrate for P-glycoprotein and MRP-1 (ABCC1). Overexpression of P-glycoprotein and MRP-1 is a leading cause of resistance to chemotherapy, which has been found to also correlate with TrkB and MYCN expression, which are of clinical importance in neuroblastoma (Blanc et al., 2003; Norris et al., 1997).

The ABC transporter activity in human SH-SY5Y neuroblastoma cells is clearly inhibited by statins. Indeed, simvastatin directly inhibited dye transport at equimolar concentrations of verapamil (Sieczkowski et al., 2010). Making use of the fluorescence behaviour of doxorubicin the accumulation of the anthracycline could also be monitored in real time confocal microscopy. The intracellular doxorubicin accumulation was immediately enhanced by statins in SH-SY5Y cells but also in a MYCN amplified neuroblastoma cell line STA-NB-10. This is a direct confirmation of immediate inhibition of transporters by statins.

However, beyond this direct inhibition statins exhibit an additional mode of action, which is
related to reduced glycosylation. As depicted in Fig. 17.1 dolichol is a side product in the mevalonate pathway, which might be reduced or depleted in a cell which is treated with statins. Dolichol represents the carrier of sugars needed for N-linked glycosylation of proteins (Marquardt and Denecke, 2003). Correct glycosylation is therefore a prerequisite for correct protein folding and protein transport to the plasma membrane. This is exemplified for the erythropoietin receptor, IGF-1 receptor and insulin receptor which need glycosylation not only for the correct topological placement, but also for proper function (Hamadmad and Hohl, 2007; Hwang and Frost, 1999).

Especially heavily glycosylated proteins might be affected by a depletion of dolichol and therefore respond to statins action more sensitive. The P-glycoprotein (ABCB1) appears as a fully glycosylated 180 kDa species and the core glycosylated 140 kDa species (Szakacs et al., 2006). Atorvastatin and simvastatin reduce the 180 kDa form of P-glycoprotein, but not verapamil. This shift to the core glycosylated species of P-glycoprotein has also functional implications. Enzymatic deglycosylation of the transporter by an application of N-glycosidase F is sufficient to enhance doxorubicin accumulation similar to the action of statins (Sicczkowski et al., 2010).

Finally, statin treatment also leads to a down-regulation of P-glycoprotein in neuroblastoma cells (Fig. 17.3). Already after 24 h incubation western blot analysis for P-glycoprotein from whole cell lysates show a marked reduction at concentration of 1–3 μM atorvastatin. Definitely, statins mechanism of action involves multiple hits on ABC transporter and therefore may be of clinical relevance, especially in conditions of multidrug resistance.

**Discussion**

ABC-transporters, also termed multidrug resistance (MDR) proteins, play a crucial role in drug efflux and clearance at the blood-brain barrier, bone marrow or blood-testis-barrier (Szakacs et al., 2006). The main function of these transporters is protection of the tissue and organ behind the barrier, and therefore it is beneficial. However, expression of these transporters in tumor-initiating cells and cancer stem cells is now believed to participate in tumor development, because of the barrier function which protects these cell types from chemotherapy (Fletcher et al., 2010). Moreover, ABC transporters are over-expressed in a wide range of tumors, as these are colon, kidney, adrenocortical or hepatocellular carcinomas.

To date, there are 13 ABC transporters associated with drug transport and drug resistance out of all known 49 transporters. Among them the P-glycoprotein and multidrug resistance associated protein 1 (MRP1, ABCC1) confer resistance to a broad range of chemotherapeutic drugs, such as vinca alkaloids, anthracyclines, taxanes and methotrexate (Szakacs et al., 2006).

In neuroblastoma, the prognostic marker MYCN enhances P-glycoprotein expression and there is emerging evidence that MYCN directly regulates also the expression of the MRP1 transporter (Blanc et al., 2003; Norris et al., 1997). MYCN oncogene amplification occurs in approximately 20% of the neuroblastoma and represents the best established clinical and biological marker which correlates with unfavourable prognosis.
Studies have shown that MRP1 expression correlates with poor clinical outcome and prognosis which is accompanied by MYCN amplification (Norris et al., 1997).

The 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors, statins, inhibit the rate limiting enzyme of the mevalonate pathway (Fig. 17.1) and thereby deplete cells of its downstream precursors and cholesterol. Due to their antiproliferative effect statins have been investigated in cancer cells and were found to induce apoptosis in the low micromolar concentration range (Jakobisiak and Golab, 2010; Minichsdörfer and Hohenegger, 2009; Sieczkowski et al., 2010; Werner et al., 2011).

A detailed concentration-response relationship of simvastatin and doxorubicin in human rhabdomyosarcoma and neuroblastoma cells revealed that the cytotoxicity of the anthracycline was more than additive (Sieczkowski et al., 2010; Werner et al., 2004) (Fig. 17.2b). Bogman et al. could show that statins like simvastatin, atorvastatin or lovastatin inhibit P-glycoprotein in high micromolar concentrations, while pravastatin is ineffective (Bogman et al., 2001). These data were confirmed by simvastatin and lovastatin induced inhibition of P-glycoprotein mediated daunorubicin and rhodamine 123 efflux.

Besides direct inhibition statins have an impact on glycosylation as this has been shown for proteins like the erythropoietin receptor, IGF-1 receptor and insulin receptor (Hamadmad and Hohl, 2007; Hwang and Frost, 1999). The mature fully glycosylated P-glycoprotein (~180 kDa) is shifted by simvastatin or atorvastatin to the 140 kDa core-glycosylated species. Importantly, such a shift in the molecular mass and motility behaviour of P-glycoprotein is only seen when the cells were exposed to statins for 24 h or longer. Impairment of endogenous glycosylation in the endoplasmic by statins is further corroborated by the fact that tunicamycin, inhibitor of protein glycosylation gave a similar P-glycoprotein patterns in Western blot analysis (Sieczkowski et al., 2010). Moreover, the overall expression of P-glycoprotein is reduced as depicted in Fig. 17.3. One possible explanation is provided by protein degradation. Reduced glycosylation of P-glycoprotein and possibly other ABC-transporters may impair the half life of the transporters and thereby lead to a successive down-regulation. Another explanation for the impaired glycosylation of the P-glycoprotein is provided by statins mechanism of action. As depicted in Fig. 17.1 inhibition of the HMG-CoA reductase may result in depletion from cellular dolichol, which is crucial for the initial step in glycosylation of proteins in the endoplasmic reticulum. However, further experimental evidence is required to support such assumptions.

Glycosylation of the P-glycoprotein is important for appropriate protein folding and plasma membrane export. Thus, on long term incubation with statins transporters like P-glycoprotein are reduced on the surface and thereby these cells might be more prone to conventional chemotherapy. Such a scenario is of particular interest in neuroblastoma. Transporter mediated multidrug resistance and amplification of MYCN correlate with poor prognosis for neuroblastoma patients. Moreover, MYCN is a transcription factor targeting the P-glycoprotein promotor and thereby evokes the protein up-regulation (Blanc et al., 2003). The fact that statins cytotoxic effect is restricted to P-glycoprotein expressing neuroblastoma cells further emphasizes the co-application of statins in patients who are refractory to chemotherapy (Dimitroulakos and Yeger, 1996). Due to the observation that statins not only inhibit P-glycoprotein, but also reduce expression of the transporter makes that compounds superior to other P-glycoprotein inhibitors. The accumulation of higher amounts of chemotherapeutic agents like doxorubicin may represent a new approach in the chemotherapy of refractory neuroblastoma and improve drug safety.

Acknowledgments This work was supported by project P22385 of the FWF-Austria to M.H.

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Abbreviations

ABC .................................................. Adenosine triphosphate (ATP)-binding cassette
ABCB1 ........................................ ATP-binding cassette subfamily B member 1
ABCC1 ........................................ ATP-binding cassette subfamily C member 1
ABCC2 ........................................ ATP-binding cassette subfamily C member 2
ABCC4 ........................................ ATP-binding cassette subfamily C member 4
ABCC6 ........................................ ATP-binding cassette subfamily C member 6
ABCG2 ........................................ ATP-binding cassette subfamily G member2
AFC ................................................. Ac-DEVD-7-amino-4-trifluoro-methylcoumarin
AFR .................................................. African region
ALK ............................................. Anaplastic lymphoma receptor tyrosine kinase
AMR .................................................. Region of Americas
ATRX ........................................... α-thalassaemia/mental retardation syndrome X-linked
B2M .................................................... β2 microglobulin
Bax .................................................. Bcl2-associated X
BiP .................................................. Binding immunoglobulin protein
BSA .................................................. Bovine serum albumine
CAD ................................................................. Coronary artery disease
CAR ............................................................... Constitutive androstan receptor
CDG ................................................................. Congenital diseases of glycosylation
CFP ................................................................. Cyan fluorescent protein
CHOP ........... CCAAT/enhancer-binding protein (C/EBP)-homologous protein
CNS ................................................................. Central nervous system
CTL ................................................................. Control
CVD ................................................................. Cardiovascular disease
DMEM ............................................................. Dulbecco’s modified Eagle’s medium
DTT ................................................................. Dithiothreitol
EDTA ............................................................... Ethylenediaminetetraacetic acid
EMR ................................................................. Eastern region
eNOS ............................................................... Endothelial nitric oxide synthase
ER ................................................................. Endoplasmic reticulum
ERAD .............................................................. Endoplasmic reticulum-associated degradation
EUR ............................................................... European region
FACS .............................................................. Fluorescence-activated cell sorting
FBS ................................................................. Fetal bovine serum
FDA ................................................................. US Food and Drug Administration
FPP .............................................................. Farnesyl-pyrophosphate
GAPDH ......................................................... Glyceraldehyde 3-phosphate dehydrogenase
GGPP .......................................................... Geranylgeranyl-pyrophosphate
GPP .............................................................. Geranly-pyrophosphate
GTPase ......................................................... Guanosine triphosphate hydrolase
HDL ............................................................ High-density lipoprotein
HDL-C ......................................................... High-density lipoprotein cholesterol
HEK-293 ...................................................... Human embryoid kidney-293
HEPES ......................................................... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA ..................................................... 3-hydroxy-3-methyl-glutaryl-Coenzyme A
HPLC ........................................................... High-performance liquid chromatography
HRP ............................................................. Horseradish peroxidase
IgG$_{2A}$ .......................................................... Immunglobulin G$_{2A}$
INPC ......................................................... The International Neuroblastoma Pathology Committee
INSS .......................................................... International Neuroblastoma Staging System
IPP ............................................................. Isopentenyl-5-pyrophosphate
JNK ............................................................ c-Jun N-terminal kinase
LDL ................................................................. Low-density lipoprotein
LDL-C ...................................................... Low-density lipoprotein cholesterol
LDL-R ...................................................... Low-density lipoprotein receptor
LLO .......................................................... Lipid-linked oligosaccharide
NBD ............................................................... Nucleotide binding domain
NF-κB ...................................................... Nuclear factor κ B
NO ................................................................. Nitric oxide
OATP ......................................................... Organic anion-transporting polypeptide
PBS .......................................................... Phosphate buffered saline
PCR ............................................................ Polymerase chain reaction
PHOX2B .................................................. Paired-like homeobox 2b
PI ................................................................. Propidium iodide
PI3K .......................................................... Phosphoinositid-3 kinase
PPAR-α ....................................................... Peroxisome proliferator-activated receptor α
PXR ............................................................. Pregnane X receptor
RD .............................................................. Rhabdomyosarcoma
RPLP0 ....................................................... 60S acidic ribosomal protein P0
RPS14 .......................................................... 40S ribosomal protein S14
SD ................................................................. Standard deviation
SDS ............................................................... Sodium dodecyl sulfate
SDS-PAGE .......... Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEAR ........................................ South East Asia region
SH-SY5Y ......................................................... Neuroblastoma
Sim ............................................................... Simvastatin
SLC ............................................................... Solute carrier
SMC .............................................................. Smooth muscle cell
SREBP-2 ........................................ Sterol response element-binding protein 2
TGF-β ................................................ Transforming growth factor β
TLC ............................................................... Thin layer chromatography
TMD ............................................................ Transmembrane domain
TR ............................................................... Thyroid hormone receptor
Tris .............................................................. 2-Amino-2-hydroxymethyl-propane-1,3-diol
TrkA .............................................................. Tyrosine kinase receptor A
TrkC .............................................................. Tyrosine kinase receptor C
UGT ......................... Uridine 5'-diphosphate-glucuronosyl transferase
VDR .............................................................. Vitamin D receptor
VEGFR ........................................ Vascular endothelial growth factor receptor
VLDL .............................................. Very low-density lipoprotein
WB ................................................ Western blot
WHO ............................................. World Health Organisation
WPR ............................................... Western Pacific region
YFP ............................................... Yellow fluorescent protein
ZnRD1 ........................................... Zinc ribbon domain containing protein 1
Acknowledgements


Mein besonderer Dank geht an Prof. Dr. Martin Hohenegger, dass er mir die Möglichkeit zu dieser Arbeit gegeben, mich in jedem Schritt dieser Arbeit betreut hat und die ganze Zeit an meiner Seite war. Genauso an all den anderen Professoren in der Institut, die uns immer das Gefühl gegeben haben, dass wir eine große „wissenschaftliche“ Familie sind. Natürlich möchte ich mich auch bei meinen PhD-Komitee-Mitgliedern Prof. Dr. Peter Chiba und Prof. Dr. Christian Nanoff für ihre wissenschaftliche Unterstützung und Ideen bedanken.

Ich danke all meinen FreundInnen und meiner ganzen Familie, die immer an mich geglaubt und mich immer unterstützt haben. Besonders meiner einzigartigen Mutter... Auf neue Abenteuer ✨

lix
1. Introduction

1.1. Cardiovascular diseases and cholesterol

Cardiovascular diseases (CVDs), including stroke, are still the leading cause of death globally. In 2008, approximately 17.3 million people died from CVDs, 7.3 million due to heart diseases and 6.2 million due to stroke (Alwan, 2011). Although tobacco use, insufficient physical activity, excessive alcohol use, unhealthy diet, and raised blood pressure play critical roles by development of heart and coronary artery diseases (CADs), raised serum cholesterol still constitutes the highest risk factor which is estimated to cause 2.6 million deaths each year (Alwan, 2011). Noteworthy, raised cholesterol level is a major problem especially in developing and developed countries (Meyer et al, 2001) (Figure 2).

Almost 20 years ago, Law et al. have shown that a 10% decrease in serum cholesterol in men led to a 20-50% reduction in heart disease events dependent on the age of patients within five years (Law et al, 1994). Since that observation, the relationship between cholesterol and ischaemic heart as well as CADs was deeply investigated.
In the early 1900s, the link between cholesterol-rich food and experimental atherosclerosis was described in rabbits by Russian researchers Anitschkow and Ignatowski (Ignatowski, 1909). Approximately 40 years later, Gofman and his colleagues got interested in previous works of Anitschkow. They performed several studies about lipid metabolism, cholesterol transport in blood and the clinical aspects of the link between lipoproteins and coronary heart diseases (CHDs) (Gofman & Lindgren, 1950).

Following these findings and supporting incidences of “lipid hypothesis”, Ancel Keys carried out in 1958 the first study in this field, called Seven Countries Study. This was the first cross-cultural comparison of CVD risk in men from Yugoslavia, Italy, Greece, Finland, Netherlands, USA and Japan, and has continued more than 50 years (Menotti et al, 2008). Several other studies were introduced in 1960s: The Paul Leren Oslo Study, The Wadsworth

Figure 2: Global prevalence of raised cholesterol levels in adults (WHO region and World Bank income group, 2008). AFR (African Region), AMR (Region of Americas), EMR (Eastern Region), EUR (European Region), SEAR (South-East Asia Region), WPR (Western Pacific Region).
Veterans Administration Hospital Study and The Finnish Mental Hospitals Study.

To the modern understanding of cholesterol research, Joseph L. Goldstein and Michael Brown have contributed with their studies and major publications during 1970s and 80s. Their work was mostly concentrated in characterisation of cholesterol metabolism, genetic basis of hypercholesterolemia and identification of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, which is a critical enzyme in cholesterol biosynthesis in the cell (Goldstein & Brown, 1977). Most importantly, they showed that elimination of low-density lipoprotein (LDL) from serum by increased LDL receptor (LDL-R) levels helps to fight against atherosclerosis and CHDs (Brown & Goldstein, 1981). Consequently, this collaboration of Goldstein and Brown led to the Nobel Prize in Physiology or Medicine in 1985.

In 1970s, the Coronary Primary Prevention Trial (1973) and the Framingham study (1977) have also proved that lowering of blood cholesterol reduces the risk of heart attacks in patients. Gordon et al. showed that high density lipoprotein cholesterol (HDL-C) is associated with the reduced incidence of CADs (Gordon et al, 1977). However, later studies have evaluated that only reduction of LDL-C levels cannot satisfyingly prevent atherosclerosis and CHDs, and that increasing levels of HDL-C are also associated with lower risk of these events (Di Angelantonio et al, 2009).

Based on these studies and findings, new targets could be identified to overcome dyslipidemia and further CVDs. The most prominent compounds of these established drug therapies are statins, niacin and fibrates. Statins are
very effective HMG-CoA reductase inhibitors and used to reduce elevated LDL-C levels. They are standard therapy in primary and secondary cardiovascular prevention. Niacin (nicotinic acid) is the oldest and most efficacious drug to raise HDL-C levels, whose effects were examined in detail by the Coronary Drug Project between 1965 and 1974 (Stamler, 1977). In addition to the raise of HDL-C levels, niacin therapy decreases elevated triglyceride levels. Fibrates (fibric acid derivates) are synthetic ligands for peroxisome proliferator-activated receptor α (PPAR-α). They are used to decrease serum LDL-C and elevated triglycerides, and are able to raise HDL cholesterol (Goldenberg et al, 2008).
1.2. Cholesterol metabolism and biosynthesis

Cholesterol is an essential structural membrane constituent of higher eukaryotes and facilitates membrane permability and fluidity (Myant, 1981). Accordingly, it is associated with function and trafficking of membrane proteins as well as transmembrane signal transduction. It also plays crucial role as the precursor molecule for the synthesis of steroid hormones, vitamin D and bile salts.

There are two sources of cellular cholesterol: dietary cholesterol and de novo synthesis within the body. In general, 900 mg cholesterol are required for human metabolim each day (Levy et al, 2007). Whereas 200-500 mg cholesterol were ingested by typical human diet, total cholesterol biosynthesis amounts up to 2000 mg per day (Cohen, 2008). The principal sites of cholesterol synthesis are the liver and the central nervous system (CNS).

**Figure 3:** Lipoprotein metabolism (Rader & Daugherty, 2008).
Cholesterol circulates as a component of lipoproteins (Figure 3), and its concentration is approximately 2.5-7.5 mmol/l in human (Mackay & Mensah, 2004). These lipoproteins comprise chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Durrington, 2007). Chylomicrons are secreted by enterocytes into the lacteals of the intestine and are found in the blood or lymphoid fluid. They consist of phospholipid, cholesterol and triglyceride complexed with Apo B_{48} (Kindel et al, 2010). After entering into the blood system, lipoprotein lipase located on the luminal site of the vascular endothelium of tissues hydrolyses the triglyceride of chylomicrons and forms cholesterol-rich remnants. Uptake of these particles by LDL receptor-like protein (LRP) pathway allows the transport of intestinal cholesterol to the liver (Cooper, 1997).

Another carrier lipoprotein is VLDL. It is assembled by cholesterol and triglyceride complexed with apolipoproteins, Apo B_{100} respectively (Olofsson et al, 2000). It enables cholesterol secretion from liver to the tissues. Similar to chylomicrons, removal of triglycerides by lipoprotein lipases results in formation of LDL (Charlton-Menys & Durrington, 2008). LDL is the major cholesterol-carrying lipoprotein in plasma. During circulation in blood, Apo B_{100} binds to LDL-R on the surface of hepatocytes. Following its receptor-mediated endocytosis and degradation in the cell, cholesterol is released into the cellular cholesterol pool (Nabel, 2003). In comparison, HDL regulates the transport of excess cholesterol arriving in the tissues back to the liver, called reverse cholesterol transport (Charlton-Menys & Durrington, 2008). HDL
receives this excess cholesterol and can transfer it to VLDL and further to LDL, from where it can complete its passage back to the liver by endocytosis.

Balancing external and internal cholesterol is a challenge for the cell in terms of cholesterol homeostasis. Mevalonate pathway is responsible for production of several isoprenoids, including cholesterol, which play crucial role in diverse cellular functions (Goldstein & Brown, 1990). As previously mentioned, Goldstein et al. have identified two enzymes for feedback regulation of mevalonate synthesis (Figure 4): HMG-CoA synthase and HMG-CoA reductase (Goldstein & Brown, 1990). This feedback activity is based on the level of LDL. These two enzymes get enhanced activated in the absence of LDL, thereby promoting mevalonate pathway.

**Figure 4:** The mevalonate pathway and its feedbacks dependent on LDL-R level (Goldstein & Brown, 1990).
Cholesterol biosynthesis in the cell occurs by mevalonate pathway. This begins with the condensation of two Acetyl-CoA molecules forming acetoacetyl-CoA and further HMG-CoA (Figure 5). Reduction of this molecule by NADPH to mevalonate is initiated by HMG-CoA reductase. Notably, this is the rate-limiting step in this process where statins target and inhibit further steps of mevalonate pathway. In next steps, mevalonate is converted further to mevalonic acid, mevalonate-5-phosphate, mevalonate-5-pyrophosphate, isopentenyl-5-pyrophosphate (IPP), dimethylallyl-PP, geranyl-PP (GPP), and farnesyl-PP (FPP). The endproducts of this pathway are ubiquinone, dolichol, geranyl-GPP (GGPP) and cholesterol.

**Ubiqinone**, also known as Coenzyme Q10, is presented in mitochondria and involved in electron transport chain and aerobic cellular respiration (Ernster
Dolichol is a lipid carrier in N-linked glycosylation of proteins in the endoplasmatic reticulum (ER) (Behrens & Leloir, 1970). The isoprenoid metabolites GGPP and FPP are responsible for posttranslational modification of several proteins which also play a role in tumorigenesis (Jackson et al, 1997). And finally, cholesterol is an important component in cellular membrane structure and integrity, and it also serves as precursor for the synthesis of steroid hormones and bile acid (Edwards & Ericssson, 1999).
1.3. Statins

As mentioned previously in section 1.1, increased cholesterol levels are related to an increased risk of CVD as a leading mortality cause in the world. Nowadays, more than 30 million people in the US and approximately 200 million people worldwide are using cholesterol-lowering drugs. Based on the obtainment of evidence that lowering total and LDL cholesterol can diminish this risk, researchers were interested in investigations to find a microbial metabolite which can inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Studies have shown that competitive inhibition of HMG-CoA reductase decreases hepatocellular cholesterol concentrations by activated translocation of sterol response element-binding protein 2 (SREBP-2) into the nucleus and leads to enhanced turnover of LDL-R (Briggs et al, 1993; Ness et al, 1996).

In 1950s and 1960s, many cholesterol-lowering agents like nicotinic acid (Altschul et al, 1955), cholestyramine (Tennent et al, 1960), neomycin (Goldsmith et al, 1960), plant sterols (Pollak, 1953) and many others were introduced into the clinics, however they showed severe side effects. After 2 years of investigation, in 1973 Akira Endo et al. have identified a component isolated from Penicillium citrinum, called mevastatin, which is a potent inhibitor of HMG-CoA reductase without showing any serious side effects (Endo et al, 1976a; Endo et al, 1976b). Following the clinical success and safety of this compound, several other mevastatin analogues were isolated and tested (Beck et al, 1990). To date, lovastatin, fluvastatin, pravastatin,
atorvastatin, rosuvastatin, pitavastatin, and simvastatin were approved by US Food and Drug Administration (FDA).

Statins are grouped in two types: natural or fungal-derived statins (lovastatin, simvastatin, pravastatin) and synthetic statins (atorvastatin, rosuvastatin, fluvastatin). These groups differ in their interaction with HMG-CoA reductase and in their lipophilicity as well as in their LDL-C-lowering activity (Weng et al, 2010). Although liver is the target organ for all statins, different solubility properties define their membrane transport ability and selectivity (Hamelin & Turgeon, 1998). Lovastatin, simvastatin, atorvastatin and fluvastatin are lipophilic, whereas pravastatin and rosuvastatin are hydrophilic (Figure 6). The lipophilicity of statins affects their passive diffusion through hepatocyte cell membranes. Thus, hydrophilic statins are more hepatoselective, and their uptake is carrier-mediated by ATP-binding cassette (ABC) and solute carrier (SLC) family (Hamelin & Turgeon, 1998). Additional to their different lipophilicity, lovastatin and simvastatin are administered as lactone pro-drugs.

Figure 6: Chemical structures of statins (Schachter, 2005).
which are first hydrolysed and converted in active form, whereas others are already in the active hydroxy acid form (Corsini et al, 1999).

From the view of structural chemistry, statins are constituted of an HMG-CoA analogue, the pharmacophore, and a ring system with different side groups responsible for their different solubility properties (Gazzerro et al, 2012) (Figure 6). Generally, synthetic statins are described as more efficient inhibitors because of their better interaction characteristics with HMG-CoA reductase. The absorption of statins occurs very rapidly reaching peak plasma concentration ($T_{\text{max}}$) within four hours, and the percentage is between 30% and 98%. However, it is postulated that race or ethnicity, food intake, concomitant diseases, age and sex are other factors that may affect statin metabolism (Gazzerro et al, 2012).

Statins enter systemic circulation through intestinal cells, and their metabolism in the liver is mediated by uridine 5’-diphospho(UDP)-glucuronosyl transferase (UGT) and enzymes of cytochrome P450 family: fluvastatin by CYP2C9 isoenzym; lovastatin, simvastatin and atorvastatin by CYP3A4 isoenzyme (Bottorff & Hansten, 2000) (Figure 7). Following the metabolism of statins, excretion proceeds from liver and kidney via the bile into the feces. Mainly, this elimination is mediated by ABC transporters like ABCB1, ABCC2 or ABCG2 (Gazzerro et al, 2012) (Figure 7).
In terms of clinical relevance of statins, recent reports of many randomized controlled trials and meta-analyses have shown that every 1 mM reduction of serum LDL-C accomplished by standard statin therapy of 20-40 mg/day led to a ~20% reduction of major CVDs in the 5-year period (Baigent et al, 2005). Expectedly, higher concentrations of statins enhanced further this effect (Baigent et al, 2010).

Figure 7: Pharmacokinetics of statins (copyright to PharmGKB) (Whirl-Carrillo et al, 2012).
1.3.1. Side effects of statins

Statins are generally well tolerated. However, they show rarely side effects. The most important clinical relevant problems are hepatotoxicity, myopathy, autoimmune reactions and liver failure (Black, 2002; Liu et al, 2010; Staffa et al, 2002). These adverse effects are mainly based on the excessive dosing or drug-drug interactions disturbing statin pathway. For example, drugs with higher affinity to CYP3A4 block statin binding to this isoenzyme leading to decreased metabolism and increased plasma level of statins, which further enhance the risk of statin-induced side effects (Jacobson, 2004).

Hepatotoxicity is mainly caused by increased levels of some components and enzymes in the serum, like bilirubin and alkaline phosphate, which are dose-dependent and occur with a frequency of 1-33% (Maron et al, 2000). Myotoxicity with myalgia and increased levels of serum creatine kinase arise in 0.5% of patients (Tobert, 1988), whereas rhabdomyolysis only in ~0.1% of patients (Farmer & Torre-Amione, 2000). Although the mechanism for toxicity is still not totally clear, it is hypothesized that these side effects are associated with membrane function, calcium regulation, effects on ubiquinone and drug-drug interactions.

One of these drug-drug interactions occurs with inhibitors of organic anion-transporting polypeptide (OATP) 1B1. Shitara et al. showed that inhibition of OATP1B1 is higher attributed by increased concentrations of cerivastatin rather than CYP3A4 or CYP2C8 inhibition (Shitara et al, 2003). In contrast, cyclosporine A increases the plasma levels of non-P450-mediated type of statins also via OATP1B1 (Launay-Vacher et al, 2005). At last, interactions
between statins and oral antidiabetic drugs (Ayalasomayajula et al, 2007; Bachmakov et al, 2008), coumarin anticoagulants (Hickmott et al, 2003), fibric acid compounds (Prueksaritanont et al, 2005) and others are also pronounced in the literature.

Moreover, several studies have shown that statin therapy elevated ubiquinone concentrations in humans (Laaksonen et al, 1996). On the other hand, statins also impact membrane physiology of skeletal muscles initiated by altered cholesterol content, membrane fluidity, membrane electrical properties (Pierno et al, 1995), Na/K pump density (Gray et al, 2000), excitation-contraction coupling (Pierno et al, 1999), and tyrosine phosphorylation in signal transduction (Mutoh et al, 1999).

1.3.2. Pleiotropic effects of statins

In addition to cholesterol-lowering effect, statins also exert pleiotropic effects. These are mainly based on the inhibition of numerous isoprenoid metabolites such as GGPP and FPP. Depletion of GGPP and FPP leads to altered posttranslational modification of cell signalling proteins, including small guanosine triphosphate hydrolase (GTPase) family members Ras, Rac and Rho (Chow, 2009). These proteins play key role in several cellular processes, which are also involved in tumorigenesis. Some of these effects can be defined as immunomodulation, anti-inflammation, anti-angiogenesis and anti-proliferation.
The most important effect of statins on immune system is impairment of T-cell activation and response. This is mainly based on the suppression of cytokine-induced maturation of dendritic cells because upregulation of costimulatory and/or adhesion molecules on lymphocytes is inhibited (Weitz-Schmidt, 2003; Yilmaz et al, 2004). Moreover, statins exhibit biphasic effects in a dose-dependent manner in terms of neovascularisation. Whereas low concentrations act proangiogenic by inducing phosphoinositid-3 kinase (PI3K)/Akt pathway and enhancing endothelial nitric oxide synthase (eNOS) activity (Kureishi et al, 2000), high doses are anti-angiogenic through RhoA-dependent inhibition of vascular endothelial growth factor receptor (VEGFR) (Park et al, 2002) (Figure 8). Reduction of LDL-C, upregulation of eNOS, production of nitric oxide (NO), activation of Akt as well as inhibition of Rho prenylation mediated by statins lead also to amelioration of endothelial dysfunction (Jarvisalo et al, 1999).

Another biphasic effect of statins was identified by smooth muscle cells (SMC). Whereas lipophilic statins sensitize SMCs to apoptotic inducers, hydrophilic ones seem to protect them from apoptosis (Makabe et al, 2010). Statins can promote the dedifferentiation of SMCs and so can upregulate the expression of calcium channels, which abolishes again the efficacy of calcium channel blockers (Clunn et al, 2010). Interestingly, studies have shown that statin users experienced Alzheimer disease 60% lower than in the total population and 73% lower than patients using other cardiovascular medications (Wolozin et al, 2000).
Pleiotropic effects of statins in term of cancer are still conflicting. They exhibit both increased and decreased cancer risk. Statins were speculated to stimulate transforming growth factor β (TGF-β) signalling induced by cholesterol reduction, and further to increase the protumor factors (Chen et al, 2008). Moreover, higher concentrations of lovastatin impacted mitosis by dysfunction of centromeres in various cell lines and enhanced so mutations and malignancies (Lamprecht et al, 1999). Other studies have shown proapoptotic and growth inhibiting effects of lovastatin by altered Rho geranylgeranylation (Bifulco, 2008). For example, lovastatin is able to upregulate the propaptotic proteins Bcl-2-associated X (Bax) and Bim, and to

Figure 8: Pleiotropic effects of statins (Gazzerro et al, 2012).
downregulate the anti-apoptotic protein Bcl2 in both haematological and solid tumors (Agarwal et al, 1999; Dimitroulakos et al, 2000). In breast cancer cells, statins suppress MEK/ERK pathway by reduced levels of nuclear factor κB (NF-κB) and decreased DNA binding of adapter protein 1. Moreover, they induce apoptosis via c-Jun N-terminal kinase (JNK) pathway (Koyuturk et al, 2007).

Another pleiotropic activity of statins is related to cell cycle and inhibition of proliferation (Figure 9). Statins are able to induce G1-S arrest by upregulation of p21^{WAF1/CIP1} and/or p27^{KIP1} in various cell lines like neuroblastoma, glioblastoma, adenocarcinoma, melanoma, mesothelioma, acute myeloid leukemia, breast cancer, gastric, pancreatic, and prostate carcinoma (Gazzerro et al, 2012).

![Figure 9: Effects of statins on cell cycle (Gazzerro et al, 2012).](image-url)
Some clinical investigations indicated that low levels of serum cholesterol might be associated with increased cancer risk and enhanced development of already present tumors (Kritchevsky & Kritchevsky, 1992). On the other hand, some human clinical trials produced no significant difference in tumor incidence by statin using patients (Coogan et al, 2007; Duncan et al, 2007; Ford et al, 2007). Although findings in this field are still controversial, recent studies have shown that statin use begun before a diagnosis might be associated with prevention of cancer or lower risk of cancer incidence. A very recent nationwide work indicated that statin usage led to a reduction of cancer-related mortality for 13 cancer types (Nielsen et al, 2012). As recently reviewed and summarized by Gazzerro et al., statins as adjuvant chemotherapy are able to potentiate the antitumor activities of some chemotherapeutics, including anthracyclines and tyrosine kinase inhibitors, in vitro as well as in vivo (Gazzerro et al, 2012). For instance, sensitizing effects of statins in clinical trials were identified in advanced cancer of the head, neck and cervix (Knox et al, 2005), in hepatocellular carcinoma (Graf et al, 2008) and in acute myeloid leukemia (AML) (Kornblau et al, 2007). In addition, statins have been shown to increase the radiosensitivity of glioblastoma, prostate and rectal cancer cells (He et al, 2012; Katz et al, 2005; Larner et al, 1998). Notably, all-cause mortality by statin users with cancer was reduced by 15% (Nielsen et al, 2012). Still, large scale and more detailed clinical trials are needed to clarify the potential of statins in oncology.
1.3.3. Dolichol and \(N\)-glycosylation of proteins

Dolichol, a downstream product of mevalonate pathway, is a lipid carrier for the glycan precursor, which is the key factor in \(N\)-linked glycosylation of proteins in the ER (Behrens & Leloir, 1970). Dolichol biosynthesis proceeds via generation of linear polymers by isoprenoids mainly on the cytoplasmic site of the endoplasmic reticulum (ER). Whereas the length of these lipids is variable and species-dependent, their configuration of the double bonds is fixed (Cantagrel & Lefeber, 2011). All tissues and almost all membranes in eukaryotic cells contain dolichol, however the amount is highly variable depending on tissue and age (Rip et al, 1985).

Dolichol phosphate as precursor is assembled in the ER. During \(N\)-linked glycosylation, dolichol phosphate was modified by addition of oligosaccharide which is initiated by GlcNAc-1-phosphotransferase (Figure 10). The elongated product, \(\text{Man}_5\text{GlcNAc}_2\)-P-P-Dol, is flipped to the inside of the ER, and three dolichol phosphates in the form of dolichol phosphate glucose (Dol-P-Glc) are used as sugar donor to complete the lipid-linked oligosaccharide (LLO) synthesis that this glycan can be transferred onto nascent proteins (Cantagrel & Lefeber, 2011).
\textit{N}-linked glycosylation occurs simultaneously with translation (Figure 11). Proteins entering ER are often modified by the addition of the glycan (GlcNAc$_2$-Man$_9$-Glc$_3$) to the amino group of Asn residues in the consensus Asn-X-Ser/Thr motif. This assembly was mediated by translocon-associated oligosaccharyl transferase (OST). Following removal of two terminal glucoses by glucosidases, monoglycosylated substrates are recognized by calnexin and calreticulin. After disulphide bond formation and folding process, the final glucose is removed, modified proteins are demannosylated and can exit the ER. Misfolded proteins are mainly targeted by ER-associated degradation (ERAD) complex, also called ER quality control, and eliminated from the

\textbf{Figure 10:} Dolichol phosphate elongation and \textit{N}-linked glycosylation (Copyright to Dept. Biol. Penn State, 2003).
glycosylation as well as protein trafficking cycle. They are imported back to the cytosol, rapidly ubiquitinated and degraded by proteasome (Vembar & Brodsky, 2008).

Several studies have shown that dolichol is involved in vesicle trafficking. Moreover, defects in glycosylation and dolichol biosynthesis cause various diseases, known as congenital disorders of glycosylation (CDG) (Cantagrel & Lefeber, 2011; Haeuptle & Hennet, 2009).

Figure 11: N-linked glycosylation of proteins (Vembar & Brodsky, 2008).
1.4. Cancer

Cancer is a disease characterized by uncontrolled growth of abnormal cells and their spreading into diverse organs (metastasis). It is one of the leading causes of death, and the incidence is increasing. By 2030, the global burden is expected to increase with 21.4 million new cancer cases and 13.2 million cancer deaths (Society, 2013). Alone in 2012, 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer within 5 year of diagnosis were stated (Cancer, 2012).

These statistics make cancer one of the most interesting diseases worldwide. Several researches and studies are performed and will be performed to increase our knowledge to fight against cancer. Though, last decades showed that the more we understand the mechanisms behind this disease, the more we confront its complexity.

In 2011, Hanahan et al. have updated their milestone review about hallmarks of cancer (Hanahan & Weinberg, 2011). They postulate ten hallmarks to be essential to develop tumor: sustaining proliferative signaling, resisting cell death, enabling replicative immortality, activating invasion and metastasis, evading growth suppressors, inducing angiogenesis, deregulating cellular energetics, genome instability and mutation, avoiding immune destruction and tumor-promoting inflammation. For the study presented here, we are mainly interested in resisting cell death of cancer cells.

Depending on the stage of disease, tumors can be classified into two groups: benign and malignant tumors. Benign tumors grow locally without
metastasis. They are mostly harmless to their hosts and can be easily removed via surgery. However they may cause clinical problems, for example when they alter hormone levels in the body. In contrast, malignant tumors exhibit cellular abnormalities, invasion and at last potential for metastasis. They grow in a much more disorganized form than benign tumors and can arise from diverse cell types throughout the body (Knowles & Selby, 2005).

Malignant tumors are classified in carcinoma (including squamous cell carcinoma and adenocarcinoma), sarcoma, leukemia, lymphoma, and neuroectodermal tumors.

1.4.1. Neuroblastoma

Neuroectodermal tumors are a major group of non-epithelial tumors causing 2.5% of cancer-related deaths and originated from various cellular components of the central and nervous system. They comprise glioma, glioblastoma, schwannoma, medulloblastoma and neuroblastoma.

Neuroblastoma is a rare malignant tumor of early childhood derived from primitive neural crest cells of the sympathetic nervous system. However, most neuroblastomas arise in the adrenal medulla or lumbar sympathetic ganglia (Cheung & Dyer, 2013). It accounts for approximately 8% of all cancer in children (Kaatsch, 2010). It grows very heterogeneously, and about 60% of patients have already metastasis in bone marrow, cortical bone, lymph nodes or liver at diagnosis (DuBois et al, 1999).
Several clinical studies investigated about the prognostic significance of many biologic and genetic abnormalities, however these mutations showed infrequency. Some oncogenes were identified, which might be involved in neuroblastoma progression, including MYCN, tyrosine kinase receptor A (TrkA), tyrosine kinase receptor C (TrkC), anaplastic lymphoma receptor tyrosine kinase (ALK), α-thalassaemia/mental retardation syndrome X-linked (ATRX) and paired-like homeobox 2b (PHOX2B).

First identified mutation in neuroblastoma was PHOX2B, which is involved in cell cycle exit and differentiation of neural crest-derived autonomic neurons (Raabe et al, 2008). Another very prominent marker of neuroblastoma is MYCN which regulates proliferation, growth, differentiation and survival of cells in the developing CNS (Grimmer & Weiss, 2006). MYCN amplification is one of the three strongest determinants of clinical outcome (Cohn et al, 2009). It has been identified in approximately 22% of tumors and indicates poor outcome. Another prominent oncogenic driver in neuroblastoma is ALK. Activating mutations or amplifications of this gene are associated with lethal disease, especially in the presence of MYCN amplification (Berry et al, 2012; Schulte et al, 2011). Moreover, mutations of ATRX lead to abnormal telomeres progression in neuroblastoma, which is one of the most important hallmarks of cancer for survival. Notably, high telomerase activity was identified in 30% of neuroblastomas at diagnosis and is predictive of reduced event-free survival and overall survival (Coco et al, 2012). Additional to these mutations, altered expression of multidrug resistance proteins like ABCB1, ABCC1 and ABCC4 has been also characterized in neuroblastoma (Norris et al, 2005). These are also prognostic markers of clinical outcome and may also
affect tumor progression through mechanisms other than drug efflux (Henderson et al, 2011).

Previously, neuroblastoma was classified according to International Neuroblastoma Staging System (INSS) in 4 stages (Table 1) (Brodeur et al, 1993). In 1999, Shimada et al. updated this classification system, in which degree of differentiation, the Schwannian stromal content and the mitotic-karyorrhexis index were regarded (Shimada et al, 1999) (Table 2). Moreover, the International Neuroblastoma Pathology Committee (INPC) has adapted and used further this classification system.

Neuroblastoma is a therapeutic challenge. These tumors require intensive combination therapy with chemotherapy, surgery and radiation, but their overall prognosis is still poor. Chemotherapy with combinations of cisplatin, doxorubicin, vincristine, cyclophosphamide, and etoposide is the most common treatment (Hara, 2012). Topoisomerase I inhibitors, topotecan and irinotecan have shown activity against neuroblastoma (Vassal et al, 2008). Additionally, myeloablative chemotherapy is associated with positive effects on neuroblastoma in phase III studies (Berthold et al, 2005). Another study has proven benefits of retinoid compounds on outcome of this disease (Reynolds et al, 2003). On the other hand, anti-GD2 antibodies are introduced as maintenance therapy, however the effect is dependent on the immune system (Cheung et al, 1998). Consequently, research and development of new therapies are still needed.
**Table 1:** International Neuroblastoma Staging System (INSS) (Brodeur et al, 1988).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Localized tumor with complete gross excision, with or without microscopic disease; representative ipsilateral lymph nodes negative for tumor microscopically</td>
</tr>
<tr>
<td>2A</td>
<td>Localized tumor with incomplete gross excision; representative ipsilateral non-adherent lymph nodes negative for tumor microscopically</td>
</tr>
<tr>
<td>2B</td>
<td>Localized tumor with or without complete gross excision; representative ipsilateral non-adherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically</td>
</tr>
<tr>
<td>3</td>
<td>Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement</td>
</tr>
<tr>
<td>4</td>
<td>Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow liver, skin and/or other organs</td>
</tr>
<tr>
<td>4S</td>
<td>Localized primary tumor (as defined for stage 1, 2A or 2B), with dissemination limited for skin, liver and/or bone marrow</td>
</tr>
</tbody>
</table>
Table 2 International Neuroblastoma Pathology Classification system (the Shimada system) (Shimada et al, 1999).

<table>
<thead>
<tr>
<th>International Neuroblastoma Pathology classification</th>
<th>Original Shimada classification</th>
<th>Prognostic group</th>
</tr>
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<tbody>
<tr>
<td><strong>Neuroblastoma</strong></td>
<td></td>
<td></td>
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<tr>
<td>Favorable</td>
<td>(Schwannian stroma-poor)</td>
<td></td>
</tr>
<tr>
<td>&lt; 1.5 yrs</td>
<td>Poorly differentiated or differentiating &amp; low or intermediate MKI tumor</td>
<td>Favorable</td>
</tr>
<tr>
<td>1.5-5 yrs</td>
<td>Differentiating &amp; low MKI tumor</td>
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<tr>
<td>Unfavorable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1.5 yrs</td>
<td>a) Undifferentiated tumor</td>
<td>Unfavorable</td>
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<tr>
<td></td>
<td>b) High MKI tumor</td>
<td></td>
</tr>
<tr>
<td>1.5-5 yrs</td>
<td>a) undifferentiated or poorly differentiated tumor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b) intermediate or high MKI tumor</td>
<td></td>
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<tr>
<td>≥ 5 yrs</td>
<td>All tumors</td>
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<tr>
<td><strong>Ganglioneuroblastoma</strong></td>
<td></td>
<td></td>
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<tr>
<td>Intermixed</td>
<td>(Schwannian stroma-rich)</td>
<td>Favorable</td>
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<tr>
<td></td>
<td>Intermixed (favourable)</td>
<td></td>
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<tr>
<td><strong>Ganglioneuroma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maturing</td>
<td>(Schwannian stroma-dominant)</td>
<td>Favorable</td>
</tr>
<tr>
<td></td>
<td>Well differentiated (favourable)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ganglioneuroma</td>
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<tr>
<td>Mature</td>
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<td></td>
</tr>
<tr>
<td><strong>Ganglioneuroblastoma</strong></td>
<td>(composite Schwannian stroma-rich/ stroma-dominate and stroma-poor)</td>
<td></td>
</tr>
<tr>
<td>Nodular</td>
<td>Stroma-rich nodular (unfavourable)</td>
<td>Unfavorable</td>
</tr>
</tbody>
</table>
1.4.2. Chemotherapy and multidrug resistance transporters

Several medical therapies are available to treat cancer depending on the type, location and stage of tumor. The most common cancer treatment options are surgery, chemotherapy and radiation therapy. Notably, chemotherapy is still the most asked method to fight against this disease.

The success of chemotherapy depends on many factors, whereas chemoresistance still constitutes the leading challenge. This is mainly caused by upregulation of ATP-binding cassette (ABC) transporters, which correlates with poor outcome. The ABC transporters are one of the largest families of the transmembrane proteins and comprise in humans at least 48 genes (Dean & Annilo, 2005). They export xenobiotics, lipids and metabolic products across the plasma and intracellular membranes by utilizing the energy of ATP hydrolysis (Fletcher et al, 2010) (Figure 12 and 13). The most prominent transporters of this family are ABC

**Figure 12:** ABC transporter-mediated drug efflux (Fletcher et al, 2010).
subfamily B member 1 (ABCB1; also known as P-glycoprotein, multidrug resistance protein 1 (MDR1)), ABC subfamily C member 1 (ABCC1, multidrug resistance-associated protein 1 (MRP1)) and ABC subfamily G member 2 (ABCG2, breast cancer resistance protein (BCRP)), which can transport a wide variety of chemotherapeutics (Fletcher et al, 2010).

From the structural view, ABC transporters possess at least four domains: two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). Whereas TMDs are responsible for specific ligand binding, NBDs possess characteristic Walker A and B motifs with the homology throughout all ABC subfamilies and play key role in ATP hydrolysis. The mechanism of action of these transporters is explained by “ATP-switch model” (Higgins & Linton, 2004).

According to this model, ligand binds first to the TMDs in the high-affinity open dimer and induces conformational changes of NBDs that affinity for ATP increases. Following binding of two ATP molecules, transporter changes conformation, forms closed dimer and translocates the ligand. Thereafter,
ATPs were hydrolysed. This triggers the dissolution of the closed NBD dimer that TMD conformation is again changed. To end the transport process, $P_i$ and ADP are released, and transporter gets back to the open dimer conformation (Figure 13).

ABC genes are organized as either full transporters containing two TMDs and two NBDs (including ABCA, ABCC and ABCD subfamilies, and some members of ABCB subfamily) or as half transporters containing one of each domain (like ABCD and ABCG subfamilies, and some members of ABCB subfamily) (Hyde et al, 1990). The half transporters, like ABCG2, assemble as either homodimers or heterodimers to build a functional transporter.

ABC transporters get most attention because of their role in the resistance against several compounds, including anthracyclines. This is mainly obtained by upregulation of such transporters. Although the most prominent ABC transporter is ABCB1, there are also other important transporters of ABC family which induce high level of resistance.

ABCC1, which is also known as multidrug resistance protein 1 (MRP1), is a full transporter. This transporter possess also a wide spectrum of compounds which can be transported by this protein, including glutathione conjugates of many toxic substrates (Dean et al, 2001). Although, similar to ABCB1, ABCC1 confers resistance to such toxic compounds, it is not essential for growth or development. Upregulation of this transporter is strongly associated with failure of chemotherapy in several types of cancer, including prostate cancer and neuroblastoma (Munoz et al, 2007).
ABCG2, a half transporter, was identified by the cell line analysis selected for high level resistance to mitoxantrone, a well known anthracycline (Miyake et al, 1999). Moreover, ABCG2 is able to export several dyes like rhodamine and Hoechst. ABCG2 is also called breast cancer resistance protein (BCRP) because of its important function in chemoresistance of breast cancer cells.

ABCB1 was first identified in 1976 in Chinese hamster ovary cell mutants (Juliano & Ling, 1976). Henceforth, ABCB1 was distinguished to be involved in several cellular processes, like hormone distribution, cell differentiation, proliferation, immune response and programmed cell death (Pavek et al, 2002). From the structural view, ABCB1 possess two similar halves, each half with a transmembrane domain consisting of six α-helical membrane spanning domains and a nucleotide binding domain (Kvackajova-Kisucka et al, 2001). Like all the other ABC transporters, ABCB1 is posttranslationally modified by N-glycosylation at asparagines 91, 94 and 99 (Gribar et al, 2000). Although glycosylation was not found to affect its drug transport activity (Seres et al, 2011), several studies have shown its importance for protein quality control in the ER (Loo & Clarke, 1998) and protein trafficking to the plasma membrane (Schinkel et al, 1993). Noteworthy, inhibition of glycosylation of ABCB1 induced increased sensitivity to different drugs (Hiss et al, 2007), whereas the hypoglycosylation contributed to the appearance of multidrug resistance (Gervasoni et al, 1991).

ABCB1 substrates have shown common features: i) They are relatively high hydrophobic; ii) they have at least one tertiary basic nitrogen atom on the drug molecule in deprotonized state at neutral pH; iii) their molecular size is
lower than 1300 g/mol; and iv) their structure is flexible with different structural conformations (Breier et al, 2000; Wang et al, 2003). Moreover, the pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D receptor (VDR), peroxisome proliferator activated receptors (PPARs), zinc ribbon domain containing protein 1 (ZnRD1), NF-κB and the thyroid hormone receptor (TR) were identified as transcriptional regulator and modulator of ABCB1 (Bentires-Alj et al, 2003; Chan et al, 2011; Harmsen et al, 2010; Hong et al, 2006; Saeki et al, 2011). Therefore, ABCB1 can also be up- or downregulated independent on ABCB1 substrate upon a ligand of PXR or other mentioned receptors.

Based on the induced chemoresistance by upregulation of especially ABCB1, several ABCB1 inhibitors were developed. In the 1980s, first generation inhibitors were introduced: verapamil and cyclosporine A. They were combined with conventional chemotherapeutics. However because of their high toxicity and unpredictable pharmacokinetic interactions, they could not be used so widely in the clinics. Second generation ABCB1 inhibitors such as dexverapamil and valspodar, which were synthesized from these lead compounds of first generation, are more potent and less toxic (Krishna & Mayer, 2000). Unexpectedly, these compounds were also disappointing and did not improve survival (Fracasso et al, 2001; Friedenberg et al, 2006). Finally, third generation inhibitors tariquidar, elacridar and others were synthesized using structure-activity relationships and combinatorial chemistry (Krishna & Mayer, 2000). These molecules showed enhanced specific binding to ABCB1 and do not interact with other ABC transporters. However, again these compounds exhibited high toxicity and many side
effects that they cannot be further used in the trials without demonstrating any improvement in the therapeutic efficacy (Cripe et al, 2010). Because of these disappointing and unsuccessful trials with ABCB1 inhibitors, it has been postulated that downregulation or inhibition of compensatory upregulation of this transporter may represent a novel pharmacological access to this problem.
2. **Aims of This Thesis**

Our previous studies showed that statins can reduce ABCB1 expression and decelerate its transporter activity in addition to apoptosis induction in several tumor cell lines (Minichsdorfer & Hohenegger, 2009; Sieczkowski et al, 2010; Werner et al, 2013; Werner et al, 2004). In this study, we are interested to explain the mode of action of simvastatin on neuroblastoma cell line SH-SY5Y. First, we wanted to identify if the downregulation and inhibition of ABCB1 could be achieved in clinically relevant concentrations at protein as well as RNA level. Second, in the literature described compensatory upregulation of other ABC transporters might also be affected by simvastatin exposure. Third, impaired glycosylation of ABCB1 could generate reduced expression and decreased activity of ABCB1 on the plasma membrane, and endogenous dolichol levels might play a role in this phenomenon that protein trafficking in the ER is impacted. At last, all the anticancer activities of simvastatin should also be reflected in murine xenograft models.
3. Results

3.1. Simvastatin downregulates ABCB1 expression

Our previous studies have proven that statins are able to downregulate ABCB1. However, these significant effects were achieved with a simvastatin concentration of 10 µM in rhabdomyosarcoma (RD) cells, in neuroblastoma cell lines STA-NB10 and in SH-SY5Y (Sieczkowski et al, 2010; Werner et al, 2013). However, these concentrations are very high to administer in the clinics. By using clinically more relevant concentrations and different methods, we wanted to identify the expression of ABCB1 following simvastatin treatment in neuroblastoma cells in more detail.

SH-SY5Y cells were treated with several concentrations of simvastatin for 48 hours. Based on the fact, that ABCB1 is a transmembrane protein and performs its activity on plasma membrane, membrane fractions were isolated following to simvastatin exposure and used for SDS-PAGE and Western blot (WB) analysis. ABCB1 expression was tested against C219 antibody, which is able to bind both ATP-binding regions of ABCB1 (Georges et al, 1991). ABCB1 expression after simvastatin exposure was compared to that of untreated SH-SY5Y cells, and α-Tubulin was used as loading control.

In addition to our previous observations, we here confirm again a significant reduction of ABCB1 protein expression in membranes from simvastatin-treated neuroblastoma cells already at a clinically relevant concentration of 0.1 µM after 48 hours (Figure 14). This effect was also found after 1 µM simvastatin treatment. Interestingly, this downregulation does not seem to
be concentration-dependent. However, to investigate dose-dependency of simvastatin effects more simvastatin concentrations should be included and examined.

**Figure 14:** WB analysis of simvastatin-treated (Sim) SH-SY5Y cells. Following 48 hours exposure, proteins were isolated, and membrane fractions were tested against C219 antibody and α-Tubulin as loading control (A). In panel B, signal intensities were identified in ImageJ software and illustrated as graph (0 µM: n=4; 0.1 µM: n=3; 1 µM: n= 4) (mean ± SD). Significance is calculated with one-way ANOVA with Holm-Sidak method.

To prove this finding, we performed another experimental approach: flow cytometry. For this assay, same conditions as in WB analysis were used with additional exposure times and concentrations. Following to simvastatin administration for 6 to 72 hours, live SH-SY5Y cells were washed and mapped with MRK16 antibody and then with a corresponding Alexa®488-conjugated antibody (Figure 15). MRK16 antibody is a well known monoclonal antibody recognizing a discontinuous extracellular epitope on the ABCB1 (Georges et
al, 1993). As negative control, unmapped and with IgG$_{2A}$-mapped SH-SY5Y cells were used, and ABCB1 expression was compared on live SH-SY5Y cells with or without simvastatin treatment.

Here, we observed again a significant reduction of ABCB1 expression at the plasma membrane of SH-SY5Y cells. This effect was detected with all concentrations (1 or 3 µM) and at all time points (from 6 to 72 hours), however only 3 µM simvastatin exposure showed a clear trend of the concentration dependency (Figure 15B and C). Noteworthy, already clinically used concentration of simvastatin (0.1 µM) induced a reduction of ABCB1 expression at the plasma membrane after 48 hours (Figure 15A).

Performing two independent experimental approaches allowed us to prove that simvastatin is able to decrease ABCB1 expression. As previously mentioned in the introduction, ABCB1 is a transmembrane protein and mainly localized on the plasma membrane of the cells. Reduced expression of ABCB1 on the plasma membrane could also be initiated by enhanced internalisation of this transporter. Therefore, we were interested to differ between enhanced internalisation and/or downregulation of ABCB1.

Hence, total ABCB1 expression was quantified in SH-SY5Y neuroblastoma cells. First, FACS analyses were performed after simvastatin exposure for 6 hours (Figure 16). For this purpose, cells were washed, fixed and permabilized following drug treatment. Fixing and permeabilization steps allow antibodies to access and map also intracellularly expressed ABCB1, not only these on plasma membrane. Fixed and permeabilized cells were mapped with C219 or p170 antibody alternatively (another intracellular binding
monoclonal antibody for ABCB1) and then with a corresponding Alexa®488-conjugated antibody. Again, as negative control unmapped and with IgG2A-mapped SH-SY5Y SY5Y cells were used after fixation and permeabilization steps. By using two different but both intracellular recognizing antibodies and IgG2A-mapped controls, we were able to eliminate unspecific antibody binding falsifying

**Figure 15:** FACS analysis using live SH-SY5Y cells exposed to several concentrations of simvastatin (Sim) for 6, 24, 48 or 72 hours. As a sample, panel A represents fluorescence signals corresponding ABCB1 expression at the plasma membrane (unmapped cells: Neg CTL, control: CTL). Time (B) and concentration (C) dependency of simvastatin are also depicted. All values are presented in mean ± SD and tested for significance using one-way ANOVA with Holm-Sidak test (6h: CTL n= 12, 1 µM n= 9, 3 µM n= 10; 24h: CTL n= 11, 1 µM n= 6, 3 µM n= 4; 48h: CTL n= 19, 1 µM n= 12, 3 µM n= 13; 72h: CTL n= 9, 1 µM n= 6, 3 µM n= 3).
results and to compare same conditions with two different antibodies in whole cell.

These FACS analyses showed a significant reduction of ABCB1 expression in whole cell already after 6 hours. Noteworthy, a significant effect was only observed with a treatment of 3 µM simvastatin, and here we were able to detect some dose-dependency.

Second, we made use another experimental approach in a different cellular system: Transfection of HEK-293 cells with YFP-ABCB1-pcDNA3 (Figure 17). HEK-293 cells were transfected with YFP-ABCB1-pcDNA3 by using TurboFect (Thermo Scientific; Massachusetts, USA) and were led to recover for 48 hours. Following simvastatin treatment for another 48 hours to several concentrations (0.1, 1 and 3 µM), they were washed, resuspended in PBS and lysed by repeated heat-shock and vortex steps. YFP fluorescence corresponding ABCB1 expression was determined using a fluorescence spectrophotometer. As positive control, transfected and doxorubicin-treated

Figure 16: FACS analysis with SH-SY5Y cells exposed to different concentrations of simvastatin for 6 hours. Cells were fixed and permeabilized to map ABCB1 with C219 or p170 antibody in whole cell (0 µM n= 10, 1 µM n= 5, 3 µM n= 7). All values are presented in mean ± SD, and the significance is calculated using one-way ANOVA with Holm-Sidak test (ns: not significant).
HEK-293 cells were used to detect the upregulation of ABCB1. As negative controls, untransfected, with empty pcDNA3 construct transfected as well as with YFP-ABCB1-pcDNA3 transfected but cycloheximide-exposed cells were quantified. Cycloheximide is a general inhibitor of transcription. Based on this fact, we expected that ABCB1 expression is negatively altered in comparison to transfected and untreated cells.

Figure 17: Quantification of ABCB1 expression with heterologously YFP-tagged ABCB1 expressing HEK-293 cells following 48 hours treatment. After drug exposure, cells were lysed, and YFP fluorescence corresponding ABCB1 expression was quantified using a fluorescence spectrophotometer. Cycloheximide (CHX) as general inhibitor of transcription was used as negative control, and doxorubicin (Dox) as positive control for upregulated ABCB1. All these samples, including simvastatin-treated cells, were compared to untreated cells (CTL). All values are presented in mean ± SEM (n= 3) and tested for significance using one-way ANOVA test with Holm-Sidak method. (*: p < 0.05).

Again, simvastatin induced a reduction of YFP signal corresponding ABCB1 expression in a concentration-dependent manner, whereas a significant effect was only achieved with 3 µM simvastatin. As expected, cycloheximide inhibited ABCB1 transcription that YFP signal was significantly decreased. Noteworthy, cotreatment with simvastatin could not additionally affect and further reduce ABCB1 expression in cycloheximide-exposed HEK-293 cells.
This indicates that cycloheximide concentration alone was high enough to totally prevent transcription process in the cell.

In summary, simvastatin treatment at clinically relevant concentrations has reduced significantly ABCB1 expression on plasma membrane in a time- and concentration-dependent manner. However, further experiments have shown that this effect was not restricted only on plasma membrane, but rather in whole cell. Findings from this part of our work let us conclude that reduced expression of ABCB1 might be based on the downregulation of this transporter, and not only by the enhanced internalisation initiated by simvastatin treatment.
3.2. Compensation of ABCB1 by other ABC transporters

Several studies have reported compensation of ABCB1 expression by other ABC transporters (Vautier et al, 2009). Moreover, we were interested to compare our previous findings from protein-based analyses, to the possible alterations at RNA level. To verify this, first we performed experiments to quantify expression of ABCB1 and several other ABC transporters at the RNA level.

SH-SY5Y cells were treated for 72 hours with several concentrations of simvastatin, and RNA was isolated. Following isolation, mRNAs were reverse-transcribed in cDNA and used for quantitative-PCR (q-PCR). q-PCR was performed with ABCB1, ABCC1, ABCC6 and ABCG2 primers. For normalization, four control genes were included in the assay: GAPDH, B2M, RPLP0 and RPS14 (Figure 18).

Figure 18: q-PCR using cDNA of reverse-transcribed mRNA isolated from simvastatin-exposed SH-SY5Y for 72 hours. (0 µM / CTL: n=6; 1 µM: n= 3; 10 µM: n= 2). These cDNA samples were tested against ABCB1, ABCC1, ABCC6 and ABCG2, and normalized to GAPDH, B2M, RPLP0 and RPS14. All values are presented in mean ± SD and tested for significance using one-way ANOVA test with Holm-Sidak method. (*: p < 0.05; **: p < 0.005; ***: p < 0.001).
Our analyses showed that simvastatin led to a significant downregulation of ABCB1 at RNA level, which was also found on ABCC1, ABCC6 and ABCG2. It is worth mentioning that already lowest concentration of simvastatin (1 µM) reduced significantly mRNA of ABCB1 and ABCC1. However, this could not be detected by ABCC6. Consequently, compensation by ABCC1, ABCC6 and ABCG2 described in the literature was in our experiments not indicative for this appearance.

To follow the compensatory regulation of other ABC transporters, similar conditions were applied for protein assays (Figure 19). SH-SY5Y cells were exposed to several concentrations of simvastatin for 48 or 72 hours. Following treatment, cells were washed and whole cell lysates were isolated. These fractions were used in SDS-PAGE and further in WB analysis. They were tested against ABCC1, ABCC4, ABCG2 and as loading control against β actin (Figure 19).

![Figure 19: WB analysis using whole cell lysates isolated from simvastatin-treated (Sim) SH-SY5Y cells for 48 or 72 hours. Whole cell lysates were tested against ABCC1, ABCC4, ABCG2 and as loading control against β actin. All WB analyses were performed independently.](image-url)

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In contrast to previous results, we could not detect a downregulation at protein level. Although ABCC1 expression was lightly decreased only after 72 hours, ABCC4 expression was definitely upregulated. Interestingly, this effect was already observed at 1 µM simvastatin after 48 hours, and did not enhance by increasing concentration. Additionally, a slight upregulation of ABCG2 was also detected, however after 72 hours simvastatin exposure.

In summary, possible compensation activity of other ABC transporters could be partly detected only at protein level, whereas at RNA level all transporters were significantly downregulated. This appearance could be explained by increased protein stability of other ABC transporters and decreased ABCB1 expression initiated by simvastatin treatment.
3.3. **Simvastatin impacts also transporter activity of ABCB1**

Based on the previous findings at protein and mRNA level of ABCB1, we postulated that it should also be reflected in its decreased function. For this purpose, we made use rhodamine 123, which is a well known ABCB1 substrate and widely used to quantify transporter activity (Mairinger et al, 2012; Parveen et al, 2011), and flow cytometry, where we could continuously monitor and follow fluorescent rhodamine 123 efflux mediated by ABCB1.

SH-SY5Y cells were exposed to several concentrations of simvastatin for 48 hours. In the literature, several studies have reported the direct inhibition of ABCB1 by statins (Goard et al, 2010; Sieczkowski et al, 2010; Werner et al, 2013). To avoid this effect, live cells were extensively washed, loaded with ABCB1 substrate rhodamine 123 for 30 minutes (Tamaki et al, 2011), and the efflux of this compound was continuously monitored by FACS with a robust signal from $10^6$ events within 5 minutes (Figure 20). To normalize the obtained signals to the ABCB1 proteins already present on the plasma membrane, live SH-SY5Y cells were mapped with MRK16 antibody and then with a corresponding Alexa®488-conjugated antibody. Again as negative control, live unmapped and with IgG$_{2A}$-mapped SH-SY5Y cells were used.

These experiments showed that already lowest concentration of simvastatin (0.1 µM) decelerated rhodamine 123 efflux which is established by many studies to be mainly mediated by ABCB1 (Figure 20A). Moreover, the amplitude of rhodamine 123 efflux was decreased (Figure 20B), and half lives
were prolonged by simvastatin treatment in a concentration-dependent manner (Figure 20C). Both impacts were significant and indicate that simvastatin is not only able to downregulate ABCB1 but also able to diminish its transporter activity.

Here it is important to remember again that signals obtained from FACS analysis were normalized to the number of ABCB1 protein expressed on the plasma membrane. The results presented here shows rhodamine 123 efflux activity per ABCB1 protein meaning that decelerated transport of ABCB1 substrate does not correlate and cannot be explained by downregulation of this transporter.
**Figure 20:** Kinetic measurements of ABCB1 transport activity in SH-SY5Y cells exposed to simvastatin (Sim) for 48 hours. The efflux of rhodamine 123 was quantified using FACS and monitored for 5 minutes. In panel A, all data points represent rhodamine efflux as exponential decay curves following global curve fitting by SigmaPlot software ($k > 0.992$). The amplitudes (B) were significantly decreased, whereas half lives ($t_{1/2}$) (C) were prolonged in a concentration-dependent manner (0 µM / CTL: n= 7; 0.1 µM: n= 4; 1 µM: n= 6; 3 µM: n= 4). All values are presented in mean ± SD, and the significance is calculated using one-way ANOVA with Holm-Sidak test (ns: not significant).
3.4. ABCB1 localisation is altered by simvastatin exposure

In addition to downregulation of ABCB1, we were also interested in the localisation of ABCB1 following simvastatin exposure. For this purpose, we made use of a plasmid construct with CFP-tagged ABCB1 and confocal microscopy. Thus, live cells with fluorescent ABCB1 can be analysed, and the localisation of this protein can be identified.

First, SH-SY5Y cells were transfected with CFP-ABCB1-pcDNA3 construct by using FuGene® HD (Promega; Madison, USA). After recovery for 48 hours, heterologously CFP-ABCB1 expressing cells were treated for 1.5 to 72 hours with various concentrations of simvastatin. Cells were washed, fresh medium was added and live cell imaging was performed using confocal microscopy LSM510 (Carl Zeiss; Jena, Germany) (Figure 22).

Images were analysed offline using ImageJ software. ABCB1 expression on the plasma membrane as well as in the cytoplasm was calculated (Figure 23). For analysis, the images were converted to 32 bit format, and the filter Rainbow RGB was used to make signal strength visible. Then, a line through the cells was drawn randomly. Signals of each pixel on the line were calculated using plot profile in ImageJ, and they were presented in arbitrary units (AUC) (Figure 21). For each condition, 10-20 cells were analysed, and ABCB1 expression was compared to that of untreated cells.

Figure 22 represents seven different exposure times with three different simvastatin concentrations. Our analyses have shown that simvastatin
exposure impaired ABCB1 protein trafficking that the localisation of this transporter in the cytoplasm was much more pronounced with increasing concentrations and in a time-dependent manner. Although only high concentrations (3 and 10 µM simvastatin) led to an increased internalisation of ABCB1 at short-time treatments (1.5, 3, 4.5 and 6 hours), already 1 µM simvastatin disturbed ABCB1 protein trafficking after long exposure times.

Our quantification analysis clearly shows that in normal conditions ~80% of ABCB1 is localized at the plasma membrane, and only ~20% in the cytoplasm (Figure 23). However, simvastatin exposure changed this appearance dramatically. Interestingly, this effect was already observed at all concentrations after 1.5 hours, which became much more relevant with increasing treatment times and concentrations of simvastatin. In comparison, at 24 hours cells seem to be less affected from simvastatin treatment that ABCB1 internalisation was not so obvious in comparison to these after 1.5, 3, 4.5 or 6 hours. Nevertheless, simvastatin exposure for 48 and 72 hours reduced again ABCB1 expression at plasma membrane up to 40% in a concentration-dependent manner. Consequently, these images might indicate another phenomenon of statins in their mode of action: short-(direct inhibition of ABCB1) and long-term effects (downregulation and altered protein trafficking).

In addition to determination of ABCB1 localisation, confocal microscopy images also allow us to detect the morphological changes of cells due to drug exposure. These images confirm again apoptosis induction by statins in tumor cells, which is also reported in the literature for many tumor cells.
Here, we were able to notice that cells shrunk and built apoptotic bodies dependent on simvastatin concentrations and exposure times. Moreover, this might also explain peculiar time dependency that “simvastatin-resistant” cells, which overcame apoptosis and were detected in higher concentrations or after long exposure times, exhibits reduced ABCB1 internalisation.

**Figure 21:** Densitometric quantification of CFP-fluorescence signals obtained from confocal microscopy. Images were analysed offline using ImageJ software. CFP signal (green in first panel) was converted using Rainbow RGB filter (second panel) in intensity coded pseudocolours. Then, a line through the cell was drawn randomly. This line helped us to identify the signals of each pixel (in AUC) on that line crossing through the plasma membrane as well as the intracellular area. These two big peaks demonstrated in the graph correspond the signal at the plasma membrane, and the area between them cytoplasm. Moreover, this process was repeated with different lines on each cell captured using confocal microscopy.
Figure 22: Live cell imaging of CFP-ABCB1-pcDNA3 transfected SH-SY5Y cells using confocal microscopy LSM510 following simvastatin (Sim) exposure for several time points. (untreated cells: CTL).
**Figure 23:** The quantification and analysis of CFP signals from live cell imaging by confocal microscopy. These analyses were performed using ImageJ software as mentioned in results and as demonstrated above in Figure 21. In panel A, ABCB1-CFP fluorescence at the plasma membrane was determined following simvastatin (Sim) short-term (left panel) or long-term (right panel) exposure, whereas ABCB1 expression in the cytoplasm was demonstrated in panel B.
3.5. **Simvastatin effects involved in endogenous dolichol level**

Statins are HMG-CoA inhibitors blocking further steps of mevalonate pathway. As mentioned before, these also comprise endproducts which cause cholesterol-independent effects in the cell. Dolichol phosphate is such a downstream product of the mevalonate pathway and critically required for glycoprotein biosynthesis (Behrens & Leloir, 1970). Relating to this, our previous studies have shown that simvastatin impacts glycosylation of proteins (Sieczkowski et al, 2010). Based on this assumption, we were interested in further determination of dolichol dependency of statin effects.

3.5.1. **Simvastatin depletes cells from endogenous dolichol**

SH-SY5Y cells were exposed to several concentrations of simvastatin for 48 hours. Following treatment, cells were extensively washed, and lipid extraction was performed. Lipid extracts were pre-separated by thin layer chromatography (TLC), and silica gel of area of interest with dolichol was scratched. Again, lipids from this area were isolated, and extracts were injected into HPLC to quantify endogenous dolichol. As standard, dolichol C_{80-105} isolated from bovine heart was used (Figure 24A). All the peak areas corresponding endogenous dolichol were added up and normalized to protein concentration of samples. Moreover, endogenous dolichol levels of simvastatin-exposed SH-SY5Y cells were compared to this of untreated cells.
Expectedly, endogenous dolichol levels were significantly reduced by simvastatin in a concentration-dependent manner (Figure 24B). Noteworthy, a significant decrease of endogenous dolichol was observed already at a clinically relevant concentration of 0.1 µM (Figure 24C and 24D).

Based on the fact that dolichol is the key component in the glycosylation process of proteins, this significant dolichol depletion of cells by simvastatin might directly impact ABCB1 trafficking in the cell, which mainly explains enhanced localization of ABCB1 in the cytoplasm by simvastatin treatment (see Section 3.4).

3.5.2. Dolichol addition abolishes simvastatin-induced glycosylation defects

Previously, we have observed a shift from the fully-glycosylated form (170 kDa) to the immature, core-glycosylated form (140 kDa) of ABCB1 in glycosylation and WB analyses with statin-exposed samples (Sieczkowski et al, 2010). This observation might be related to our finding of dolichol depletion of cells by simvastatin treatment. Based on the fact that dolichol plays a crucial role in the glycosylation process of proteins, we performed add-back assays by exogenous addition of dolichol C$_{80-105}$.

To examine this, SH-SY5Y cells were treated with simvastatin for 24 hours in absence or presence of dolichol C$_{80-105}$ (Figure 25). Following treatment cells were washed, lysed, and whole cell extracts were collected for SDS-PAGE and WB analysis. Lysates were tested against C219 antibody, and as loading
Figure 24: HPLC analysis of endogenous dolichol after simvastatin (Sim) exposure for 48 hours. Dolichol C\textsubscript{80-105} isolated from bovine heart was used as standard (A). Endogenous dolichol isolated from untreated (CTL) (C) and simvastatin exposed (D) SH-SY5Y cells were determined by HPLC. In panel B, all samples exposed to different concentrations of simvastatin were summarized. All values are presented in mean ± SD (n= 5), and the significance is tested using one-way ANOVA with Holm-Sidak test.
control α-Tubulin was used (Figure 25).

Again, simvastatin treatment decreased ABCB1 expression in general, and led to a slight shift to the immature form of ABCB1 (140 kDa) in comparison to untreated SH-SY5Y cell. Worth mentioning, dolichol alone led to intensify the ABCB1 band indicating the full saturation of glycosylation pattern. Indeed, the addition of dolichol restored the glycosylation pattern of ABCB1 which was impacted by simvastatin exposure. However, 5 µM dolichol in combination with 3 µM simvastatin was less effective to prevent this altered glycosylation of ABCB1 in comparison to coadministration of 1 µM dolichol and 3 µM simvastatin. This can be explained by higher concentrations of DMSO (0.6%) in drugs leading to cytotoxicity in the cell. Consequently, these data confirm that statin-altered glycosylation is mainly based on the depletion of endogenous dolichol levels.

**Figure 25:** Prevention of simvastatin (Sim)-induced glycosylation alterations of ABCB1 by addition of dolichol. Cells were exposed to simvastatin (Sim) in the absence or presence of dolichol C80-105 for 24 hours. Using C219 antibody, ABCB1 expression in the whole cell was determined in WB analysis (A). α-Tubulin was used as loading control. Intensities of these signals were quantified in ImageJ software (B). (untreated cells: CTL)
3.5.3. Exogenous dolichol restores apoptosis triggered by simvastatin

Defects in glycosylation process of proteins seem to be caused by dolichol depletion raised by simvastatin treatment. Based on our previous works and the literature, we know that statins are able to trigger apoptosis (Minichsdorfer & Hohenegger, 2009; Sieczkowski et al, 2010; Wasinger et al, 2014; Werner et al, 2004), which may be partially induced via ER stress because of this dolichol depletion established in previous results.

For this purpose, highly sensitive upregulation of typical ER stress markers binding immunoglobulin protein (BiP, also called GRP78) and CCAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP) was tested on mRNA level (Figure 26). SH-SY5Y cells were exposed to simvastatin for 48 hours. RNA from these cells was isolated, reverse-transcribed in cDNA, and PCR was performed. Expression of these two genes was compared to that of untreated cells. As control gene, GAPDH was used.

Our analyses have shown that simvastatin treatment for 48 hours triggered significantly upregulation of both genes, corresponding induced ER stress. Moreover, this was already obtained at lowest concentration (0.1 µM) of simvastatin. Interestingly, we could not identify any concentration-dependency in this experimental approach. However to identify this, more concentrations should be introduced. It seems that already 0.1 µM simvastatin is sufficient to induce ER stress, and this effect was not further enhanced by 1 µM simvastatin.
Based on the fact that ER stress and dolichol are important factors in simvastatin-induced apoptosis, we were interested to test if dolichol coadministration is able to prevent apoptosis mediated by statins.

First, we made use fluorometric caspase 3 assay (Figure 27A). SH-SY5Y cells were treated with several concentrations of simvastatin for 48 hours in the absence or presence of dolichol C_{80-105}. Then, cells were washed, lysed to collect cytoplasmic fractions and used in fluorometric caspase 3 assay. As expected, caspase 3 activity, which is a prominent marker for apoptosis induction, was dramatically enhanced following simvastatin exposure in a concentration-dependent manner. Moreover, this increase was significantly abolished and inhibited by coadministration of dolichol, again in a concentration-dependent manner.

Second, apoptosis induction was determined by FACS analysis with Annexin V/PI-staining. Annexin V/PI-staining is a widely used experimental approach for detection of apoptosis that binding of Annexin V/PI corresponds apoptosis induction in the cell (Figure 27B). SH-SY5Y cells were treated with
same conditions as in the caspase assay performed before. All cells (live and apoptotic) were washed, stained with Annexin V/PI and analysed using FACS. Only Annexin V-positive cells were considered as pre-apoptotic, whereas Annexin V- and PI-positive cells were identified as dead. Unstained and only Annexin V- or PI-stained cells were used as controls.

Likely to previous findings from fluorometric caspase 3 assay, simvastatin alone led to increased number of apoptotic cells in a concentration-dependent manner. Moreover, increasing concentrations of dolichol C_{80-105} significantly prevented apoptosis induction mediated by simvastatin.

In conclusion, two independent experimental approaches have proven that add-back of dolichol was able to prevent apoptosis induction by simvastatin. Thus, these findings together with depletion of endogenous dolichol confirm that dolichol dependency of simvastatin plays a crucial role in the mode of action of this HMG-CoA reductase inhibitor.
Figure 27: Prevention of simvastatin-induced apoptosis by dolichol C_{80-105} coadministration on SH-SY5Y cells after 48 hours. In panel A, caspase 3 activity was quantified using fluorometric measurements. As a second method, the number of Annexin V/PI-positive cells, corresponding apoptotic cells, were detected using FACS analysis (B). In both experiments, simvastatin-induced apoptosis was inhibited by dolichol coadministration. All these experiments were repeated and performed independently using different samples. All values are presented in mean ± SD (n= 5), and the significance is tested using one-way ANOVA with Holm-Sidak test (*: p < 0.05; **: p < 0.01; ***: p < 0.005).
3.6. **Murine xenograft model**

Our *in vitro* experiments confirm that simvastatin exhibits several activities like downregulation as well as impaired trafficking of ABCB1 and induction of apoptosis. Moreover, we have proved that these effects are at least partly based on the altered dolichol levels in the cell. In order to confirm these findings and to identify the relevance of our results, we performed *in vivo* experiments using murine xenograft model. 8 CD-1 Nu/Nu mice were inoculated with SH-SY5Y neuroblastoma cells (1 x 10^7 cells) on their right and left flanks, and simvastatin was applied orally (4.25 mg/kg/day) only to 4 mice. The other 4 mice did not got simvastatin and used as control group. In both groups, one tumor was not detectable. The animals were controlled every day, and their weight and tumor size were measured every second day. They were sacrificed by neck dislocation due to critical tumor size or end of the experiment (day 61). Isolated organs and tumors were shock-frozen or fixed, and used for further analysis.

3.6.1. **Simvastatin reduces tumor growth**

As mentioned before, weight and tumor progression of mice were examined every second day. Tumor volumes were calculated and normalized to the body weight of mice on that day. Based on these measurements, control group (untreated mice) was compared to simvastatin-treated animals in their body mass and tumor weight (Figure 28). First, no significant difference in body mass as well as in organ weights was detected between these two
groups (Figure 28A, Table 3). Tumor weight in simvastatin-administered mice was slightly diminished, although this effect was not significant (Figure 28B). These analyses have shown that tumor progression was abolished over time and obviously decelerated after 3 weeks of simvastatin treatment (Figure 28C).

**Table 3**: Analysis of the murine neuroblastoma xenograft models. Post mortem, the organs of four mice in each group are presented here as mean ± SD and tested with Students t-test (ns: not significant).

<table>
<thead>
<tr>
<th>Organs</th>
<th>Control (g)</th>
<th>Simvastatin (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (n= 4)</td>
<td>1.49 ± 0.14</td>
<td>1.59 ± 0.24 (ns)</td>
</tr>
<tr>
<td>Heart (n= 4)</td>
<td>0.14 ± 0.02</td>
<td>0.13 ± 0.01 (ns)</td>
</tr>
<tr>
<td>Spleen (n= 4)</td>
<td>0.19 ± 0.06</td>
<td>0.18 ± 0.05 (ns)</td>
</tr>
<tr>
<td>Kidney (n= 8)</td>
<td>0.53 ± 0.2</td>
<td>0.52 ± 0.1 (ns)</td>
</tr>
<tr>
<td>Lung (n= 4)</td>
<td>0.21 ± 0.05</td>
<td>0.2 ± 0.02 (ns)</td>
</tr>
</tbody>
</table>

In summary of this first part, we can conclude based on the unchanged body mass and organ weights that simvastatin treatment did not affect mice’s overall welfare. However, simvastatin administration impacted tumor progression and finally tumor weight measured post mortem. Expectedly,
mice with statin therapy developed smaller tumors, and their tumor progression was also diminished in comparison to control group.

**Figure 28:** SH-SY5Y inoculated murine xenograft model on their both flanks. 4 mice with each two SH-SY5Y inoculations were used as control (CTL) as well as simvastatin (Sim) group. Simvastatin treatment did not change body mass of mice (A). In panel B, simvastatin effects on tumor weight is demonstrated. In addition, the comparison of tumor progression exhibited decelerated tumor progression in simvastatin group (C). All values are presented in mean ± SD and tested for significance using Students t-test (ns: not significant).
3.6.2. **ABCB1 is downregulated by simvastatin also *in vivo***

Our *in vitro* analyses from previous sections have proven that statins impact ABCB1 expression as well as its glycosylation in SH-SY5Y neuroblastoma cells. As mentioned before, ABCB1 possess mature (170 kDa) and immature (140 kDa) form, where simvastatin led to a shift from mature to immature form indicating altered glycosylation of ABCB1.

We were interested to verify these results in our murine xenograft model. Noteworthy, it is described in the literature that simvastatin reaches the highest concentrations in the liver (Gazzerro et al, 2012). Therefore, we made use for these analyses tumor as well as liver samples.

Small tumor as well as liver pieces were extensively washed, homogenized and lysed. Following isolation of membrane fractions, proteins were separated by SDS-PAGE and used further in WB analysis. They were tested against C219 antibody, and β-actin was used as loading control (Figure 29A).

Similar to our *in vitro* analyses, membrane extracts from liver as well as tumor samples have shown that ABCB1 was reduced in the simvastatin-treated group compared to control group. However, this effect was only significant in liver tissues (Figure 29B). Moreover, in all control samples higher band (170 kDa) corresponding the mature form ABCB1 was prominent, although simvastatin treatment initiated a significant reduction of this form. This observation was made in both liver and tumor samples. Consequently, these data confirm our *in vitro* results and their relevance *in vivo* in terms of ABCB1 regulation as well as its glycosylation.
Figure 29: WB analysis using membrane fractions of liver and neuroblastoma. Post mortem, membrane fractions of tissue from liver and neuroblastoma were probed for ABCB1 using C219 antibody (A). Each lane represents a different animal. The intensities were analysed offline using ImageJ software (B). All values are presented here as mean ± SD and tested with Students t-test.
3.6.3. Simvastatin triggers apoptosis in tumor

As described before, statins are able to induce apoptosis in several cell lines (Minichsdorfer & Hohenegger, 2009; Sieczkowski et al, 2010; Wasinger et al, 2014; Werner et al, 2013; Werner et al, 2004). Additionally, in this study we were also able to show in two different and independent experimental approaches that simvastatin induces apoptosis in SH-SY5Y neuroblastoma cells, and this effect can be prevented by addition of dolichol (see Section 3.5.3). Here, we were interested to reflect this activity in vivo, in our murine xenograft model (Figure 30).

For this purpose, we used cytoplasmic fractions extracted from tumor. They were separated by SDS-PAGE, and WB analysis was performed. Cytoplasmic proteins were probed against PARP. PARP cleavage is a prominent indicator for induction of apoptosis and therefore widely used to detect apoptosis in cells. This can be easily detected in WB analysis that uncleaved PARP have a size of 110 kDa representing the upper band. However, if PARP is cleaved following apoptotic stress, another product will be visible in addition to uncleaved PARP, namely cleaved PARP with a size of 89 kDa representing the lower band. As loading control, α-Tubulin was used (Figure 30A).

Expectedly, in control samples no relevant PARP cleavage was detected. However, in simvastatin group PARP cleavage was significantly higher indicating apoptosis induction in tumor cells (Figure 30B). Thus, we can conclude that apoptosis triggered by simvastatin in our xenograft model is another evidence that the effect of statins are not only relevant for in vitro analysis, but also in vivo.
Figure 30: WB analysis using cytoplasmic fraction of neuroblastoma. Post mortem, cytoplasmic fractions isolated from neuroblastoma tissues were probed for cleaved and uncleaved PARP (A). Each lane represents a different animal. The intensities were analysed offline using ImageJ software (B). All values are presented here as mean ± SD (n=5) and tested with Student's t-test.
4. Discussion

4.1. General discussion

Chemoresistance is still one of the most difficulties in the treatment of several cancer types and results in the treatment failure in over 90% of patients with metastatic disease. There are several mechanisms leading to decreased effectiveness of chemotherapy: drug activation and inactivation, alterations in drug target, DNA methylation, processing of drug-induced damage, evasion of apoptosis and accelerated drug efflux (Wilson et al, 2006).

However, chemoresistance initiated by enhanced drug efflux is mostly triggered by upregulation of ABC transporters, which is directly correlated with poor outcome (Gottesman et al, 2002). These transporters are ATP-dependent efflux transporters. They protect different tissues from a variety of endogenous and exogenous substances, including a wide range of structurally unrelated cytotoxic compounds, also many anticancer drugs. Moreover, they are also responsible in several processes in the body, like regulation of hormone distribution, cell differentiation, proliferation, immune responses and apoptosis (Pavek et al, 2002).

Three well known members of this transporter family were predominantly focused and investigated about their role in drug resistance: ABCB1 (P-glycoprotein, P-gp), ABCC1 (MRP1) and ABCG2 (BCRP). The most prominent and most studied one among these transporters is ABCB1. It was hypothesized that drug resistance could be overcome by inhibiting this
transporter. However, several clinical trials with disappointing results concluded that this “P-gp hypothesis” might be incorrect or inefficient to overcome chemoresistance (Tamaki et al, 2011). Therefore, recent investigations are mainly focused in the regulation of ABCB1. Several receptors and other factors were identified to play an important role in the regulation of ABCB1. Some of these components are the transcription factor NF-κB or nuclear receptors, like PXR or PPARγ (Bentires-Alj et al, 2003; Chan et al, 2011; Harmsen et al, 2010; Zhou et al, 2009). This proves again the complexity of chemoresistance that not only ABCB1 substrates are able to up- or downregulate this transporter, but also other ligands or inducers.

Statins, HMG-CoA reductase inhibitors, are well established and safely used compounds in the treatment of hypercholesterolemia. It has been proven that they also possess pleiotropic effects (Gazzerro et al, 2012). However, antitumor activities caught most attention in pharmacological studies and oncological researches. Some investigations have shown that low levels of serum cholesterol might be associated with increased cancer risk and enhanced development of already present tumors (Kritchevsky & Kritchevsky, 1992). On the other hand, some human clinical trials produced no significant difference in tumor incidence by statin using patients (Gazzerro et al, 2012). Although findings in this field are still controversial and conflicting, recent studies proved that statin use begun before a cancer diagnosis might be associated with prevention of cancer or lower risk of cancer incidence (Ahern et al, 2011; Boudreau et al, 2010). Moreover, a very recent nationwide work indicated that statin use led to a reduction of cancer-related mortality for 13 cancer types, and all-cause mortality by statin users
with cancer was reduced by 15% (Nielsen et al, 2012). As recently reviewed and summarized by Gazzerro et al., statins as adjuvant therapy are able to potentiate the antitumor activities of some chemotherapeutics, including anthracyclines and tyrosine kinase inhibitors, in vitro as well as in vivo (Gazzerro et al, 2012). Still, large scale and more prospective clinical trials are needed to clarify the potential of statins as adjuvant chemotherapy.

Based on this relationship of this HMG-CoA reductase inhibitor and cancer, our previous studies have confirmed that statins are able to induce apoptosis and inhibit ABCB1 in melanoma, neuroblastoma and rhabdomyosarcoma cells (Atil et al, 2015; Minichsdorfer & Hohenegger, 2009; Sieczkowski et al, 2010; Wasinger et al, 2014; Werner et al, 2013; Werner et al, 2004). To examine clinical evidence of this approach, here we expanded these findings by observations in SH-SY5Y neuroblastoma cells and identified that already clinical relevant concentrations (0.1 µM) of simvastatin was effective to downregulate ABCB1 expression on plasma membranes after 48 hours (Figure 14). Notably, higher concentrations of simvastatin decelerated the expression of ABCB1 on plasma membrane already as well as in whole cell already after 6 hours (Figure 15 and 16). This finding with such low concentrations is important and makes statins a promising candidate to use in the clinics against chemoresistance without any side effects.

Statins exhibited their regulatory activity also at RNA level. Here, we tested also other ABC transporters because of their compensatory actions by inhibition or downregulation of ABCB1 described previously in the literature (Vautier et al, 2009). Hence, we found that ABCB1 with other ABC
transporters (ABCC1, ABCC6 and ABCG2) were significantly downregulated following simvastatin treatment (Figure 18), which was also found in WB analyses against ABCC1, whereas ABCG2 and ABCC4 were upregulated (Figure 19).

Consequently, all these effects on protein and RNA level were, as expected, mostly in a time- and concentration-dependent manner. Worth mentioning, these observations at protein level could be reproduced by different experimental approaches and could be mirrored also in HEK293 cells and indicate that downregulation of ABCB1 mediated by statins could also be adjusted in an experimentally transformed cell system (Figure 17).

To understand the underlying mechanisms for this downregulation, we should pay attention to the regulation of ABCB1 and targets as well as effects of statins in the cell. ABCB1 expression can be regulated posttranslationally or transcriptionally. Many reports suggest that certain signalling pathways are involved in the regulation of ABCB1. By posttranslational regulations, the most important pathways are Mitogen-Activated Protein Kinase (MAPK) signalling and ubiquitin-proteasomal degradation. Moreover, ABCB1 expression can be also affected by trafficking and recycling as well as stability and glycosylation, phosphorylation or ubiquitination of this protein (Katayama, 2014).

The study from Katayama et al. showed that inhibition of ERK pathway, one of the three MAPK signaling pathways responsible for cell growth, by inhibitors of MEK and HSP90 is able to downregulate the ABCB1 expression, whereas other inhibitors of PI3K and p38 MAPK were not effective to the
regulation of ABCB1 (Katayama et al, 2007). However, some other studies reported that the ABCB1 expression can be reduced by activation or inhibition p38 MAPK (Guo et al, 2008; Lu et al, 2013).

On the other hand, the JNK pathway is also reported to be involved in the regulation of the promoter of the MDR1 gene (Liu et al, 2008). In another study, a cyclosporine analogue PSC833, downregulated the expression of MDR1 gene by activating JNK/c-Jun/AP-1 and suppressing nuclear factor kappa B (NF-κB) (Bark & Choi, 2010), although some other groups demonstrated that AP-1 is the gene activator of MDR-1 (Chen et al, 2014; Guo et al, 2008). In addition, another study has shown that ABCB1 expression can be increased with the upregulation of phosphorylated ERK1/2 (Maitra & Hamilton, 2005). However, there are also some findings that MDR1 gene expression is increased by downstream transcription factors in the ERK pathway (Shen et al, 2011; Tomiyasu et al, 2013; Zhao et al, 2013). Thus, these factors are able to dually regulate ABCB1 expression at both the transcriptional and posttranscriptional levels.

NF-κB proteins play an important role in controlling both innate and adaptive immunity. Activation of NF-κB in cancer cells by chemotherapy impair the effectiveness of chemotherapeutics, and inhibition of this factor induces apoptosis (Baldwin, 2001). Bentires-Alj et al. showed that upregulation of NF-κB results in enhanced ABCB1-mediated chemoresistance (Bentires-Alj et al, 2003). Sequences for NF-κB and many other transcription factors have been identified in the promoter region of the MDR1 gene. AP-1 and NF-κB among these factors have been shown to bind directly to the promoter region of this
gene (Barancik et al, 2001; Bark & Choi, 2010; Chen et al, 2014; Guo et al, 2008; Liu et al, 2008; Thevenod et al, 2000). Kanagasabai et al. have shown that heat shock factor 1 and heat shock protein 27 are able to suppress MDR1 mRNA expression through inactivation of NF-κB (Kanagasabai et al, 2011). Furthermore, statins have been shown by different groups that they are able to inhibit NF-κB activation and further this inflammatory pathway (Koyuturk et al, 2007; Shen et al, 2010). Consequently, this inhibition mediated by statins can explain the downregulation of ABCB1 and decelerated multidrug resistance in cancer cells, which might be based on the inhibition of NF-κB.

In contrast, statins increase expression and activation of PPARs (PPARα and PPARγ), a nuclear hormone receptor superfamily, in several cell lines as well as in vivo (Qin et al, 2010; Shen et al, 2010; Yano et al, 2007). Martin et al. has shown that inhibition of Rho signaling pathway following statin incubation decreases its transcriptional activity (Martin et al, 2001). Moreover, a recent study supposed that activation of PPARγ in SGC7901/VCR cells (a gastric cancer cell line selected by vincristine) reversed ABCB1-mediated multidrug resistance by downregulation at both mRNA and protein levels in a dose-dependent manner (Chen et al, 2010). Although the mechanism of PPARγ activation downregulating ABCB1 is still unknown, PPARγ ligands have been shown to markedly inhibit NF-κB expression in some cell lines suggesting to regulate target gene expression (Chen et al, 2002; Gupta et al, 2001).

In terms of its function, reduced expression on the plasma membrane would mean enhanced accumulation of ABCB1 substrates with less administered
drug concentrations, in other words reduced chemoresistance. As mentioned above, chemoresistance plays the key role in failure of cancer therapy. Because all ABCB1 inhibitors generated in several generations have shown severe side effects and because they alone could not really overcome chemoresistance, reduced expression of ABCB1 expression on plasma membrane could make statins good candidates in adjuvant therapy. At this point, some studies have already shown that statins are able to interact with ABCB1 and to inhibit it directly (Bogman et al, 2001; Wang et al, 2001). Moreover, many ABC transporters like ABCC2, ABCG2 and ABCB1 have been identified to be involved in statin transport (Chen et al, 2005; Huang et al, 2006).

To characterize the effects of simvastatin on transport activity of ABCB1, we examined efflux rates of the ABCB1 substrate rhodamine 123 by flow cytometry (Figure 20). The half life of rhodamine efflux was significantly augmented, and less substrate was exported from SH-SY5Y cells. Importantly, these significant effects were obtained already at a clinical relevant concentration of simvastatin (0.1 µM). Noteworthy, we have quantified efflux activity per each ABCB1 molecule, as whole fluorescence signal of accumulated rhodamine 123 was normalized to the amount of expressed ABCB1 proteins on the cell surface, which was again determined via FACS by mapping with an extracellular binding antibody (MRK16). Thus, these observations could not be explained by reduced ABCB1 expression on plasma membrane and were not correlated to ABCB1 downregulation. Beside downregulation of ABCB1, statins also impact the functionality of this transporter that its efflux activity, which can play an important role in the
treatment of resistant cancer cells, is diminished with already clinical relevant concentrations. Taken together, these findings have demonstrated that low concentrations of statins are able to inhibit transport activity of ABCB1 resulting in higher effectivity of chemotherapeutical drugs with lower concentrations and less side effects. These and previous findings make statins very promising candidates especially in oncology.

In addition to downregulation and reduced transport activity, we were interested in effects on the localisation pattern of ABCB1 following simvastatin treatment. As previously mentioned, ABCB1 is a transporter located on the plasma membrane. Decelerated activity could also be based on the impaired protein trafficking that ABCB1 cannot be transported from ER to the membrane to perform its efflux activity. For this purpose, we made use of SH-SY5Y cells transfected with CFP-ABCB1-pcDNA3 plasmid construct (Figure 22 and 23). Indeed, already after 1.5 hours of simvastatin administration ABCB1 expression was increased in the cytoplasm (>30%) in comparison to untreated cells, whereas its expression on the plasma membrane was direct proportionally decreased. Again, this disturbed trafficking of ABCB1 was expectedly in a time- and concentration-dependent manner.

However, it is important to mention that longer exposure times and highest concentration (10 µM) of simvastatin were cytotoxic, as expected. These conditions have induced apoptosis, caused changes in the morphology of SH-SY5Y cells, and led to building of apoptotic buddies. Based on these, the time- and concentration-dependent effect could not be really followed after longer
incubation times at highest concentration. Under these conditions, the correlation of altered protein trafficking of ABCB1 and statin treatment could not be achieved.

In our previous work, we made the observation that statin treatment changed the glycosylation pattern of ABCB1 (Sieczkowski et al, 2010). We have speculated that glycosylation is involved in simvastatin effects, since the fully glycosylated form of the ABCB1 transporter is reduced, similar to tunicamycin treatment (Sieczkowski et al, 2010; Werner et al, 2013). Application of PNGase F confirmed deglycosylation of the fully glycosylated form (170kDa) compared to the core glycosylated form (140 kDa), where this deglycosylation pattern was similar to that of statin treated cells. Moreover, application of PNGase F to intact neuroblastoma cells enhanced doxorubicin accumulation similar to simvastatin treatment (Sieczkowski et al, 2010).

More than 20 years ago, Richert et al. studied that ABC transporters are posttranslationally modified by N-glycosylation (Richert et al, 1988). N-glycosylation has been shown to play a critical role in the regulation of intracellular targeting, in protein folding and maintenance of protein stability (Urquhart et al, 2005). In relation to the glycosylation, dolichol plays a crucial role in N-linked glycosylation of proteins in the ER (Behrens & Leloir, 1970). Thus, we were interested to investigate the effects of simvastatin on endogenous dolichol level, which may cause the glycosylation defects leading to decreased activity and downregulation as well as altered localisation of ABCB1. Indeed, simvastatin exposure at already lowest concentration (0.1 µM) led to a significant decrease of endogenous dolichol in SH-SY5Y cells.
Dependent on reduced dolichol levels in the cell, ABCB1 could not be fully-glycosylated, which is detected by the shift of the mature form to the core-glycosylated form in our protein analysis. Expectedly, addition of dolichol prevented this altered glycosylation of ABCB1 mediated by simvastatin treatment (Figure 25).

For the function and translocation of ABCB1, glycosylation is a very important posttranslational process in the cell. ABCB1 has a phosphorylation site for Pim-1. Phosphorylation of this site by Pim-1 stabilizes the core glycosylated from of ABCB1, protects from degradation and allows its glycosylation (Xie et al, 2010). This glycosylation promotes the translocation of ABCB1 to the cell surface. Similarly, studies with glycosylation-deficient ABCB1 have shown that this deficiency decreases expression levels of this transporter at the cell surface, although the transport function seems to be unaffected (Gribar et al, 2000). Thus, our findings correspond with these previous studies that impaired glycosylation induced by dolichol depletion following statin treatment inhibits the translocation of ABCB1 to the cell surface (Figure 22 and 23) and affects its transport activity on neuroblastoma cells (Figure 20).

In addition to the effects on ABCB1, dolichol depletion in the cell also seems to be an important factor for cell death in simvastatin-exposed SH-SY5Y cells. Three different and independent experimental approaches were performed to confirm this assumption. First, ER stress was corroborated by detection of the typical ER stress markers BiP and CHOP. The upregulation of BiP and CHOP following simvastatin treatment was observed already at clinically relevant concentration of 0.1 µM (Figure 26). Second, simvastatin-induced
apoptosis was prevented by coadministration with dolichol, which were quantified by FACS analysis using Annexin V/PI staining method (Figure 27A). Third, activity of caspase 3, which is a typical executor of apoptosis, was again reduced by addition of dolichol compared to simvastatin alone, which has significantly enhanced caspase 3 activity and triggered apoptosis (Figure 27B).

All together, these results led us to suggest that dolichol and its depletion by statins is a key factor in the mode of action of these HMG-CoA reductase inhibitors. Inhibiton of HMG-CoA reductase by statins impacts the glycosylation process of proteins and further the protein quality. Dolichol depletion might impact protein quality control contributing ER stress and apoptosis. However, this can only partly explain the effects of statins on neuroblastoma cells. Although decreased expression of ABCB1 on plasma membrane could be explained by glycosylation defects ending in altered ER trafficking and increased ubiquitination or proteasomal degradation (Loo & Clarke, 1998; Zhang et al, 2004), it has been shown in the literature that glycosylation does not really affect its drug transport activity (Seres et al, 2011), although the inhibition of glycosylation has been found to be associated with increased drug sensitivity (Werno et al, 2008).

Subsequently, we wanted to prove antitumor activities of simvastatin also in vivo using xenograft models. For this purpose, we made use of CD-1 Nu/Nu mice inoculated with neuroblastoma cells in the presence or absence of 4.25 mg/kg/day simvastatin treatment orally applied. This concentration is approximately 4 times higher than in humans but accounts for 5-10 times
higher turnover rates of murine metabolism, corresponding to a clinical relevant concentration (Gazzerro et al, 2012). Following simvastatin therapy, there were no differences in body weight and in the weight of organs of both mice groups (Figure 28A and Table 3). However, we identified a remarkable decrease in tumor weight and progression, respectively (Figure 28B and 28C). Moreover, our WB analysis indicated similarly to our in vitro analysis that simvastatin reduces ABCB1 expression in tumor as well as in liver, however this effect in liver was not significant (Figure 29). In our opinion, this observation makes statins much more interesting as adjuvant chemotherapy.

Finally, we were able to compare apoptotic potential of statins in vitro to that in vivo (Figure 30). PARP is a typical inducer, whose cleavage initiates further steps of apoptosis (Simbulan-Rosenthal et al, 1998). WB analysis displayed significantly enhanced PARP cleavage in SH-SY5Y tumor samples isolated from simvastatin-administered mice. Thus, we can confirm our previous in vitro findings and also other studies that simvastatin can induce apoptosis in tumor tissue (Minichsdorfer & Hohenegger, 2009; Sieczkowski et al, 2010; Wasinger et al, 2014; Werner et al, 2013; Werner et al, 2004).

These pleiotropic benefits and their safety make statins good candidates for combination therapies in cancer. In addition to their inherent anticancer activities, they may also permit administration of lower concentrations of chemotherapeutics and thereby help to reduce side effects. Statins per se are well tolerated and may therefore improve antitumor efficiency, which is now further highlighted by the fact that ABCB1 is downregulated in vitro and in
vivo. Thus, this study shows that statins may act as a lead compound in novel anticancer therapies in addition to their cardiovascular prevention.
4.2. Conclusion & Future prospects

First, here we show downregulation and inhibition of the most prominent chemoresistance initiating protein, ABCB1, by simvastatin. These data were obtained already at clinical relevant concentrations of simvastatin. Although several ABCB1 inhibitors were still in focus of pharmacological research to overcome chemoresistance, they cannot really be used in clinics because of their severe side effects, like high cytotoxicity or cross-reactivity with other transporters. In contrast, statins can differ from these inhibitors as they are safe, widely-used and do not possess any severe side effects, besides rare myotoxicity and elevated liver parameters. Second, the anticancer activities of statins are associated with altered endogenous dolichol levels leading to ER stress and facilitate apoptosis in vitro as well as in vivo. Consequently, statins constitute a promising evidence for a combination therapy in different tumor types. Notably, indications from epidemiological studies indicate cardiovascular-independent benefits in statin users, which include improved overall health and reduced cancer incidence or progression. However, further investigations are strongly needed to introduce statins in cancer therapy.
5. Materials and Methods

5.1. Chemicals and reagents

Simvastatin was purchased from Merck (NJ, USA), all other reagents and chemicals from Sigma Aldrich (St. Louis, USA) or Carl Roth (Karlsruhe, Germany), if not mentioned otherwise.

5.2. Cell culture

Study was performed with human neuroblastoma (SH-SY5Y) and human embryonic kidney (HEK)-293 cells (ATCC – LGC Standards; Wesel, Germany). SH-SY5Y cells were maintained in DMEM/Ham’s F12 medium, HEK-293 cells in Dulbecco’s modified Eagle’s medium (DMEM) High Glucose medium, all supplemented with 10% fetal bovine serum (FBS) with humidified atmosphere of 5% CO₂ at 37°C. Cell culture chemicals were purchased from PAA Laboratories (Austria).

5.3. Lipid extraction and chromatographic analysis

According to the simvastatin treatment (0.1 µM, 1 µM and 3 µM) for 48 hours, 1 x 10⁷ SH-SY5Y cells were lysed in methanol with 3% acetic acid (v/v). The lysate was supplemented with the same volume of hexane and vortexed vigorously for 15 seconds. The upper phase containing lipids was collected.
Again, the lower phase was supplemented with hexane for a second lipid extraction step. Second upper phase was added to the first one, and the organic solvents were evaporated from the collected lipids, which were stored at -80°C.

Collected lipids were dissolved in CHCl₃ and grossly separated by thin layer chromatography (TLC) using TLC Silica gel 60 plates, which were pre-washed with hexane (Merck; Darmstadt, Germany). Chromatography was performed with hexane including 20% ethyl acetate as mobile phase. As standards, C₈₀₋₁₀₅ dolichol isolated from bovine heart and C₅₅ were used. The separated lipids were visualized by CuSO₄ and heating the plate. Samples corresponding to the relative motility of dolichol standards were scratched and transferred to a glass column retaining the matrix of the TLC. Dolichol was eluted by 500 µl CHCl₃ from the silica gel. This step was repeated four times. All eluates were collected, evaporated, dissolved in 20 µl 2-propanol/methanol/n-hexane (45:45:10) and applied to high performance liquid chromatography (HPLC). Dolichol fractions were separated with the running buffer (propanol/methanol/n-hexane, 45:45:10) at 1.7 ml/min (Hitachi pump L-2130 and UV detector L-2400; Tokyo, Japan). Data were analyzed with Elite LaChrom software.

5.4. **Cell lysis**

Untreated and simvastatin-exposed SH-SY5Y cells were washed with PBS, shock-frozen with liquid nitrogen and lysed with RIPA buffer (50 mM Tris-HCl,
pH= 8; 150 mM NaCl; 10 mM glycerolphosphate; 0.1% SDS; 1% NP-40) containing protease inhibitors aprotinin (2 µg/ml), leupeptin (10 µg/ml) and pefablock (1 mM). Following the incubation on ice for 10 minutes, lysates were again shock-frozen in liquid nitrogen and centrifuged with 30000 x g for 30 minutes at 4°C. The supernatant fractions were stored at -80°C and used for Western blot analysis.

5.5. Membrane extraction

According to the simvastatin treatment, SH-SY5Y cells were washed with PBS and shock-frozen with liquid nitrogen. Lysis was performed in a hypotonic buffer (10 mM Tris-HCl, pH=7.4; 250 mM sucrose; 2 mM CaCl₂; 10 mM EDTA) supplemented with protease inhibitors aprotinin (2 µg/ml), leupeptin (10 µg/ml) and pefablock (1 mM). The cell slurry was gently resuspended and centrifuged at 600 x g for 10 minutes at 4°C to separate nuclei. The supernatant was centrifuged again with 11600 x g for 20 minutes at 4°C to remove microsomes. At last, another centrifugation step was performed with 100000 x g for 45 minutes at 4°C to separate cytosolic fraction from membrane fraction. The pellet containing membrane fraction was recovered in 100-200 µl resuspension buffer (20 mM Tris-HCl, ph= 7.4; 150 mM NH₄Cl; 5 mM MgCl₂) with protease inhibitors mentioned before. Lysates were shock-frozen in liquid nitrogen and stored at -80°C. All steps were performed on ice.
5.6. Caspase 3 activity

Following drug exposure, SH-SY5Y cells were washed with PBS and disrupted with liquid nitrogen. Cells were lysed in caspase lysis buffer (25 mM HEPES, pH= 7.5; 1 mM EGTA; 5 mM EDTA; 5 mM MgCl2) containing protease inhibitors aprotinin (2 µg/ml), leupeptin (10 µg/ml) and pefablock (1 mM). Subsequently, the cell slurry was again shock-frozen in liquid nitrogen and incubated on ice for 10 minutes. After ultrasound treatment for 1 minute, cell lysates were centrifuged with 30000 x g for 30 minutes at 4°C. Supernatant corresponding cytosolic fraction was shock-frozen in liquid nitrogen and stored at -80°C.

The fluorescence-based caspase 3 assay was carried out as previously described using 50 µM of the Ac-DEVD-7-amino-4-trifluoro-methylcoumarin (AFC)-conjugated caspase 3 substrate according to the manufacturer’s protocol (Alexis; Vienna, Austria) (Sacher et al, 2005).

5.7. ER-stress PCR

After simvastatin exposure, SH-SY5Y cells were washed, trypsinated, and total RNA was isolated using RNeasy Mini Kit following manufacturer’s protocol (Qiagen, Germany). Isolated RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific; Massachusetts, USA) as described in manufacturer’s protocol. PCR was performed by a step of denaturation at 95°C for 5 minutes, followed by 26 (for GAPDH), 28 (for CHOP) or 30 cycles (for BiP) of denaturation at 95°C, annealing at 60°C for 1
minute and DNA synthesis at 72°C for 1 minute. The final step included elongation at 72°C for 7 minutes. For this experiment, BiP and CHOP primer pairs were used (Thermo Scientific; Massachusetts, USA). As control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used (Table 4). Following PCR, samples were loaded to 1% agarose gels with ethidium bromide (1%). Gels were run with 135 V for approximately 25 minutes. Signals were quantified from gel images, and analyses were performed offline using ImageJ software (http://rsbweb.nig.gov/ij/).

**Table 4:** PCR primer pairs used for ER-stress PCR.

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Forward (5´ - 3´)</th>
<th>Reverse (5´ - 3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BiP</td>
<td>CGAGGAGGAGGACAAGAAGG</td>
<td>CACCTTGACGGCAAGAACT</td>
</tr>
<tr>
<td>CHOP</td>
<td>GCACCTCCCAGAGCCCTCCTCCTCC</td>
<td>GTCTACTCCAAGCCTTCCCCCTGCG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CAAGGTCATCCATGACAACHTTG</td>
<td>GTCCACCACCTGTTGCTGTAG</td>
</tr>
</tbody>
</table>

### 5.8. Quantitative PCR

Following drug exposure, cells were handled and prepared for quantitative (real time) polymerase chain reaction (PCR) as described in Section 5.7. q-PCR was performed by using SensiMix SYBR & Fluorescein (GenXpress, Austria), and specific primers for genes of interest were listed in Table 5. Quantitative PCR was performed by a step of denaturation at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 30 seconds and DNA synthesis at 72°C for 30 seconds. The final melting step
included denaturation at 95°C for 15 seconds, 60°C for 15°C seconds, a linear temperature gradient to 95°C in 20 minutes and 95°C for 15 seconds. \( C_T \) values were normalized to the four control genes (\( \beta 2 \) microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 60S acidic ribosomal protein P0 (RPLP0) and 40S ribosomal protein S14 (RPS14)), and the quantification was performed using the comparative \( C_T \) method.

**Table 5**: PCR primer pairs used for quantitative PCR.

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Forward (5´ - 3´)</th>
<th>Reverse (5´ - 3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABCB1</strong></td>
<td>GCGCCTCGAGATGGATTTGGAAGGGACC</td>
<td>GCGGATGGCTGGGTGTTCCAGC</td>
</tr>
<tr>
<td><strong>ABCC1</strong></td>
<td>CTGACAAGCTAGACCATGAATGTT</td>
<td>TCACACGCGGCTCCTTT</td>
</tr>
<tr>
<td><strong>ABCC4</strong></td>
<td>GGATCCAAGAAACTGATGAGTTAAT</td>
<td>TCACAGTGCTGCTGAAAATAG</td>
</tr>
<tr>
<td><strong>ABCC6</strong></td>
<td>CACTGGCTCCAGGATCAGC</td>
<td>CAGACCAGGCTGACTCCTG</td>
</tr>
<tr>
<td><strong>ACBG2</strong></td>
<td>CTAGATGGGGTTTTCCAAGCGTTCATAA</td>
<td>TGAAACACTGTTGGTGTGTCAGGAAGA</td>
</tr>
<tr>
<td><strong>B2M</strong></td>
<td>GTGCTCGGCCTACTCTTCTC</td>
<td>GTCACTTCAATGTGGGAT</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>CAAGGTCAATCCATGACACATT</td>
<td>GTCCACCACCTGTGCTGTAG</td>
</tr>
<tr>
<td><strong>RPLP0</strong></td>
<td>GCAATGTGGCCAGTGCTTG</td>
<td>GCCTGACCTTTTCAGCAA</td>
</tr>
<tr>
<td><strong>RPS14</strong></td>
<td>GGCAGACCGAGATGAATCCTCA</td>
<td>CAGGTCGCCGTTGTCAGTCGG</td>
</tr>
</tbody>
</table>

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5.9. **FACS analysis of ABCB1**

ABCB1 expression was analyzed on cell surface and as total in native and fixed SH-SY5Y cells. For cell surface expression, SH-SY5Y cells (5 x 10^5) treated with various concentrations of simvastatin were washed with PBS and mapped with MRK16 antibody (0.5 µl MRK16 in 50 µl PBS for 30 minutes at room temperature under gentle shaking) (Kamiya Biomedical Company; Seattle, USA). It was visualized with a corresponding Alexa®488-conjugated goat anti-mouse antibody (1:100 in PBS for 30 minutes at 4°C) (Invitrogen; CA, USA). After incubation, cells were washed with PBS, and percentage of positive cells was analyzed on a FACSCanto II (Becton Dickinson; NY, USA).

For total ABCB1 expression, cells were prepared as mentioned before. Cells were fixed in 100 µl 0.9% NaCl with 4% formaldehyde and incubated for 15 minutes at room temperature. For permeabilisation, 100 µl 0.5% Tween-20 in PBS were added and incubated for another 15 minutes at room temperature. After washing with PBS, fixed and permabilized cells were mapped with C219 or p170 antibody (1:50 in 100 µl PBS with 10% FBS and 1% NaN₃ for 30 minutes at room temperature under gentle shaking). The visualization and measurement were performed as mentioned above via fluorescence-activated cell sorting (FACS).

As control, unstained cells as well as cells mapped with mouse immunoglobulin G₂A (IgG₂A) (Becton Dickinson; NY, USA) with corresponding Alexa®488-conjugated goat anti-mouse antibody were used in both experimental setups. All analyses were performed using Flowing software (http://www.flowingsoftware.com).
5.10. FACS analysis of Annexin V/PI staining

Apoptosis was determined with biparametric FACS analysis using FITC-conjugated Annexin V (Ebioscience; San Diego, USA) and propidium iodide (PI) (Sigma Aldrich; St. Louis, USA). After drug exposure for 48 hours, SH-SY5Y cells (5 x 10^5) were washed and treated with Accutase® (Sigma Aldrich; St. Louis, USA). Following cell detachment, cells were washed again with PBS and stained in binding buffer (10 mM HEPES, pH= 7.4; 140 mM NaCl) containing 2.5 µg/ml Annexin V and 0.5 µg/ml PI for 10 minutes at room temperature in the dark. After incubation, cells were washed with PBS, and percentage of positive cells was analyzed on a FACSCanto II (Becton Dickinson; NY, USA) and Flowing software (http://www.flowingsoftware.com).

5.11. FACS analysis of rhodamine 123 efflux

SH-SY5Y cells (1 x 10^6) cells were exposed to several concentrations of simvastatin for 48 hours. Cells were washed and stripped with Accutase® (Sigma Aldrich; St. Louis, USA). Rhodamine 123 efflux were determined with previously described method (Chiba et al, 1996). Loading was performed with 0.53 µM rhodamine 123 for 30 minutes at 37°C under gentle shaking. After washing, cells were kept on ice until the measurement, and the measurement was performed with a FACSCalibur (Becton Dickinson; NY, USA) at 37°C. Efflux was continuously monitored for 5 minutes with approximately 10^6 events. Additionally, ABCB1 expression on plasma membrane was quantified against MRK16 antibody. This facilitates to normalize the efflux
rates to ABCB1 available on the cell surface. The analyses were done with CellQuest software (Becton Dickinson; NY, USA) as previously described (Chiba et al, 1996) and SigmaPlot (Jandl; Erkrath, Germany).

5.12. Protein turnover of YFP-ABCB1 fusion protein

HEK-293 cells were seeded in 6-well plates and transfected with 0.4 µg yellow fluorescent protein (YFP)-ABCB1-pcDNA3 plasmid using TurboFect according the manufacturer’s protocol (Thermo Scientific; Massachusetts, USA). The NH₂-terminal tagged YFP-ABCB1 construct was kindly provided by Prof. Peter Chiba (Institute of Medical Chemistry – Medical University of Vienna) and Dr. Oliver Kudlacek (Institute of Pharmacology – Medical University of Vienna). After recovery for 48 hours, cells were exposed for another 48 hours in the absence or presence of cycloheximide (10 µg/ml), doxorubicin (0.1 µM), simvastatin (1 or 3 µM) or vehicle (empty pcDNA3 plasmid). Subsequently, cells were shock-frozen in liquid nitrogen and resuspended in PBS. YFP fluorescence (excitation: 515 nm, emission: 530 nm) corresponding to ABCB1 expression was measured with a fluorescence spectrophotometer (FL-4500 Hitachi; Tokyo, Japan). Signals were corrected for protein concentration and background signal of the vehicle, and analysed using FL-Solutions 2.0 (Hitachi; Tokyo, Japan).
5.13. Confocal microscopy

SH-SY5Y cells were seeded in 4-well chamber slides (Nunc™ Lab-Tek™ Chamber Slide System – Thermo Scientific; Massachusetts, USA) and transfected with 0.5 µg cyan fluorescent protein (CFP)-ABCB1-pcDNA3 plasmid using FuGene® HD (Promega; Madison, USA) with a ratio of transfection reagent to DNA 4:1 according the manufacturer’s protocol (Thermo Scientific; Massachusetts, USA). As mentioned before, The NH₂-terminal tagged YFP-ABCB1 construct was kindly provided by Prof. Peter Chiba (Institute of Medical Chemistry – Medical University of Vienna) and Dr. Oliver Kudlacek (Institute of Pharmacology – Medical University of Vienna). After recovery, cells were treated with 1, 3 or 10 µM simvastatin for several time points. Cells on chamber slides were washed with PBS, and pictures were taken at an excitation wavelength of 458 nm and an emission of 514 nm on a confocal microscope (LSM410 – Carl Zeiss; Jena, Germany). A 100x objective was used with 1.6 zoom for live imaging of the cells, and ABCB1 expression was monitored using CFP signal. The offline analyses were performed using LSM Image Browser (Carl Zeiss; Jena, Germany) and ImageJ software (http://rsbweb.nig.gov/ij/).

5.14. Murine xenograft experiments

Xenograft experiments were approved by the Animal Welfare Committee of the Medical University of Vienna and the Austrian Science Ministry. 6 weeks old female CD-1 Nu/Nu mice were purchased from Charles River (Sulzfeld,
Germany). SH-SY5Y cells (1 x 10^7 in PBS) were subcutaneously inoculated into the left and right flank. A day-night rhythm was emulated by light every 12 hours. The welfare of the animals was checked every day, weight and tumor volume were monitored every other day. Twelve days after inoculation, groups of 4 mice were assigned to water or water supplemented with simvastatin (4.25 mg/kg/day). The animals were sacrificed by neck dislocation due to critical tumor size on day 61 or earlier due to critical tumor size. Organs and tumors were excised, weighted, and aliquots were rapidly frozen in liquid nitrogen and stored at -80°C.

For tissue analysis, liver and tumor samples (25-50 mg) were homogenized in solution A (10 mM HEPES, pH= 7.5; 10% sucrose; 5 mM EDTA; 1 mM DTT) with protease inhibitors: aprotinin (100 µM), leupeptin (100 µM), pefablock (1 mM), calpain inhibitors I and II (each 10 µM) using a glass/Teflon homogenizer. Lysates were centrifuged at 100 x g for 5 minutes to remove gross tissue particles. Next centrifugation of supernatant (600 x g, 10 minutes) was performed to remove nuclei. At last, the supernatant was centrifuged again at 100000 x g for 45 minutes. Pellet containing membrane fraction was resuspended in 100-200 µl solution B (10 mM HEPES, pH= 7.5; 10% sucrose; 2 mM EDTA; 1 mM DTT) with protease inhibitors listed above. Supernatant (cytosolic fraction) and membrane fractions were stored at -80°C. All steps were carried out at 4°C.
5.15. Western blot analysis

Dependent on the protein of interest, they (15-30 µg) were resolved on a 7% or 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to nitrocellulose membranes (GE Healthcare, UK), blocked with 5% bovine serum albumin (BSA) and incubated overnight at 4°C with the primary antibodies listed in Table 6. Proteins of interest were visualized by ECL system (Amersham ECL Plus or ECL Prime Western Blotting Detection Reagent; GE Healthcare; UK) using a species-corresponding horse radish peroxidise (HRP)-conjugated secondary antibody (1:10000 in 2% BSA, for 1 hour at room temperature) (Cell Signaling Technology; Massachusetts, USA) following manufacturer’s instructions. All signals were quantified and analysed using ImageJ software (http://rsbweb.nig.gov/ij/).
Protein concentrations were determined according to Bradford protein assay using BSA as a protein standard (Bradford, 1976). All data presented here are in mean ± standard deviation (SD). Statistical analyses were performed using scientific software SigmaPlot (Jandl; Erkrath, Germany) with either unpaired Students t-test or for multiple comparisons with ANOVA and Holm-Sidak test. A p-value of < 0.05 is considered statistically significant.

### Table 6: Antibodies used in Western blot analyses.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Antibodies (Clone)</th>
<th>Dilution</th>
<th>Purchased by</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>C219</td>
<td>1:300</td>
<td>Merck-Millipore (Darmstadt, Germany)</td>
</tr>
<tr>
<td>ABCB1</td>
<td>p170</td>
<td>1:50</td>
<td>Neomarkers (Fremont, USA)</td>
</tr>
<tr>
<td>ABCC1</td>
<td>QCRL1</td>
<td>1:1000</td>
<td>GeneTex (Irvine, USA)</td>
</tr>
<tr>
<td>ABCC4</td>
<td>EB06538</td>
<td>1:1000</td>
<td>Abgent (San Diego, USA)</td>
</tr>
<tr>
<td>ABCG2</td>
<td>5D3</td>
<td>1:100</td>
<td>Santa Cruz (Santa Cruz, USA)</td>
</tr>
<tr>
<td>PARP</td>
<td>9542</td>
<td>1:1000</td>
<td>Cell Signaling Technology (Massachusetts, USA)</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>B-5-1-2</td>
<td>1:10000</td>
<td>Sigma Aldrich (St. Louis, USA)</td>
</tr>
</tbody>
</table>

5.16. **Miscellaneous procedures**

Protein concentrations were determined according to Bradford protein assay using BSA as a protein standard (Bradford, 1976). All data presented here are in mean ± standard deviation (SD). Statistical analyses were performed using scientific software SigmaPlot (Jandl; Erkrath, Germany) with either unpaired Students t-test or for multiple comparisons with ANOVA and Holm-Sidak test. A p-value of < 0.05 is considered statistically significant.
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**Study in Biology and Microbiology – Genetics**, University of Vienna, Vienna – Austria (2004 - 2010)


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- **Atil B.**, Sieczkowski E., Hohenegger M. Statins reduce endogenous dolichol levels in neuroblastoma cell line SH-SY5Y. *SFB35 Symposium* (2012), Vienna, Austria.
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- **Atil B.**, Sieczkowski E., Hohenegger M. Statins modulate the

- **Atil B.,** Sieczkowski E., Hohenegger M. Statins: Inhibition of ATP-binding cassette (ABC) transporters in neuroblastoma cells. YSA – Young Scientist Association of the Medical University of Vienna (2011), Vienna, Austria.

- **Atil B.,** Sieczkowski E., Hohenegger M. Statins affect on ATP-binding cassette B1 transporter turn over in human neuroblastoma cells. EMBO (2011), Vienna, Austria.


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- **Atil B.,** Werner M., Sieczkowski E., Chiba P., Hohenegger M. Transporter-mediated interaction of simvastatin and doxorubicin translates into topoisomerase II inhibition in human
rhabdomyosarcoma cells. SFB35 Symposium (2010), Vienna, Austria.


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