Published in final edited form as:

Biochim Biophys Acta. 2018 September ; 1865(9): 1211–1229. doi:10.1016/j.bbamcr.2018.06.006.

Prazosin induced lysosomal tubulation interferes with cytokinesis and the endocytic sorting of the tumour antigen CD98hc

Robert Fuchs^{1, #}, Anika Stracke¹, Viktoria Holzmann^{1, 2}, Gerfried Luschin-Ebengreuth¹, Nathalie Meier-Allard¹, Nadine Ebner¹, Teresa Maria Lassacher¹, Markus Absenger-Novak³, Eleonore Fröhlich³, Matthias Schittmayer^{4, 5}, Sara Cano Crespo⁶, Manuel Palacin⁶, Beate Rinner⁷, and Ruth Birner-Gruenberger^{4, 5}

¹Chair of Immunology and Pathophysiology, Otto Loewi Research Center, Medical University of Graz, Heinrichstraße 31, 8010 Graz, Austria

²FH JOANNEUM – University of Applied Sciences, Alte Poststraße 149, 8020 Graz, Austria

³Centre for Medical Research, Medical University of Graz, Stiftingtalstrasse 24, 8010 Graz, Austria

⁴Research Unit Functional Proteomics and Metabolic Pathways, Institute of Pathology, Medical University of Graz, Auenbruggerplatz 25, 8036 Graz, Austria

⁵Omics Center Graz, BioTechMed-Graz, Stiftingtalstrasse 24, 8010 Graz, Austria

⁶Institute for Research in Biomedicine (IRB-Barcelona), Barcelona Institute of Science and Technology (BIST), Department of Biochemistry and Molecular Biomedicine, University of Barcelona, and CIBERER. Parc Científic de Barcelona. Baldiri I Reixac 10-12, 08028 Barcelona, Spain

⁷Biomedical Research, Medical University of Graz, Roseggerweg 48, 8036 Graz, Austria

Abstract

The quinazoline based drug prazosin (PRZ) is a potent inducer of apoptosis in human cancer cells. We recently reported that PRZ enters cells via endocytosis and induces tubulation of the endolysosomal system. In a proteomics approach aimed at identifying potential membrane proteins with binding affinity to quinazolines, we detected the oncoprotein CD98hc. We confirmed shuttling of CD98hc towards lysosomes and upregulation of CD98hc expression in PRZ treated cells. Gene knockout (KO) experiments revealed that endocytosis of PRZ still occurs in the

[#]Corresponding author: Robert Fuchs, Heinrichstrasse 31, 8010 Graz, Austria, robert.fuchs@medunigraz.at, Tel.: +43/699/12096633.

Authors' contributions

RF designed the study, performed experiments, analysed and interpreted data and wrote the manuscript. AS, MS, RBG, and BR contributed to conception, interpretation of data, design of the study and development of methodology. AS, VH, GLE, NMA, NE, TML, MS, and BR performed experiments and contributed to data analysis and interpretation. SCC and MP generated and provided CD98hc knockout cells and discussed obtained data. MAN contributed to the acquisition of data. AS, TML, EF, SCC, MP, BR and RBG contributed to reviewing of the manuscript. EF contributed to study supervision and provided essential materials. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

absence of CD98hc - suggesting that PRZ does not enter the cell via CD98hc but misroutes the protein towards tubular lysosomes. Lysosomal tubulation interfered with completion of cytokinesis and provoked endoreplication. CD98hc KO cells showed reduced endoreplication capacity and lower sensitivity towards PRZ induced apoptosis than wild type cells. Thus, loss of CD98hc does not affect endocytosis of PRZ and lysosomal tubulation, but the ability for endoreplication and survival of cells. Furthermore, we found that glutamine, lysomototropic agents – namely chloroquine and NH₄Cl – as well as inhibition of v-ATPase, interfere with the intracellular transport of CD98hc. In summary, our study further emphasizes lysosomes as target organelles to inhibit proliferation and to induce cell death in cancer. Most importantly, we demonstrate for the first time that the intracellular trafficking of CD98hc can be modulated by small molecules. Since CD98hc is considered as a potential drug target in several types of human malignancies, our study possesses translational significance suggesting, that old drugs are able to act on a novel target.

Keywords

Cancer; CD98hc/4F2/SLC3A2; Prazosin; Lysosomes; Lysomototropic agents; Apoptosis

1 Introduction

Quinazoline based α 1-adrenergic antagonists like prazosin (PRZ), doxazosin and terazosin were introduced into medicine for treatment of hypertension and benign prostate hyperplasia. Surprisingly, several studies have shown that quinazolines induce apoptosis in various types of malignant cells. Kyprianou et al. were the first to demonstrate that quinazoline based α 1-adrenergic antagonists are able to induce apoptosis in prostate cancer cells [1]. Most interestingly, it turned out that the pro-apoptotic action of quinazolines is completely independent of α 1-adrenergic receptors [1–7]. This fact is supported by the drug doses required to induce apoptosis in cancer cells. Only nM concentrations of quinazolines are needed to block adrenergic receptors, whereas μ M concentrations are required to stop proliferation and to induce apoptosis in cancer cells. To date, cancer research on quinazolines in the human system thus has been limited to *in vitro* studies, because various severe side effects, primarily concerning the regulation of blood pressure, are suspected *in vivo*. Several animal studies using xenograft models have, however, proven that quinazolines are also able to inhibit human tumour growth *in vivo* [7–10]. Since the first demonstration of the pro-apoptotic action of quinazolines in the prostate, numerous studies have demonstrated similar pro-apoptotic activity in breast cancer cells, leukaemia, pituitary adenoma, bladder cancer, renal cancer and most recently in glioblastoma as well [1–5,7–12].

Our group discovered that the α 1-adrenergic antagonist PRZ is able to induce apoptosis in leukaemia cells via a mechanism independent of adrenergic receptors [5,12]. We further found that PRZ is also able to induce apoptosis in cells derived from medullary thyroid carcinoma - a malignancy characterized by high resistance against chemotherapy - which further emphasises the potent anti-tumour effects of PRZ [13]. Besides direct anti-proliferative and pro-apoptotic activity further studies have revealed that quinazolines activate anoikis - a protective effect against metastasis – and pronouncedly inhibit tumour-angiogenesis [8,9,14]. In summary, data in the literature highlight manifold anti-tumour

actions of quinazolines *in vitro* and *in vivo*. Even though some details about the effects of quinazolines on apoptotic signalling pathways are known, the main drug targets triggering pro-apoptotic effects still need to be defined. We found that quinazolines enter cells via endocytosis and subsequently induce a tubular morphologic reorganisation of the LAMP1 positive endolysosomal system [15]. In order to discover proteins with affinity to quinazolines, we performed native gel electrophoresis of proteins bound to the fluorescent PRZ-derivative BODIPY® FL Prazosin (QAPB) and subsequently performed mass spectrometric analysis of fluorescent protein bands after in-gel tryptic digestion [15]. We identified up to 700 different proteins (unpublished results), initially questioning the specificity of our approach. As a starting point we focused on membrane proteins since PRZ was shown to enter cells via endocytosis and to interfere with endocytic sorting [6,15]. Among those we recurrently found CD98 heavy chain (CD98hc, SLC3A2, 4F2, 4F2hc), an oncoprotein controlling cellular amino acid homeostasis and integrin function [16]. CD98hc acts as a chaperone for various amino acid transporters like LAT1, LAT2, y+LAT1, y+LAT2 and xCT, which import essential amino acids (AA) in antiport with glutamine into the cell and thereby provide AA for protein synthesis and production of the antioxidant glutathione [16,17]. Furthermore, CD98hc regulates autophagy and promotes cell growth and protein synthesis via the PI3K-AKT-mechanistic Target Of Rapamycin (mTOR) pathway [16,18]. Santiago-Gómez et al. discovered recently that CD98hc participates in tumour progression by inhibition of β -catenin proteasomal degradation via AKT/GSK-3 β signalling [19]. In earlier studies Feral et al. had already shown that CD98hc is associated with β 1 integrins and contributes to integrin-dependent cell spreading, cell migration and protection from apoptosis [20,21]. Poettler et al. demonstrated that CD98hc drives integrin-dependent renal cancer cell behaviour [22]. In line with the observations of Poettler et al., Kyprianou's group found that quinazolines interrupt intracellular survival signals and induce anoikis in cancer by targeting integrin mediated cell-cell and cell-extracellular matrix interactions [14,23]. These results concerning integrin signalling represent an obvious link between CD98hc and the pro-apoptotic signalling of quinazolines.

Based on our findings in proteomics analysis and the multifactorial significance of CD98hc in cancer cells, we investigated a possible role of CD98hc in quinazoline induced apoptosis. We also tested whether the lysomototropic agents chloroquine and ammonia [24–26] and the v-ATPase inhibitor bafilomycin A1, which attenuate the cytotoxicity of PRZ [15], interfere with the trafficking of CD98hc. Since CD98hc is currently seen as potential drug target in several types of human malignancies [16,21], the primary motivation and goal of our study was to uncover possible interactions of old drugs with this novel target.

2 Materials and Methods

2.1 Cancer Cell Culture

K562 chronic myeloid leukaemia cells, obtained from ATCC (Manassas, VA, USA) and LNCaP prostate cancer cells (CLS Cell Lines Service GmbH, Eppelheim, Germany) were cultivated in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS, Merck/Biochrom, Berlin, Germany), 2 mM L-glutamine (Sigma) and 100 U/ml penicillin and 0.1mg/ml streptomycin ("Penstrep", Sigma). In some

experiments the standard medium was supplemented with MEM amino acid concentrate (Sigma) or additional AA mainly obtained from Carl Roth (Karlsruhe, Germany). In all cell culture experiments with PRZ the described RPMI-1640 standard medium was also used.

2.2 Drugs and Reagents

Bafilomycin A1 (BafA1), bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide (BPTES), chloroquine diphosphate salt (CHQ), cytochalasin-D (Cyto-D), 6-Diazo-5-oxo-L-norleucine (DON), *E. coli* derived asparaginase (L-ASP), glutathione (GSH), L-methionine sulfoximine (MSO), n-acetylcysteine (NAC), nocodazole (NDZ), Pit-1 and prazosin hydrochloride (PRZ) were purchased from Sigma. Rapamycin was obtained from LC Laboratories (Woburn, MA, USA) and NVP-BEZ235 (BEZ235) from the Cayman Chemical Company (Ann Arbor, MI, USA). Depending on solubility, drugs were either solved in cell culture proved dimethyl sulfoxide (DMSO, Sigma) or double distilled water (Fresenius Kabi, Graz, Austria).

2.3 Analysis of cellular proliferation and viability

Proliferation of K562 cells was assessed with a CASY[®] Cell Counter and Analyser System (OMNI Life Science, Bremen, Germany). For proliferation assays, K562 cells were cultivated in 24 well plates with a starting cell number of 2×10^4 cells/ml. Every condition was analysed in duplicate. For flow cytometry assays, western blotting experiments and qPCR 1×10^6 K562 cells were cultivated in 10 ml medium in 25 cm^2 cell culture flasks. Proliferation and viability of LNCaP cells were assessed using the WST-1 reagent (Roche, Mannheim, Germany) following the manufacturer's instructions. Cells were harvested by trypsinisation, washed once in medium without glutamine and were cultivated in 96 well tissue culture plates, starting with a cell number of 1×10^4 cells in 50 μl medium without glutamine. Cells were allowed to attach to the surface of the plate overnight, before addition of different concentrations of glutamine or NH_4Cl +/- prazosin to reach a final volume of 100 μl /well. Afterwards, cells were cultivated for 24h or 48h. Absorption at 450nm and as a reference at 650nm was determined with a Sunrise[™] absorbance reader (Tecan, Männedorf, Switzerland) and/or with a BMG Labtech SPECTROstar Nano microplate reader (Ortenberg, Germany). All conditions were tested in triplicates. Cell death of K562 and HEK293T cells was also tested with the WST-1 assay analysing samples of cell suspensions in sextuplicates (K562) or triplicates (HEK293T) following cultivation.

2.4 CRISPR/Cas9-mediated CD98hc knockout in HEK293T cells

The cell line HEK293T, obtained from ATCC, was maintained in DMEM medium with high glucose (Sigma) supplemented with 10% FBS, "Penstrep" and 100 μM 2-mercaptoethanol (βME , Sigma). To generate CD98hc knockout (KO) cells, the CRISPR/Cas9 system was used according to the manufacturer's instructions (Santa Cruz Biotechnology/SCBT, Dallas, TX, USA). HEK293T cells were co-transfected with the CD98hc CRISPR/Cas9 KO plasmid (sc-400501; SCBT) and the CD98hc homology-directed repair (HDR) plasmid (sc-400501-HDR; SCBT). The CD98hc CRISPR/Cas9 KO plasmid consists of a pool of three plasmids designed to disrupt gene expression by causing a double-strand break in 5'-GATTCTCTATGTCCCGAACC-3', 5'-TCGGGACATAGAGAATCTGA-3' and 5'-TCATCCCCGTAGCTGAAAAC-3'. The CD98hc HDR plasmid contains a puromycin

resistance gene to allow selection of stably transfected cells with successful integration. Briefly, cells (1×10^5 cells per well) were seeded onto 6-well culture plates in 2 ml of DMEM per well, 24h prior to transfection and grown to 80% confluency. Cells were transfected with 1 μ g of CD98hc CRISPR/Cas9 KO plasmid and 1 μ g of CD98hc HDR plasmid (SCBT) using polyethylenimine (Polysciences Europe GMBH, Eppelheim, Germany) and NaCl and incubated at 37 °C, 5% CO₂ for 48h. Successful co-transfection of the CRISPR/Cas9 KO plasmid and HDR plasmid was visually confirmed by detection of the green fluorescent protein (GFP) and the red fluorescent protein (RFP), respectively, by fluorescence microscopy. DMEM medium was exchanged with fresh medium containing puromycin (1 μ g/ml, SCBT). Expanded pools of cells resistant to puromycin were maintained for three passages in the corresponding selective medium and were further enriched for cells expressing high levels of RFP by sorting on a Becton Dickinson (BD) FACS Aria™ Fusion Cell Sorter (San Jose, CA, USA) at the Scientific and Technological Centers of the University of Barcelona/CCiTUB). The same sorter was used to deposit single cells into 96-well plates for clonal selection. Several clones were obtained, expanded and assayed for CD98hc expression. In all HEK293T cell culture experiments with prazosin, RPMI-1640 standard medium with addition of β ME was used.

2.5 Real Time Quantitative PCR

Expression of human CD98hc (SLC3A2) at the mRNA-level was analysed versus 18S rRNA using TaqMan® chemistry (Thermo-Fisher/Applied Biosystems, Foster City, CA, USA). RNA was extracted with TRI-Reagent RT (MRC Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. Extracted RNA was quantified and analysed using a NanoDrop ND-1000 spectrophotometer (Thermo-Fisher/Peqlab, Erlangen, Germany) and RNA-gel electrophoresis. Extracted RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Thermo-Fisher/Applied Biosystems). For TaqMan®-analysis inventoried assays of Thermo-Fisher/Applied Biosystems (SLC3A2: assay number: Hs00374243_m1 and Eukaryotic 18S rRNA Endogenous Control, assay number: Hs99999901_s1) were used. Quantitative RT-PCR was performed on a CFX96™ - Real-Time PCR Detection System (Bio-Rad) using the 2-^{-CT} algorithm for quantification of relative gene expression of controls versus treated samples. Each sample was measured in triplicates.

2.6 Western Blotting

Cells were harvested and washed twice with calcium/magnesium free phosphate buffered saline (CMF-PBS) at 4°C. Cells were lysed with a lysing buffer containing 50mM Tris base, 10mM EDTA, 1% Triton X-100 and the cOmplete™ Protease Inhibitor Cocktail (Roche). Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For SDS-PAGE, 10% separating gels and 4% stacking gels were used. Ten μ g of total protein mixed with Laemmli buffer were loaded on SDS-gels per lane. Before loading, the samples were denatured at 95°C for 5min. PageRuler™ Prestained Protein Ladder (Thermo Fisher) was used as molecular weight marker. For the blotting procedure a Bio-Rad Wet/Tank Blotting System and PVDF membranes (Amersham™ Hybond® P western blotting membranes, GE-Healthcare, Piscataway, NJ, USA) were used. The membrane was blocked in 5% non-fat milk in Tris

Buffered Saline (T-BST) containing 0.1% Tween[®] 20 detergent for 1h at room temperature (RT). After blocking, the membranes were incubated with the respective primary antibody overnight at 4°C. The following primary antibodies were used: mouse CD98hc (E-5, SCBT), rabbit GSK-3 α (D80E6, Cell Signaling Technology/CST, Danvers, MA, USA), rabbit phospho (Ser21) - GSK-3 α (36E9, CST), polyclonal rabbit GAPDH (BioLegend), polyclonal rabbit AMPK α (BioLegend), rabbit phospho (Thr172) - AMPK α (40H9, CST) and polyclonal rabbit β -actin (Sigma). After washing, the membrane was incubated with the corresponding horseradish peroxidase (HRP) labelled secondary antibody (goat anti-rabbit IgG (H+L)-HRP conjugate/Bio-Rad, or F(ab')₂-goat anti-mouse IgG (H+L), HRP/Sigma) for 1h at RT. To visualise the antigen-antibody-antibody complex, we used the HRP substrate Clarity[™] Western ECL (Bio-Rad) and a ChemiDoc[™] Touch Imaging System (Bio-Rad). ImageJ was used for semi-quantitative analysis of band intensity.

2.7 Flow Cytometry (FACS) assays

FACS analysis was performed to assess surface expression of CD98hc and CD107a (LAMP1) in K562 cells. After they were harvested and washed once with CMF-PBS buffer, cells were resuspended in CMF-PBS supplemented with 10% FBS (staining buffer) and cell numbers were assessed. For each tested condition, 2×10^5 cells in a total volume of 200 μ l staining buffer were incubated with a fluorescein isothiocyanate (FITC) labelled anti-human CD98hc mouse antibody (MEM-108, BioLegend) in parallel with an allophycocyanin (APC) labelled anti-human CD107a mouse antibody (H4A3, BioLegend) in the dark for 1h at 4°C. In parallel, cells for each condition were stained with the same concentration of matching isotype-antibodies (FITC-mouse IgG1, κ /BioLegend and APC-mouse IgG1, κ /Thermo Fisher/eBioscience). Afterwards, cells were washed twice with CMF-PBS and resuspended in staining buffer. Until analysis samples were shielded from light and kept on ice. Flow cytometry was performed with a BD LSRFortessa[™] flow cytometer running BD FACSDiva[™] software. Light scattering characteristics of cells were used to discriminate dead cells as described previously [27]. Emitted mean fluorescence-area (FL-A) was used for data analysis. To assess the mode of cell death, FACS analysis of cleaved (activated) caspase-3 and Annexin V (AV)/cell-impermeant DNA binding dye - double staining was done. To stain active caspase-3 5×10^5 cells were fixed with Cytofix/Cytoperm (BD) for 20min at 4°C and stained with an Alexa Fluor 647-labelled antibody against active caspase-3 obtained from BD according to the manufacturer's instructions. For AV staining cells were harvested and washed once with staining buffer. Then, 1×10^5 cells were stained in AV staining buffer (BioLegend), either with AV-APC (BioLegend) and propidium iodide (PI, Thermo Fisher/eBioscience), or Pacific Blue[™] AV (BioLegend) combined with the SYTOX Green Nucleic Acid Stain (Thermo Fisher) at RT and shielded from light for 20min. For analysis of DNA-content, cells were fixed with ice cold 70% ethanol and stained with PI according to standard protocols. Cells were analysed either with a BD LSR II or a BD LSRFortessa flow cytometer.

2.8 Fluorescence labelling of adherent growing living cells

Cells were stained as recently described with the fluorescent prazosin derivative BODIPY[®] FL Prazosin (QAPB) and the red fluorescent lysomototropic dye Lysotracker[®] Red (LT), both obtained from Thermo Fisher/Molecular Probes (Eugene, OR, USA) [15]. QAPB was

added directly to the culture at a final concentration of 100nM (LNCaP) or 200nM (HEK293T) to survey drug-trafficking in living cells. LT-staining was performed after cell cultivation. To identify apoptotic cells directly in cell cultures at the endpoint of the experiment, the CellEvent™ Caspase-3/7 Green ReadyProbes® Reagent or the Hoechst 33342 nuclear dye (both obtained from Thermo Fisher/Molecular Probes) were used according to the manufacturer's instructions. LAMP1-GFP, LAMP1-RFP or Rab5-RFP fusion proteins were expressed transiently in LNCaP cells using the CellLight® BacMam 2.0 technology (Molecular Probes/Thermo Fisher). The fluorescence of stained cells was analysed with an Eclipse TE300 (Nikon, Tokyo, Japan) inverted microscope, the LSM 510 META scanning laser confocal microscope (Zeiss, Jena, Germany) and/or a ZOE™ Fluorescent Cell Imager (Bio-Rad). Live cell imaging studies observing lysosomal tubulation in LAMP1-RFP expressing LNCaP cells were performed using a Zeiss Cell Observer.

2.9 Immunofluorescence staining

Indirect immunofluorescence (IF) staining followed standard protocols as described in previous studies [15]. The following primary antibodies were used: mouse CD98hc (either MEM-108/BioLegend, or UM7F8/BD Pharmingen), mouse LAMP1 (H4A3, BioLegend), rabbit LAMP1 (D2D11, CST), mouse CD44 (BJ18, BioLegend), mouse CD59 [p282 (H19)] (BioLegend), mouse CD147 (HIM6, BioLegend) and mouse alpha tubulin (DM A1, Thermo Fisher). For fluorescent visualisation of primary antibody binding, matching secondary antibodies labelled with either Cy3 or Alexa Fluor® 555 or DyLight® 488 obtained from Jackson ImmunoResearch (West Grove, PA, USA) or BioLegend were used. 4', 6-Diamidin-2-phenylindol (DAPI, 1µg/mL, Sigma) was routinely used to counterstain nuclei but is not shown in every image compilation. In some experiments actin fibres were visualised with phalloidin staining using phalloidin-tetramethylrhodamine B isothiocyanate (Sigma). A Leica DM 4000 fluorescence microscope (Wetzlar, Germany) was used to monitor and document IF-stained cells.

2.10 Statistics

Data are presented as mean values + or +/- standard deviation calculated with Microsoft Excel. Significance was calculated using Sigma Plot 13 (Systat Software Inc., San Jose, CA, USA). Normal distribution of data was tested with the Shapiro-Wilk test. Multiple testing or testing of several treatment groups versus a single control group was done with one-way ANOVA with Holm-Sidak post hoc testing, assuming normal distribution of data. For multiple testing or testing several treatment groups versus a single control group in data sets lacking normal distribution, one-way ANOVA on the ranks and Student-Newman-Keuls (NKS) post hoc test or Dunnett's method were used as appropriate. The overall significance level was set at $p = 0.05$. To test for possible treatment interactions, the free software tool CombeneFit 2.021 (Cancer Research UK/University of Cambridge, Cambridge, UK) was employed, following the Bliss independence model and one sample t-test for testing significance of possible drug interactions.

3 Results

3.1 Prazosin reroutes CD98hc towards tubular lysosomes

Because we had detected CD98hc in a BODIPY[®] FL Prazosin (QAPB) positive protein fraction [15] (unpublished results), we initially analysed the pattern of CD98hc in prazosin (PRZ) treated LNCaP prostate cancer cells with indirect immunofluorescence (IF). We found CD98hc mainly located at the cell membrane in untreated cells, whereas in PRZ treated cells, CD98hc appeared in tubular structures (Fig. 1). Since one of the main cytotoxic mechanisms of PRZ is lysosomal tubulation [15], we investigated a possible association of CD98hc with LAMP1 positive tubules. In fact, double IF assays showed perfect analogy of CD98hc with morphologically altered lysosomes in PRZ treated cells (Fig. 1). We obtained similar effects in the leukaemia cell line K562, which was used as a second model in our study [5,12,15] (Suppl. Fig. 1). Because CD98hc is a known cargo of clathrin independent endocytosis (CIE) which employs tubular carriers for the endocytic recycling of proteins back to the cell membrane [28], we wanted to investigate whether the trafficking of other CIE cargo proteins such as CD44, CD59, and CD147 (basigin) was affected by PRZ as well [28,29]. CD147 was of particular interest, since CD98hc is associated with basigin [30]. As reported by Verkaik et al. [31], we also saw no expression of CD44 in LNCaP cells (Suppl. Fig. 2). We found CD59 expressed in LNCaP cells, but the fluorescence pattern of CD59 was not affected by PRZ treatment (Suppl. Fig. 2). Basigin showed a pronounced association with the cell membrane in untreated and PRZ treated cells, but in contrast to CD98hc, no striking tubular pattern appeared in PRZ treated cells (Suppl. Fig. 2). This data suggests that PRZ does not affect clathrin independent endocytosis in general but specifically targets CD98hc to newly formed lysosomal tubular structures.

3.2 Membrane derived CD98hc is shuttled towards tubular lysosomes

To investigate whether membrane derived CD98hc is shuttled towards lysosomes in PRZ treated cells, we directly added the CD98hc (MEM-108) antibody routinely used in our IF experiments to cultures of LNCaP cells and treated them with PRZ for 24h without prior removal of the antibody solution. Following fixation and incubation with the secondary antibody we observed an almost identical pattern of CD98hc as in cells stained according to the standard IF protocol (Fig. 2). In cells treated with PRZ, but not in control cells, we observed lysosomal accumulation of CD98hc and the formation of the tubular LAMP1/CD98hc pattern. In contrast, addition of an anti CD44 antibody, used as isotype control for the CD98hc antibody, to the culture gave no specific signal. Because the antibody solution contained a low concentration of NaN₃, we also tested the effects of NaN₃ on the pattern of CD98hc in PRZ treated cells. Interestingly, the supplementation of the medium with azide equivalent to the antibody solution showed no effect on untreated cells but resulted in polar accumulation of CD98hc and attenuation of the formation of the tubular CD98hc fluorescence pattern in PRZ treated cells, suggesting that the tubulation process depends on ATP (not shown). In summary, the main outcome of these experiments is that the CD98hc protein that accumulates in the lysosomes of PRZ treated cells at least partially originates from the cell membrane.

3.3 Prazosin induces de novo synthesis of CD98hc

IF-assays have shown that the localization of CD98hc changes from a surface to a lysosomal pattern in response to PRZ treatment. To test whether PRZ also induces expression changes of CD98hc in K562 cells, we used qPCR to quantify CD98hc mRNA levels, flow cytometry to assess cell membrane bound CD98hc protein and western blotting (WB) to screen for changes in total CD98hc-expression. qPCR and WB assays showed a dose dependent increase of CD98hc-transcription and translation (Fig. 3). But even though the accumulation of CD98hc in the lysosomes of PRZ treated cells suggested a possible lack of CD98hc at the cell membrane, CD98hc surface expression in PRZ treated cells remained almost constant (Fig. 3). There was only a slight (~20%) reduction of CD98hc expression in cells treated with 20µM PRZ. WB experiments in the LNCaP cell line as well showed a dose dependent up-regulation of CD98hc in response to PRZ treatment (Fig. 3), whereas the level of membrane bound CD98hc remained almost constant as assessed by flow cytometry (data not shown).

3.4 CRISPR/Cas9-knockout of the SLC3A2 gene in HEK293T cells does not inhibit endocytosis of quinazolines and lysosomal tubulation but interferes with pro-apoptotic signalling in response to prazosin

In order to establish a functional connection between CD98hc, PRZ induced lysosomal tubulation and apoptosis, a CRISPR/Cas9-mediated gene knockout (KO) of SLC3A2 - the gene coding for CD98hc - was realized in the HEK293T (HEK) cell line. The HEK cell line is an established model for research on CD98hc [30]. Successful KO of CD98hc was confirmed with WB (Fig. 4A), IF (Fig. 4C) and PCR/qPCR (not shown). Using this model, we observed that the adhesive behaviour of CD98hc KO cells was impaired compared to wild type (WT) cells. KO cells showed adhesive growth, but were very prone towards detachment in response to vibration or agitation. There was also upregulation of CD98hc expression (Fig. 4A, B) and lysosomal tubulation (Fig. 4D) in response to PRZ treatment in HEK cells, confirming that the HEK cell line is an appropriate model for our purposes. Experiments with CD98hc KO cells clearly showed that the uptake of QAPB and lysosomal tubulation were not disabled in the absence of CD98hc (Fig. 4D); however, we observed fundamental differences in the response of HEK cells towards PRZ treatment when CD98hc was deleted. First, we saw that PRZ treatment already induced rounding of CD98hc KO clones at concentrations of 15µM, whereas WT cells mostly maintained their typical morphology. As a consequence, PRZ treated KO clones preferentially formed floating cell aggregates and tubular lysosomes showed pronounced perinuclear localization in these cells (Fig. 4D). Second, staining of living cells with the Hoechst nuclear dye showed the accumulation of complex nuclei in WT cells treated with 15µM PRZ due to inhibition of cytokinesis [6,15], but this was only sparse in cultures of CD98hc KO cells (Suppl. Fig. 3). Most interestingly, within 48h there was pronounced nuclear fragmentation in response to 30µM PRZ in WT cells whereas nuclear fragmentation was much lower in CD98hc KO cells (Suppl. Fig. 3). To confirm these preliminary observations, we tested proliferation, viability, DNA content and apoptosis in KO clones. Cell counts showed that proliferation of CD98hc KO cells was decreased compared to WT cells (Fig. 5). In HEK cells treated either with 15µM or 30µM PRZ there was no increase in cell counts over a total period of 72h, irrespective of CD98hc expression. But even though total cell numbers of PRZ treated WT

cells remained constant over time, the cell volume and the DNA content of these cells were still increasing, indicating endoreplication (Fig. 5C and Suppl. Fig. 3). Within 24h both WT cells and CD98hc KO clones treated with 15 μ M PRZ showed an accumulation of cells with 4N and >4N DNA content (Fig. 5C). In cells treated with 30 μ M PRZ, the 4N cell fraction was dominant. In the course of time, cells treated with 15 μ M PRZ showed a continuous increase in DNA content and the parallel appearance of a pronounced Sub-G1 peak, indicating nuclear fragmentation and hence apoptosis. It was evident that the progress of the cell cycle and subsequently the accomplishment of the multinucleated phenotype and the induction of apoptosis were clearly attenuated in CD98hc KO cells. WT cells treated with 30 μ M PRZ showed ongoing DNA synthesis while cytokinesis was inhibited; there was a time dependent increase of the Sub-G1 peak, which conforms to our microscopic observations of massive karyorrhexis under these conditions (Suppl. Fig. 3). Most interestingly, CD98hc KO cells treated with 30 μ M PRZ were almost completely arrested in a 4N-state at 24h and persisted in that state for up to 72h (Fig. 5C). WST-1-viability assays (Fig. 5B) showed only minor differences between WT cells and CD98hc KO clones concerning measured optical density (OD) 450 values. At 24h after addition of 15 μ M PRZ to cultures OD values of CD98hc KO clones 1 and 2 were significantly ($p < 0.05$) lower compared to WT cells. These differences disappeared with drug dose and time. Cell death analysis using annexin V and the SYTOX Green Dead Cell Stain of cells (Fig. 5D) treated with 15 μ M PRZ showed only slight differences in viability between WT and CD98hc KO clones. However, with 30 μ M PRZ treatment, KO clones showed a significantly ($p < 0.001$) higher level of viability (i.e. a higher percentage of annexin V/SYTOX negative cells) than WT cells. The mean percentage ($n=3$) of viable WT cells following 48h treatment with 30 μ M PRZ was 14 $\pm$ 5% compared to 40 $\pm$ 2% (Clone 1), respectively 41 $\pm$ 2% (Clone 2). Thus, loss of CD98hc does not affect endocytosis of PRZ and lysosomal tubulation, but does affect the progress of endoreplication and the survival of cells.

3.5 Ammonia - a by-product of glutaminolysis - preserves growth and viability of cancer cells in the presence of prazosin

Since it is known that besides AA, several xenobiotics also enter the cell via LAT1 (=CD98 light chain/CD98lc) which is coupled to CD98hc by a disulphide bridge [16,17,32], we tested whether an excess of distinct AA might interfere with the effects of 10 μ M PRZ on K562 cells. In fact, among tested AA an antagonistic effect against the anti-proliferative effect of PRZ was observed for glutamine (Gln) (Fig. 6B) but not for alanine, arginine, asparagine, glycine, histidine, leucine, phenylalanine, serine, threonine, or supplementation of the growth medium with complex MEM amino acid concentrate (not shown). However, increasing Gln levels in the medium did not fully restore the growth of cells reduced in response to 10 μ M PRZ. We confirmed that high Gln-concentrations in the medium ameliorated the anti-proliferative effect of low doses of PRZ over a total period of 72h (Fig. 6C). Proliferation of untreated K562 cells was not significantly influenced by either the omission of Gln supplementation or the presence of Gln in a concentration range (0.5-10mM) employed in common growth media formulations.

Furthermore, there was at least a modest protective effect for cysteine, which is most possibly associated with its antioxidant properties [33]. Addition of glutathione or also its

antioxidant precursor N-acetylcysteine to the medium resulted in a similar effect as seen for cysteine (not shown). Glucose deprivation or increasing levels (2.75-33mM) of glucose did not significantly influence the growth of K562 cells treated with 10 μ M PRZ (data not shown).

In the light of CD98hc, the protective role of Gln against the cytotoxicity of PRZ was not surprising, because CD98hc is a crucial component for the maintenance of cellular Gln homeostasis, especially in malignant cells (Fig. 6A) [16, 34–37]. Since our results suggested PRZ induced “glutamine addiction” of K562 cells, we tested whether targeting cellular Gln metabolism at several points - as illustrated in Fig. 6 - might enhance the cytotoxicity of PRZ. We therefore tested whether the enzyme asparaginase (L-ASP) and the glutaminase (GLS) inhibitors BPTES and DON were able to interfere with the protective effect of Gln against the cytotoxicity of PRZ on K562 cells. As a competitive Gln-analogue, DON also inhibits other Gln-metabolizing enzymes such as glutamine fructose-6-phosphate amidotransferase (GFAT) that use Gln as a nitrogen donor for the synthesis of several biomolecules [36] (Fig. 6A).

L-ASP, which deaminates the AA asparagine and to a lesser extent Gln [38] (Fig. 6A), was used to eliminate Gln in the medium. As reported by Song et al. L-ASP inhibited the growth of K562 cells dose dependently [39] and surprisingly attenuated the antiproliferative effects of PRZ (Fig. 6 and Suppl. Fig. 4A). In contrast, glutaminase inhibitors also inhibited cell growth dose dependently, but solely exhibited an additive but no synergistic inhibitory effect with PRZ and did not cancel the protective effect of high Gln doses (data not shown).

To further investigate the protective effect of L-ASP, we tested its products ammonia, glutamate and aspartate on PRZ treated cells. We observed a pronounced protective role of NH₄Cl (without affecting the pH of the medium) on PRZ treated cells but no effects with either glutamate or aspartate (Fig. 6 and Suppl. Fig. 4A). Just 1mM NH₄Cl completely neutralized the effect of 10 μ M PRZ. In order to test whether ammonia is utilized as a substrate to generate Gln via glutamate-ammonia-ligase (GLUL) [37], we used the GLUL-inhibitor methionine sulfoximine (MSO) either alone or in a cocktail with PRZ. However, even though used in concentrations up to 4mM, MSO neither inhibited the growth of K562 cells alone, nor did it show a synergistic growth inhibitory effect with PRZ irrespective of the Gln concentration in the medium (not shown).

We also tested the effects of Gln and ammonia on cell viability to confirm results seen with cell counting. As expected, we observed a significant ($p < 0.001$) decrease in cell viability following treatment with pro-apoptotic 15 μ M PRZ [12] in standard medium (without Gln) using WST-1 viability assays (Suppl. Fig. 4). In the presence of ammonia the harmful effects of PRZ were almost completely neutralized. Supplementation of the medium with 10mM Gln also significantly ($p < 0.001$) attenuated the cytotoxicity of PRZ but did not completely restore the viability of cells. Caspase-3 activation occurred in at least a low percentage of cells treated with 15 μ M PRZ without addition of Gln or ammonia, but not when supplemented with 10mM Gln or ammonia (Suppl. Fig. 4). Annexin V/propidium iodide (AV/PI) assays confirmed ongoing apoptosis in Gln deprived PRZ treated cells (Suppl. Fig. 4). Addition of ammonia to the medium fully protected the cells against the induction of

apoptosis, whereas supplementation of the medium with 10mM Gln appeared only to slow down the progression of apoptosis.

To reproduce our results obtained in the K562 cell line in LNCaP cells, we treated the latter for a total period of 72h with increasing concentrations of PRZ without or with 2mM or 10mM Gln. As reported by Dong and Yung [40] we observed that Gln deprivation inhibits the proliferation of LNCaP cells, but surprisingly, high doses of Gln also interfered with cell growth (Suppl. Fig. 5). Thus - in contrast to K562 cells - the growth behaviour of LNCaP cells is clearly influenced by Gln supply. With PRZ concentrations $>15\mu\text{M}$ high doses of Gln exerted a protective effect on LNCaP cells. Microscopic analysis of cells treated with $30\mu\text{M}$ PRZ under Gln free conditions additionally stained with the caspase-3/7 green reagent showed rounding, massive detachment and caspase activation, indicated by green fluorescent staining of the nuclei (Suppl. Fig. 5). Supplementation of the medium with high doses of Gln at least partially preserved the adhesive character of the cells and suppressed the induction of apoptosis although 10mM Gln could not completely neutralize the cytotoxicity of PRZ. NH_4Cl also significantly ($p<0.05$) interfered with the cytotoxic effect of PRZ (Suppl. Fig. 5). Microscopic analysis of cells showed dose dependent rounding, detachment and activation of effector caspases, attenuated by supplementation of the medium with NH_4Cl .

3.6 Ammonia maintains membrane expression and total expression of CD98hc in PRZ treated cells

To assess CD98hc cell membrane and total protein expression of PRZ treated cells in dependence of Gln and ammonia, we analysed cells by flow cytometry (FACS) and WB. To test whether tubular LAMP1 positive structures are in contact with the cell membrane, we used FACS to assess (in parallel to CD98hc) the expression of LAMP1 (CD107a), which is a marker for lysosomal exocytosis [41]. FACS analysis of PRZ [$15\mu\text{M}$] treated cells showed a constant 20% reduction of membrane bound CD98hc under Gln free conditions, which could be reversed by supplementation of the medium with either Gln or NH_4Cl (Fig. 7). We detected weak expression of CD107a at the cell membrane of untreated cells that was approximately doubled by 5mM NH_4Cl (Fig. 7). With $15\mu\text{M}$ PRZ a fivefold increase of CD107a at the cell membrane was evident that was almost completely abolished by NH_4Cl but not by Gln.

These results imply that lysosomal tubules are indeed in contact with the cell membrane, providing a route via late endosomes/lysosomes back to the cell membrane (via lysosomal exocytosis).

Analysis of total CD98hc protein expression showed that NH_4Cl but not Gln significantly interfered with PRZ induced stimulation of CD98hc expression (Fig. 7). The supplementation of Gln free medium with NH_4Cl maintained CD98hc expression in the presence of PRZ at the same level as in untreated cells, further confirming an intriguing relationship between ammonia and PRZ.

For preliminary information on whether PRZ might alter the activation state of cellular energy sensors AMPK α and GSK3 α , which are proteins downstream from CD98hc [30,42],

we assessed the phosphorylation state of these proteins in K562 cells following 24h treatment with PRZ (Fig. 7). These analyses were done in parallel with CD98hc WB as described above. GSK3 α showed a significantly ($p < 0.05$) higher phosphorylation level at Ser21 in PRZ treated cells in the absence of Gln or ammonia, indicating inactivation of GSK3 α via active PI3K-AKT-signalling upstream of GSK3 α [43]. In the presence of ammonia PRZ induced phosphorylation of GSK3 α was abolished. We also observed significant alterations in the activation state of AMPK α . Interestingly, within 24h ammonia and PRZ reduced the level of phosphorylation of AMPK α at Thr172 compared to untreated control (without NH₄Cl/Gln/PRZ) indicating inactivation of AMPK α and therefore a sufficient level of cellular energy. Thus, even though we only analysed a single time point, our results suggest that PRZ influences the metabolic state of the cell, but surprisingly causes no lack of energy.

3.7 Ammonia, glutamine, chloroquine and the v-ATPase-inhibitor bafilomycin A1 interfere with lysosomal tubulation and the trafficking of CD98hc

To assess the pattern of CD98hc and lysosomes in LNCaP cells in dependence on ammonia or Gln, we performed CD98hc/LAMP1 IF analysis, and QAPB/LT vital staining's. In the presence of ammonia, the localization and morphology of LAMP1 positive endosomes/lysosomes was only slightly affected and the pattern of CD98hc remained mostly unchanged (Fig. 8). Interestingly, the supplementation of the medium with ammonia (2mM or 5mM) completely abolished PRZ induced tubulation of lysosomes and lysosomal accumulation of CD98hc in the absence (not shown) or presence of Gln (Fig. 8). Analysis of lysosomes in living cells using LT and QAPB confirmed the results obtained in fixed cells (Suppl. Fig. 6). In the low concentrations used NH₄Cl did not prevent the accumulation of LT in obviously still acidic lysosomes. Over the total incubation time of 24h, NH₄Cl did not prevent cellular uptake of QAPB but provoked a more diffuse fluorescence pattern and completely abolished the formation of QAPB positive tubules. Gln was also able to interfere with lysosomal tubulation and PRZ induced trafficking of CD98hc but the potency of Gln was much less pronounced than ammonia (Suppl. Fig. 7). We so conclude that ammonia causally interferes with PRZ induced lysosomal tubulation and resultant misrouting of CD98hc.

Since we demonstrated in our previous study that the FDA approved lysomototropic drug chloroquine (CHQ) and the v-ATPase-inhibitor bafilomycin A1 (BafA1) are able to restore the growth of cells in the presence of PRZ [15], we also tested whether these drugs are able to influence the trafficking of CD98hc either alone or in mutual reaction with PRZ. As already shown previously for CHQ [15], BafA1 also induced a dose dependent accumulation of enlarged LAMP1 positive endosomes/lysosomes in the cytoplasm (Suppl. Fig. 8) and completely eliminated PRZ induced lysosomal tubulation in LNCaP cells. We further showed that in response to either CHQ or BafA1 treatment CD98hc also accumulates in cytoplasmic vesicles in LNCaP (Suppl. Fig. 8) as well as K562 cells (not shown). In K562 cells low concentrations of both drugs (CHQ: 10-20 μ M, BafA1: 1-5nM) induced the formation of enlarged LAMP1 positive vesicles that showed co-localization with CD98hc and efficiently interfered with PRZ induced lysosomal tubulation. Furthermore, in CHQ and BafA1 treated cells there was a dose dependent increase of CD107a expression at the cell membrane, indicating lysosomal exocytosis. Interestingly, CHQ treatment alone resulted in a

dose dependent decrease of CD98hc membrane expression (with 20 μ M CHQ: 70 $\pm$ 5% CD98hc expression compared to untreated control, n=7, p<0.001), whereas BafA1 only marginally altered CD98hc membrane expression.

Experiments using HEK293T SLC3A2 KO cells showed that both CHQ and ammonia preserved the adhesive behaviour of KO cells in the presence of PRZ and also completely inhibited lysosomal tubulation, suggesting that the protective effects of these lysomototropic agents are not causally dependent on CD98hc expression (not shown).

3.8 Prazosin induced lysosomal tubulation is reversible, depending on an interplay of actin and microtubules but is non-sensitive towards inhibition of the canonical PI3K-AKT-mTor pathway

Since lysosomal tubulation is one of the main features of the cytotoxicity of PRZ, we aimed to further characterize this process. Besides LAMP1 positive tubules a further typical sign of PRZ-treated cells is vacuolization. We tested, whether these vacuoles represent early (sorting) endosomes or, lysosomes. We observed the appearance of Rab5 positive vacuoles parallel to Rab5 tubules, but no LAMP1 positive tubules within 10min of treatment of LNCaP cells with 20 μ M PRZ (Fig. 9A). In cells treated for 24h with PRZ, Rab5 positive tubules were replaced by LAMP1 positive tubules. Live cell imaging studies revealed that LAMP1+ vacuoles swell and fuse with each other (Suppl. Video File 1A). Most interestingly, time-lapse microscopy also showed that LAMP1+ vacuoles arose in the cytoplasm and accumulated in the perinuclear region of PRZ treated cells. LAMP1+ tubules were obviously “growing” towards vacuoles originating from lysosomes located at the cell poles (Suppl. Video Files 1B/C). Although vacuoles also became LAMP1 positive, they did not accumulate LysoTracker® (Fig. 9B) suggesting that vacuoles are non-acidic and not physically connected with LAMP1+ tubules. Confocal microscopy showed that following 24h PRZ treatment a minority of (small) vacuoles were positive for both Rab5 and LAMP1, whereas huge vacuoles were only positive for LAMP1 (Fig. 9C). Live cell imaging also confirmed that LAMP1 positive tubules interfered with cytokinesis, as indicated by LAMP1+ intercellular bridges between dividing cells (Suppl. Video Files 1A-C).

In experiments investigating whether PRZ induced tubulation is still reversible [6] when late endosomes/lysosomes are affected, we observed that the formation of QAPB/LAMP1 positive tubules was indeed reversible. Interestingly, tubules disappeared, but QAPB/LAMP1 positive vacuoles remained and were still visible 24h after removal of PRZ (Fig. 9D). In summary, these experiments clearly show that PRZ induced vacuoles derive from swollen, obviously functionally impaired, early endosomes; this triggers the subsequent tubulation of lysosomes.

Zhang et al. also tested the role of the cytoskeleton in PRZ induced endosomal tubulation, but their results were inconclusive [6]. In our hands, using either cytochalasin-D (Cyto-D) or nocodazole (NDZ) to disrupt the actin cytoskeleton or microtubules, respectively, both the morphology of the cells and PRZ induced lysosomal tubulation were affected. Depolymerisation of F-actin with Cyto-D, which affects most endocytic pathways [44], interfered with the cellular transport of QAPB, indicated by QAPB-positive vesicles spread all over the cytoplasm with and without additional PRZ treatment, and also abolished the

formation of the QAPB positive tubular pattern within 3h (not shown). Surprisingly, within 24h we observed not solely polar protrusions as usual with PRZ, but the appearance of fine LAMP1 positive tubules all around the cell (Fig. 9E). Disruption of microtubules resulted in loss of the spindle shaped appearance of LNCaP cells. Before NDZ treated cells finally showed rounding and detached within 24h, they spread on the surface of the flask and formed cell clusters (Fig. 9E). In NDZ treated cells, lysosomes were localized perinuclear in vesicular form in control cells, or in tubular form in the presence of PRZ (Fig. 9E). This observation is in good accordance with findings that lysosomal positioning and tubulation depend on microtubules [45–47].

A recent study by Saric et al. showed that lipopolysaccharide induced lysosomal tubulation in antigen presenting cells is abrogated by PI3K and mTor-inhibitors [46]. We tested the ability of the novel dual PI3K/mTOR inhibitor BEZ235 and the phosphatidylinositol-3,4,5-triphosphate (PIP3) antagonist Pit-1 [48] to interfere with PRZ induced lysosomal tubulation in LNCaP cells. In the presence of Pit-1 (but not BEZ235) LNCaP cells lost their spindle like morphology and formed cell clusters without visible contours of single cells. Similar effects were observed in cells treated with NDZ, but cells treated with Pit-1 showed no detachment within 24h.

Interestingly, using these drugs either alone or in combination with QAPB/PRZ, we observed opposing results. Pre-treatment of cells with BEZ235 resulted in enhanced accumulation of QAPB and Lysotracker® (LT) in lysosomes, whereas Pit-1 inhibited the uptake of QAPB and lysosomal trapping of LT (Suppl. Fig. 9). Furthermore, lysosomal tubulation was not inhibited by BEZ235 up to concentrations of 500nM, whereas in cells treated with 150µM Pit-1 no lysosomal tubulation was evident with QAPB or LT. Additional IF staining showed that the morphology of LAMP1 positive endosomes/lysosomes was not altered by treatment of cells with either BEZ235 or Pit-1, suggesting a pH-dependent effect of those drugs on LT accumulation (Suppl. Fig. 9). LAMP1-IF staining further confirmed that lysosomal tubulation was completely stopped by Pit-1, but not by BEZ235. Parallel CD98hc staining showed a less pronounced tubular CD98hc fluorescence pattern and enhanced polar accumulation in PRZ/BEZ235 treated cells. In cells co-treated with Pit-1 and PRZ, the CD98hc staining remained at the cell membrane with no evident tubular structures, suggesting that endocytosis of PRZ/QAPB and subsequent lysosomal tubulation and trapping of CD98hc depend on PIP3. Experiments using the selective mTor inhibitor rapamycin (up to 1µM) showed similar results as with BEZ235, ruling out the canonical PI3K-AKT-mTor pathway as the force driving PRZ induced lysosomal tubulation.

4 Discussion

4.1 Prazosin induced lysosomal tubulation interferes with cytokinesis

Prazosin (PRZ), the first clinically approved substance of the drug class of quinazoline-based α 1-adrenoceptor antagonists, had a greater pro-apoptotic effect on malignant cells than other agents of this drug class, which is why we also focused on it [49,50]. PRZ recently attracted great attention for its anticancer potential, since Kahn et al. showed that PRZ can kill glioblastoma, a highly aggressive brain tumour with a poor prognosis [7]. Already in 2012 Zhang et al. were the first to associate PRZ with alterations in the

endolysosomal system [6]. The authors showed that PRZ disturbs endocytic sorting due to an off-target interaction with dopamine receptor DRD3, causing tubulation of endosomes and subsequent inhibition of cytokinesis [6]. We further found out more about the effects of PRZ on the endolysosomal system demonstrating that during long-term treatment of cells with PRZ the lysosomes also form a tubular pattern, which is reversible by lysomototropic agents and blockade of v-ATPase [15]. Live cell imaging showed that tubular LAMP1 positive structures did indeed interfere with the process of cytokinesis. This observation goes along with the finding of Rajamanoharan et al. that lysosomal activity in close proximity to the intercellular bridge plays an essential role in cytokinesis [51]. In 2001, Bergeland et al. have already shown that during cytokinesis lysosomes accumulate in the vicinity of the microtubule organization center [52]. Concerning the kinetics of PRZ induced lysosomal tubulation (modelled in Fig. 10) we observed that vacuoles deriving from early endosomes (EE) trigger the formation of LAMP1 positive tubules. This hypothesis is supported by the appearance of swollen EE, which are the “distribution centres” of intracellular vesicle trafficking [53], convincing hints that PRZ interferes with endocytic sorting [6] and the observation that huge QAPB positive vacuoles remain in the cytoplasm of cells following removal of PRZ. The pronounced swelling of EE is most probably due to osmotic imbalances in the vesicle lumen [54]. During long-term treatment of cells with PRZ Rab5 positive tubules disappeared, but LAMP1 positive tubules arose instead. In the light of the observation that vacuoles acquire LAMP1, maturation of EE towards late endosomes is thinkable, which is known to be accompanied by loss of EE tubular domains [55]. Tubular lysosomes obviously grow towards vacuoles but do not fuse with them. Because of the dynamics of vacuoles regarding their volume and their interaction with LAMP1 positive tubules we hypothesize that vacuoles attract lysosomes and subsequently exchange quinazoline bound proteins with tubular lysosomes via a “kiss and run” mechanism [56]. Tubular lysosomes subsequently grow towards and fuse with the cell membrane, as indicated by increased expression of the lysosomal membrane protein LAMP1 at the cell surface - technically representing a kind of lysosomal exocytosis [41]. However, we have no proof to date as to whether CD98hc is also shuttled back to the membrane via this pathway. Several research groups suggested a similar mode of action for antigen presenting cells (APC) of the immune system [46,47,57]. Antigen loaded class II MHC molecules were shown to be shuttled via LAMP1 positive tubular structures towards the cell membrane using microtubules as trafficking route. Moreover, the canonical PI3K-AKT-mTor pathway was found to be essential for lysosomal tubulation in APC [46]. We did not, however, observe that BEZ235, which targets multiple members of the mTor pathway simultaneously [58], interferes with the effects of PRZ on lysosomes. This means that physiological lysosomal tubulation in APC may be somehow functionally different compared to drug (PRZ) induced lysosomal tubulation. Even though, the sensitivity of PRZ induced lysosomal tubulation towards the PIP3 antagonist Pit-1 suggests that PIP3 has an essential role in this process. A common feature of lysosomal tubulation in APC and PRZ treated cells is its dependence on microtubules. When microtubules were disrupted in PRZ treated cells, perinuclear tubular lysosomes accumulated, suggesting that PRZ induced lysosomal tubulation not in general but that the anterograde trafficking of tubular lysosomes depends on microtubules. Furthermore, the formation of QAPB positive tubules was also sensitive towards disruption

of actin fibres. PRZ induced lysosomal tubulation therefore is conducted via interaction of vesicles of the endolysosomal system with actin filaments and microtubules.

4.2 CD98hc paves the way towards Mitotic Catastrophe of PRZ treated cells

Besides the characterization of the kinetics of PRZ induced lysosomal tubulation, we aimed primarily to identify further protein targets of PRZ responsible for the pro-apoptotic effects of quinazolines. We recently described that QAPB showed a relatively stable interaction with a protein complex that provided a possible window of opportunity for the identification of proteins involved in the cytotoxicity of PRZ [15]. We identified CD98hc, a protein whose trafficking and expression is modulated by PRZ and that is also a crucial factor for growth and survival of PRZ treated cells. We demonstrated that in the presence of PRZ, CD98hc is shuttled from the cell membrane towards tubular lysosomes. This is quite interesting because it is known that CIE cargo proteins normally traffic directly into Arf6-associated tubules after internalization and avoid lysosomal degradation [59]. LAT1 is also a known CIE cargo [16,60]. Unfortunately, due to unspecific binding of the LAT1 antibody we used, we cannot yet discriminate whether PRZ shuttles solely CD98hc-monomers and/or CD98hc-LAT1 heterodimers towards lysosomes, although we have also consistently identified LAT1 in the QAPB positive protein fraction (results not shown). Enhanced shuttling of CD98hc towards lysosomes is a phenomenon that was already described in 2002 to occur in cells under withdrawal of growth factors [61]. But even though we also confirmed that CD98hc in lysosomes of PRZ treated cells originates from the cell membrane, total CD98hc expression levels were highly elevated whereas expression at the cytoplasmic membrane was only slightly reduced or remained almost constant under these conditions. Accordingly, our hypothesis was that PRZ either acts as a ligand of CD98hc, stimulating endocytosis (and recycling) of its receptor, or interferes with endocytic sorting of CD98hc in an unspecific manner. Using HEK293T CD98hc knock out (KO) clones we observed that KO cells were still able to accumulate QAPB and also showed tubular lysosomes. It was then clear that CD98hc is not required for endocytosis of quinazolines per se and is also not causally responsible for PRZ induced lysosomal tubulation. We therefore conclude that CD98hc is rerouted towards tubular lysosomes when PRZ blocks endocytic sorting (Fig. 10).

Nevertheless, we found that CD98hc is a protein that significantly influences the fate of PRZ treated cells regarding growth and survival. When treated with high doses of PRZ, HEK293T WT cells, but CD98hc KO cells only to a limited extent, were able to synthesize DNA, favouring the genesis of a multinucleated phenotype. Interestingly, CD98hc was already associated manifold with the formation, due to induction of cell fusion, and occurrence of multinucleated giant cells in various cell types, including monocytes, trophoblasts and glioblastoma cells [62–64]. Since HEK293T CD98hc KO clones showed an overall reduced proliferative potential compared to WT cells, we hypothesize that lack of CD98hc delays the induction of apoptosis in cells in which cytokinesis is inhibited. It is well known that cytokinesis failure and the subsequent appearance of a binucleated (and multinucleated) phenotype cause genetic instability and apoptosis via so-called Mitotic Catastrophe [65]. This hypothesis is supported by the findings of Lin et al. that PRZ induces DNA damage stress in PC-3 prostate cancer cells [49]. Furthermore, Spencer et al. have demonstrated recently that PRZ sensitises prostate cancer cells to docetaxel [66]. The latter

is a chemotherapeutic agent which targets microtubules and induces cell death in cancer cells through induction of Mitotic Catastrophe [67]. Since highly proliferative cells and especially cancer cells are characterized by high CD98hc expression [16], CD98hc might pave the way towards Mitotic Catastrophe in cells treated with PRZ.

In the context of CD98hc and PRZ, cellular proliferation and survival, further studies should pay special attention to the role of integrins. Similar to CD98hc [60], it was shown in cancer cells expressing p53 mutants that recycling of β 1-integrin (via EE) is accelerated, whereas degradation in lysosomes is bypassed [68]. As mentioned in the introduction, on the one hand quinazolines target integrins [14,22] and on the other hand CD98hc is associated with integrins and promotes integrin-signalling [16,20,21]. In line with these results it was recently shown that CD98hc plays a central role in acute myelogenous leukaemia (AML) via integrin binding [69]. In 2009 we had already shown that PRZ induces pronounced apoptosis in the AML cell line HEL [12]. But an open question still remains, as to whether solely inhibition of cytokinesis [12], which is a common feature of PRZ treated cells, and/or misrouting of CD98hc and subsequent adverse effects on integrin-signalling cause PRZ induced apoptosis in HEL cells. Most interestingly, it was shown by Sachlos et al. in 2012 that AML cells express DRD3 (and other dopamine receptors), which serves as an atypical receptor for PRZ [6,70]. This finding establishes a possible connection between DRD3, PRZ and CD98hc. Generally, the pro-proliferative and anti-apoptotic roles of CD98hc seem to be strongly cell and tissue dependent. For instance, CD98hc in AML cells seems to be crucial for integrin signalling, whereas the loss of CD98hc in the colon cell line LS174T sensitizes the cells towards inhibition of LAT1, suggesting an essential role of CD98hc as chaperone for LAT1 in this cells line [71].

Besides the role of CD98hc, our data clearly shows that PRZ also induces apoptosis independent of CD98hc suggesting alternative pro-apoptotic mechanisms.

4.3 Prazosin, Ammonia and CD98hc: An intriguing love triangle

We found that ammonia, a by-product of glutaminolysis and amino acid metabolism in general, is able to completely neutralize the cytotoxicity of PRZ (Fig. 10). Routinely, commercial growth media are provided without Gln, since Gln is known to break down to ammonia and pyroglutamate during storage (Fig. 6) [72,73]. In a recent study, Zhang et al. demonstrated that during incubation of DMEM-medium (without cells) under standard cell culture conditions, approximately 1mM ammonia is generated within 24h [73]. In preliminary experiments (not shown) we compared the cytotoxicity of PRZ in dependence on the growth medium since the standardized recipes of DMEM and RPMI significantly differ regarding their supply of AA. Interestingly, growth of cells was inhibited in RPMI medium as described here, but surprisingly, the growth inhibitory effect of PRZ was weak or absent in DMEM medium. Catalysing the breakdown of Gln with L-ASP or supplementation of the (RPMI-) medium with NH_4Cl protected the cells against the cytotoxicity of PRZ, proving that the protective effect of Gln occurs via generation of ammonia. Supplementation of the medium with NH_4Cl also completely inhibited lysosomal tubulation and subsequent misrouting and upregulation of CD98hc. We therefore assume that when there is no misrouting of CD98hc, there is also no need to upregulate CD98hc. A

limitation of our study is that so far, we can only speculate about the underlying protective mechanisms of NH_4Cl . There is validated information that ammonia, similarly to CHQ and BafA1, alkalizes the pH in vesicles of the endolysosomal system. However, in order to alkalize lysosomes, much higher concentrations are routinely used than in our study [25]. At the moment, we can offer a highly speculative but interesting hypothesis that PRZ and ammonia compete for the same binding site at the dopamine receptor DRD3. Interestingly, Zhang et al., who have already shown that PRZ enters the cell via DRD3, also recently demonstrated that DRD3 acts as a cellular ammonia sensor but as yet no studies on the interaction of ammonia and PRZ at DRD3 have been published [73]. Our study is the first to partially close this gap, demonstrating an antagonistic effect of ammonia on the cytotoxicity of PRZ. It is well known that ammonia is a metabolite that is highly enriched in tumours, whereby enhanced glutaminolysis of tumour cells is seen as major source of ammonia [25,26]. Therefore, the antagonistic effects of ammonia might be important to overcome for the possible future use of quinazolines in chemotherapy.

Regarding the trafficking of CD98hc we also observed that treatment with CHQ and BafA1 results in accumulation of CD98hc in the cytoplasm. It is well known that luminal acidification of the endocytic pathway is required for vesicle trafficking and associated cellular functions [74]. Nonetheless, our study shows for the first time that the trafficking of CD98hc, which has an important role in cellular amino acid homeostasis, is sensitive towards manipulation of endocytic pH. Our study so revealed a new link between cellular pH control and bioenergetic mechanisms [75].

4.4 Conclusions

In synopsis, our study sheds new light on the process of PRZ induced lysosomal tubulation and shows for the first time that the endocytic recycling of the oncoprotein CD98hc can be modulated *in vitro* using FDA approved drugs. Our study further spotlights lysosomes as a hopeful new target for the treatment of cancer [76], thanks to the novel mechanism by which tubular lysosomes inhibit cytokinesis. Since CD98hc is seen as potential drug target in several types of human malignancies [16,21,70,71], our study can be expected to have high translational significance. Nevertheless, intensive research is still needed to translate our research into clinical practice and to fully understand the interactions between the intriguing love triangle of prazosin, ammonia and CD98hc.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We wish to thank Eugenia Lamont for proofreading the manuscript and Katharina Meditz and Marie-Therese Frisch for competent technical support. Furthermore, we want to thank Andrea Groselj-Strele for her help concerning analysis of drug interaction studies.

Funding

This work was supported by a grant from the Austrian Science Fund (FWF), P24006 (to RF); the Franz Lanyar Foundation (to RF) and by private donations obtained with a fund-raising campaign kindly initiated by MEFOgraz (to RF). The identity of donors remained completely anonymous for all authors involved, excluding any conflict of

interest in this regard. Furthermore, our study was supported by the Spanish Ministry of Science and Innovation, SAF2015-64869-R-FEDER (to MP) and the Government of Catalonia, Grant SGR2009-1355 (to MP). SCC is a recipient of a Severo Ochoa Fellowship.

References

- [1]. Benning CM, Kyprianou N. Quinazoline-derived alpha1-adrenoceptor antagonists induce prostate cancer cell apoptosis via an alpha1-adrenoceptor-independent action. *Cancer research*. 2002; 62:597–602. [PubMed: 11809715]
- [2]. Gonzalez-Juanatey JR, Iglesias MJ, Alcaide C, Pineiro R, Lago F. Doxazosin induces apoptosis in cardiomyocytes cultured in vitro by a mechanism that is independent of alpha1-adrenergic blockade. *Circulation*. 2003; 107:127–31. [PubMed: 12515754]
- [3]. Fernando MA, Heaney AP. Alpha1-adrenergic receptor antagonists: novel therapy for pituitary adenomas. *Molecular endocrinology*. 2005; 19:3085–96. [PubMed: 16020484]
- [4]. Hui H, Fernando MA, Heaney AP. The alpha1-adrenergic receptor antagonist doxazosin inhibits EGFR and NF-kappaB signalling to induce breast cancer cell apoptosis. *European journal of cancer*. 2008; 44:160–6. [PubMed: 18042375]
- [5]. Fuchs R, Schraml E, Leitinger G, Stelzer I, Allard N, Haas HS, et al. alpha1-Adrenergic drugs modulate differentiation and cell death of human erythroleukemia cells through non adrenergic mechanism. *Experimental cell research*. 2011; 317:2239–51. [PubMed: 21781962]
- [6]. Zhang X, Wang W, Bedigian AV, Coughlin ML, Mitchison TJ, Eggert US. Dopamine receptor D3 regulates endocytic sorting by a Prazosin-sensitive interaction with the coatomer COPI. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:12485–90. [PubMed: 22802617]
- [7]. Kahn SA, Costa SL, Gholamin S, Nitta RT, Dubois LG, Fève M, et al. The anti-hypertensive drug prazosin inhibits glioblastoma growth via the PKC-dependent inhibition of the AKT pathway. *Embo Mol Med*. 2016; 8:511–26. [PubMed: 27138566]
- [8]. Park MS, Kim BR, Dong SM, Lee SH, Kim DY, Rho SB. The antihypertension drug doxazosin inhibits tumor growth and angiogenesis by decreasing VEGFR-2/Akt/mTOR signaling and VEGF and HIF-1alpha expression. *Oncotarget*. 2014; 5:4935–44. [PubMed: 24952732]
- [9]. Chiang CF, Son EL, Wu GJ. Oral treatment of the TRAMP mice with doxazosin suppresses prostate tumor growth and metastasis. *Prostate*. 2005; 64:408–18. [PubMed: 15789364]
- [10]. Kyprianou N, Benning CM. Suppression of human prostate cancer cell growth by alpha1-adrenoceptor antagonists doxazosin and terazosin via induction of apoptosis. *Cancer Res*. 2000; 60:4550–5. [PubMed: 10969806]
- [11]. Siddiqui EJ, Shabbir M, Thompson CS, Mumtaz FH, Mikhailidis DP. Growth inhibitory effect of doxazosin on prostate and bladder cancer cells. Is the serotonin receptor pathway involved? *Anticancer research*. 2005; 25:4281–6. [PubMed: 16309229]
- [12]. Fuchs R, Stelzer I, Haas HS, Leitinger G, Schauenstein K, Sadjak A. The alpha1-adrenergic receptor antagonists, benoxathian and prazosin, induce apoptosis and a switch towards megakaryocytic differentiation in human erythroleukemia cells. *Annals of hematology*. 2009; 88:989–97. [PubMed: 19241077]
- [13]. Fuchs R, Schwach G, Stracke A, Meier-Allard N, Absenger M, Ingolic E, et al. The Anti-hypertensive Drug Prazosin Induces Apoptosis in the Medullary Thyroid Carcinoma Cell Line TT. *Anticancer research*. 2015; 35:31–8. [PubMed: 25550532]
- [14]. Sakamoto S, Schwarze S, Kyprianou N. Anoikis disruption of focal adhesion-Akt signaling impairs renal cell carcinoma. *Eur Urol*. 2011; 59:734–44. [PubMed: 21269758]
- [15]. Fuchs R, Stracke A, Ebner N, Zeller CW, Raninger AM, Schittmayer M, et al. The cytotoxicity of the alpha1-adrenoceptor antagonist prazosin is linked to an endocytotic mechanism equivalent to transport-P. *Toxicology*. 2015; 338:17–29. [PubMed: 26449523]
- [16]. Cantor JM, Ginsberg MH. CD98 at the crossroads of adaptive immunity and cancer. *J Cell Sci*. 2012; 125:1373–82. [PubMed: 22499670]
- [17]. de la Ballina LR, Cano-Crespo S, Gonzalez-Munoz E, Bial S, Estrach S, Caillaudeau L, et al. Amino Acid Transport Associated to Cluster of Differentiation 98 Heavy Chain (CD98hc) Is at

- the Cross-road of Oxidative Stress and Amino Acid Availability. *J Biol Chem.* 2016; 291:9700–11. [PubMed: 26945935]
- [18]. Milkereit R, Persaud A, Vanoaica L, Guetg A, Verrey F, Rotin D. LAPT4b recruits the LAT1-4F2hc Leu transporter to lysosomes and promotes mTORC1 activation. *Nat Commun.* 2015; 6:7250. [PubMed: 25998567]
- [19]. Santiago-Gomez A, Barrasa JJ, Olmo N, Lecona E, Burghardt H, Palacin M, et al. 4F2hc-silencing impairs tumorigenicity of HeLa cells via modulation of galectin-3 and beta-catenin signaling, and MMP-2 expression. *Bba-Mol Cell Res.* 2013; 1833:2045–56.
- [20]. Feral CC, Nishiya N, Fenczik CA, Stuhlmann H, Slepak M, Ginsberg MH. CD98hc (SLC3A2) mediates integrin signaling. *Proc Natl Acad Sci U S A.* 2005; 102:355–60. [PubMed: 15625115]
- [21]. Prager GW, Feral CC, Kim C, Han J, Ginsberg MH. CD98hc (SLC3A2) interaction with the integrin beta subunit cytoplasmic domain mediates adhesive signaling. *J Biol Chem.* 2007; 282:24477–84. [PubMed: 17597067]
- [22]. Poettler M, Unseld M, Braemswig K, Haitel A, Zielinski CC, Prager GW. CD98hc (SLC3A2) drives integrin-dependent renal cancer cell behavior. *Mol Cancer.* 2013; 12:169. [PubMed: 24359579]
- [23]. Garrison JB, Kyprianou N. Doxazosin induces apoptosis of benign and malignant prostate cells via a death receptor-mediated pathway. *Cancer research.* 2006; 66:464–72. [PubMed: 16397262]
- [24]. Manic G, Obrist F, Kroemer G, Vitale I, Galluzzi L. Chloroquine and hydroxychloroquine for cancer therapy. *Mol Cell Oncol.* 2014; 1:e29911. [PubMed: 27308318]
- [25]. Eng CH, Yu K, Lucas J, White E, Abraham RT. Ammonia derived from glutaminolysis is a diffusible regulator of autophagy. *Sci Signal.* 2010; 3:ra31. [PubMed: 20424262]
- [26]. Merhi A, Delree P, Marini AM. The metabolic waste ammonium regulates mTORC2 and mTORC1 signaling. *Sci Rep.* 2017; 7 44602.
- [27]. Fuchs R, Schraml E, Leitinger G, Letofsky-Papst I, Stelzer I, Haas HS, et al. alpha1-adrenergic drugs exhibit affinity to a thapsigargin-sensitive binding site and interfere with the intracellular Ca²⁺ homeostasis in human erythroleukemia cells. *Experimental cell research.* 2011; 317:2969–80. [PubMed: 21851819]
- [28]. Eyster CA, Higginson JD, Huebner R, Porat-Shliom N, Weigert R, Wu WW, et al. Discovery of New Cargo Proteins that Enter Cells through Clathrin-Independent Endocytosis. *Traffic.* 2009; 10:590–9. [PubMed: 19302270]
- [29]. Capestrano M, Mariggio S, Perinetti G, Egorova AV, Iacobacci S, Santoro M, et al. Cytosolic phospholipase A(2)epsilon drives recycling through the clathrin-independent endocytic route. *Journal of Cell Science.* 2014; 127:977–93. [PubMed: 24413173]
- [30]. Xu DS, Hemler ME. Metabolic activation-related CD147-CD98 complex. *Mol Cell Proteomics.* 2005; 4:1061–71. [PubMed: 15901826]
- [31]. Verkaik NS, Trapman J, Romijn JC, Van der Kwast TH, Van Steenbrugge GJ. Down-regulation of CD44 expression in human prostatic carcinoma cell lines is correlated with DNA hypermethylation. *Int J Cancer.* 1999; 80:439–43. [PubMed: 9935187]
- [32]. Geier EG, Schlessinger A, Fan H, Gable JE, Irwin JJ, Sali A, et al. Structure-based ligand discovery for the Large-neutral Amino Acid Transporter 1, LAT-1. *P Natl Acad Sci USA.* 2013; 110:5480–5.
- [33]. Kerkick C, Willoughby D. The Antioxidant Role of Glutathione and N-Acetyl-Cysteine Supplements and Exercise-Induced Oxidative Stress. *J Int Soc Sport Nutr.* 2005; 2
- [34]. Nicklin P, Bergman P, Zhang BL, Triantafellow E, Wang H, Nyfeler B, et al. Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy. *Cell.* 2009; 136:521–34. [PubMed: 19203585]
- [35]. Pochini L, Scalise M, Galluccio M, Indiveri C. Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. *Front Chem.* 2014; 2
- [36]. Hensley CT, Wasti AT, DeBerardinis RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. *J Clin Invest.* 2013; 123:3678–84. [PubMed: 23999442]
- [37]. Krall AS, Christofk HR. Rethinking glutamine addiction. *Nat Cell Biol.* 2015; 17:1515–7. [PubMed: 26612572]

- [38]. Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A, et al. L-Asparaginase Treatment in Acute Lymphoblastic Leukemia. *Cancer-Am Cancer Soc.* 2011; 117:238–49.
- [39]. Song P, Ye L, Fan JJ, Li YB, Zeng X, Wang ZY, et al. Asparaginase induces apoptosis and cytoprotective autophagy in chronic myeloid leukemia cells. *Oncotarget.* 2015; 6:3861–73. [PubMed: 25738356]
- [40]. Dong YS, Yung HC. Glutamine Deprivation Induces Growth Inhibition in Human Prostate Carcinoma LnCap Cells. *J Cancer Prev.* 2011; 16:41–50.
- [41]. Samie MA, Xu H. Lysosomal exocytosis and lipid storage disorders. *J Lipid Res.* 2014; 55:995–1009. [PubMed: 24668941]
- [42]. Fei F, Li XF, Xu L, Li DY, Zhang ZP, Guo X, et al. CD147-CD98hc Complex Contributes to Poor Prognosis of Non-Small Cell Lung Cancer Patients Through Promoting Cell Proliferation Via the PI3K/Akt Signaling Pathway. *Ann Surg Oncol.* 2014; 21:4359–68. [PubMed: 25084765]
- [43]. Ruvolo PP, Qiu YH, Coombes KR, Zhang N, Neeley ES, Ruvolo VR, et al. Phosphorylation of GSK3 alpha/beta correlates with activation of AKT and is prognostic for poor overall survival in acute myeloid leukemia patients. *Bba Clin.* 2015; 4:59–68. [PubMed: 26674329]
- [44]. Dutta D, Donaldson JG. Search for inhibitors of endocytosis: Intended specificity and unintended consequences. *Cell Logist.* 2012; 2:203–8. [PubMed: 23538558]
- [45]. Pu J, Guardia CM, Keren-Kaplan T, Bonifacino JS. Mechanisms and functions of lysosome positioning. *J Cell Sci.* 2016; 129:4329–39. [PubMed: 27799357]
- [46]. Saric A, Hipolito VE, Kay JG, Canton J, Antonescu CN, Botelho RJ. mTOR controls lysosome tubulation and antigen presentation in macrophages and dendritic cells. *Mol Biol Cell.* 2016; 27:321–33. [PubMed: 26582390]
- [47]. Li X, Rydzewski N, Hider A, Zhang X, Yang J, Wang W, et al. A molecular mechanism to regulate lysosome motility for lysosome positioning and tubulation. *Nat Cell Biol.* 2016; 18:404–17. [PubMed: 26950892]
- [48]. Miao B, Skidan I, Yang J, Lugovskoy A, Reibarkh M, Long K, et al. Small molecule inhibition of phosphatidylinositol-3,4,5-triphosphate (PIP3) binding to pleckstrin homology domains. *Proc Natl Acad Sci U S A.* 2010; 107:20126–31. [PubMed: 21041639]
- [49]. Lin SC, Chueh SC, Hsiao CJ, Li TK, Chen TH, Liao CH, et al. Prazosin displays anticancer activity against human prostate cancers: targeting DNA and cell cycle. *Neoplasia.* 2007; 9:830–9. [PubMed: 17971903]
- [50]. Forbes A, Anoopkumar-Dukie S, Chess-Williams R, McDermott C. Relative cytotoxic potencies and cell death mechanisms of α_1 -adrenoceptor antagonists in prostate cancer cell lines. *Prostate.* 2016; 76:757–66. [PubMed: 26880388]
- [51]. Rajamanoharan D, Mccue HV, Burgoyne RD, Haynes LP. Modulation of phosphatidylinositol 4-phosphate levels by CaBP7 controls cytokinesis in mammalian cells. *Molecular Biology of the Cell.* 2015; 26:1428–39. [PubMed: 25717182]
- [52]. Bergeland T, Widerberg J, Bakke O, Nordeng TW. Mitotic partitioning of endosomes and lysosomes. *Curr Biol.* 2001; 11:644–51. [PubMed: 11369227]
- [53]. Jovic M, Sharma M, Rahajeng J, Caplan S. The early endosome: a busy sorting station for proteins at the crossroads. *Histol Histopathol.* 2010; 25:99–112. [PubMed: 19924646]
- [54]. Sonawane ND, Szoka FC, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J Biol Chem.* 2003; 278:44826–31. [PubMed: 12944394]
- [55]. Huotari J, Helenius A. Endosome maturation. *Embo J.* 2011; 30:3481–500. [PubMed: 21878991]
- [56]. Duclos S, Corsini R, Desjardins M. Remodeling of endosomes during lysosome biogenesis involves 'kiss and run' fusion events regulated by rab5. *Journal of Cell Science.* 2003; 116:907–18. [PubMed: 12571288]
- [57]. Vyas JM, Van der Veen AG, Ploegh HL. The known unknowns of antigen processing and presentation. *Nat Rev Immunol.* 2008; 8:607–18. [PubMed: 18641646]
- [58]. Yu ZY, Xie GF, Zhou GT, Cheng Y, Zhang GT, Yao GM, et al. NVP-BEZ235, a novel dual PI3K-mTOR inhibitor displays anti-glioma activity and reduces chemoresistance to temozolomide in human glioma cells. *Cancer Lett.* 2015; 367:58–68. [PubMed: 26188279]

- [59]. Dutta D, Donaldson JG. Sorting of Clathrin-Independent Cargo Proteins Depends on Rab35 Delivered by Clathrin-Mediated Endocytosis. *Traffic*. 2015; 16:994–1009. [PubMed: 25988331]
- [60]. Maldonado-Baez L, Williamson C, Donaldson JG. Clathrin-independent endocytosis: A cargo-centric view. *Exp Cell Res*. 2013; 319:2759–69. [PubMed: 23954817]
- [61]. Edinger AL, Thompson CB. Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Molecular Biology of the Cell*. 2002; 13:2276–88. [PubMed: 12134068]
- [62]. Tajima M, Higuchi S, Higuchi Y, Miyamoto N, Uchida A, Ito M, et al. Suppression of FRP-1/CD98-mediated multinucleated giant cell and osteoclast formation by an anti-FRP-1/CD98 mAb, HBJ 127, that inhibits c-src expression. *Cell Immunol*. 1999; 193:162–9. [PubMed: 10222058]
- [63]. Dalton P, Christian HC, Redman CWG, Sargent IL, Boyd CAR. Membrane trafficking of CD98 and its ligand galectin 3 in BeWo cells - implication for placental cell fusion. *Febs J*. 2007; 274:2715–27. [PubMed: 17451431]
- [64]. Takeuchi H, Kubota T, Kitai R, Nakagawa T, Hashimoto N. CD98 immunoreactivity in multinucleated giant cells of glioblastomas: An immunohistochemical double labeling study. *Neuropathology*. 2008; 28:127–31. [PubMed: 18021193]
- [65]. Vitale I, Galluzzi L, Castedo M, Kroemer G. Mitotic catastrophe: a mechanism for avoiding genomic instability. *Nat Rev Mol Cell Bio*. 2011; 12:384–91.
- [66]. Spencer BH, McDermott CM, Chess-Williams R, Christie D, Anoopkumar-Dukie S. Prazosin but Not Tamsulosin Sensitises PC-3 and LNCaP Prostate Cancer Cells to Docetaxel. *Pharmacology*. 2018; 17:10–8.
- [67]. Morse DL, Gray H, Payne CM, Gillies RJ. Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. *Mol Cancer Ther*. 2005; 4:1495–504. [PubMed: 16227398]
- [68]. Mellman I, Yarden Y. Endocytosis and Cancer. *Csh Perspect Biol*. 2013; 5
- [69]. Bajaj J, Konuma T, Lytle NK, Kwon HY, Ablack JN, Cantor JM, et al. CD98-Mediated Adhesive Signaling Enables the Establishment and Propagation of Acute Myelogenous Leukemia. *Cancer Cell*. 2016; 30:792–805. [PubMed: 27908736]
- [70]. Sachlos E, Risueno RM, Laronde S, Shapovalova Z, Lee JH, Russell J, et al. Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell*. 2012; 149:1284–97. [PubMed: 22632761]
- [71]. Cormerais Y, Giuliano S, LeFloch R, Front B, Durivault J, Tambutte E, et al. Genetic Disruption of the Multifunctional CD98/LAT1 Complex Demonstrates the Key Role of Essential Amino Acid Transport in the Control of mTORC1 and Tumor Growth. *Cancer Res*. 2016; 76:4481–92. [PubMed: 27302165]
- [72]. Heeneman S, Deutz NEP, Buurman WA. The Concentrations of Glutamine and Ammonia in Commercially Available Cell-Culture Media. *J Immunol Methods*. 1993; 166:85–91. [PubMed: 8228290]
- [73]. Li ZY, Ji XM, Wang WC, Liu JJ, Liang XF, Wu H, et al. Ammonia Induces Autophagy through Dopamine Receptor D3 and MTOR. *Plos One*. 2016; 11
- [74]. Brett CL, Tukaye DN, Mukherjee S, Rao RJ. The yeast endosomal Na⁺(K⁺)/H⁺ exchanger Nhx1 regulates cellular pH to control vesicle trafficking. *Molecular Biology of the Cell*. 2005; 16:1396–405. [PubMed: 15635088]
- [75]. Parks SK, Chiche J, Pouyssegur J. Disrupting proton dynamics and energy metabolism for cancer therapy. *Nat Rev Cancer*. 2013; 13:611–23. [PubMed: 23969692]
- [76]. Piao SF, Amaravadi RK. Targeting the lysosome in cancer. *Ann Ny Acad Sci*. 2016; 1371:45–54. [PubMed: 26599426]

Fig. 1.

Prazosin (PRZ) induced endosomal/lysosomal tubular structures in the prostate carcinoma cell line LNCaP are positive for CD98hc. LNCaP cells were treated without (**A**) or with 15µM PRZ (**B**) for a total time of 24h. Then, patterns of LAMP1 positive late endosomes/lysosomes and CD98hc were assessed in parallel by indirect immunofluorescence. Nuclei of cells were counterstained with DAPI. In untreated cells, CD98hc is mainly located at the cell membrane, whereas in PRZ treated cells CD98hc accumulates in LAMP1 positive tubular lysosomes (arrows).

Fig. 2.

CD98hc associated with the lysosomes originates from the cell membrane. LNCaP cells were incubated 15min at 37°C without (w/o) or with addition of an anti CD98hc (MEM-108) antibody (Ab) or an anti CD44 Ab as isotype control before treatment with 15μM prazosin (PRZ) for 24h. Afterwards, LAMP1/CD98hc immunofluorescence (IF) analysis was performed (w/o primary CD98hc antibody staining). Nuclei of cells were counterstained with DAPI. w/o Ab: Control cells cultivated w/o addition of PRZ/Ab and stained with both secondary Ab only. Treatment of cells with PRZ in the presence of the CD98hc Ab resulted in the typical tubular CD98hc pattern, suggesting that the CD98hc protein associated with the lysosomes in PRZ treated cells derives from the cell membrane.

Fig. 3.

Prazosin (PRZ) induces de novo synthesis of CD98hc. **A-D:** K562 cells were treated for 24h with PRZ. Afterwards, CD98hc-expression was analysed by qPCR to assess CD98hc-mRNA-expression (in relation to 18sRNA) (**A**), flow cytometry to assess CD98hc expression at the cell membrane (**B**) and western blotting (WB) to analyse total CD98hc expression (**C, D**). **A:** PRZ induces transcription of CD98hc. $n=4$. P-value according to Dunnett's test. A.U. = arbitrary units, C = control. **B:** Cell surface expression of CD98hc is only slightly reduced due to treatment with PRZ. $n=4$, except PRZ [20 μ M]: $n=3$. *: $p < 0.01$,

₁: $p < 0.001$ versus control (C) according to ANOVA and Holm-Sidak post hoc analysis: **C-D: Total expression of CD98hc in response to 24h treatment with PRZ as assessed by WB. $n=4$. P-value according to ANOVA and Holm-Sidak post hoc analysis. β -Actin was used as loading control. **E-F**: Total expression of CD98hc in LNCaP cells in response to a 24h treatment with PRZ as assessed by WB. P-value according to ANOVA and Holm-Sidak post hoc analysis. $n=3$.

Fig. 4.

Prazosin (PRZ) induced lysosomal tubulation occurs independent of CD98hc. To test whether CD98hc is required for PRZ induced lysosomal tubulation, the SLC3A2 gene - coding for CD98hc - was knocked out (KO) in HEK293T (HEK) cells. **A-B:** Western blotting (WB) analysis of PRZ treated HEK cells. Wild type (WT) and two KO clones were treated with PRZ for 24h. **A:** A representative HEK-CD98hc plot out of four experiments is shown. As expected, no CD98hc signals were obtained in KO clones. WT cells typically presented two CD98hc bands, but only the respective lower band showed an increase of

intensity in response to PRZ. GAPDH was used as loading control. **B:** Densitometry analysis of CD98hc lower bands. *: $p < 0.01$ compared to untreated control according to ANOVA and Holm-Sidak post-hoc testing. $n=4$. **C:** Demonstration of KO of SLC3A2 in HEK293T cells by indirect immunofluorescence (IF) against CD98hc. Nuclei of cells were counterstained with DAPI. Two independent SLC3A2 KO clones (Clone1 and Clone2) were used in our study. WT Neg.Control = Negative Control (secondary antibody only). **D:** Cells were either treated for 6h with $15\mu\text{M}$ PRZ and lysosomes were visualised by IF staining against LAMP1 or for 24h and stained with the fluorescent PRZ derivative QAPB. PRZ induced LAMP1+ tubular structures in both WT and KO cells. Both PRZ treated KO clones mainly lost their spindle shaped morphology and exhibited perinuclear LAMP1+ tubules. Similar results were obtained when cells were stained with QAPB.

Fig. 5.

The role of CD98hc in the cytotoxicity of prazosin (PRZ) on HEK293T (HEK) cells. SLC3A2 (CD98hc) was knocked out (KO) in HEK cells using CRISPR/Cas9 technology. Proliferation (A), viability (B), DNA content (C) and apoptosis/necrosis (D) of two KO clones, herein referred as Clone1 and Clone2 were analysed following 24h or 48h treatment with PRZ and compared to wild type (WT) cells. **A:** Cell counting showed an overall lower proliferative capacity of CD98hc KO clones compared to WT cells and no increase in cell counts in cultures treated with PRZ irrespective of CD98hc expression. $n=4$. **B:** WST-1

viability assays showed a significant (*: $p < 0.05$) lower optical density (OD) at 450nm of CD98hc KO clones compared to WT cells at 24h according to ANOVA and Holm-Sidak post hoc testing. $n=3$. **C:** DNA content profiles of HEK cells exposed to PRZ. HEK cells were treated with PRZ up to 72h and stained afterwards with propidium iodide (PI) to assess DNA content or apoptosis. Treatment of HEK cells with PRZ resulted in the genesis of cells with 4N or >4N DNA content and apoptosis, indicated by the appearance of a Sub-G1-peak. SLC3A2 KO cells treated with 30 μ M PRZ were arrested in a binucleated (4N) state and persisted in that state for up to 72h. In contrast, WT cells did not cease DNA-synthesis in the presence of PRZ but also reached an >4N state and underwent apoptosis. The percentages in the upper right corner of the histograms depict the relative portions of cells with distinct DNA-content; respectively apoptotic cells (Sub-G1). The assignment of DNA content peaks is exemplified in the Wild Type-24h-PRZ [15 μ M] histogram. The presented results are representative for both SLC3A2 KO clones tested in a total of three independent experiments with similar outcome. **D:** Cell death analysis of PRZ treated HEK cells. HEK cells (WT versus CD98hc KO clones) were treated with PRZ and stained with Annexin V and the cell membrane impermeable DNA dye SYTOX Green. The presented plots (WT versus Clone1) are representative for three independent experiments with similar outcomes for Clone1 and Clone2.

Fig. 6.

Glutamine (Gln) and its metabolite ammonia preserve proliferation of K562 cells treated with prazosin (PRZ). **A:** Gln-metabolism of cancer cells according to Hensley et al. and Krall et al. [36,37] and description of how Gln-metabolism was manipulated in our study to test the influence of Gln-supply on the cytotoxicity of PRZ. First (1) we cultivated cells +/- addition of Gln or NH_4Cl or (2) enzymatically depleted Gln using asparaginase. Spontaneous break down of Gln *in vitro* is a known phenomenon in cell culture. Gln is imported into the cell via various Gln-transporters. As a non-essential amino acid Gln can

also be synthesized by glutamate-ammonia ligase (GLUL), inhibited by **(3)** methionine sulfoximine (MSO). In the cell Gln is used for the synthesis of glutathione (GSH, together with cysteine/Cys and glycine/Gly) and as nitrogen donor for various enzymatic reactions such as glutamine fructose-6-phosphate amidotransferase (GFAT) inhibited by the Gln analogue 6-Diazo-5-oxo-L-norleucin (DON). Gln is imported into mitochondria and undergoes anaplerotic reactions to generate α -ketoglutarate (α -KG) that enters the Krebs cycle [36]. The Gln degrading enzyme glutaminase (GLS) is inhibited by DON or the GLS inhibitor bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide (BPTES). **B-F:** Proliferation of PRZ treated cells depending on Gln supply. Cell counts were assessed at given time points using an automated cell counter. **B:** K562 cells were treated for 48h without (control) or with 10 μ M PRZ and increasing concentrations of Gln. Control w/o Gln/PRZ = 100%, n=7, *: p<0.01, **: p<0.001 versus cultivation with PRZ w/o (0) Gln according to Dunnett's test. **C:** K562 cells were cultivated with supply of 2mM or 10mM Gln for 48h, or 72h respectively. **D:** Supplementation of the medium with asparaginase restores proliferation of PRZ treated K562 cells. n=3. *: p<0.05, **: p<0.001 versus PRZ [10 μ M], #: p<0.05, ##: p<0.01 versus PRZ [15 μ M] according to ANOVA and Holm-Sidak post hoc analysis. **E:** Supplementation of the medium with NH₄Cl restores proliferation of PRZ treated K562 cells. n=3. P-values according to ANOVA and Holm-Sidak post hoc analysis. **F:** Replenishment of the growth medium with additional glutamate (Glu) or aspartate (Asp) does not influence the anti-proliferative effect of PRZ. n=3. **G-H:** Combeneft synergy-mapping of combined treatments PRZ versus asparaginase (**G**), or PRZ versus ammonia (**H**).

Fig. 7.

PRZ modulates expression of CD98hc depending on the presence of Gln, or ammonia. **A-B:** CD107a (LAMP1) and CD98hc were analysed in parallel by flow cytometry following cultivation of cells with or without (w/o) 15µM PRZ and varying supplementation of the medium with Gln or NH₄Cl. **A:** CD98hc/CD107a histograms of one representative experiment series. The grey peaks represent the emitted fluorescence of cells stained with an appropriate isotype-antibody. **B:** Statistical analysis of CD98hc/CD107a expression. *: p<0.01, **: p<0.001 versus PRZ-w/o, #: p<0.05, ##: p<0.001 versus Control-w/o according

to ANOVA and Holm-Sidak post hoc analysis (CD98hc), respectively ANOVA on ranks and NKS post hoc analysis (CD107a). n=5. **C-D:** Expression of CD98hc was assessed by western blotting in parallel to phosphorylation analysis of energy sensors AMPK α (p-AMPK α , Thr172) and GSK α (p-GSK3 α , Ser21) following 24h treatment of K562 cells with 15 μ M PRZ either with or without (w/o) addition of Gln or NH₄Cl to the medium. **C:** Representative blots of one out of four independent experiment series. **D:** Densitometry results using ImageJ presented as area under the curve (AUC) values. n=4 except AMPK-analysis: n=3. *: p<0.05, **: p<0.001 versus PRZ-w/o, #: p<0.01 versus Control-w/o, according to ANOVA or ANOVA on ranks and Holm-Sidak, or NKS post hoc analysis. A.U. = arbitrary units.

Fig. 8.

Ammonia completely abrogates prazosin (PRZ) induced lysosomal tubulation. LNCaP cells were cultivated with/without (w/o) 15μM PRZ with/w/o supplementation of the medium with 2mM NH_4Cl for 24h. Afterwards, cells were fixed and stained parallel with antibodies against CD98hc/LAMP1 and appropriate secondary antibodies. Nuclei were counterstained with DAPI. Within 24h PRZ induces shuttling of CD98hc towards LAMP1 positive tubular endosomes/lysosomes. Lysosomal tubulation is completely neutralised by supplementation of the medium with NH_4Cl .

Fig. 9.

Dynamics of prazosin (PRZ) induced lysosomal tubulation. **A:** PRZ induced vacuoles derive from Rab5 positive early endosomes. Rab5-RFP expressing LNCaP cells were treated with 20µM PRZ for 10min. Vacuoles (rectangles) are positive for Rab5. **B:** LAMP1-GFP expressing LNCaP cells were treated with 20µM PRZ for 24h and stained with LysoTracker® Red (LT). LAMP1 positive tubules, but not vacuoles (area shown in higher magnification in the rectangle) accumulate LT. **C:** LNCaP cells expressing Rab5-RFP and LAMP1-GFP were exposed to 10µM PRZ for 24h and analysed by confocal microscopy. LAMP1 positive

vacuoles appeared, but only a minority of them were Rab5 and LAMP1 double positive (arrow). **D:** Lysosomal tubulation is reversible. LNCaP cells were treated for 3h (with/without addition of QAPB) with PRZ and incubated for further 6h after removal of PRZ. Lysosomes were visualised by immunofluorescence staining; QAPB staining was observed with an inverted fluorescence microscope. Within 6h post withdrawal of PRZ, LAMP1/QAPB positive tubules disappeared, but vacuoles remained. **E:** The role of microtubules (MT) and actin in PRZ induced lysosomal tubulation. In order to interfere with the functionality of MT or the actin cytoskeleton, LNCaP cells were either treated +/- PRZ with 5µM nocodazole (NDZ) for 2h or 2µg/ml cytochalasin-D (Cyto-D) for 24h. Cells treated with NDZ showed a diffuse tubulin pattern. Cyto-D treatment clearly disrupted actin fibres as visualised by phalloidin staining. NDZ or combined PRZ/NDZ treatment resulted in perinuclear accumulation of vesicular or tubular (with PRZ) lysosomes. Long-term treatment of cells with Cyto-D/PRZ resulted in the formation of fine LAMP1+ protrusions all around the cell. The Cyto-D+PRZ picture was intentionally overexposed to visualize those structures.

Fig. 10.

Model of prazosin (PRZ) induced lysosomal tubulation and its interference with the trafficking of CD98hc. The model was created based on results obtained in our lab in the light of data obtained by Zhang et al. showing that PRZ interferes with endocytic sorting due to an interaction with dopamine receptor DRD3 [6]. PRZ enters the cell via endocytosis through interaction with DRD3 or other still unknown receptors ending up in Rab5 positive early (=sorting) endosomes. This process results in obviously functionally impaired endosomes that swell and form huge LAMP1 positive vacuoles. According to our observations tubular lysosomes grow towards vacuoles but do not fuse with them suggesting a “kiss and run” exchange mechanism between vacuoles and lysosomes. Finally, tubular lysosomes, which interfere with the successful completion of cytokinesis, get in touch with the cell membrane, a process referred to as lysosomal exocytosis, indicated by increased expression of the lysosomal membrane protein LAMP1 at the cell membrane. Lysosomal tubulation interferes with the endocytic sorting of the oncoprotein CD98hc, which is misrouted towards lysosomal tubules. Transcription and translation of CD98hc are induced in response to PRZ treatment to maintain surface expression of CD98hc. Depending on PRZ dose CD98hc expression favours endoreplication and apoptosis. Lysomototropic agents – i.e. ammonia as by-product of glutaminolysis, chloroquine (CHQ) and the v-ATPase inhibitor bafilomycin A1 (BafA1) interfere with PRZ induced lysosomal tubulation and the intracellular trafficking of CD98hc. Ammonia was defined as a potent antagonist of PRZ

able to completely neutralize the cytotoxicity of PRZ and obviously independent of its documented alkalizing functions on the endolysosomal system. Pit-1 inhibits endocytosis of QAPB/PRZ, PRZ induced lysosomal tubulation and subsequent lysosomal trapping of CD98hc, indicating PIP3 dependent endocytosis of quinazolines.