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

Robert Fuchs[a,*], Anika Stracke[a], Viktoria Holzmann[a,b], Gerfried Luschin-Ebengreuth[a], Nathalie Meier-Allard[a], Nadine Ebner[a], Teresa Maria Lassacher[a], Markus Absenger-Novak[c], Eleonore Fröhlich[c], Matthias Schittmayer[d,e], Sara Cano Crespo[f], Manuel Palacin[f], Beate Rinner[g], Ruth Birner-Gruenberger[d,e]

[a] Chair of Immunology and Pathophysiology, Otto Loewi Research Center, Medical University of Graz, Heinrichstraße 31, 8010 Graz, Austria
[b] FH JOANNEUM – University of Applied Sciences, Alte Poststraße 149, 8020 Graz, Austria
[c] Centre for Medical Research, Medical University of Graz, Stiftingtalstrasse 24, 8010 Graz, Austria
[d] Research Unit Functional Proteomics and Metabolic Pathways, Institute of Pathology, Medical University of Graz, Auenbruggerplatz 25, 8036 Graz, Austria
[e] Omics Center Graz, BioTechMed-Graz, Stiftingtalstrasse 24, 8010 Graz, Austria
[f] Institute 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
[g] Biomedical Research, Medical University of Graz, Roseggerweg 48, 8036 Graz, Austria

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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 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 NH_4Cl – 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.

1. Introduction

Quinazoline based α1-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 α1-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 α1-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 μM 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

* Corresponding author.
E-mail address: robert.fuchs@medunigraz.at (R. Fuchs).

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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 α1-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 emphasizes 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-3β signalling [19]. In earlier studies Feral et al. had already shown that CD98hc is associated with β1 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 0.1 mg/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 trypsination, 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 24 h or 48 h. Absorption at 450 nm and as a reference at 650 nm 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′-TCGGGACATAG AGAATCTGA-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