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The cytotoxicity of the α1-adrenoceptor antagonist prazosin is linked to an endocytotic mechanism equivalent to transport-P

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Abstract

Since the α1-adrenergic antagonist prazosin (PRZ) was introduced into medicine as a treatment for hypertension and benign prostate hyperplasia, several studies have shown that PRZ induces apoptosis in various cell types and interferes with endocytotic trafficking. Because PRZ is also able to induce apoptosis in malignant cells, its cytotoxicity is a focus of interest in cancer research. Besides inducing apoptosis, PRZ was shown to serve as a substrate for an amine uptake mechanism originally discovered in neurones called transport-P. In line with our hypothesis that transport-P is an endocytotic mechanism also present in non-neuronal tissue and linked to the cytotoxicity of PRZ, we tested the uptake of QAPB, a fluorescent derivative of PRZ, in cancer cell lines in the presence of inhibitors of transport-P and endocytosis. Early endosomes and lysosomes were visualised by expression of RAB5-RFP and LAMP1-RFP, respectively; growth and viability of cells in the presence of PRZ and uptake inhibitors were also tested. Cancer cells showed co-localisation of QAPB with RAB5 and LAMP1 positive vesicles as well as tubulation of lysosomes. The uptake of QAPB was sensitive to transport-P inhibitors bafilomycin A1 (inhibits v-ATPase) and the antidepressant desipramine. Endocytosis inhibitors pitstop® 2 (general inhibitor of endocytosis), dynasore (dynamin inhibitor) and methyl-β-cyclodextrin (cholesterol chelator) inhibited the uptake of QAPB. Bafilomycin A1 and methyl-β-cyclodextrin but not

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Conflict of interests
None.

Appendix A. Supplementary data
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desipramine were able to preserve growth and viability of cells in the presence of PRZ. In summary, we confirmed the hypothesis that the cellular uptake of QAPB/PRZ represents an endocytotic mechanism equivalent to transport-P. Endocytosis of QAPB/PRZ depends on a proton gradient, dynamin and cholesterol, and results in reorganisation of the LAMP1 positive endolysosomal system. Finally, the link seen between the cellular uptake of PRZ and cell death implies a still unknown pro-apoptotic membrane protein with affinity towards PRZ.

Keywords

Prazosin; Endocytosis; Transport-P; Lysosomes; Apoptosis

1. Introduction

BODIPY® FL Prazosin (QAPB), a fluorescent derivative of the quinazoline based α1-adrenergic antagonist prazosin (PRZ), was on the one hand demonstrated to be a useful tool for in vitro tracking of α1-adrenoceptors but on the other hand also for a non-adrenoceptor-mediated uptake mechanism for amines in neurones, called transport-P (TP) (Al-Damluji et al., 1997; Mackenzie et al., 2000; Pediani et al., 2005; Daly et al., 2010; Segura et al., 2013). We previously analysed the uptake of QAPB in the human erythroleukemia cell lines K562 and HEL, because we had observed that PRZ inhibits proliferation and induces apoptosis in these cell lines through a non-adrenergic mechanism (Fuchs et al., 2009; 2011a,b). The uptake of QAPB into erythroleukemia cells was not inhibited even by high (μM) concentrations of the physiological α1-adrenoceptor-agonist norepinephrine and no expression of α1-adrenoceptors was found in our tested cell lines (Fuchs et al., 2011a, 2011b). The first evidence for TP was provided in 1993 by Al-Damluji et al. (1993). TP was shown to be a unique transport mechanism, distinguishable from known amine transporters with PRZ as a substrate (Al-Damluji and Kopin, 1996a). The uptake of PRZ was shown to be non-linear and to increase exponentially in concentrations greater than 10^{-7} M, similar to observations in erythroleukemia cells (Al-Damluji and Kopin, 1996a; Fuchs et al., 2011b). The TP mechanism showed sensitivity towards treatment with the lysomototropic drug chloroquine (CHQ) and the v-ATPase inhibitor bafilomycin A1 (BAF), suggesting a pH-gradient driven uptake process (Al-Damluji and Kopin, 1996a). Further, the uptake of PRZ through TP was sensitive towards treatment with antidepressants, such as desipramine (DESI) (Al-Damluji and Kopin, 1996b). The group around Al-Damluji et al. focused primarily on neurones and showed that TP occurs in post-synaptic peptidergic neurones, but not in pre-synaptic neurones or non-neuronal tissue, such as kidney cells or muscle cells (Al-Damluji and Kopin, 1996a; Al-Damluji et al., 2001). The identity of the main proteins involved in TP is still completely unknown, so that the TP mechanism is mainly characterized by its kinetics, substrate specificity and its sensitivity to pharmacological inhibitors. So far PRZ on the one hand and phenylethylamines and antidepressants on the other have been identified as substrates for TP (Al-Damluji and Kopin, 1998; Zunszain et al., 2005; da Silva et al., 2008). da Silva et al. (2008) analysed related structural analogues of PRZ and tested the activation of TP by these drugs. They found that only quinazolines are able to activate TP and the chemical structure of PRZ appears to be very specific for the TP mechanism.

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Based on the similarity in the uptake process of QAPB between erythroleukemia cells and neurones and the vesicular fluorescence pattern observed (Al-Damluji and Kopin, 1996a; Fuchs et al., 2011b), we hypothesised that the TP mechanism also exists in non-neuronal tissue and may represent an endocytotic uptake process of PRZ/QAPB.

A recent study by Zhang et al. (2012) showed that PRZ interferes with endocytotic sorting through an off-target effect, providing a first hint of cellular trafficking of PRZ and the mechanism of endocytosis.

Since PRZ and other quinazolines in μM-concentrations have a pronounced pro-apoptotic effect on different kinds of cells, including several malignant cell types such as leukaemia cells, prostate cancer cells, medullary thyroid carcinoma (MTC) cells or breast cancer cells (Benning and Kyprianou, 2002; Garrison and Kyprianou, 2006; Lin et al., 2007; Fuchs et al., 2009, 2015), the cytotoxic mechanism of quinazoline based drugs is in the focus of interest of current cancer research (Desiniotis and Kyprianou, 2011). Apoptosis induced by quinazoline based α1-adrenergic antagonists, including PRZ, doxazosin and terazosin, was proven to be independent of α1-adrenoceptors as shown for TP (Gonzalez-Juanatey et al., 2003; Fernando and Heaney, 2005; Hui et al., 2008; Fuchs et al., 2011a; Zhang et al., 2012). We thus raised the hypothesis that the TP mechanism and the pro-apoptotic effect of PRZ are linked.

Though we focussed on K562 cells, we also tested our hypothesis with other malignant cell lines such as the prostate carcinoma cell line LNCaP and the MTC cell line TT, as well as lymphocytes/monocytes obtained from peripheral blood. We investigated the effect of endocytosis inhibitors and known modulators of TP on cellular uptake of QAPB, analysed the effects of these drugs on the pro-apoptotic and anti-proliferative action of PRZ and assessed potential protein binding of QAPB. These drugs were the TP inhibitors BAF, CHQ and DESI and the endocytosis inhibitor pitstop® 2 (PIT), the dynamin inhibitor dynasore (DYN) and the cholesterol depleting agent methyl-β-cyclodextrin (MD). PIT was originally introduced as a drug specifically interfering with clathrin-mediated endocytosis, but recent studies have shown that PIT also inhibits clathrin-independent endocytosis (Dutta et al., 2012; Willox et al., 2014). DYN is a non-competitive inhibitor of the GTPase dynamin (Macia et al., 2006), which plays an important role in endocytotic processes as it is involved in endocytotic vesicle scission events in clathrin-dependent and clathrin-independent endocytosis (Macia et al., 2006; Sandvig et al., 2011). We tested MD because of the lipophilic nature of PRZ and QAPB. The main documented function of MD is to deplete cholesterol from cell membranes (Kabouridis et al., 2000; Segura et al., 2013). As a consequence, the integrity of membrane lipid rafts, which are characterized by a special lipid composition including sphingolipids and cholesterol, is impaired. Lipid rafts function as special signalling platforms of the cell membrane and are also the entry site for bacterial toxins - e.g. the cholera toxin - and viruses (Pike, 2003; Chinnapen et al., 2007; Ewers and Helenius, 2011). Finally, cellular imaging was used to track QAPB in cancer cells and to assess the morphology of endosomes and lysosomes in drug-treated cells.

2. Material and Methods

2.1. Cells and Cell Culture

K562 cells, obtained from ATCC (Manassas, VA, USA) and human erythroleukemia (HEL) cells (DSMZ, Braunschweig, Germany) were maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS, Biochrom, Berlin, Germany), 2 mM L-glutamine (Sigma–Aldrich), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma–Aldrich). LNCaP cells, obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany) were cultivated in RPMI-1640 medium with the same composition of supplements as for K562 cells. The cell line TT was cultivated in Ham's F12 medium (Biochrom) supplemented with 10% FBS and 2 mM L-glutamine. Mononuclear cells from peripheral blood (MNCs), donated by the principal investigator, were isolated by density centrifugation using Histopaque-1077 (Sigma–Aldrich) following a standard protocol. Blood was collected in EDTA tubes.

2.2. Drugs

Bafilomycin A1, chloroquine diphosphate salt, dynasore hydrate, methyl-β-cyclodextrin and prazosin hydrochloride were purchased from Sigma–Aldrich. Desipramine hydrochloride was obtained from Santa Cruz Biotechnology (Dallas, TX, USA), pitstop® 2 from Abcam (Cambridge, UK).

2.3. Analysis of cellular proliferation and cell death

Proliferation of suspension cells (K562, HEL) was assessed with a CASY® Cell Counter and Analyser System (Roche, Reutlingen, Germany). For proliferation assays, K562 and HEL cells were cultivated for 48 h with a starting cell number of 2xE4 cells/ml. Every condition was analysed in duplicate. Proliferation of TT cells was assessed using the WST-1 reagent (Roche, Mannheim, Germany) following the instructions of the producer. Cells were cultivated in 96 well tissue culture plates, starting with a cell number of 1xE4 cells in 100 µl medium. All conditions were tested in triplicate. Cell death of K562 cells treated with prazosin, desipramine, bafilomycin A1 or combinations of individual drugs was further assessed by annexin V/ 7-aminoactinomycin (7-AAD) staining. In total, 2xE5 K562 cells were cultivated in 10 ml medium in 25 cm^2 cell culture flasks. Cells were co-stained with annexin V-FITC (Becton Dickinson (BD)/ Pharmingen, Franklin Lakes, NJ, USA) and 7-AAD (Biolegend, San Diego, CA, USA) using a standard protocol. Since bafilomycin A1 was dissolved in dimethyl sulfoxide (DMSO), the same amount of DMSO was added to each cell culture flask. Cells were analysed with a BD LSRFortessa™ Flow Cytometer System running with the BD FACSDiva™ software. Based on emitted fluorescence, cells were assigned to the viable (Annexin V low/ 7-AAD−), apoptotic (Annexin V high/ 7-AAD −) or the late apoptotic/necrotic (Annexin V high/ 7-AAD+) cell fraction. Activation of initiator caspases 8 and 9 was assessed in K562 cells by luminescence based Caspase-Glo® assays (Promega, Fitchburg, WI, USA) following the instructions of the manufacturer. All measurements were performed in triplicate. Luminescence was analysed with a GloMax®-Multi Detection System (Promega).

2.4. Analysis of cellular uptake of BODIPY® FL Prazosin with fluorescence microscopy and flow cytometry

K562 cells were harvested and washed once with RPMI-1640 medium without (w/o) phenol red. For staining with BODIPY® FL Prazosin (QAPB, Life Technologies/Molecular Probes, Eugene, OR, USA), cells were re-suspended in RPMI-1640-medium w/o phenol red, supplemented with L-glutamine, penicillin/streptomycin, 25 mM HEPES (Sigma–Aldrich) and ITS Liquid Media Supplement (Sigma–Aldrich). Cells (4xE5/ml) were incubated with/w/o endocytosis inhibitors for 1 h at 37 °C in a water bath. Then, QAPB (100 nM) was added and cells were further incubated for 30 min at 37 °C. After incubation, cells were washed twice with magnesium/calcium free phosphate buffered saline (CMF-PBS) and re-suspended in CMF-PBS. Until analysis by fluorescence microscopy and/or flow cytometry, samples were shielded from light and kept on ice. MNCs obtained from peripheral blood were treated the same way. For fluorescence microscopy, K562 cells were analysed with a Leica DM 4000 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with the Leica L5 filter cube. QAPB stained cells were analysed with the LSRFortessa™ Flow Cytometer. The QAPB fluorescence emitted by cells was analysed in the FITC channel of the flow cytometer, assessing FL-A to quantify the total amount of the dye bound by the cells. Because the fluorescence peaks did not show Gaussian distribution, the median fluorescence intensity (FL) was chosen as analysis parameter. To stain adherent LNCaP cells with QAPB, cells were cultivated in chamber slides (Discovery Labware/Corning, Billerica, MA, USA) coated with poly-D-lysine hydro bromide (Sigma–Aldrich). LNCaP cells were stained with QAPB the same way as K562 cells without washing steps. The fluorescence of LNCaP cells was analysed with an Eclipse TE300 (Nikon, Tokyo, Japan) inverted microscope or a Cell Observer based on an Axiovert 200 M inverted fluorescence microscope (Zeiss, Jena, Germany) for live cell imaging, whereby the cells were observed for 20 min. Early endosomes or lysosomes were visualised in LNCaP cells by expression of Rab5-RFP fusion proteins or LAMP1-RFP fusion proteins using the CellLight® BacMam 2.0 technology (Life Technologies/Molecular Probes). Lysosomes were further identified with the lysomototropic agent Lysotracker® Red (Life Technologies/Molecular Probes) following the instructions of the producer. In some co-localisation experiments nuclei were stained with Hoechst 33342 dye (Life Technologies/Molecular Probes). Co-localisation experiments of endosomes or lysosomes with QAPB employed a LSM 510 META scanning laser confocal microscope (Zeiss). Indirect immunofluorescence staining of LAMP1 was done as described previously (Fuchs et al., 2011a). A monoclonal anti LAMP1 antibody (clone H4A3) and as secondary antibody an Alexa Fluor® 555 labelled goat anti mouse IgG antibody obtained from Biolegend were used to detect LAMP1. Slides were analysed with the Leica DM 4000 fluorescence microscope. To track ganglioside-GM1 rich lipid rafts and caveolae, cells were incubated with 2 μg/ml cholera toxin subunit B, Alexa Fluor® 555 conjugate (CTB, Life Technologies/Molecular Probes).

2.5. Analysis of QAPB binding at protein level

To detect proteins with affinity towards QAPB, K562 cells were harvested, washed once with RPMI-1640 medium w/o phenol red and stained with QAPB (30 min) following pre-treatment with/without endocytosis inhibitors for 1 h in a tissue culture incubator at 37 °C. Following drug treatment, cells were harvested and washed twice in CMF-PBS buffer. Total

cellular protein was isolated using a protein lysis buffer containing Trisbase [50 mM], EDTA [10 mM], Triton X 100 (1%), and Complete Protease Inhibitor cocktail tablets (Roche, Indianapolis, IN, USA). All working steps were performed under dim light and on ice. Protein concentration was assessed with the BCA assay (Thermo Scientific/Pierce, Rockford, IL, USA) following the manufacturer 's instructions. A total of 10 μg of each protein sample was mixed with Native PAGE 4X Sample Buffer (Life Technologies), loaded on Criterion™ TGX™ 4–20% precast gels (Biorad, Hercules, CA, USA) without sodium dodecyl sulphate and separated first for 20 min at 70 V followed by 12 h at 100 V. Following separation of proteins, gels were scanned for fluorescent protein bands with a Molecular Imager FX laser scanner (Biorad). The total protein on the gel was visualised with Coomassie staining.

2.6. Statistics

Data are presented as mean values +/− standard deviation. Significance was calculated with Sigma Plot 12.5 (Systat Software Inc., San Jose, CA, USA). Normal distribution of data was tested with the Shapiro–Wilk test. Depending on data distribution, the Mann–Whitney Rank Sum Test was used to test the significance of differences observed between two datasets. Multiple testing was done with One Way ANOVA with Holm-Sidak post hoc testing. The overall significance level was set at $p \leq 0.05$.

3. Results

3.1. Modulators of transport-P and general endocytosis inhibitors interfere with the uptake of QAPB in K562 cells

Since transport-P has been described as a cellular uptake mechanism for amines sensitive towards treatment with BAF and the antidepressant drug DESI, we tested the effect of those drugs on the uptake of QAPB in the K562 cell line by flow cytometry (FACS) and fluorescence microscopy. In the flow cytometry assays QAPB stained cells emitted a bright fluorescent signal (Figs. 1 and 2). FACS analysis showed almost 100% staining efficiency of K562 cells and fluorescence peaks with non Gaussian distribution. Typically, there were two overlying fluorescence peaks. BAF as well as DESI reduced the emitted QAPB fluorescence, but not beyond a certain level. Even in the presence of BAF or DESI, QAPB stained cells emitted a stronger fluorescence signal than unstained cells. The same inhibitory effect was observed in mononuclear cells obtained from peripheral blood, where monocytes exhibited a brighter QAPB fluorescence signal than lymphocytes (Supplementary material Fig. S1).

A 24 h pre-treatment of K562 cells with CHQ resulted in enhanced fluorescence, which decreased with increasing CHQ concentrations (Figs. 1 and 2). DYN treatment reduced the emitted fluorescence, but the effect of DYN was not strong enough to reach statistical significance. MD reduced the emitted fluorescence of K562 cells as well, whereas PIT surprisingly enhanced the emitted fluorescence (Figs. 1 and 2). Auto fluorescence of the drug was excluded in a control experiment (not shown). Fluorescence microscopy analysis of the fluorescence pattern of cells pre-treated with endocytosis inhibitors BAF, DYN, PIT and MD showed that the formation of vesicular structures in K562 cells was impaired or

even completely abolished (Fig. 1). A paradox was seen with cells treated with PIT. Similar to BAF-treated cells, these cells did not show a vesicular fluorescence pattern, and they emitted an even stronger fluorescence signal than control cells in FACS analysis. Cells treated with DESI showed vesicular structures, but the cells ' overall emitted fluorescence was less intense (Figs. 1 and 2). CHQ-treated cells exhibited pronounced fluorescent vesicular structures. The effect of CHQ treatment on the uptake of QAPB was particularly clear in the LNCaP cell line, where the fluorescence signal of CHQ-treated cells was much more intense than in normal cells, as will be shown below (Fig. 7). DYN exposed K562 cells sporadically showed vesicular structures (Fig. 1). Typically, these vesicles, most likely representing endocytotic pits, were localized at the periphery of the cells and stuck at the cell-membrane due to inhibition of the GTPase dynamin.

To analyse possible QAPB binding at the protein level, total protein obtained from QAPB stained cells was initially separated by conventional SDS-PAGE. As laser scanning of the gel showed no fluorescent protein bands (not shown), the experiment was repeated under non denaturing conditions; this revealed a distinctive fluorescence band at the upper section of the gel (Supplementary material Fig. S2) and total protein staining showed a pronounced protein band at the same height. To link the protective effect of endocytosis inhibitors to protein binding of QAPB, we compared the intensity of the fluorescent band of untreated cells to that of cells that were pre-treated with endocytosis inhibitors before staining with QAPB. We observed that the fluorescent protein bands of cells pre-treated with MD or BAF were less intense than untreated or vector treated cells (Supplementary material Fig. S2). CHQ, PIT and DYN-treated cells exhibited no change or only slightly less intense fluorescence. Most interestingly, DYN and PIT influenced the separation behaviour of the proteins with affinity towards QAPB: DYN decelerated whereas PIT accelerated the electrophoretic mobility of the fluorescent protein(s).

3.2. Particular endocytosis inhibitors are able to preserve proliferation and viability of K562 cells in the presence of prazosin

To test the effect of endocytosis inhibitors on PRZ-treated K562 cells, proliferation and/or viability assays were performed with endocytosis inhibitors +/− addition of PRZ. Pre-treatment of K562 cells with CHQ for 24 h or MD for 1 h, as well as parallel addition of BAF, protected K562 cells against the growth inhibitory effect of PRZ (Fig. 3A). The protective role of CHQ was also successfully reproduced in the HEL erythroleukemia cell line (Supplementary material Fig. S3). Multiple screening experiments with DESI (1–30 μM) showed no protection against the growth-inhibitory effect of PRZ (Fig. 3B). DYN, PIT and DMSO—used as a solvent for some of the endocytosis inhibitors—did not further influence the growth behaviour of K562 cells in the presence of PRZ (Fig. 3A).

To confirm that the protective effect of BAF, CHQ and MD is not limited to K562 cells, the experiments were reproduced in the TT cell line using the WST-1 assay. This confirmed the protective effect of endocytosis inhibitors on growth inhibition induced by PRZ (Fig. 3C).

Annexin V/7-AAD viability assays of K562 cells treated with PRZ, DESI, BAF or combinations of PRZ + BAF or PRZ + DESI showed that PRZ and DESI also induce apoptosis of K562 cells (Fig. 4). Induction of apoptosis by PRZ and DESI was evident

through enhanced cellular binding of fluorescent annexin V and subsequent accumulation of annexin V/7-AAD double positive cells (Fig. 4). BAF at a concentration of 1 nM was able to preserve the viability of K562 cells in the presence of PRZ (10–20 μM) (Fig. 4), whereas DESI showed no protective effect. Interestingly, there was no statistically significant difference in the percentage of living cells between cells treated with DESI alone versus combinations of DESI + PRZ; on the contrary, cell viability was lower in DESI− + PRZ-treated cells versus PRZ-treated cells. The interfering effect of BAF with PRZ was already visible when the light scattering characteristics of cells treated with PRZ were compared with cells co-treated with BAF and PRZ (Supplementary material Fig. S4). Typically, PRZ-treated cells exhibited an increase in the side scatter signal, which is abolished by parallel BAF treatment. A similar effect was apparent with addition of DESI.

Since the signalling of death receptors is closely linked to endocytosis (Schneider-Brachert et al., 2013), we analysed the activation of caspase 8, the key caspase in the extrinsic apoptotic pathway, in comparison to caspase 9, the key caspase in the intrinsic apoptotic pathway. Activation of the effector caspases 3 and 7 in PRZ-treated cells is already well documented (Fuchs et al., 2009, 2015). In fact, our analysis showed a time and dose dependent activation of caspase 8 in PRZ-treated K562 cells within 24 h (Supplementary material Fig. S5), but there also was concomitant activation of caspase 9, again time and dose dependent (Supplementary material Fig. S5).

3.3. QAPB shows co-localisation with RAB5-RFP and LAMP1-RFP in LNCaP cells

As reported by Al-Damluji et al. (2001) for neurones, following QAPB staining single cells exhibited a punctate fluorescence pattern in the LNCaP (Figs 5–7), the K562 and the HEL cell line (Supplementary material Fig. S6). Co-localisation experiments of QAPB with the early endosome marker Rab5 in the LNCaP cell line demonstrated the presence of endosomes positive for QAPB (Fig. 5). Live cell imaging revealed that fluorescent vesicles are shuttled towards the cell poles (Supplemental Video 1). Co-localisation of QAPB with the lysomototropic substance Lysotracker® Red and LAMP1-RFP fusion proteins (Fig. 5) suggests that QAPB is transported towards the LAMP1 positive late endosome/lysosome compartment. Co-localisation of QAPB and Lysotracker® was further evident in the K562 and HEL cell line (Supplementary material Fig. S6).

3.4. Prazosin induces tubulation of LAMP1 positive late endosomes/lysosomes

As shown for the first time in 2011 in erythroleukemia cells (Fuchs et al., 2011b), parallel PRZ/QAPB treatment of LNCaP cells produced a change from a vesicular QAPB fluorescence pattern towards a fibre-like fluorescence pattern (Fig. 6A). Further experiments clarified that this phenomenon is already visible within a period of 10 min.

In 2012, Zhang et al. demonstrated that PRZ induces tubulation of Rab5 positive endosomes in HeLa cells, and we could confirm these results for the most part in the LNCaP cell line. As reported by Zhang et al. also within 10 min tubular structures appeared that were positive for Rab5 (not shown). The authors further reported that PRZ induced tubular structures were negative for LAMP1. However, they solely analysed LAMP1 positive organelles in short time experiments. Since the pH in late endosomes and lysosomes is low, and CHQ, which

raises the pH in late endosomes/lysosomes, is able to preserve the growth of cells in the presence of PRZ, we analysed the fluorescence pattern of QAPB and LAMP1 in LNCaP and TT cells following 24 h treatment with PRZ. Within 24 h there was complete reorganisation of LAMP1 positive organelles in the form of tubular structures in both of these cell lines (Figs. 6 and 7 and Supplementary material Fig. S7). These LAMP1 positive structures showed co-localisation with QAPB and were also stained with Lysotracker® (Fig. 6, Supplementary material Fig. S7). Most interestingly, polar protrusions, which are formed in response to PRZ treatment in several tested cancer cell lines (Fuchs et al., 2009, 2015), were positive for QAPB and LAMP1 (Fig. 6C, Supplementary material Fig. S7C). Because these needle-like protrusions are so fragile and small, they are hard to detect by conventional light microscopy.

To test whether the uptake of QAPB occurs through ganglioside-GM1 rich lipid raft membrane domains, we co-treated LNCaP cells for 10 min with QAPB, PRZ and a fluorescence labelled version of the ganglioside-GM1 binding cholera toxin subunit B (CTB). Whereas in untreated cells CTB mostly accumulated in the perinuclear region (not shown), in PRZ-treated cells CTB appeared in tubular structures almost identical with the fluorescence pattern of QAPB (Fig. 6D). As reported by Zhang et al., the morphology of the Golgi apparatus and the endoplasmic reticulum—organelles CTB transits during the retrograde transport—was not altered in PRZ-treated LNCaP cells (not shown).

3.5. Prazosin induced tubulation of LAMP1 positive late endosomes/lysosomes is reversible by pre-treatment with chloroquine

To test the effect of CHQ on PRZ-induced tubulation of LAMP1-positive structures, LNCaP cells were pre-treated with CHQ and exposed to PRZ/QAPB for 2 h and 24 h, respectively. In CHQ-treated cells, QAPB accumulated in enlarged vesicles and there was no tubulation of the QAPB fluorescence pattern after 2 h addition of PRZ (Fig. 7). Further, CHQ treatment inhibited the formation of huge vacuoles, which are normally formed in PRZ-treated cells within 10 min, as well as the outgrowth of polar protrusions in long-term (24 h) experiments (Fig. 7). LAMP1-immunofluorescence assays of cells treated with PRZ for 24 h showed that the tubulation of lysosomes was almost completely abrogated in CHQ-pre-treated cells (Fig. 7).

4. Discussion

Several studies have shown that quinazoline based α1-adrenoceptor antagonists exhibit affinity towards off-targets, such as cyclic nucleotide phospodiesterases (Hess, 1975), HERG channels (Thomas et al., 2004) or DRD3 (Zhang et al., 2012). So far, these targets could not, however, be related to two major off-target effects of quinazolines. First, the ability of PRZ to act as a substrate for TP, an uptake process for amines, and second, the ability of quinazolines to induce apoptosis. Our study is the first to link these off-target effects. As mentioned in the introduction, the TP mechanism is mainly defined by its kinetics, substrate specificity (QAPB/PRZ, phenylethylamines and antidepressants) and the sensitivity against bafilomycin and chloroquine. In previous studies using erythroleukemia cells and several α1-adrenergic ligands, we showed that PRZ is able to induce apoptosis in

these cells (Fuchs et al., 2009; 2011a,b). The uptake of QAPB into erythroleukemia cells showed kinetics similar to neurones and was inhibited by PRZ but not by norepinephrine (Fuchs et al., 2011b). In the light of these results and a recent hint by Zhang et al. that PRZ interferes with endocytosis (Zhang et al., 2012), we tested the hypothesis that TP is an endocytotic process that is linked to apoptosis and also occurs in non-neuronal cells. (Fig. 8)

The endocytosis hypothesis was confirmed by the observation that QAPB co-localises with early endosomes and late endosomes/lysosomes. Since the vesicular fluorescence pattern was similar in K562 cells, and tested negative for α1-adrenoceptor expression (Fuchs et al., 2011a) and LNCaP cells expressing α1-adrenoceptors (Thebault et al., 2003), our results confirm that the trafficking of QAPB via TP is independent of α1-adrenoceptors. The involvement of α1-adrenoceptors in LNCaP cells cannot, however, be excluded, since QAPB acts as a functional α1-adrenoceptor antagonist and is a proven tool for tracking α1-adrenoceptors (Mackenzie et al., 2000; Pediani et al., 2005; Daly et al., 2010; Segura et al., 2013). Segura et al. recently described endocytotic shuttling of α1-adrenoceptors that was pronouncedly similar to our work with regard to co-localisation of QABP with Lysotracker® and the sensitivity towards MD treatment. The trafficking of QAPB through the unknown off-target thus shows at least an intersection with the trafficking of α1-adrenoceptors. Here, the drug concentration might be an important criterion for the selectivity of QAPB in binding adrenoceptors and/or the unknown off-target involved in TP. But to the best of our knowledge, the effect of tubulation of endosomes and lysosomes has never yet been described in any study on α1-adrenergic receptors, which have been under intensive investigation for decades. In the results section we mention a difference between our results and Zhang et al. (2012), i.e. that they did not see any tubulation of lysosomes. This discrepancy is most likely due to an insufficient period of incubation with PRZ. Zhang et al. explain the tubulation process of endosomes by perturbation of endocytic sorting. A further possible explanation for this phenomenon could be a process of disturbed recycling of a receptor back to the cell membrane that entered the cell through endocytosis. However, we could not detect a pronounced association of Rab4 or Rab11 with PRZ-induced tubules in LNCaP cells following 24 h of incubation (unpublished observation).

Since Al-Damluji et al., who discovered TP, demonstrated that it is sensitive to treatment with pH-modulators and antidepressants (Al-Damluji and Kopin, 1996a,b), we investigated whether these drugs inhibit the uptake of QAPB. In fact, bafilomycin, chloroquine and the antidepressant drug desipramine significantly influenced the uptake and the response of K562 cells towards QAPB/PRZ, so that besides the kinetics all so far known criteria for transport-P were fulfilled. Here, this leads us to set the uptake of QAPB in erythroleukemia cells (and other tested cells) as equivalent to transport-P.

In flow cytometry experiments with K562 cells, we observed two overlapping QAPB-fluorescence peaks, one emitting a (relatively) low fluorescence signal, and the other emitting a strong one. In BAF and PIT treated cells we detected either a decrease of emitted QAPB fluorescence (BAF) or an increase (PIT). These changes in fluorescence intensity were due to the obvious disappearance of either the high-fluorescence peak (BAF), or the low-fluorescence peak (PIT). Interestingly, when we evaluated these cells with fluorescence microscopy, we did not find fluorescent vesicles in either BAF- or in PIT-treated cells, so

that the appearance of the two overlapping FL peaks cannot be explained by the occurrence or absence of QAPB-positive endosomes in the cells. As yet, there is no satisfactory explanation for the enhanced fluorescence signals in PIT-treated cells. This situation is aggravated by the fact that the molecular target of PIT still needs to be defined. At least, the autofluorescence of the drug could be excluded by a control experiment analysing cells exposed to PIT only. Generally, the exposure time in the fluorescence microscopy experiments was set so as to optimise the visualisation of bright QAPB-positive vesicles in the cells, whereas total emitted fluorescence of the cells was assessed by flow cytometry, meaning that one possible cause of this obvious discrepancy is to be found in the methodology.

When we analysed the protein binding capacity of QAPB, we saw that BAF reduced the FL signal of the protein band, whereas PIT mainly interfered with electrophoretic mobility of this protein band. We thus hypothesize that the high fluorescence peak derives from cells where QAPB is profoundly bound to the protein target, whereas the low fluorescence peak represents cells in which the dye predominantly interacts with the membrane and/or has diffused passively into the cells. This assumption is to some extent supported by the observation of Pediani et al. (2005) that showed that QAPB remains non-fluorescent in the aqueous phase, but fluoresces when bound to (α1-adrenergic) receptors. However, bearing in mind the non-identity of α1-adrenoceptors and the protein(s) responsible for TP, this is just a hypothesis. A limitation of our study is that it is unclear whether the fluorescent bands in the native gels are exclusively binding proteins of QAPB. It is most likely that there is some nonspecific protein aggregation, since the protein bands are visible with Coomassie blue staining. However, our strategy using native gels is a first step towards the identification of new quinazoline-affine proteins.

Recent results (Gzyl-Malcher et al., 2012, 2013) demonstrated that the lipophilic drug PRZ is able to interact with artificial phospholipid monolayers. Emission of cellular fluorescence despite the lack of QAPB positive vesicular structures therefore suggests that the QAPB-FL signal may originate from the plasma membrane. Experiments with the cholesterol depletor MD showed that cholesterol obviously favours the interaction of QAPB with the membrane and the protein target. Since cholesterol is an important component of lipid rafts, sensitivity of endocytotic uptake towards treatment with MD is mainly attributed to lipid raft mediated endocytosis in the literature (Kabouridis et al., 2000; Segura et al., 2013). Short time experiments with the lipid raft marker CTB showed that QAPB positive tubules were clearly positive for the GM1-ganglioside, which further supports the hypothesis that lipid rafts and/or caveolae mediate the uptake of QAPB/PRZ (Chinnapen et al., 2007). At least, the trafficking of QAPB/PRZ interferes with the retrograde transport of CTB.

Our results also showed that prazosin is able to activate caspase 8, which suggests pro-apoptotic signals derived from the cell membrane and/or endosomes, even though we have not as yet tested the functional link between transport-P/endocytosis and caspase activity. Caspase 8 activation due to PRZ treatment may reflect activation of death receptors on the cell membrane or disturbed integrin signalling (Garrison and Kyprianou, 2006; Schneider-Brachert et al., 2013). The integrin hypothesis is supported by the well documented negative impact of quinazolines on integrin signalling and studies by Stupack et al. demonstrating

caspase 8 activation in cells due to unligated integrins (Stupack et al., 2001; Garrison and Kyprianou, 2006). Although activation of caspase 8, which is a key player in the extrinsic apoptotic pathway, implies that the PRZ-induced proapoptotic signals may derive from the cell membrane and/or endosomes, parallel activation of caspase 9 suggests that PRZ activates further apoptotic pathways. These mechanisms may reflect the inhibition of cytokinesis by PRZ (Fuchs et al., 2009; Zhang et al., 2012) and/or modulation of important signal transduction pathway components such as AKT, AMPK and mTor (Yang et al., 2011).

Our results showed that BAF interferes with the binding of QAPB at the protein level, most likely implicating a protein localized at the plasma membrane with affinity towards QAPB and PRZ, whose uptake into the cell is driven by a proton gradient. BAF (and MD as well) not only inhibited endocytosis of QAPB, but also preserved viability of K562 and TT cells in the presence of PRZ, confirming that QAPB and PRZ share the same cell uptake mechanism. Even though BAF alone inhibited the growth of both tested cell lines as observed in other studies (Nakashima et al., 2003; von Schwarzenberg et al., 2013; Schempp et al., 2014), BAF obviously overruled the growth inhibitory effect of PRZ. In contrast to BAF, CHQ, which also increases the pH of acidic organelles, did not inhibit the uptake of QAPB, as reported by Al-Damluji and Kopin (1996a). In contrast, CHQ significantly enhanced the QAPB signal of K562 and LNCaP cells. Nevertheless, cells treated with CHQ concentrations that raise the cellular QAPB signal were partially protected against the toxicity of PRZ. These data imply that the toxicity of PRZ depends not only on a pH gradient over the plasma membrane, but also on the acidic pH in the endolysosomal system. pH regulation in late endosomes and lysosomes is essential for optimal enzymatic activity within these organelles, is required for the recycling of receptors and the maturation of lysosomes (Maxfield and McGraw, 2004; Carnell et al., 2011). A possible explanation for the discrepancy we see between our results with CHQ and the results presented by Al-Damluji is the fact that Al-Damluji et al. did not pre-treat cells with CHQ as described by several other studies (Sasaki et al., 2010; Dunmore et al., 2013; Liang et al., 2014), but exposed cells to CHQ in parallel with QAPB. CHQ was found to act as a chemosensitizer when used in combination with anti-cancer drugs (Sasaki et al., 2010); however, with PRZ the opposite is the case, making the pro-apoptotic process of PRZ unique in this regard. In LNCaP cells pre-treated with CHQ, the tubulation of lysosomes was abrogated, providing the first functional evidence that the tubulation process of lysosomes is directly linked to the anti-proliferative effect of PRZ. Since QAPB accumulated in CHQ pre-treated cells without formation of tubules, we think that these tubular structures indeed represent misrouted recycling endosomal structures. In summary, our data suggest that endocytosis of PRZ as well as the induction of apoptosis is linked to pH regulation across the plasma membrane and/or the membranes of the endolysosomal system.

The inhibition of endocytosis of QAPB by DYN implies that TP is a dynamin driven process. The QAPB fluorescence pattern of DYN treated cells, characterized by sporadic fluorescent pits stacked at the periphery of the cells, reflected the mechanism of action of dynamin, whose function is to execute the abscission of endocytotic pits from the plasma membrane. Similar to PIT, DYN interfered with the electrophoretic mobility of the QAPB affine protein band, suggesting either a change in charge and/or molecular weight of the

protein complex. As observed with PIT, DYN inhibited endocytosis of QAPB but was not able to preserve the growth of PRZ treated cells when concentrations were used that inhibited the uptake of QAPB. It may be that on the one hand, DYN does not interfere with protein-binding of QAPB/PRZ itself, and on the other hand with the essential role of dynamin-GTPases in cellular processes like cytokinesis and mitochondrial fission (Konopka et al., 2006; Qian et al., 2012).

While the action of DYN on the endocytosis of QAPB can be related to a distinct protein, the mechanism by which DESI interferes with the uptake of QAPB remains unclear. DESI is a reuptake inhibitor of norepinephrine, but Al-Damluji and Kopin (1996b) excluded this mechanism of action as the underlying effect of DESI on PRZ uptake. We demonstrated that DESI induces apoptosis in K562 cells, as is already documented in the literature for cells from other tissues (Arimochi and Morita, 2008; Ma et al., 2011). As with PRZ, the main protein target of DESI, responsible for the induction of apoptosis, is still obscure. Based on the observation that DESI interferes with the uptake of QAPB, a common pro-apoptotic target of DESI and PRZ is possible.

Our work with MNCs has shown that the uptake of QAPB into lymphocytes and monocytes is sensitive to DESI and BAF as well, implying that TP is not limited to malignant cells. Since PRZ also induces apoptosis in fibroblasts and several other cell types (Lin et al., 2007; Fuchs et al., 2009,2015), it can be expected that TP is a more general process not exclusive to neurones. So far, the physiological significance of transport-P is still unknown.

Considering the link between TP and apoptosis, and the affinity of TP for phenylethylamines, which are ingredients of common foods like wine or chocolate (Sengupta and Mohanakumar, 2010), the pathophysiological significance of our observations could be worth further study. Even though the data of Zhang et al. (2012) suggest a role of DRD3 in the interference of PRZ with endocytotic sorting, this might not be the complete story, since DRD3 is rarely expressed in humans (Uhlen et al., 2005, 2015). A daring hypothesis for future research would be that the protein complex with affinity to QAPB represents a kind of death receptor located at the plasma membrane.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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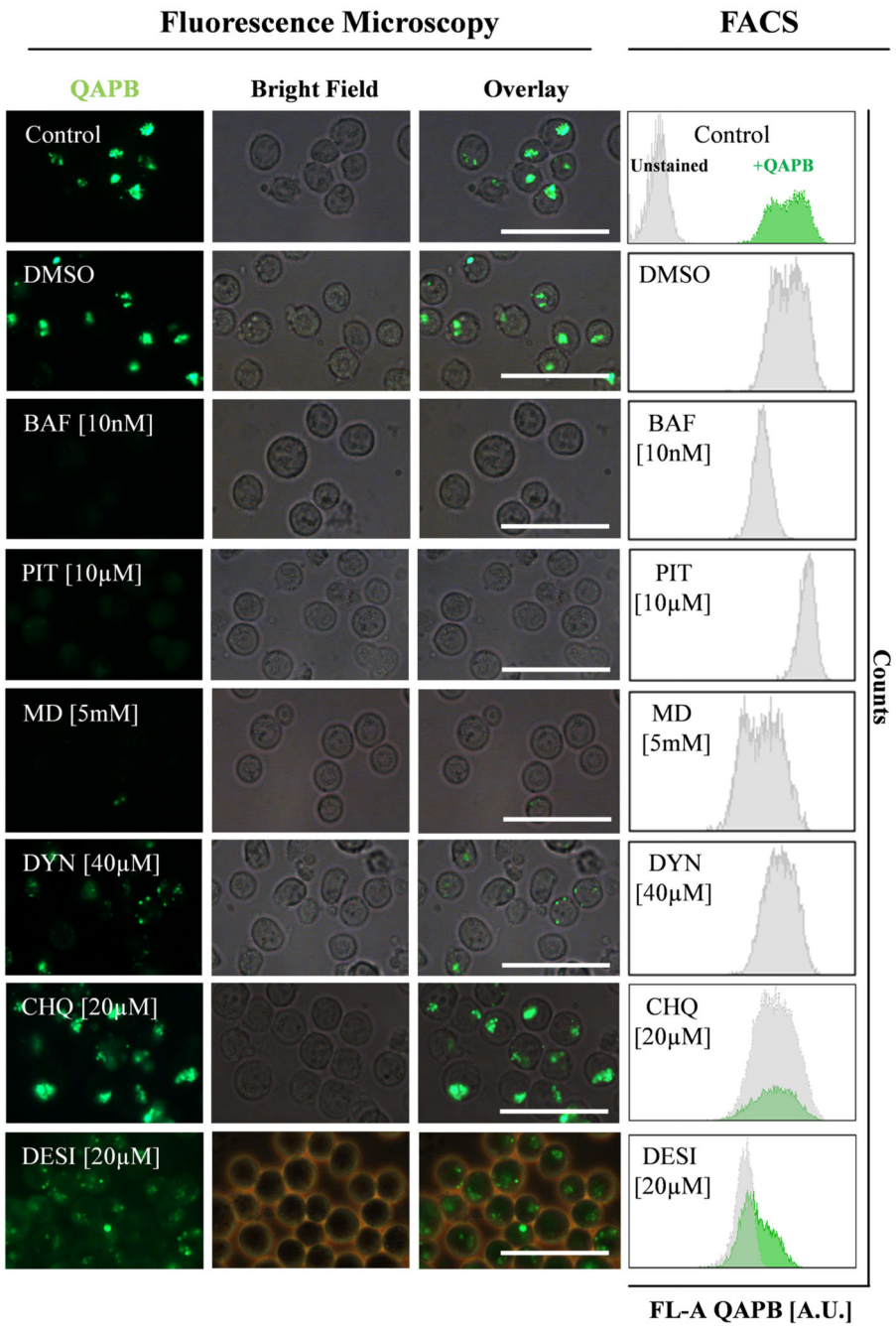

Fig. 1.

QAPB fluorescence pattern analysed by fluorescence microscopy and fluorescence intensity assessed by flow cytometry (FACS) following staining of K562 cells with/without endocytosis inhibitors. Typically, K562 cells stained with QAPB show a vesicular fluorescence pattern. Treatment of cells with bafilomycin A1 (BAF), pitstop® 2, (PIT) and methyl-β-cyclodextrin (MD) interferes with the occurrence of these vesicles. Dimethyl sulfoxide (DMSO), which was used as solvent for some of the drugs, and chloroquine (CHQ) did not interfere with vesicle formation. In cells treated with dynasore (DYN), fewer

vesicles were found, typically localized at the periphery of the cells. FACS analysis essentially confirmed the results obtained by microscopy, except for cells treated with PIT. These cells did not show a vesicular fluorescence pattern, but emitted a stronger fluorescence signal than control cells. The data presented are representative for at least three experiments per analysed condition, except microscopic analysis of desipramine treated cells, which was done only twice. The control histogram represents an overlay of unstained cells and cells stained with QAPB. Since the images and the FACS histograms of CHQ- and desipramine (DESI)-treated cells come from separate experimental series, the respective control is shown as a green overlay peak in the FACS histograms. All other samples derive from the same experimental series. The bars in the pictures represent 50 μm.

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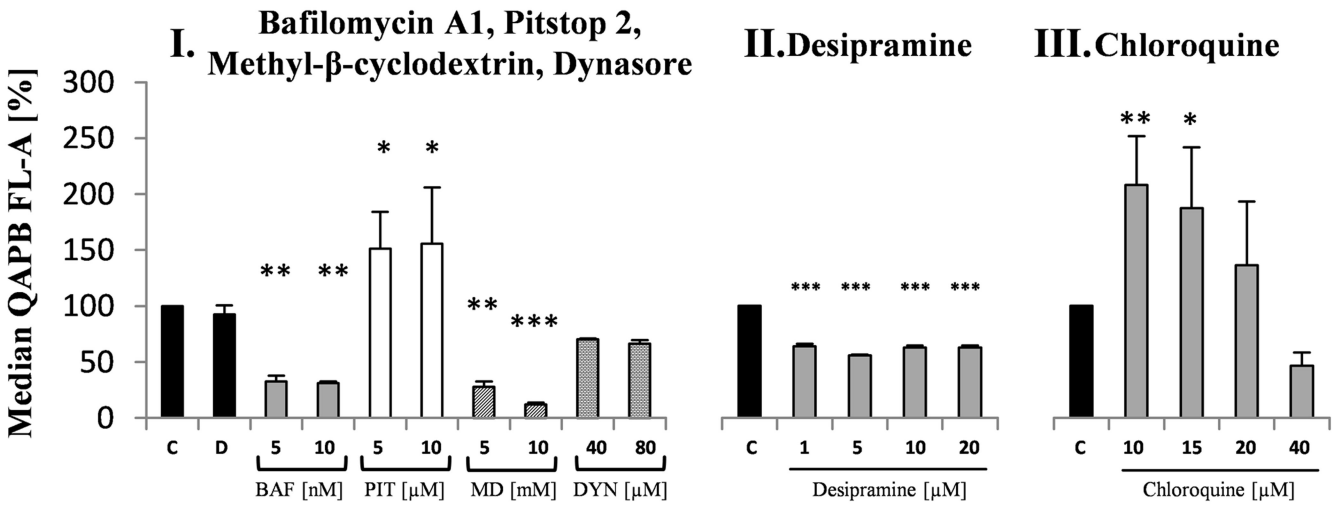

Fig. 2.

The uptake of QAPB into the cell is sensitive to endocytosis inhibitors and known modulators of transport-P. K562 cells were pre-treated with/without endocytosis inhibitors for 1 h and exposed to QAPB for a further 30 min. After staining, the fluorescence of cells was assessed by flow cytometry. The graphs show the normalized median QAPB FL-Area (FL-A) of K562 cells. C: Untreated Control, D: Dimethyl sulfoxide as vector control, BAF: Bafilomycin A1, PIT: Pitstop® 2, MD: Methyl-β-cyclodextrin, DYN: Dynasore, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ versus control according to ANOVA testing, I: C: $n = 4$, D: $n = 4$, BAF: $n = 3$, PIT: $n = 3$, MD: $n = 3$, DYN: $n = 3$, II: $n = 3$, III: $n = 4$.

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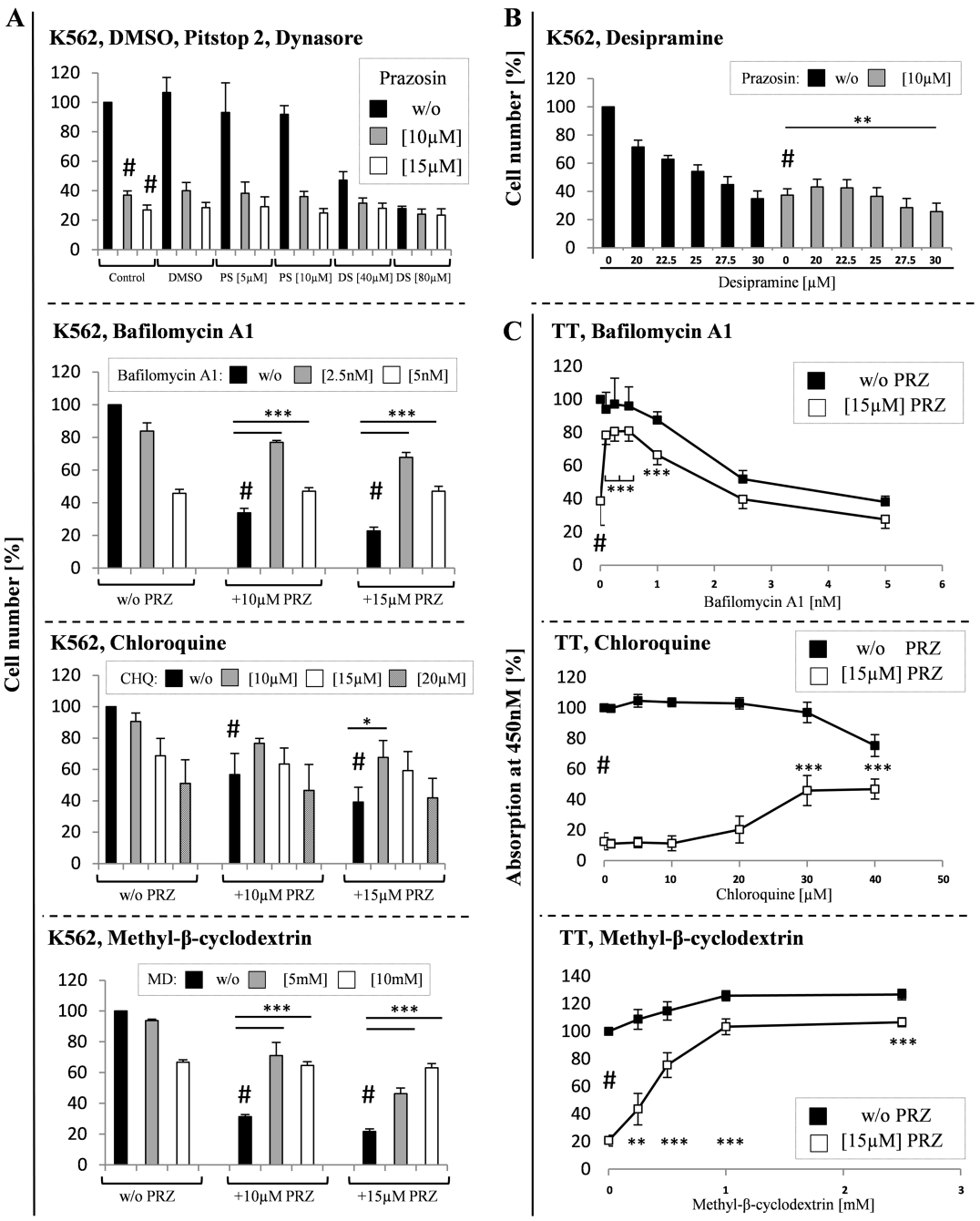

Fig. 3.

Effect of transport-P inhibitors and endocytosis inhibitors on the growth of K562 and TT cells in the presence of prazosin (PRZ). (A, B) K562 cells were treated for 48 h with either PRZ, uptake-inhibitors, the vector dimethyl sulfoxide (DMSO) or combinations of inhibitors and PRZ. Following incubation, cell counts were assessed with an automated cell counter. Bafilomycin A1 (BAF) was added in parallel to cultures with PRZ or cells were pre-treated overnight (chloroquine/CHQ) or 1 h (desipramine/DESI, dynasore/DS, methyl-β-cyclodextrin/MD, pitstop® 2/PS,) before the addition of PRZ. *: $p < 0.05$, **: $p < 0.01$, ***:

$p < 0.001$; #: $p < 0.001$ versus untreated control. DMSO, PS, DS: $n = 3$; BAF: 2.5 nM: $n = 3$, 5 nM: $n = 4$, w/o PRZ: $n = 4$; CHQ: $n = 4$; MD: $n = 3$; DESI: $n = 6$. (C) The medullary thyroid carcinoma cell line TT was incubated 48 h with PRZ in parallel with BAF, or after pre-treatment of cells with CHQ (overnight) or MD (1 h). Following incubation, growth and viability of cells were assessed, analysing the absorption of the WST-1 reagent at 450 nM. #: $p < 0.001$ versus untreated control, **: $p < 0.01$, ***: $p \leq 0.001$ versus PRZ [15 μM]. BAF: $n = 4$, CHQ: $n = 3$, MD: $n = 4$, except 1 mM, 2.5 mM: $n = 3$. w/o: without.

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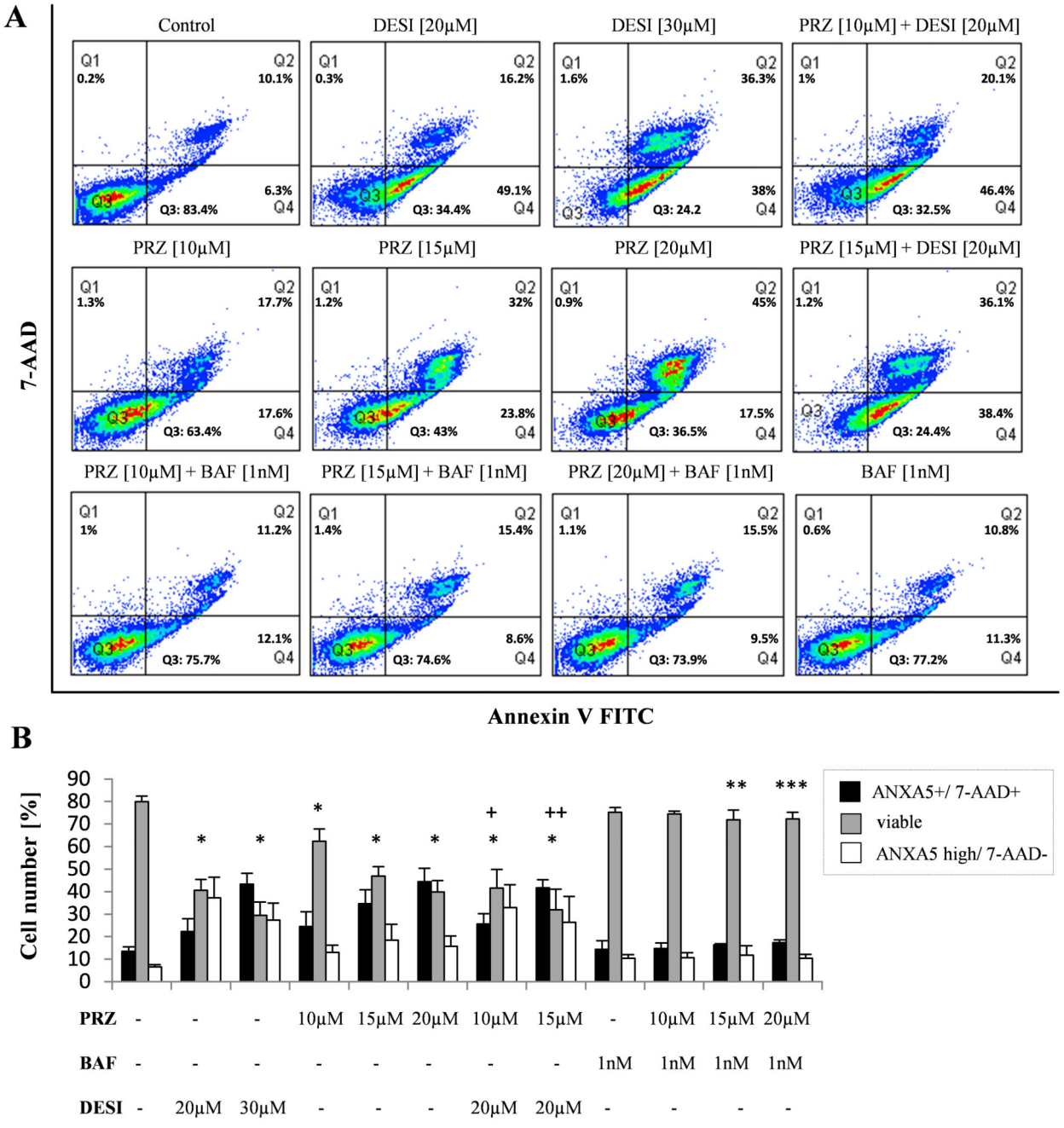

Fig. 4.

Prazosin, as well as desipramine, induces apoptosis in K562 cells. K562 cells were incubated 48 h in the presence of prazosin (PRZ), desipramine (DESI), bafilomycin A1 (BAF) or combinations of individual drugs. Cells were harvested and stained with 7-aminoactinomycin D (7-AAD) and annexin V (ANXA5)-FITC and were analysed by flow cytometry. (A) Density plots of one representative experiment are shown. The percentage of cells in each quadrant (Q1-4) is presented. (B) Statistical analysis of cell fractions. The percentage of living cells was assessed as statistical analysis parameter for cell death

analysis. *: $p < 0.001$ versus untreated control, **: $p < 0.001$ versus PRZ [15 μM], ***: $p < 0.001$ versus PRZ [20 μM], +: $p < 0.001$ versus PRZ [10 μM], ++: $p < 0.01$ versus PRZ [15 μM], Control: $n = 5$, DESI/DESI+PRZ: $n = 4$, PRZ: $n = 5$, BAF/BAF + PRZ: $n = 3$.

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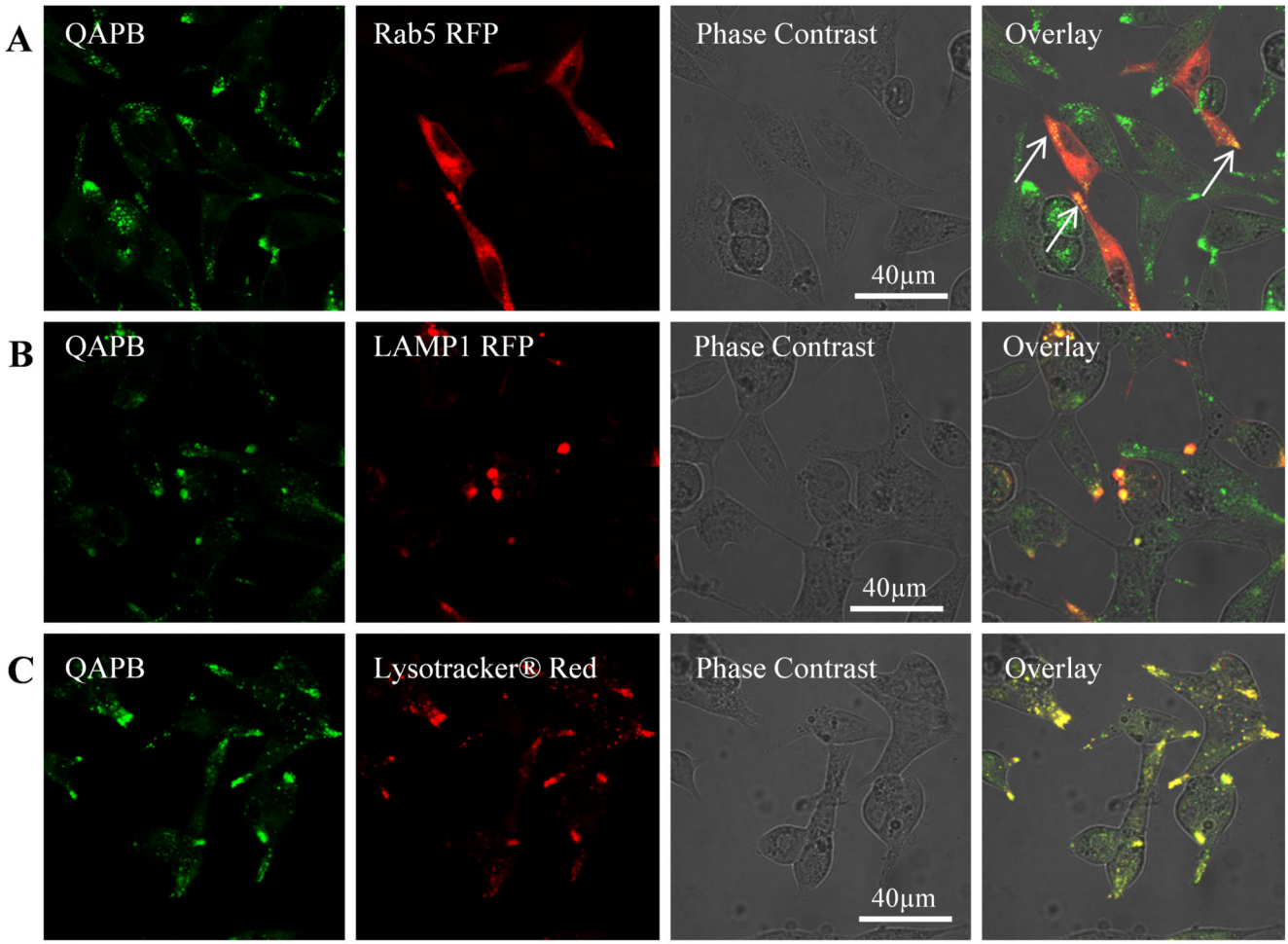

Fig. 5.

Visualisation of the trafficking of BODIPY® FL Prazosin (QAPB) in living cells using confocal microscopy. (A) QAPB shows co-localisation with the early endosome marker Rab5. A Rab5-RFP fusion protein construct was expressed in LNCaP cells using a baculovirus vector system. Cells were treated with 100 nM QABP and analysed by confocal microscopy. Yellowish vesicular structures (arrows) in the generated overlay picture indicate QAPB positive endosomes. (B/C) QAPB shows co-localisation with the lysosome marker LAMP1 and the lysomototropic reagent Lysotracker(R) Red. (B) LNCaP cells were transduced with baculovirus particles in order to express LAMP-RFP fusion protein and stained with QAPB. Yellowish vesicular structures in the picture overlay indicate that QAPB ends up in the late endosome/lysosome compartment of the cells. (C) LNCaP cells were co-stained with QAPB and Lysotracker® Red, which preferentially accumulates in the acidic late endosome/lysosome compartment of the cells. Co-localization of QAPB and Lysotracker® was evident in the generated overlay, indicated by yellow signals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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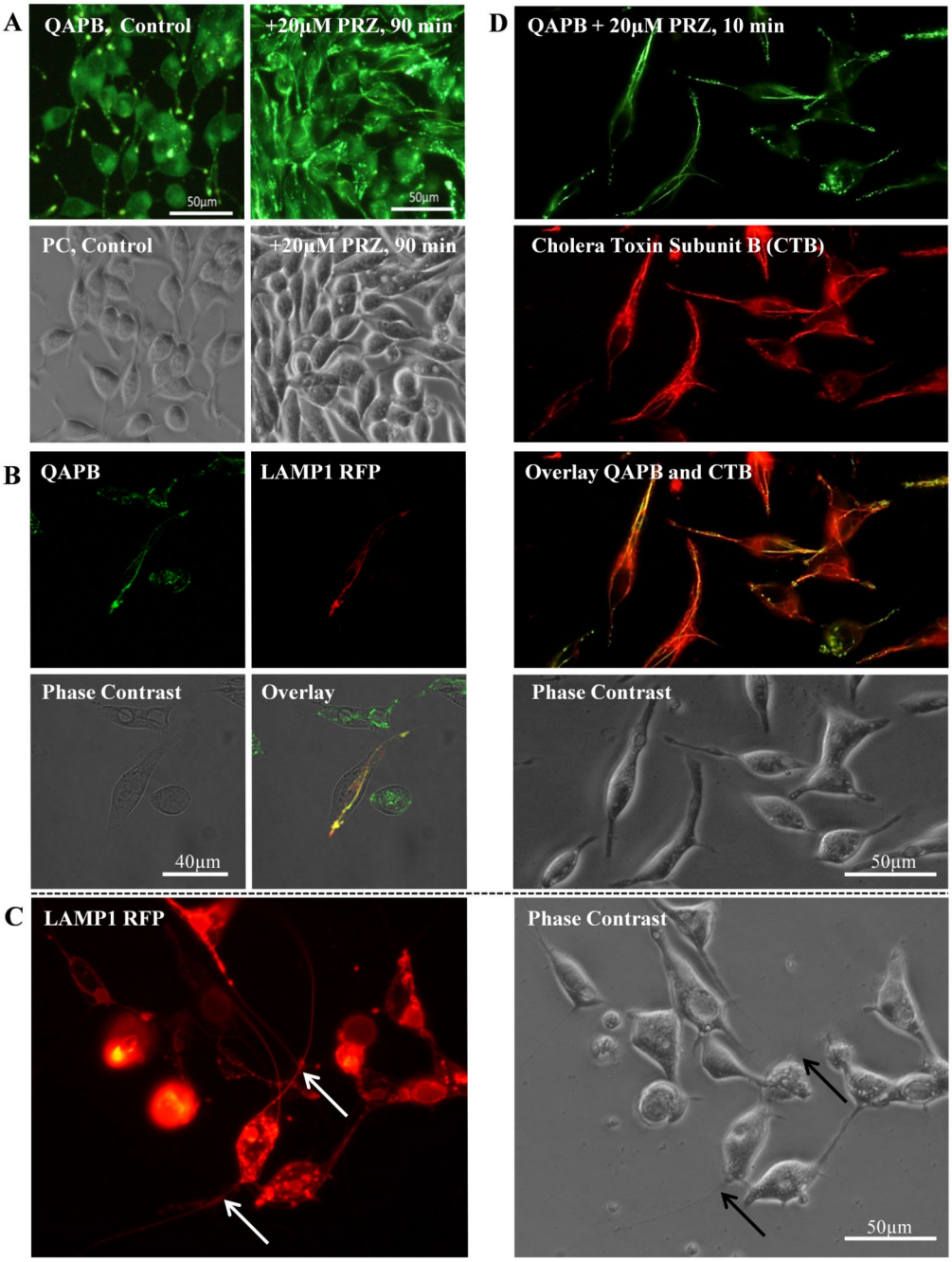

Fig. 6.

Prazosin (PRZ) induces tubulation of LAMP1 positive late endosomes and lysosomes in LNCaP cells. (A) LNCaP cells were treated +/− PRZ and 100 nM BODIPY® FL Prazosin (QAPB) for 90 min. Following incubation, tubulation of the QAPB fluorescence pattern was evident. (B) LNCaP cells expressing LAMP1 RFP were treated with 10 μM PRZ for 24 h and stained with QAPB. Co-localisation of QAPB with LAMP1 RFP suggests tubulation of lysosomes due to PRZ treatment. (C) LNCaP cells expressing LAMP1 RFP were treated with 10 μM PRZ for 24 h and analysed by fluorescence microscopy. Our results showed that

the formation of needle like polar protrusions (arrows), which are formed by PRZ treated cells, is the consequence of tubulation of lysosomes. (D) The uptake of QAPB shows interference with the trafficking of cholera toxin subunit B (CTB). Cells were exposed to QAPB, Alexa® Fluor 555 labelled CTB and 20 μM PRZ for 10 min and were analysed by fluorescence microscopy. Tubulation of the QAPB and the CTB fluorescence pattern was evident with clear interference of both signals (overlay). PC = Phase Contrast.

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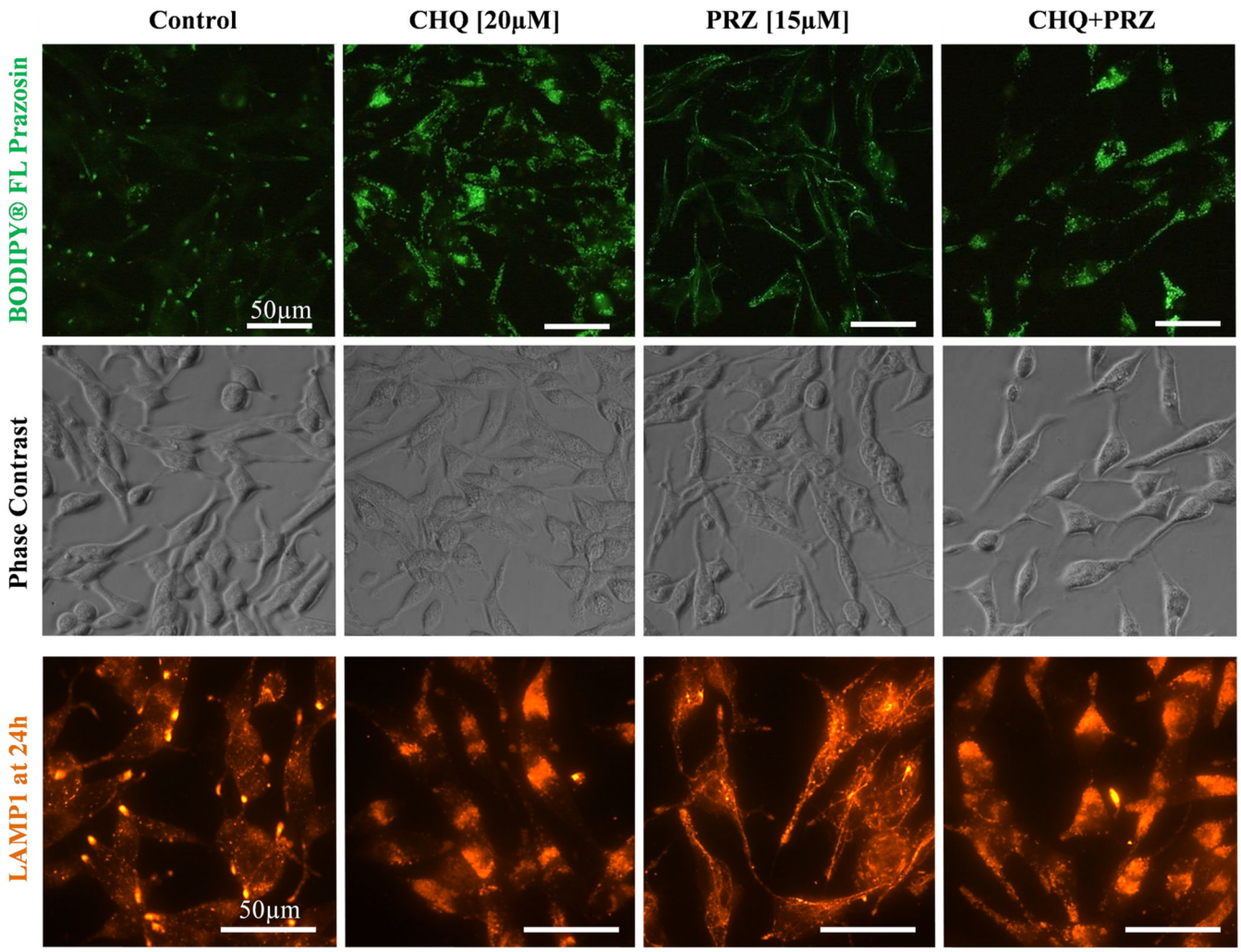

Fig. 7.

Pre-treatment with chloroquine (CHQ) results in an accumulation of BODIPY® FL Prazosin (QAPB) in LNCaP cells and interferes with the formation of prazosin (PRZ) induced lysosomal tubules. Cells were pre-treated over night with CHQ and exposed to 100 nM of QAPB and 15 µM PRZ for a total time of 2 h (upper and middle row). In CHQ treated cells QAPB accumulates in large vesicles without appearance of QAPB positive tubules. The morphology of lysosomes was assessed by LAMP1 immunofluorescence staining 24 h after addition of PRZ to the cultures (lower row), and no tubulation of lysosomes was observed 24 h after addition of PRZ to CHQ pre-treated cultures.

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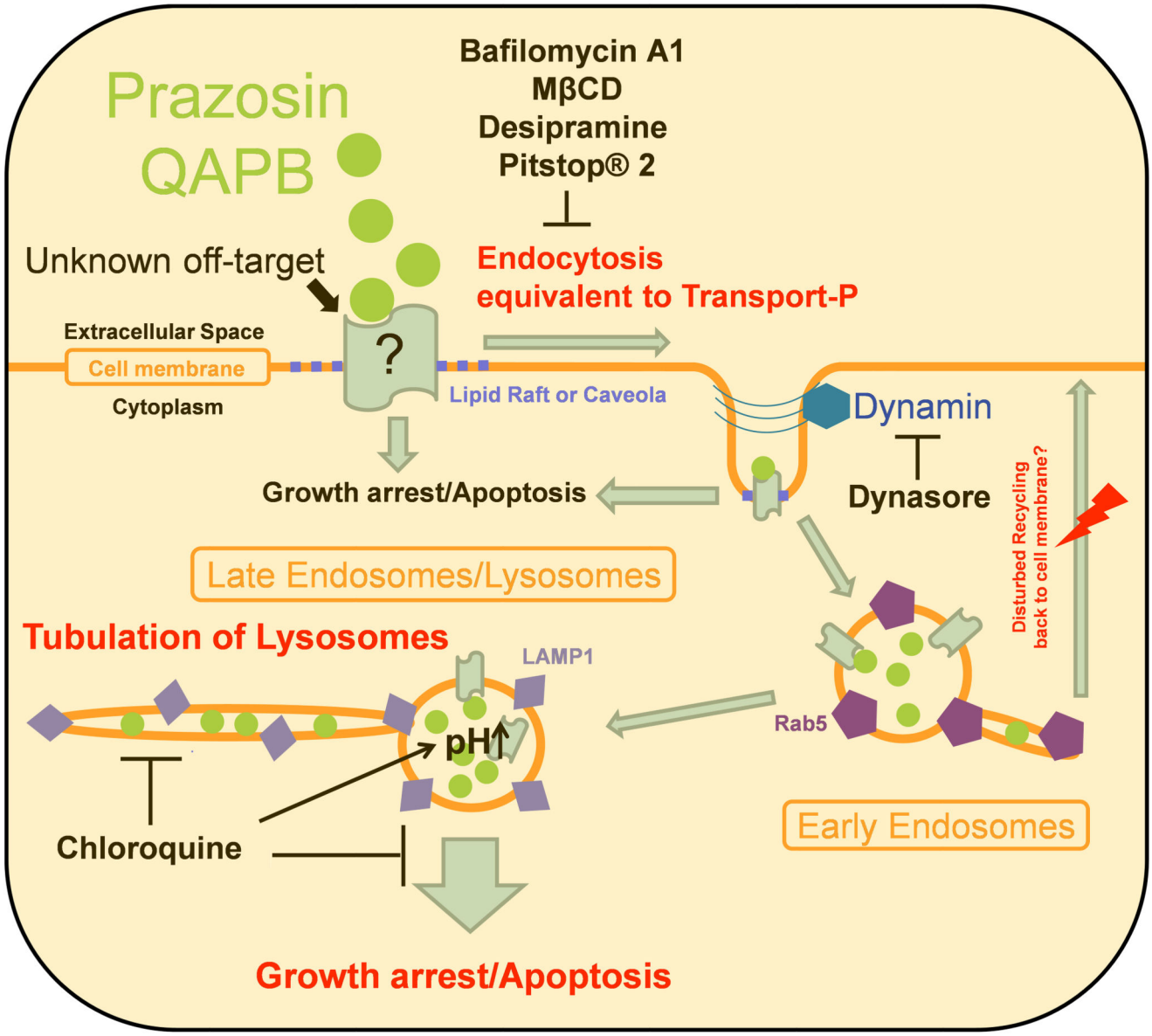

Fig. 8.

Proposed mechanisms of the link between the uptake of prazosin/QAPB and the induction of apoptosis. The results of our study suggest that prazosin/QAPB enter the cell through endocytosis equivalent to transport-P via an unknown target localised at the cell membrane. Finally, the unknown target ends up in the lysosomes as they form tubular structures that develop into long polar cellular protrusions. Since the lysomototropic agent chloroquine can inhibit the formation of tubulating lysosomes and also restores the growth of cells, the cytotoxicity of prazosin seems to be closely related to the endolysosomal system. For detailed explanation please see the discussion section. MβCD = methyl-β-cyclodextrin.