The Anti-hypertensive Drug Prazosin Induces Apoptosis in the Medullary Thyroid Carcinoma Cell Line TT

ROBERT FUCHS1*, GERT SCHWACH1*, ANIKA STRACKE1, NATHALIE MEIER-ALLARD1, MARKUS ABSENGER2, ELISABETH INGOVIC3, HELGA SUSANNE HAAS1, ROSWITHA PFRAGNER1 and ANTON SADJAK1

1Institute of Pathophysiology and Immunology, Center of Molecular Medicine, Medical University of Graz, Graz, Austria;
2Center for Medical Research, Medical University of Graz, Graz, Austria;
3Research Institute for Electron Microscopy, Graz University of Technology, Graz, Austria

Abstract. Background/Aim: Medullary thyroid carcinoma (MTC) is a tumor associated with poor prognosis since it exhibits high resistance against conventional cancer therapy. Recent studies have shown that quinazolines exhibit a pro-apoptotic effect on malignant cells. The aim of the present study was to elucidate whether MTC cells are affected by quinazolines, in particular prazosin. Materials and Methods: Proliferation, apoptosis and cell morphology of the MTC cell line TT were analyzed by WST-1 assay, caspase 3/7 activation tests and microscopy. Fibroblasts were used as control for non-malignant cells. Results: Prazosin potently inhibited the growth of TT cells, induced apoptosis and caused vacuolization, as well as needle-like filopodia. Fibroblasts were affected by prazosin in the same way as MTC cells. Conclusion: MTC cells are responsive to prazosin treatment similar to other malignancies. The fact that fibroblasts also respond to prazosin further highlights the importance to identify the unknown pro-apoptotic target of quinazolines.

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*These Authors contributed equally to this work.

Correspondence to: Robert Fuchs, Institute of Pathophysiology and Immunology, Center of Molecular Medicine, Medical University of Graz, Heinrichstrasse 31A, 8010 Graz, Austria. Tel: +43 3163801953, Fax: +43 3163809640, e-mail: robert.fuchs@medunigraz.at

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apoptosis in glandular epithelial and smooth muscle cells of the prostate in benign prostatic hyperplasia (BHP) patients (12). However, the group around Kyrianou et al. discovered in a follow-up study that the pro-apoptotic mechanism of α1-adrenergic antagonists on prostate cells is independent of α1-adrenoceptors (13). This is in line with our observation in leukaemia cells in which α1-adrenergic blockers induce apoptosis in the absence of α1-adrenoceptors (14-16). Further studies have revealed that α1-adrenergic antagonists also induce apoptosis in malignant prostate carcinoma cells (13).

Based on observations in prostate cancer cells, other research groups investigated the impact of α1-adrenergic drugs on further human malignancies, such as pituitary adenoma, breast cancer, bladder cancer, as well as mesothelioma (17-20). The results of these investigations were promising since a pronounced pro-apoptotic effect of α1-adrenoceptor blockers on malignancies was documented in the respective studies (17-20).

The aim of the present study was to test, whether the MTC cell line TT is also sensitive towards treatment with quinazoline-based α1-adrenergic antagonists in a similar way, as already shown for other malignancies. For our study we have chosen prazosin, which exhibits a significantly higher potency to induce apoptosis in the K562 cell line than doxazosin according to recent studies in our lab (Zeller C, unpublished observation, 2013). This is in line with results obtained from human prostate cancer cell lines where prazosin exhibited supremacy against other common clinically used α1-adrenergic antagonists regarding the induction of apoptosis (21). Since information about possible growth inhibitory actions of quinazolines on non-malignant cells are sparse, we compared the effects of prazosin on TT cells with that on normal human skin fibroblasts.

Materials and Methods

Detection of α1-adrenergic receptor expression in TT cells using TaqMan® gene expression assays. Expression of α1-adrenergic receptors ADRA1A, ADRA1B and ADRA1D was assessed at the level of mRNA by TaqMan® gene expression assays (Life/Applied Biosystems, Foster City, CA, USA) using inventoried assays (ADRA1A: Hs00169124_m1, ADRA1B: Hs00171263_m1, ADRA1D: Hs00169865_m1). β-actin (ACTB, Hs03023943_g1) was used as internal control. Isolation of RNA, reverse transcription and real time polymerase chain reaction (PCR) were done as described previously (15). PCR reactions were run in a CFX96 Real Time PCR machine (Biorad, Hercules, CA, USA).

Cultivation of TT cells and skin fibroblasts. The human MTC cell line TT (presented for the first time at the congress “Advances in thyroid neoplasia”, Rome, 1981 by Leong) was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). TT cells were cultivated in Ham’s F-12 medium (Lonza, Verviers, Belgium) supplemented with 10% FBS (PAA, Pasching, Austria). The normal human skin fibroblast cell line HF-SAR, which was established at our institute (22), was maintained in Eagle’s MEM (Lonza) supplemented with 10% FBS. Both cell lines were cultivated under antibiotic-free conditions at 37°C, 5% CO₂ in a fully humidified atmosphere in a cell culture incubator.

Analysis of growth of cells by means of the WST-1 proliferation assay. Relative cell proliferation, respectively viability, was analysed using the WST-1 cell proliferation reagent 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate (Roche Diagnostics, Mannheim, Germany) following the instructions of the manufacturer. For the WST-1 assay, cells were cultivated in 96-well plates for different periods of time, starting with a cell number of 1x10⁴ cells in 100 µl medium. Absolute cell counts were assessed prior to seeding by a CASY® Cell Counter and Analyser System (Roche).

Detection of caspases 3/7 activation. Activation of effector caspases 3 and 7 was assessed by a luminescence-based Caspase-Glo® 3/7 assay (Promega, Fitchburg, WI, USA) according to the instructions of the manufacturer. Emitted luminescence was analysed with a GloMax®-Multi Detection System (Promega). Caspase-3/7-activation was further analysed using the fluorescent CellEvent® Caspase-3/7 Green ReadyProbes® Reagent (Life/Molecular Probes, Eugene, OR, USA). For this assay, cells were grown on glass chamber slides (Discovery Labware, Billerica, MA, USA) and surveyed for apoptotic cells, characterized by green fluorescent nuclei, with an Eclipse TE300 (Nikon, Tokyo, Japan) inverted microscope equipped with a FITC filter set.

Scanning electron microscopy (SEM) and live cell imaging. Preparation of cells for SEM was done following standard procedures (23, 24). Cells were grown on glass inserts in 24-well cell culture plates. After drug treatment, cells were fixed in 3% glutaraldehyde dissolved in 0.1 M cacodylate buffer, pH 7.2, overnight at 4°C and were post-fixed with osmium tetroxide. Cells were processed by critical point drying, finally coated with Au-Pd and viewed by an Ultra 55 SEM (Zeiss, Jena, Germany).

Live cell imaging was done using a Cell Observer based on an Axiovert 200M inverted fluorescence microscope (Zeiss). Cells were cultivated on glass chamber slides and observed for a total period of 24 h. Every 10 min, a series of photos was taken of different positions on the slide.

Figure 1. α1-adrenoceptor expression in the TT cell line. By means of real-time qRT-PCR we detected expression of α1-adrenergic receptors (ADRA) ADRA1A, ADRA1B but not ADRA1D. β-actin (ACTB) was analyzed as internal control (housekeeping gene). RFU: Relative fluorescence units.
Statistics. All data are presented as mean values and standard deviation (SD). Significance of obtained data was calculated using Sigma Plot 12.5 (Systat Software Inc., San Jose, CA, USA). Gaussian distribution of values was tested using the Shapiro-Wilk test. Multiple testing was performed by means of one way analysis of variance (ANOVA) with the Holm-Sidak post hoc testing procedure with an overall significance level of \( p \leq 0.05 \).

Results

**TT cells express α1-adrenergic receptors.** Even though it is well-documented that quinazoline-based α1-adrenergic antagonists induce apoptosis independent of adrenoceptors, we tested whether TT cells express α1-adrenoceptors. Using
highly specific and sensitive TaqMan® gene expression assays, we were able to show that TT cells express the α1-adrenoceptor sub-types ADRA1A and ADRA1B but not ADRA1D (Figure 1).

Prazosin induces significant morphological changes and apoptosis in TT cells. During and following a 24-h treatment with 15 μM prazosin, we observed vacuolization of cells, enhanced detachment and rounding, as well as the formation of typical spindle-like polar protrusions, most likely reminiscent to filopodia (Figure 2 and Supplementary Files, Video 2). In general, the phenotype of TT cells became more spindle-shaped following treatment with prazosin. The protrusions seemed not to originate from contraction of cells, but were mainly outgrowing from the polar endings of the cells. Ultrastructure analysis confirmed the fragile nature of these structures, which seemed to protrude from the spindle-like body of the cell (Figure 2). Further ultrastructure analysis revealed a highly complex surface structure of TT cells, which might reflect the secretory nature of these neuroendocrine cells (Figure 2). Live cell imaging of untreated and treated TT cells showed unexpected motility in vitro and the reversible formation of round multicellular aggregates (Supplementary Files, Video 1 and 2).

By means of the WST-1 assay, we observed a dose-dependent reduction of metabolic activity in prazosin-treated TT cells-containing wells indicating that prazosin induced growth inhibition and/or cell death. Methods to detect apoptosis, by means of caspase activation, showed that apoptosis occurs at prazosin concentrations ≥15 μM but not at 10μM concentrations (Figures 2 and 3). By using the fluorescence-based caspase activation assays, we confirmed the data obtained by luminescence assays clearly demonstrating that only cells with round phenotype – highly

Figure 3. Effector caspases 3/7 were activated in rounded cells but not in cells exhibiting prazosin-induced protrusions. TT cells were cultivated for 24 or 48 h with increasing concentrations of prazosin and stained with the CellEvent® Caspase-3/7 Green ReadyProbes reagent. In cells with activated caspases 3/7, a DNA-affine fluorescent dye is released from a substrate for caspases 3/7 and stains the nuclei of apoptotic cells green.
enriched in prazosin-treated wells – exhibited caspase-mediated fluorescence (Figure 3). Noticeably, cells with pronounced formation of protrusions were clearly devoid of fluorescence.

The pro-apoptotic action of prazosin is not limited to malignant cells. Since information about the pro-apoptotic action of prazosin on normal cells is sparse in comparison to malignant cells, we tested whether prazosin shows toxic effects on normal human skin fibroblasts. We observed no significant difference in the WST-1 assay, when testing untreated cells versus prazosin-treated cells over time (Figure 4). On the contrary, we found significantly ($p<0.05$) elevated effector caspase activity in prazosin-treated cells (15 μM), a dose that induced apoptosis in the TT cell line (Figure 4). Furthermore, normal human skin fibroblasts exhibited similar dramatic morphological alterations as the TT cell line (Figure 4). Cytoplasmic vacuolization was evident following prazosin treatment in the HF-SAR cells but, in contrast to the tested MTC cell line, HF-SAR cells showed no long protrusions as the TT cells did. Ultra-structural analysis revealed a more complex cell surface than untreated cells (Figure 4).

**Discussion**

To the best of our knowledge, our study is the first to show that prazosin can induce apoptosis in a MTC cell line. This is an interesting observation in the light of the fact that MTC cells are described to be resistant towards conventional chemotherapy and radiotherapy (25).

Even though it is well-documented that prazosin induces apoptotic cell death independent of adrenergic receptors, we...
analyzed the expression of several known α1-adrenergic receptors in the TT cell line because no data exist in the literature assessing ADRA1-expression for this cell line. Indeed, we could demonstrate the expression of ADRA1A and ADRA1B but not ADRA1D in TT. This expression profile is similar, but not identical, to that shown for the rat MTC cell line 6-23, where the expression of ADRA1B and ADRA1D was postulated based on the results of ligand binding assays (26). Esbenshade et al. have shown that norepinephrine (NE) can induce a voltage-gated influx of calcium in 6-23 cells through α1-adrenergic receptors (26). Nevertheless, NE-induced calcitonin release was shown to be caused through β-adrenergic receptors but not through α-adrenergic receptors in MTC cells (27). Since calcium is an important cellular second messenger (28), we hypothesize that even though prazosin exerts its pro-apoptotic action mainly independent of adrenoceptors, blockade of α1-adrenoceptors may additionally negatively affect the cells.

When observing prazosin-treated cells under the microscope, large vacuoles and long polar needle-shaped polar protrusions could be seen. According to the literature, huge vacuoles can originate from different organelles of the endomembrane system including the endoplasmic reticulum, endosomes, lysosomes, Golgi stacks, as well as the trans-Golgi network (29, 30). Since prazosin is a weak base (31), it is most likely that the vacuoles originate from an organelle with acidic pH (mainly late endosomes or lysosomes) because weak bases are known to accumulate in an acidic environment (30). Accumulation of weak bases in acidic organelles causes swelling due to influx of water (30).

The formation of prazosin-induced fibres, which was also observed in an earlier study in the HEL erythroleukemia cell line (14), indicates that prazosin treatment may influence the cytoskeleton, which is responsible for the shape and movement of cells. Live cell imaging experiments revealed that TT cells are very mobile under in vitro conditions. The formation of fibres seemed to interfere with this mobility. Interestingly, cells exhibiting fibres were negative for caspase activation in the fluorescent caspase-3/7 assay. We assume that fibre formation is either an event prior to apoptosis induction or cells with fibres are more resistant towards prazosin treatment.

It is the desire of every oncologist to treat patients with drugs that are highly selective against malignant cells, and do not harm normal cells. Thus, in order to test the effect of prazosin on normal cells, we employed HF-SAR skin fibroblasts. Interestingly, when analyzing cells with the WST-1 assay, we could not observe any significant differences in the measured WST-1 absorption. These results suggested that prazosin exerts no significant toxic impact on normal skin fibroblasts, but this suggestion was dismissed when the analysis involved the caspase assay and microscopy. Prazosin induced apoptosis and dramatic morphological alterations in HF-SAR cells too. Our WST-1 results are in line with recommendations of the guidelines for the use and interpretation of assays for monitoring cell death of eukaryotic cells presented by a consortium around Galluzzi et al. (32). According to these guidelines, the conversion of WST-1 by mitochondrial enzymes may reflect metabolic alterations that do not necessarily correlate with the number of viable cells (32).

We conclude that prazosin is generally not selective for malignant cells concerning apoptosis induction, albeit normal prostate or sperm cells seem to be not or only slightly impacted by prazosin (13, 33). The sensitivity of cells towards prazosin-induced apoptosis may depend on differential expression of a still unknown protein with affinity towards prazosin and other quinazoline-based drugs. In the light of the high concentrations (μM-range) of prazosin to induce apoptosis, it would be careless to recommend the use of this drug for the treatment of cancer patients, since strong adverse effects may arise. Adverse effects of the quinazoline doxazosin were already observed in the ALLHAT study when doxazosin was tested at normal concentrations used for the treatment of hypertension (34, 35). The obtained results revealed that the combined cardiovascular disease risk had significantly increased in the doxazosin arm of the study compared to the chlorthalidone arm. Furthermore, the risk of heart failure was doubled (34).

Some research groups have already started to design new quinazoline-based anti-cancer drugs with higher efficiency (36). This approach, and also attempts for the identification of the still not identified main pro-apoptotic target of quinazoline-based α1-adrenergic antagonists, will hopefully pave the way of quinazoline-based drugs for use in cancer therapy in the future.

Supplemental Material (http://tiny.cc/livecell14)

Video 1: Live cell imaging of the human MTC cell line TT. Cells were cultivated in chamber slides and observed using a Cell Observer for a total time of 24 h. Every 10 minutes a photo was taken of different positions on the slide.

Video 2: Live cell imaging of prazosin-treated TT cells. Cells were cultivated in chamber slides with 15 μM prazosin and observed using a Cell Observer for a total time of 24 h. Every 10 minutes a photo was taken of different positions on the slide.

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