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

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**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.*

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor of the thyroid that accounts for 5-10% of all thyroid malignancies (1). MTC was described for the first time more than 100 years ago by Jaquet (2). Development of MTC can result from a spontaneous mutation (sporadic form) or can

be transmitted hereditarily (25%) either alone (multi-generational familial MTC) or as part of a multiple endocrine neoplasia (MEN) type 2A or 2B syndrome (1-4). Even though it is well-known nowadays that MTC derives from calcitonin-producing para-follicular cells and that mutations in the *RET* proto-oncogene are the main driver mutations in MTC, widespread clinical expertise is so far limited because of the low incidence of MTC (2). Thus, no effective treatment for MTC with distant metastasis is as yet available (1-4). Knowledge about the importance of the *RET* kinase for the pathogenesis of MTC led to the development and investigation of multi-targeted inhibitors, which cross-react with *RET* (5, 6). Indeed, some clinical studies testing *RET* inhibitors were promising, but showed only modest results (5). Therefore, new therapeutic options are still needed in the therapy of MTC.

In order to find new therapeutic options for the treatment of malignancies, several strategies are nowadays pursued, including screening for new cancer drugs in natural products (7, 8) or the re-evaluation of known drugs – already authorized for other diseases – for potential anti-cancer effects. A good example for such a drug is thalidomide, which was initially authorized as a barbiturate but is nowadays used as an anti-cancer agent against multiple myeloma (9). Other examples for unexpected anti-cancer action of proved drugs are quinazolin-based α1-adrenergic antagonists, which are in the focus of the current study.

α1-adrenergic antagonists, including the quinazolines prazosin and doxazosin, are used in the treatment of hypertension. Furthermore, based on the observation that smooth muscle cells of the prostate predominately express α1-adrenoceptors (10), α1-adrenergic antagonist therapy was introduced for the first time in 1978 in order to reduce the muscle tonus of the prostate in patients with benign prostate hyperplasia (11). Kyprianou *et al.* demonstrated for the first time that α1-adrenergic antagonists are able to induce

apoptosis in glandular epithelial and smooth muscle cells of the prostate in benign prostatic hyperplasia (BHP) patients (12). However, the group around Kyprianou *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
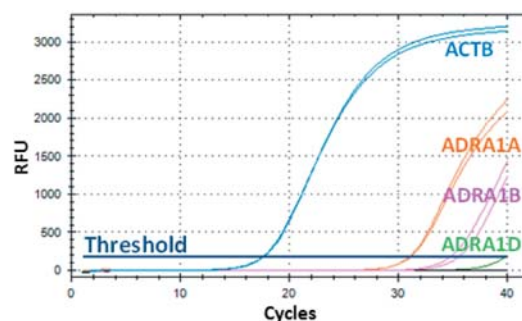


Figure 1. *α1-adrenoceptor expression in the TT cell line. By means of real-time qRT-PCR we detected expression of α1-adrenergic receptors (ADRA) ADRA1A, ADRA1B but not ADRA1D. β-actin (ACTB) was analyzed as internal control (housekeeping gene). RFU: Relative fluorescence units.*

(Lonza) supplemented with 10% FBS. Both cell lines were cultivated under antibiotic-free conditions at 37°C, 5% $CO_2$ 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 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 $1 \times 10^4$ 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 (23) 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.
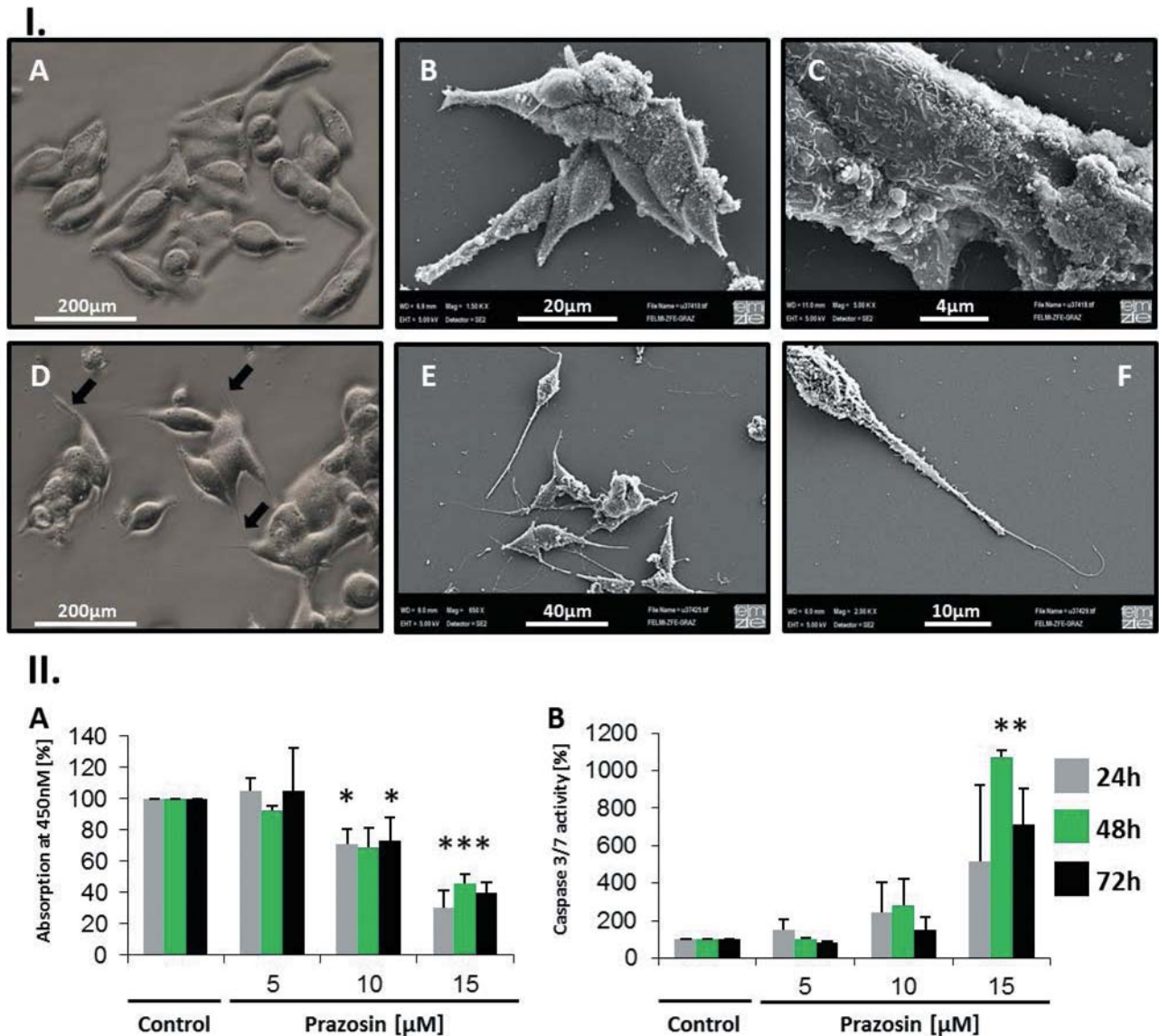
Figure 2. *Prazosin induced morphological alterations and apoptosis in the TT cell line. I. A: Light microscopic view of TT cells: Typical TT cells are small, spindle-shaped and growing in small clusters. B/C: Scanning electron microscopic (SEM) analysis of TT cells exhibited a highly complex cytoplasmic membrane showing many membrane-bound vesicular structures, which may reflect exocytotic processes. D: Light microscopic view of 24 h prazosin (15 μM)-treated TT cells: Prazosin-treated cells were generally more spindle-shaped and exhibited long filopodia like polar fibres (arrows). E/F: SEM analysis of 24 h prazosin-treated cells (15μM) confirmed the enhanced spindle-like character of TT cells upon prazosin treatment and showed that the formed fibres are very fragile structures. The fibres seem to protrude from the polar endings of the cells (F). II: A: Growth, respectively viability, of TT cells was tested using the WST-1 reagent over 72 h. A dose-dependent decrease of the optical density (OD) at 450 nm was evident when prazosin was added to the culture; n=4. B: Activation of effector caspases 3/7 indicates that prazosin induces apoptosis in TT cells; n=3. \*p<0.05 according to one-way ANOVA with the Holm-Sidak post hoc test.*

*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
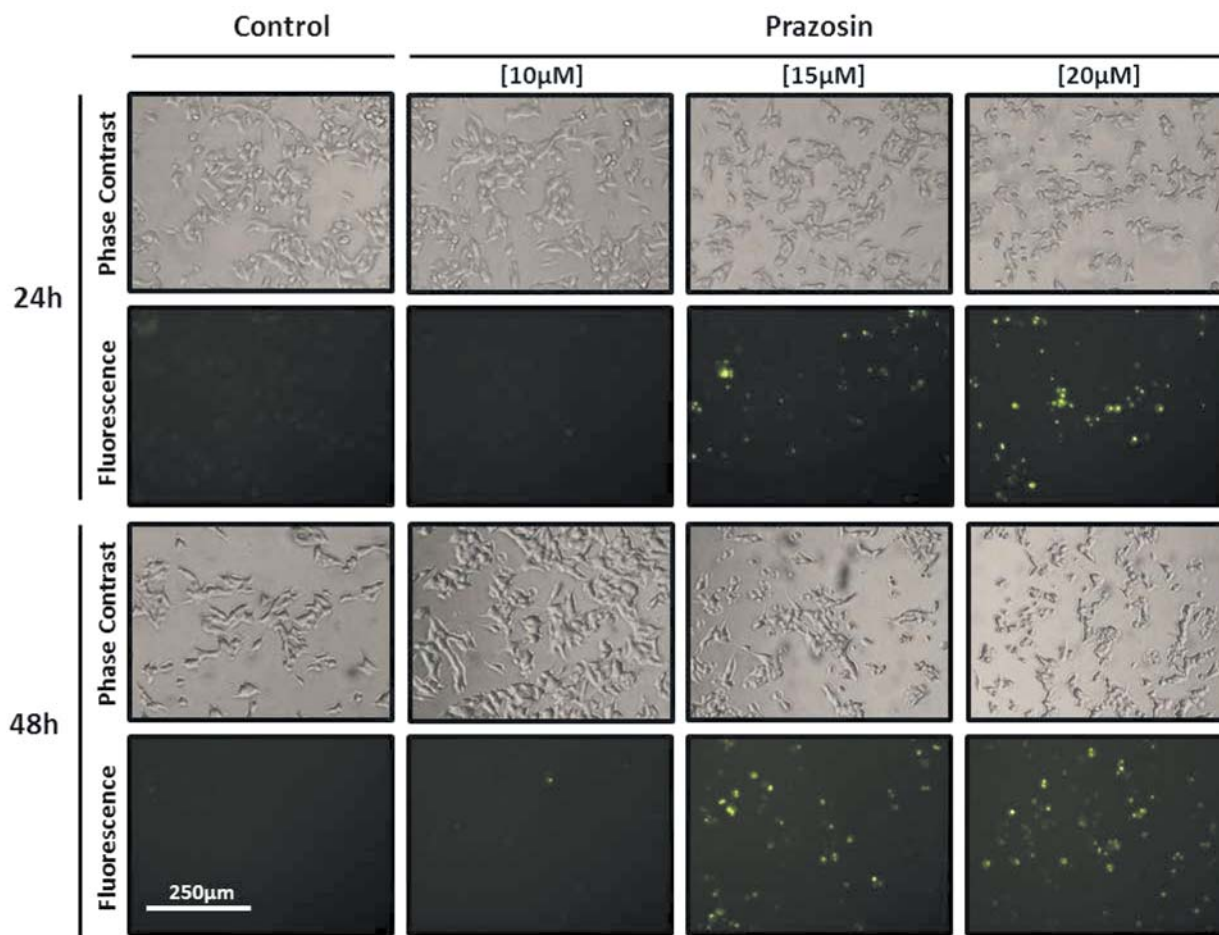
Figure 3. *Effector caspases 3/7 were activated in rounded cells but not in cells exhibiting prazosin-induced protrusions. TT cells were cultivated for 24 or 48 h with increasing concentrations of prazosin and stained with the CellEvent® Caspase-3/7 Green ReadyProbes reagent. In cells with activated 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.*

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
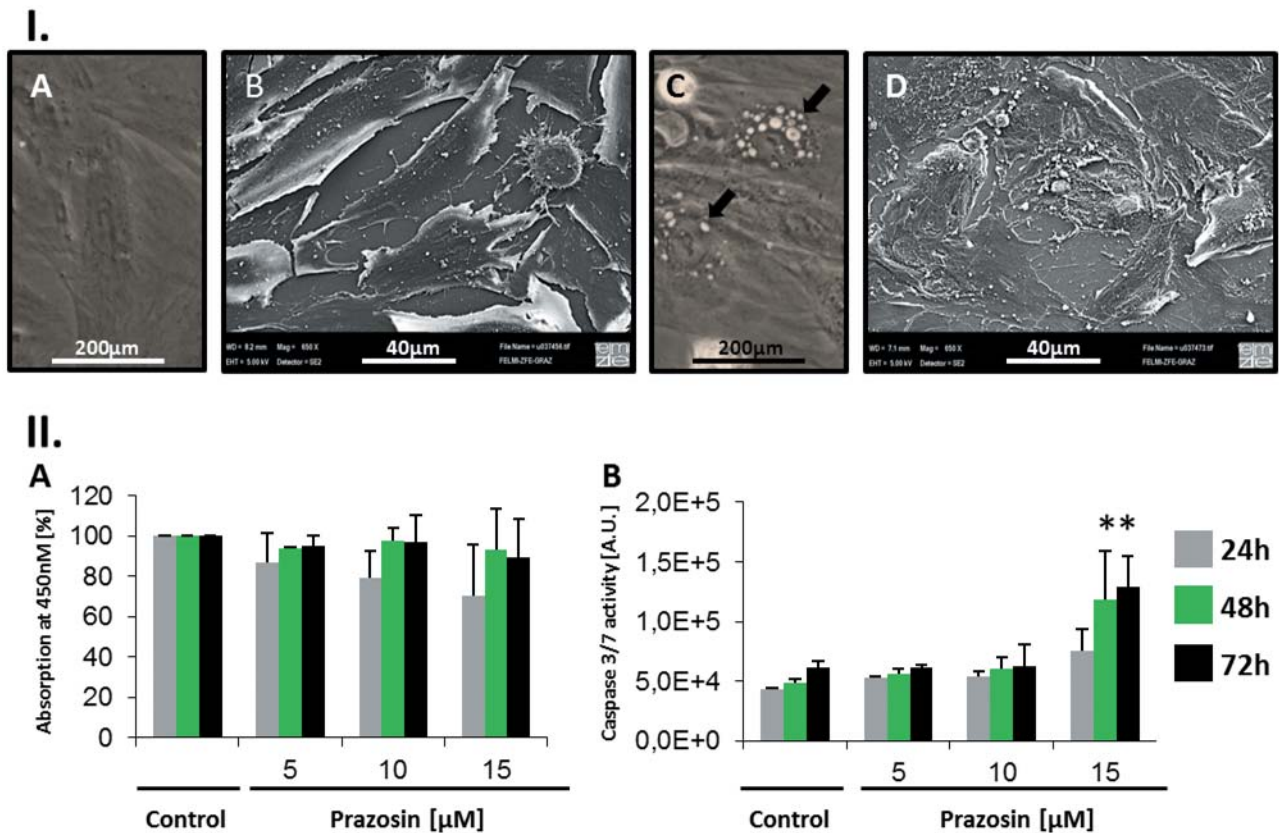
**I.**



**II.**



Figure 4. *The human skin fibroblast cell line HF-SAR is also sensitive towards prazosin treatment. I: A/B: Light (A), respectively scanning electron microscopy (SEM, B), view of HF-SAR cells. Fibroblasts exhibited a flattened phenotype with low contrast under the light microscope. C/D: Light (C), respectively SEM (D), view of 15μM prazosin-treated HF-SAR cells (24 h). Drug-treated cells exhibited huge vacuoles in the cytoplasm (C, arrows), fringy cell edges and an overall more complex cytoplasmic membrane. II: A: Growth, viability, of HF-SAR cells was tested using the WST-1 reagent over 72 h. No significant change of the OD at 450nm was seen when prazosin was added to the culture; n=3. B: Nevertheless, activation of effector caspases 3/7 indicates that prazosin induces apoptosis in HF-SAR cells too. The data presented in the graph show the mean values of triplicate luminescence measurements of one experiment. A second experiment ran under the same conditions confirmed the outcomes of the presented results. \*p<0.05 according to one way ANOVA with the Holm-Sidak post hoc test. A.U.=arbitrary units.*

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.

## Acknowledgements

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