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Maria Joana Pereira Catarata

Selective Angiotensin Type 1 Receptor Antagonists as Peroxisome Proliferator – Actived Receptor modulators

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Selective Angiotensin Type 1 Receptor Antagonists as Peroxisome Proliferator – Actived Receptor modulators

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Área: Farmacologia e Terapêutica

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Prof. Doutor Hélder Pinheiro

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Dedicatória

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Abstract

Stimulation of the peroxisome proliferator-activated receptor-gamma (PPAR-γ) promotes neuronal differentiation. Pioglitazone, a thiazolidinedione compound with antidiabetic actions, is a full agonist at PPAR-γ and promotes neurite outgrowth in human neuroblastoma cells SH-SY5Y. Furthermore, it is known that some selective angiotensin type 1 (AT-1) receptor antagonists such as telmisartan are also PPAR-γ agonists, albeit partial. We tested whether two other AT-1 antagonists, candesartan and eprosartan, also cause PPAR-γ-mediated neurite outgrowth in the SH-SY5Y cell line.

Candesartan was shown to cause a marked increase in neurite length, in a concentration-dependent manner. In the presence of 10 μM candensartan the neurite length in SH-SY5Y cells was significantly longer (42.8±1.2 μm, mean±SEM, n=321) than in the drug-free cell culture (26.9±1.1, n=140). GW9662, a PPAR-γ antagonist, at a concentration of 3 μM blocked the neurite length-promoting effect of candesartan. Eprosartan at concentrations up to 10 μM had no effect on neurite outgrowth.

Results show that candesartan promotes neurite outgrowth in SH-SY5 human neuroblastoma cells. Since GW9662 blocked the outgrowth of neurites induced by candensartan, it is concluded that candesartan is a PPAR-γ agonist, similarly to telmisartan. Agonism at PPAR-γ is independent from AT-1 receptor antagonism since eprosartan does not promote neuronal differentiation of SH-SY5Y cells.

Keywords: candesartan; eprosartan; neurites; PPAR-γ agonists; AT-1 antagonist receptors.

Resumo

Vários estudos indicam que a estimulação do recetor gama ativado por proliferadores dos peroxissomas (PPAR-γ) promove a diferenciação neuronial. A pioglitazona, uma tiazolidinediona antidiabética é um agonista total dos PPAR-γ e promove o crescimento de neurites em células de neuroblastoma humano SH-SY5Y. Para além disto, sabe-se que alguns antagonistas seletivos dos recetores do tipo 1 da angiotensina (AT-1) têm um efeito agonista nos PPAR-γ, embora o façam de forma parcial. Verificámos neste trabalho se o candesartan e eprosartan (antagonistas AT-1) tinham o efeito mediado pelo PPAR-γ sobre o crescimento de neurites em células SH-SY5Y.

O candesartan causou um aumento do comprimento das neurites, de forma dependente da concentração. Na presença de candesartan 10 µM o comprimento das neurites foi significativamente maior do que nas células cultivadas na ausência de fármaco (42.8±1.2 µm, média±erro padrão da média, n=321 e 26.9±1.1 µm, n=140, respetivamente). Verificou-se que este efeito do candesartan foi bloqueado pelo antagonista do PPAR-γ, GW9662, usado na concentração de 3 µM. O eprosartan em concentrações até 10 µM não teve efeito na diferenciação neuronial.

Os resultados mostram que o candesartan promove o aumento do comprimento de neurites nas células de neuroblastoma humano SH-SY5Y. O bloqueio deste efeito pelo antagonista do PPAR-γ, GW9662, mostra que o candesartan tal como o telmisartan é um agonista do PPAR-γ. Esta ação é independente do antagonismo dos recetores AT-1 uma vez que o eprosartan é desprovido de efeito mediado pelo PPAR-γ de diferenciação neuronial das células de neuroblastoma humano SH-SY5Y.

Palavras-chave: candesartan; eprosartan; neurites; PPAR-γ agonistas; antagonistas dos recetores AT-1.

Introduction

The renin-angiotensin-aldosterone system (RAAS) was initially seen as a regulator of arterial pressure, electrolytic balance and blood volume, in equilibrium with other humoral systems. Nowadays an equally important physiopathological intervention in arterial hypertension, heart failure, coronary disease, diabetes and chronic renal failure, has been attributed to the RAAS (Aulakh et al 2007).

Recently, a local pancreatic renin-angiotensin system (RAS) has been identified and found to be up-regulated in animal models of type 2 diabetes mellitus (Aulakh et al 2007). The link between angiotensin II and the pathophysiological changes associated with various cardiovascular diseases prompted the development of pharmaceutical agents, capable of blocking the actions of angiotensin II and reversing the associated pathologies. Angiotensin-converting-enzyme (ACE) inhibitors were firstly available, later followed by the selective angiotensin type 1 (AT-1) receptor antagonists (Unger 2002).

The improvement of beta-cell secretory function and cell mass by RAS blockers in experimental animal models of type 2 diabetes mellitus, indicate that the inhibition of RAS plays a pivotal role in protecting islet cell function, and possibly prevents the development type 2 diabetes mellitus (Leug 2007). Most of the cardiovascular actions of angiotensin II, namely vasoconstriction, cardiac hypertrophy, aldosterone synthesis and secretion, are mediated through AT-1 receptor. On the other hand, beneficial effects such as neuronal regeneration, apoptosis, cell differentiation and vasodilatation, appear to be mediated through the AT-2 receptor. Thus, it is possible that selective AT-1 receptor antagonists may drive angiotensin II to activate the unopposed AT-2 receptor (Unger 2002).

RAS blockers are widely used in patients with hypertension and metabolic syndrome. Clinical trials have demonstrated that RAS blockers lower the risk for type 2 diabetes (Scheen 2004). Some antagonists of angiotensin type 1 receptor induce peroxisome proliferator-activated receptor-gamma (PPAR-γ) activity which may be the underlying mechanism of the antidiabetic effect of pioglitazone and rosiglitazone (Yamagishi and Takeuchi 2005; Scupp et al 2004; Clemenz et al 2008).

PPARs are a family of ligand-dependent nuclear transcription factors that modulate gene expression in a many physiological processes. Different types of these receptors have been identified and shown to be encoded by different genes in both rodents and humans: PPAR-γ, PPAR-α e PPAR-δ (Berger and Moller 2002; Miglio et al 2009).

PPARs regulate the expression of the target gene by the interaction with specific PPREs (peroxisome proliferator response elements). The receptor binds to PPRE as a heterodimer with an additional proteic factor, the receptor of 9-*cis* retinoic acid (RXR). The conformation of PPAR is stabilized and altered in the presence of an agonist agent, creating a biding local, with recruitment of transcriptional coactivators that results in an increasing in gene transcription (Berger and Moller 2002).

The members of the PPAR family are known therapeutical targets. The first member of this family, PPAR-α, has an important role in fatty acid oxidation, lipid and lipoprotein

metabolism, inflammatory responses, and oxidative stress. PPAR-α is distributed in metabolically active tissues namely liver, most prominently in hepatocytes (Li et al 2012). One example of an agonist of PPAR-α are fibrates, used to prevent atherosclerosis and in patients with hypertriglyceridemia.

The other type of PPAR family is PPAR-γ that exists as three sub-types, PPAR-γ1, PPAR-γ2 and PPAR-γ3. The gene of PPAR-γ was mapped in chromosome 3, region 3p25 (Tavares et al 2007). PPAR-γ was primarily recognized in adipose tissues which PPAR-γ2 predominates (Pershadsingh 2006), but nowadays it is well established its expression in cardiovascular tissues, namely heart, endothelium, vascular smooth muscle and macrophages (Bishop-Bailey et al 2000), where they play an important role in the control of blood pressure, cell growth and inflammation.

The thiazolidinediones (TZDs) were reported to be PPAR-γ agonists. They were found to induce adipocyte differentiation and increase expression of adipocyte genes, including the adipocyte fatty acid-binding protein aP2 (Berger and Moller 2002). The agonistic effect of TZDs in PPAR-γ allows the interaction with a regulatory element within the 5' flaking region of the aP2 gene that controlled its adipocyte-specific expression (Tontonoz et al 1994).

These observations were the driving force for further experiments. Nowadays it is well known the agonistic effect of TZDs in PPAR-γ, which promote the increasing in insulin sensitivity, lower blood glucose, decrease circulation free fatty acids and triglycerides, lower blood pressure, reduce inflammatory markers, and reduce atherosclerosis in insulin-resistant patients and animal models (Duan et al 2008).

Furthermore, functional changes in PPAR-γ are implicated in the pathogenesis of cardiovascular diseases such as hypertension, atherosclerosis, cardiac hypertrophy, cardiac remodeling in the context of myocardial ischemia and proinflammatory state present in metabolic syndrome (Duan et al 2008).

Telmisartan, an antagonist of AT-1 receptor, was found to act as a partial agonist at PPAR-γ, reducing glucose, insulin, and triglyceride levels in rats fed a high-fat, high-carbohydrate diet (Yamagishi and Takeuchi 2005). In another study (Schupp et al 2004) it was demonstrated that specific AT-1 blockers (telmisartan, irbesartan, losartan) induce PPAR-γ activity, thereby promoting PPAR-γ dependent differentiation in adipocytes. Eprosartan (antagonist AT-1 receptor) has no effect in PPAR-γ activation. They observed that activation of these receptors was independent of blocking the AT-1 receptor. Telmisartan is also a partial PPAR-α agonist and seems to be restricted to the liver. Hepatic PPAR-α activation may provide an explanation for the antidyslipidemic actions of telmisartan, observed in recent clinical trials (Clemenz et al 2008).

It was also found the expression of PPAR-γ in embryonic midbrain cells rat and it has been demonstrated that a natural ligand of this receptor, 15-desoxy-Prostaglandin J2, stimulates differentiation of cultured rat embryonic midbrain cells. The cells differentiated into dopaminergic neuronal cells and its effect may exert through activation of the c-Jun N-terminal kinase (JNK) signal pathway (Park et al 2004).

The presence of the three isoforms of PPARs (PPAR-γ1, PPAR-γ2 e PPAR-γ3) in mouse adult neural stem cells has been established for the first time by Loreto et al

(2007). They have a nuclear localization in agreement with their function as transcription factors. These studies demonstrate that PPAR-γ is widely expressed in the nervous central system and play an important role such as neuronal differentiation, anti-inflammatory processes and inhibitory effect in neoplasic cell growth (Miglio et al 2009; Fuenzalida et al 2004; Valentiner et al 2005).

For example, PPAR-γ research in brain cells, demonstrated that PPAR-γ activation in glial cells, which results in diminished production of glial-derived pro-inflammatory molecules, has been involved in the protective effect of TZDs and anti-inflammatory drugs in several neurodegenerative diseases (Fuenzalida et al 2004).

According with a study made by Miglio et al (2009), pioglitazone, a PPAR-γ agonist, promotes morphological changes consistent with neuronal differentiation in SH-SY5Y human neuroblastoma cells. In their model, pioglitazone was capable to promote neurite outgrowth at nanomolar concentrations and cell differentiation as indicated by the sprouting of cell processes, during a relative long-term cell exposure. Thus we tested in these SH-SY5Y cells whether two other AT-1 receptor antagonists, candesartan and eprosartan would also show PPAR-γ mediated effects in neuronal differentiation.

All these evidences indicate the opportunity to further explore the effects of PPAR-γ, using antagonists of AT-1 receptor that have a partial agonistic action on these receptors. Due to PPAR-γ role in neuronal differentiation, we investigated whether AT-1 blockers have also an important action in neurite outgrowth through PPAR-γ agonistic action.

Materials and methods

Experiments were carried out in SH-SY5Y human neuroblastoma cells. This clonal cell line expresses PPAR-γ (Valentiner et al 2005), representing a good model to study neurite outgrowth. SH-SY5Y cells were cultured in Dulbecco modified Eagle´s medium/Nutrient Mixture Ham´s F12 (1:1) supplemented with 10% FCS, penicillin streptomycin and L-glutamine (Miglio et al, 2009). Cells lines were used in the 10^{th} passage. Cells cultures were maintained at 37 ºC in a 95% air – 5% CO2 humidified incubator and cell medium culture were replaced every two days. In order to start the experiment, one day earlier, cells were placed on four plates (A, B, C and D) with twenty four well cultures each. A volume of 200 µl, loaded with cells, were added to 300 µl of medium culture in each well (celular density was approximately $0.4x10^6$ cells per well).

In plates A and B, cells were treated for five days using the following chemicals: all-*trans*-retinoic acid 10 µM; pioglitazone 100 µM; candesartan 100 nM and 10 µM; eprosartan 100 nM and 10 µM. In plates C and D, GW9662 (PPAR-γ antagonist) 3 µM was added into wells with cells treated by candesartan 100 nM. The drug concentration and the duration of treatment was the same as in plates A and B.

All-*trans*-retinoic acid and pioglitazone were dissolved in dimethylsulfoxide (DMSO) and the final drugs concentrations were obtained by dilution of stock concentrations in the cells culture medium. The final concentration of pioglitazone was 1% of stock solution, and the final concentration of retinoic acid was less than 0.1%, to avoid actions on cell viability (Miglio et al 2009).

Candesartan and eprosartan were also dissolved in DMSO with a stock concentration of 10 mM. Afterwards, these were diluted 1000x in the cells culture medium so that the final concentration of cadesartan and eprosartan reached 10 µM. The concentration 100 nM of candesartan and eprosartan were obtained by dilution of 1% of candesartan 10 µM and eprosartan 10 µM, respectively, in the cells culture medium.

In order to obtain GW9662 in the concentration of 3 µM, stock concentration of 10 mM was diluted in the culture medium. Thus, the final concentration of GW9662 was 0.03% of stock solution.

Neurite outgrowth was evaluated as described by Miglio et al (2009). Phase-contrast images (200× magnification) were obtained from each well on an inverted microscope (Leica DMIL, Japan) equipped with a CCD camera (Ikegami, Japan). Fields were selected randomly. Digitized images of dispersed cells were collected and morphometric analysis was performed using Image J 1.40 software (NIH, USA). Cell processes were traced manually and their length was automatically measured. For this analysis, cell processes shorter than 4 µm were arbitrarily excluded due to the difficulty of tracing too short processes.

Statistics

The results are expressed as arithmetic means ± SEM (standard error of the mean). A probability level of 0.05 was considered statistically significant. One-way ANOVA followed by Newman-Keuls correction for multiple comparisons was used for statistical analyses (GraphPad Prism Software Inc, La Jolla, CA, USA).

Drugs

The drugs used in this study were: penicillin (100IU/ml; Sigma-Aldrich); streptomycin (100µg/ml; Sigma-Aldrich); L-glutamine (2mM; Sigma-Aldrich); all-trans-retinoic acid (Sigma-Aldrich); pioglitazone hydrochloride (3-(2-aminoethyl)-5-((4ethoxyphenyl)methylene)-2,4-thiazolidinedione hydrochloride, Tocris); candesartan (Takeda, Osaka, Japan); eprosartan hydrochloride (SmithKline-Beecham, King of Prussia, Pa., USA); GW9662 (2-chloro-5-nitro-N-phenylbenzamide, Sigma-Aldrich).

Results

In order to study the effects of selective AT-1 receptor antagonists on neuronal differentiation mediated by activation of PPAR-γ, we exposed SH-SY5Y human neuroblastoma cells to different concentrations of candesartan and eprosartan. Retinoic acid is a well known pro-differentiating agent for SH-SY5Y (Miglio et al 2009) and was used as a positive control. Cells that were untreated during the five days where used as a negative control.

Pioglitazone is a PPAR-γ full agonist that promotes neuronal differentiation (Miglio et al 2009), and it was used as a direct comparator to candesartan and eprosartan. The outcome with retinoic acid, as expected, was a considerable neurite outgrowth of these cells (Fig. 1 and fig. 3).

Pioglitazone also promoted cell differentiation as indicated by the sprouting of neurites (Fig. 1 and fig. 5). In SH-SY5Y cells treated with candesartan 100 nM and 10 µM, a concentration-dependent increase in neurite growth was observed (Fig. 1 and fig. 2). At the higher concentration, candesartan was as effective as pioglitazone (Fig. 1). To test whether the increase on neurite outgrowth caused by candesartan was mediated by activation of PPAR-γ experiments were done with candesartan 100 nM in the presence of the PPAR-γ antagonist GW9662.

A significant reduction in the effect of candesartan on the sprouting of neurites was observed (Fig. 2). In contrast to candesartan, eprosartan in concentrations up to 10 µM did not promote neurite growth. At the lower concentration eprosartan even caused a slight, although significant, decrease in neurite growth in comparison to drug-free control (Fig. 2).

Discussion

Several evidences show that PPAR-γ plays an important role in the central nervous system, namely in metabolic and anti-inflammatory actions. Moreover, this receptor also promotes neuronal differentiation (Kapadia et al 2009; Miglio et al 2009). PPAR-γ activation by thiazolidiediones (TZD) prior to or immediately after an insult increases PPAR-γ DNA-binding and the transcription of several anti-inflammatory target genes. TZD administration has been shown to exert neuroprotection by decreasing uncontrolled microglial activation and macrophage infiltration, decreasing proinflammatory gene expression, and also, by increasing anti-oxidant enzymatic activity and heat-shock gene expression (Kapadia et al 2009).

Furthermore, other data (Miglio et al 2009) indicate that pioglitazone (from the family of TZD) promotes neuronal differentiation and neurite outgrowth in SH-SY5Y human neuroblastoma cells. Studies suggest a direct effect of neuronal PPAR-γ activation in modulating neuron survival and differentiation (Park et al 2004; Fuenzalida et al 2005).

In our research we also studied the effect of a PPAR-γ full agonist, pioglitazone, in SH-SY5Y human neuroblastoma cells. Our results show an increase in the length of neurites, consistent with prior research and suggest neuronal differentiation promoted by the activation of PPAR-γ.

Other studies demonstrate that some AT-1 blockers act as partial PPAR-γ agonist (Yamagishi and Takeuchi 2005; Scupp et al 2004; Clemenz et al 2008). This was one of the main driving forces behind our research.

It has been found that telmisartan acts as a partial agonist of PPAR-γ, reducing glucose, insulin, and triglyceride levels in animal models of metabolic syndrome (Yamagishi and Takeuchi 2005). Schupp et al (2004) demonstrated that specific AT-1 blockers (telmisartan, ibesartan, losartan) induce PPAR-γ activity, thereby promoting PPAR-γ dependent differentiation in adipocytes. Eprosartan has no effect in PPAR-γ activation. It was suggested by Garrido-Gil et al (2012) that telmisartan provides effective neuroprotection against dopaminergic cell death and that the neuroprotective effect is mediated by PPAR-γ activation.

In so far as it can be ascertained, there are few studies about the effect on neuronal differentiation by AT-1 blockers through the PPAR-γ activation. Therefore, we felt it would be pertinent to investigate this effect using candesartan and eprosartan in SH-SY5Y human neuroblastoma cells.

Our data show that candesartan in a concentration-dependent manner promotes neuronal differentiation, as indicated by the sprouting of neurites (Fig. 1 and fig. 2). Moreover, results indicate that this effect was similar with pioglitazone.

We inquired about the mechanism of PPAR-γ activation in neuronal differentiation by candesartan. For this propose we added, to the solution with candesartan, GW9662. This drug is identified as a potent irreversible PPAR-γ ligand and profiles as a functionally selective PPAR-γ antagonist (Chen et al 2004). The results show a significant reduction on neurite outgrowth in the presence of GW9662. This suggests a mechanism dependent on PPAR-γ activation by candesartan.

Regarding eprosartan at a micromolar concentration, no relevant differences, when compared to the control group, were detected (fig. 1) indicating that eprosartan has no effect on neuronal differentiation. Moreover, candesartan at a micromolar concentration caused a considerable increase in neurite outgrowth unlike eprosartan at the same concentration. As described above, eprosartan has no effect in PPAR-γ activation (Schupp et al 2007), suggesting that this result may be due to the incapacity of the eprosartan to activate this receptor.

Furthermore, according to the obtained results, we verified that eprosartan (100 nM) caused a slightly reduction in the length of neurites (fig. 2). Also, it was noted a similar effect of candesartan (100 nM) in the presence of GW9662, and a reduction of neurite outgrowth when compared to the control group. This may suggest that, at this concentration, it produces an antagonist effect of PPAR-γ, as inferred by the results.

This was an unexpected result which requires further experiments in order to confirm this antagonistic effect.

Briefly, our present data show that candesartan has a differentiating action dependent on concentration and, also, on PPAR-γ activation. Due to the fact that PPAR-γ is a nuclear receptor, for its activation it will be necessary to use more lipofilic drugs or use candesartan in higher concentrations.

Moreover eprosartan does not seem to act in the PPAR, once it did not promote neuronal differentiation. Surprisingly, at lower concentrations, eprosartan revealed a small inhibiting differentiation effect which, at this time, we cannot explain.

The obtained results can help to clarify the underlying effect of PPAR-γ on neuronal differentiation. It was also important to verify that not all of the AT-1 blockers have an agonistic effect on this receptor.

In conclusion, these results may contribute to the understanding of the effects of PPAR-γ stimulation on neurobiological events related to neuronal differentiation and the importance role of candesartan in these processes. The development of more potent PPAR-γ agonists might provide further improvements in the treatment of acute central nervous system insults and increase functional recovery.

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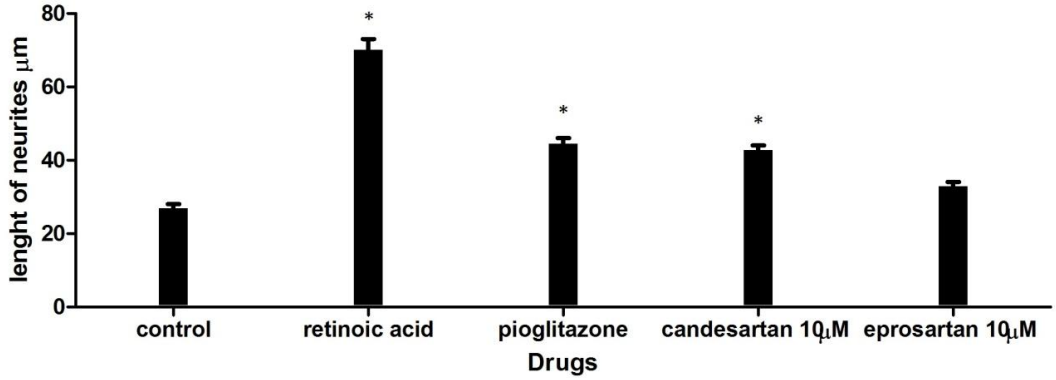
Figure 1


lenght of neurites μm
80
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retinoic acid
pioglitazone
candesartan 10μM
eprosartan 10μM
Drugs

Figure 2

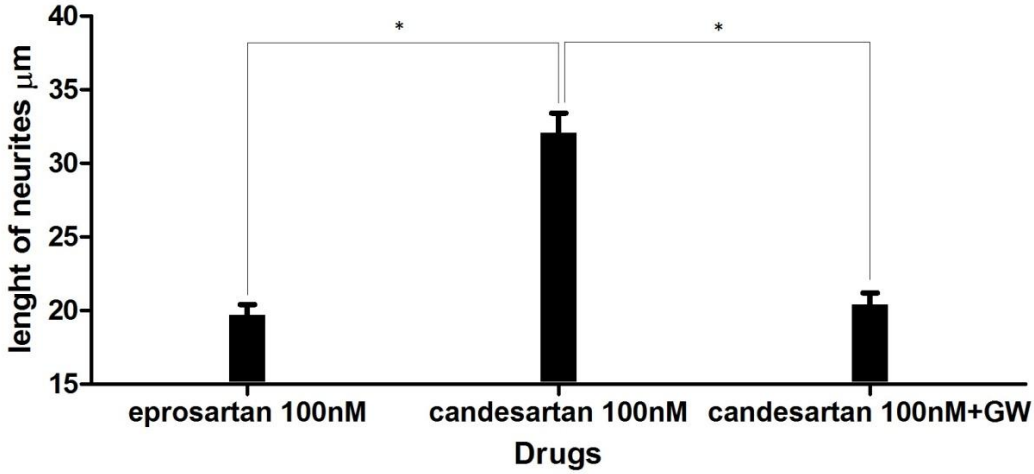
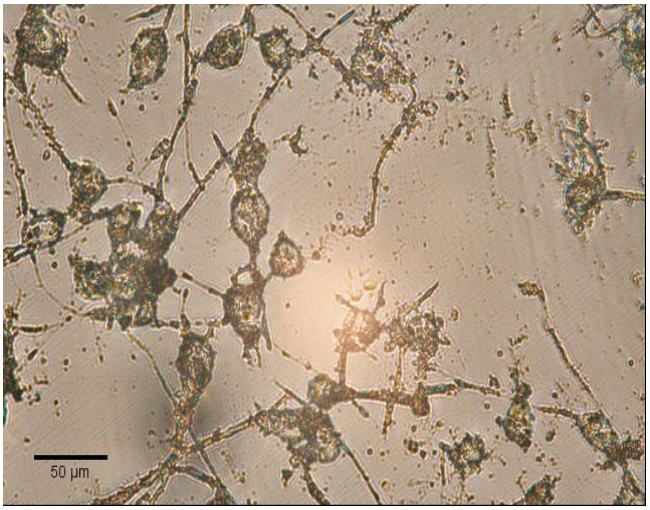

40
35
30
25
20
15
lenght of neurites μm
*
*
eprosartan 100nM
candesartan 100nM
candesartan 100nM+GW
Drugs

Figure 3



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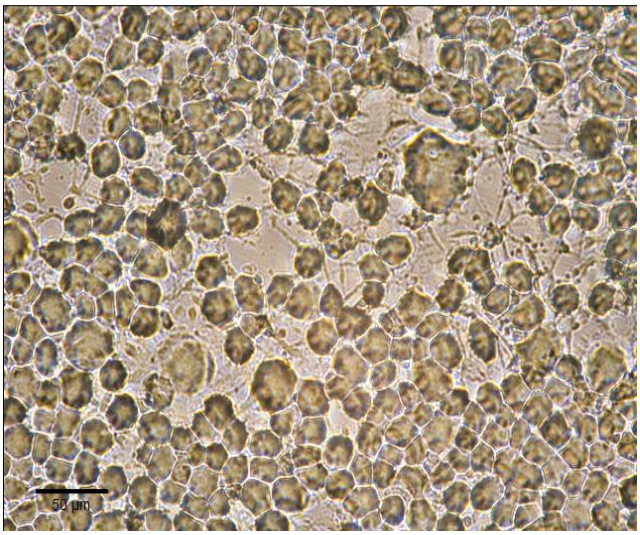

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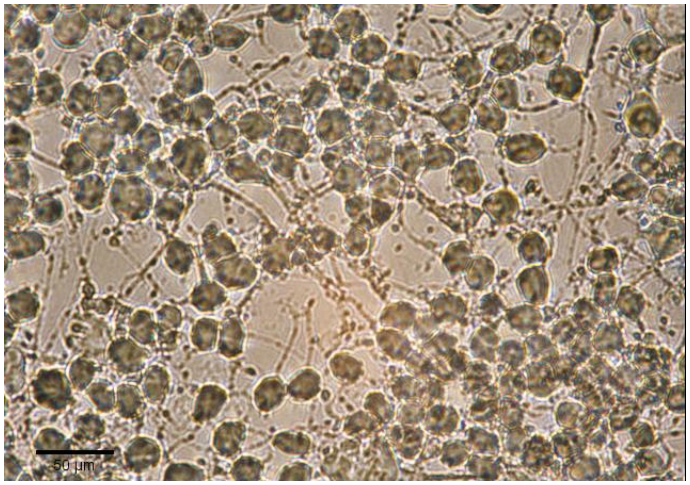

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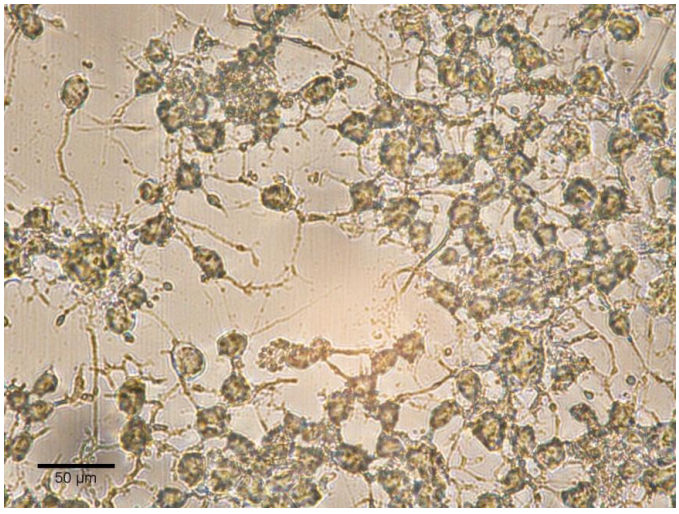

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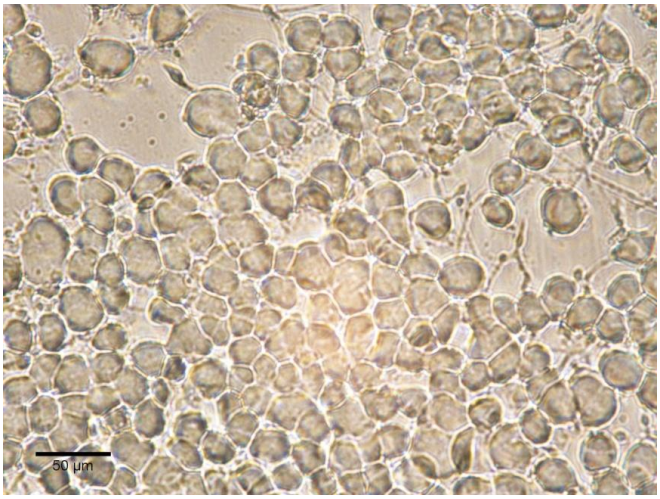


Figure 8

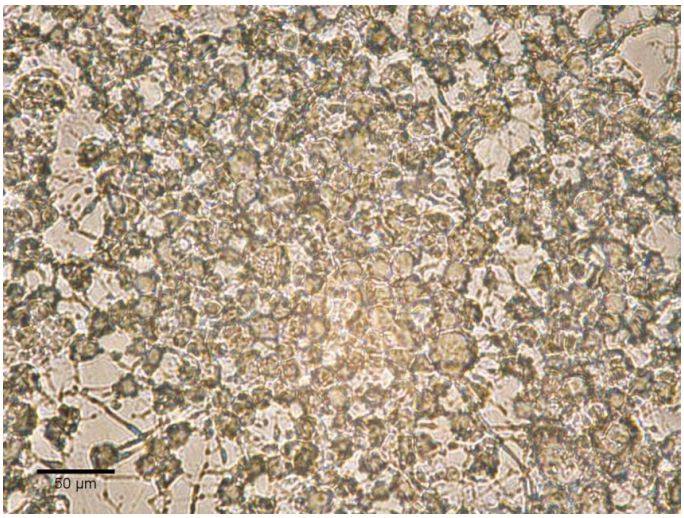


Figure 9

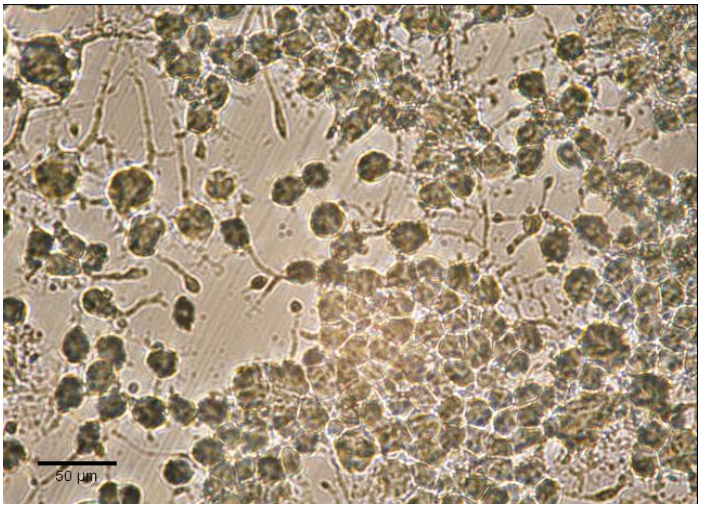
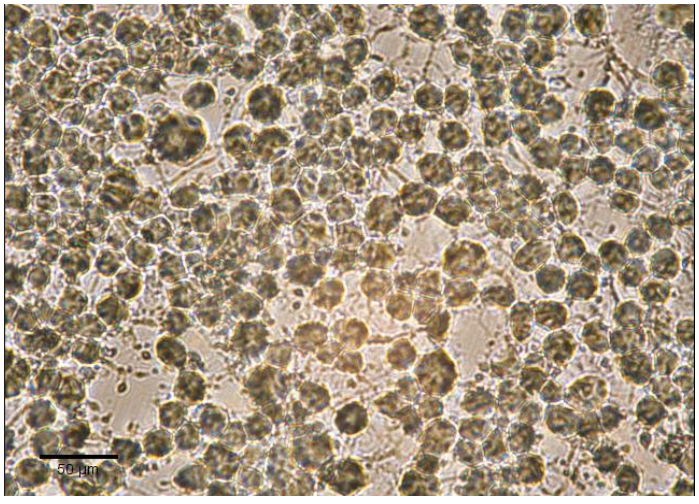


Figure 10



Legends

Fig. 1 Quantification of the average length of SH-SY5Y cells neurites, treated with different drugs. Cell neurites were traced manually and their length was automatically measured using Image J 1.40 software. For this analysis, cell processes shorter than 4 µm were arbitrarily excluded due to the difficulty of tracing too short processes. Shown are means±SEM. Sample size for control was n=140. Retinoic acid (n=200). Pioglitazone 100 µM (n=260). Candesartan 10 µM (n=321). Eprosartan 10 µM (n=169). Bar graph was done with the software GraphPad Prism. One-way ANOVA followed by Newman-Keuls correction for multiple comparisons was used for statistical analyses.

*Statistically different from the control ($p<0.05$).

Fig. 2 Quantification of the average length of SH-SY5Y cells neurites, treated with different drugs. Cell neurites were traced manually and their length was automatically measured using Image J 1.40 software. For this analysis, cell processes shorter than 4 µm were arbitrarily excluded due to the difficulty of tracing too short processes. Shown are means±SEM. Sample size for eprosartan 100 nM was n=207. Candesartan 100nM (n=295). Candesartan 100 nM+GW9662 (n=261). Bar graph was done with the software GraphPad Prism. One-way ANOVA followed by Newman-Keuls correction for multiple comparisons was used for statistical analyses.

*Statistically different from the respective column ($p<0.05$).

Fig. 3 Representative phase-contrast micrograph of SH-SY5Y cells treated with retinoic acid (10 µM) during 5 days. Scale bar 50 µm.

Fig. 4 Representative phase-contrast micrograph of untreated SH-SY5Y cells during 5 days. Scale bar 50 µm.

Fig. 5 Representative phase-contrast micrograph of SH-SY5Y cells treated with pioglitazone (100 µM) during 5 days. Scale bar 50 µm.

Fig. 6 Representative phase-contrast micrograph of SH-SY5Y cells treated with candesartan (10 µM) during 5 days. Scale bar 50 µm.

Fig. 7 Representative phase-contrast micrograph of SH-SY5Y cells treated with eprosartan (100 nM) during 5 days. Scale bar 50 µm.

Fig. 8 Representative phase-contrast micrograph of SH-SY5Y cells treated with eprosartan (10 µM) during 5 days. Scale bar 50 µm.

Fig. 9 Representative phase-contrast micrograph of SH-SY5Y cells treated with candesartan (100 nM) during 5 days. Scale bar 50 µm.

Fig. 10 Representative phase-contrast micrograph of SH-SY5Y cells treated with candesartan (100 nM) and GW9662 (3 µM) during 5 days. Scale bar 50 µm.

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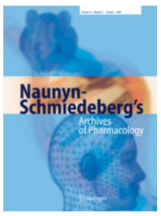

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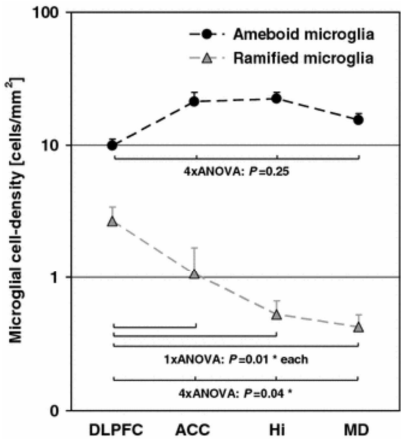

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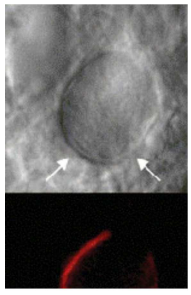

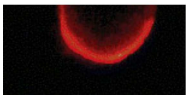

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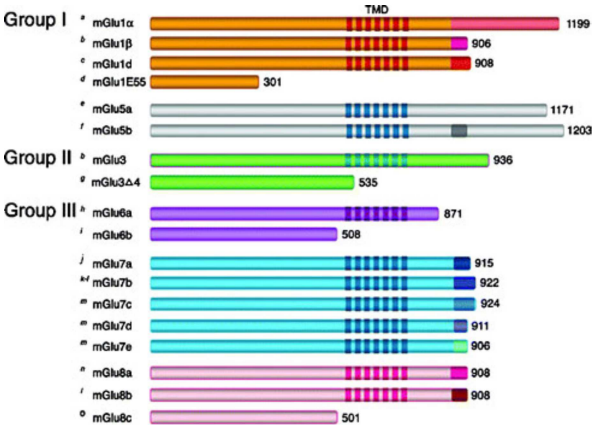

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