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Evolving views on the first two ligands of the angiotensin II type 2 receptor. From putative antagonists to potential agonists?

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ABSTRACT

The renin–angiotensin system is one of the most complex regulatory systems that controls multiple organ functions. One of its key components, angiotensin II (Ang II), stimulates two G-protein coupled class A receptors: the Ang II type 1 (AT1) receptor and the Ang II type 2 (AT2) receptor. While stimulation of the AT1 receptor causes G-protein-dependent signaling and arrestin recruitment, the AT2 receptor seems to have a constitutively active-like conformation and appears to act via G-protein-dependent and -independent pathways. Overstimulation of the AT1 receptor may lead to unwanted effects like inflammation and fibrosis. In contrast, stimulation of the AT2 receptor leads to opposite effects thus restoring the balance. However, the role of the AT2 receptor has become controversial due to beneficial effects of putative AT2 receptor antagonists. The two first synthetic AT2 receptor-selective ligands, peptide CGP42112 and small molecule PD123319, were initially both considered antagonists. CGP42112 was subsequently considered a partial agonist and it was recently demonstrated to be a full agonist. Based on the search-term PD123319 in Pubmed, 1652 studies have investigated putative AT2 receptor antagonist PD123319. Here, we put forward literature that shows beneficial effects of PD123319 alone, even at doses too low for antagonist efficacy. These beneficial effects appear compatible with agonist-like activity via the AT2 receptor. Taken together, a more consistent image of a therapeutic role of stimulated AT2 receptor emerges which may clarify current controversies.

1. The angiotensin II type 2 receptor

The renin-angiotensin system (RAS) has a major impact on a variety of serious diseases (Unger et al., 2015; Vargas et al., 2022). The protease renin is produced by the kidneys and cleaves the liver-derived protein angiotensinogen. This cleavage liberates the decapeptide angiotensin I (Angiotensin-(1–10)), which in turn can be cleaved by (combinations of) angiotensin converting enzyme (ACE), angiotensin converting enzyme-2 (ACE-2) and other peptidases yielding different angiotensin variants of which the octapeptide angiotensin II (Ang II) is the most important and most studied one. ACE converts Ang I into Ang II by removing the two carboxyterminal amino acids. Ang II stimulates both the angiotensin II type 1 (AT1) receptor and the angiotensin II type 2 (AT2) receptor. Overstimulation of the AT1 receptor may lead to vasoconstriction and adverse effects like inflammation and fibrosis (Fig. 1).

Stimulation of the AT1 receptor is thought to be counterbalanced by stimulation of receptors with protective effects (Li et al., 2017). Stimulation of the AT2 receptor by Ang II, Ang-(2–8), Ang-(1–9), Ang-(1–7) (Bosnyak et al., 2011), or allosterically enhancing AT2 receptor activity by dipeptide Ang-(3–4) (Dias et al., 2017), generally leads to effects that are opposite to those resulting from AT1 receptor stimulation. In addition, stimulation of the Mas receptor (Santos et al., 2003) by Ang-(1–7) gives protective effects similar to those resulting from AT2 receptor stimulation. Furthermore, stimulation of Mas-related G-protein coupled receptor D by alamandine also leads to protective effects opposite to those from the AT1 receptor (Lautner et al., 2013).

Several G protein-dependent and -independent signaling pathways for the AT2 receptor appear to exist. AT2 receptor signaling can involve the Gi/o protein (Hansen et al., 2000; Hayashida et al., 1996; Kang et al., 1994), or a G-protein different from Gi/o (Buisson et al., 1995). Also, G-protein independent pathways have been reported (Brechler et al., 1994), as well as signaling via adaptor proteins that directly interact with the AT2 receptor (Horiuchi et al., 2012; Mogi et al., 2007; Namsolleck et al., 2023a; Nouet et al., 2004; Reinemund, 2010). For a graphical schedule of AT2 receptor-linked pathways please see the excellent review of Bottari et al., (2015).

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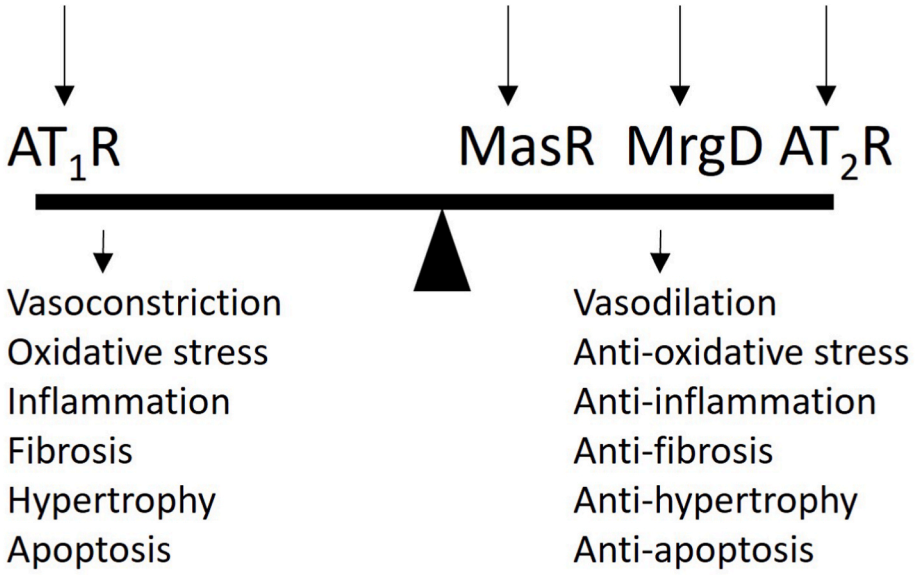

Fig. 1. Opposite biological effects of AT1 and AT2 receptor stimulation.
Ang II stimulates both the AT1 receptor and the AT2 receptor. Ang-(1–7) seems to stimulate Mas receptor and alamandine stimulates Mas-related G-protein coupled receptor D, indicated as MrgD. The adverse effects that result from (over)stimulation of AT1 receptor and the beneficial effects that result from stimulation of Mas receptor, Mas-related G-protein coupled receptor D and AT2 receptor are opposite and thus contribute to a balanced renin-angiotensin system.

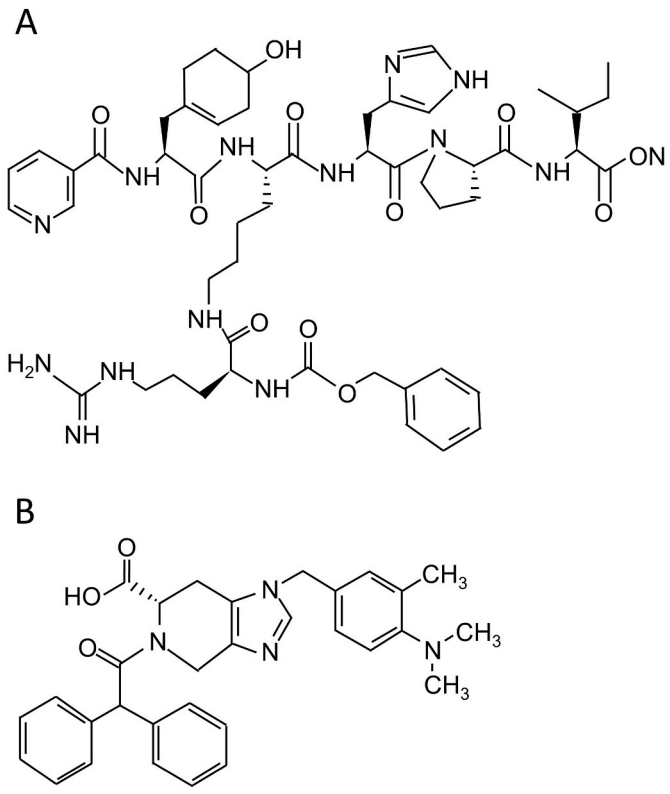

Fig. 2. Structures of CGP42112 (A) and PD123319 (B).

The two first AT2 receptor-selective ligands CGP42112 (Fig. 2A) and PD123319 (Fig. 2B) were both initially considered antagonists (reviewed in: Recarti and Bottari, 2015). The peptide CGP42112 was subsequently considered a partial agonist (Recarti and Bottari, 2015) and more recently firmly identified as a full agonist while comprehensively explaining the history of the molecule (Restrepo et al., 2022). The small molecules PD123319 and PD123177 were synthesized with the intention of obtaining antagonists of the AT2 receptor and these compounds have been extensively studied (de Gasparo et al., 2015). Superposition of CGP42112 and PD123177 shows that the diphenyl system of PD123177 superimposes on the lipophilic pocket of isoleucine in CGP42112 (de Gasparo et al., 2015). Well-characterized AT2 receptor-specific agonists have been obtained such as small molecule C21 (Wan et al., 2004), lanthipeptide LP2 (Namsolleck et al., 2021a, 2023a, 2023b; Villela et al., 2023; Wagenaar et al., 2013) and peptide NP-6A4 (Toedebusch et al., 2018).

Characterization of the functional role of the AT2 receptor is complicated by its (level of) expression on membranes of multiple organs, cell types and cytoplasmic located organelles, receptor activation, internalization and trafficking, the formation and dissociation of homo- and heterodimers on these membranes and the availability of ligands in cells and their compartments. The AT2 receptor is not only present on the plasma membrane but also on the mitochondrial membrane and on the nuclear envelope (Escobales et al., 2019). Opposing data were published on the internalization of the AT2 receptor from the plasma membrane in LLC-PK1 cells (Ferrão et al., 2017) and absence of the AT2 receptor internalization in human embryonal kidney 293 cells (Hein et al., 1997). The AT2 receptor can form constitutively active homodimers (Miura et al., 2005), but also heterodimers with the AT1 receptor (Anguiano-Robledo et al., 2007), bradykinin B2 receptor (Abadir et al., 2006), Mas receptor (Leonhardt et al., 2017; Patel and Hussain, 2018) and possibly the relaxin family peptide receptor 1 (Chow et al., 2014) and the insulin receptor (Nouet et al., 2004). Such heterodimerization may enhance or decrease the receptor response after its stimulation (Recarti and Bottari, 2015). Interestingly, dipeptide Ang-(3–4) induces the dissociation of AT1 - AT2 receptor dimers in renal proximal tubular cells of spontaneously hypertensive rats (Dias et al., 2014).

The AT2 receptor has also been suggested to be a direct inhibitor of the AT1 receptor (Abdalla et al., 2001). In general, the AT2 receptor is expressed at low levels except in fetal and neonatal tissues, but its expression is increased under pathological conditions. An exception is observed in human colorectal carcinoma cells in which AT2 receptor expression is decreased (Namsolleck et al., 2023a). The AT2 receptor-binding protein of 50 kDa (ATBP50) regulates the transport of the AT2 receptor to the cell membrane by binding to a specific motif within the cytoplasmic carboxy terminus of the AT2 receptor and

thereby enables the antiproliferative effects of the receptor (Mogi et al., 2007; Wruck et al., 2005). At least one AT2 receptor agonist, NP-6A4, induces upregulation of its own expression (Toedebusch et al., 2018). AT2 receptor expression levels are modulated by cross-talk via the AT1 receptor (Savoia and Volpe, 2015). The AT2 receptor may have constitutive activity and induces apoptosis even in the absence of Ang II stimulation (Miura and Karnik, 1999, 2000). Constitutive activity fits with the observed active-like conformation of the seven-transmembrane helical bundle (Zhang et al., 2017). The non-canonical positioning of the amphipathic helix VIII might block recruitment of intracellular G proteins and β-arrestins, and is consistent with the lack of AT2 receptor signaling responses in standard cellular assays (Zhang et al., 2017). An open, active-like structure was also reported for the AT2 receptor bound to the antagonist EMA401 with helix VIII blocking G-protein or β-arrestin recruitment (Perryman et al., 2022). In AT2 receptor signal transduction upon Ang II and [Sar1, Ile8]-Ang II binding, G protein and β-arrestin signaling could not be verified by transforming growth factor shedding or β-arrestin recruitment assays (Asada et al., 2020). The AT2 receptor appears to primarily signal via non-canonical and G protein- and β-arrestin-independent pathways (Guimond and Gallo-Payet, 2012; Porrello et al., 2009). Adaptor proteins of the AT2 receptor, such as AT2 receptor interacting protein which recruits the protein tyrosine phosphatase that contains an SH2 domain, and promyelocytic leukemia zinc finger protein may play a role in AT2 receptor signaling (Horiuchi et al., 2012; Mogi et al., 2007; Namsolleck et al., 2023a; Nouet et al., 2004; Recarti and Bottari, 2015; Reinemund, 2010). Also other proteins may directly interact with the AT2 receptor such as erb-b2 receptor tyrosine kinase 3 (ErbB3) (Knowle et al., 2000), connector enhancer of Ksr1 (CNK1) (Fritz and Radziwill, 2005), sodium-proton exchanger 6 (NHE6) (Pulakat et al., 2005) and tissue inhibitor of metalloproteinase 3 (TIMP3) (Kang et al., 2008). AT2 receptor-mediated signaling has been comprehensively reviewed (Recarti and Bottari, 2015).

The crystal structures of the AT2 receptor in complex with quinazolinone-biphenyltetrazole derivatives 1 and 2 have been reported (Zhang et al., 2017). However, it is not clear whether these ligands are agonists or antagonists. Also, the crystal structures of human AT2 receptor in complex with the partial agonist [Sar1, Ile8]Ang II (Asada et al., 2018) and endogenous Ang II (Asada et al., 2020) have been elucidated. [Sar1, Ile8]Ang II interacts with both the core binding domain, which is a binding site of small-molecule ligands of AT1 and AT2 receptors (Zhang et al., 2017), and the extended binding domain, which is the allosteric modulator binding site of muscarinic acetylcholine receptor (Asada et al., 2018). An informative graphical comparison of the helical shifts in the AT1 receptor from the inactive to the active state versus the AT2 receptor with the noncanonical helix 8 position was recently published by Speck et al. (2022). The crystal structure of AT2 receptor bound to putative antagonist EMA401 strongly confirms that the receptor is in an active-like conformation with helix 8 blocking G-protein or β-arrestin recruitment (Perryman et al., 2022).

Stimulation of the AT2 receptor has therapeutic potential in many acute and chronic diseases and was studied extensively in multiple animal models (Unger et al., 2015). Also, multiple (putative) antagonists of the AT2 receptor have been studied extensively, including PD123319, which, based on the search-term PD123319 in Pubmed, has been investigated in more than 1652 publications. PD123319 inhibited binding of Ang II to specific binding sites, but unequivocal functional blocking of the AT2 receptor by PD123319 has not been demonstrated (Dudley et al., 1990; de Gasparo et al., 2015).

2. Functional effects of putative AT2 receptor antagonists

Biological responses to receptor ligands in functional studies related to the RAS are complex and show in general a wide range of variation among experimental studies. This may be caused by the differential expression of receptors in multiple tissues and cells, receptor dimerization, ligand-specific AT2 receptor pathways, other regulatory proteins of

Table 1
In vitro and *in vivo* systems with altered AT2 receptor gene (*AT_2R*) expression indicate beneficial roles of AT_2R. Deletion/knockdown of *AT_2R* results in adverse effects. Overexpression of *AT_2R* results in beneficial effects.

Biological System	Modulated *AT_2R*	Biological effect	Reference
mice	*AT_2R^-*	Impaired drinking response to water deprivation; reduction in spontaneous movements; increased vasopressor response to Ang II	Hein et al. (1995)
mice	*AT_2R^-*	Increased blood pressure; increased sensitivity to pressor action by Ang II; increased AT_1R expression	Ichiki et al. (1995)
mice	*AT_2R^-*	Intracerebroventricular Ang II caused elevation of blood pressure	Li et al. (2003)
in vitro aorta rings	*AT_2R^-*	Enhanced vasoconstrictor response	Akishita et al. (1999)
rats	Knockdown *AT_2R*	Elevated blood pressure	Wang et al. (2004)
pregnant mice	*AT_2R^-*	Significantly increased mean arterial pressure during late gestation	Mirabito et al. (2014)
rats	Peripheral overexpression of *AT_2R*	Potentiation of the antihypertensive action of losartan	Li et al. (2007)
mice	*AT_2R^-*	Arterial thickening; perivascular fibrosis	Akishita et al. (2000)
mice	*AT_2R^-*	Adverse effects in cardiac remodeling	Wu et al. (2002); Oishi et al. (2003); Adachi et al. (2003); Ichihara et al. (2002)
rats	Overexpressed *AT_2R* in cardiac tissue	Prevention of cardiac hypertrophy in spontaneously hypertensive rats	Metcalfe et al. (2004)
mice	Overexpressed *AT_2R*	Benefits during post-MI remodeling via nitric oxide pathway; improved contractile function in adjacent noninfarcted myocardium	Bove et al. (2004); Bove et al., (2005)
in vitro myocytes; *in vivo* rats	Cardiac-selective overexpression of *AT_2R*	Protection of cardiac functions from ischemic injury	Qi et al. (2012)
mice	Overexpressed *AT_2R*	Reduced atherogenesis	Hu et al. (2008)
rats	*AT_2R*-over-expressing bone mesen-chymal stem cells	Exosomes derived from AT_2R-overexpressing bone mesen-chymal stem cells prevent restenosis	Zou et al. (2023)
mice	Smooth muscle cell specific overexpression of *AT_2R*	Stimulated bradykinin; enhanced nitric oxide/ cGMP system	Tsutsumi et al. (1999)
mice	*AT_2R^-*	Exacerbated aortic disease compared with Marfan syndrome mice with AT_2R	Habashi et al. (2011)
mice	*AT_2R^-*	Reduced urinary sodium secretion	Siragy et al. (1999)
mice	*AT_2R^-*	Increased renal fibrosis; increased fibrocytes in bone marrow	Sakagawa et al., 2000
mice	*AT_2R^-*	Impaired nephrogenesis; impaired expression of Pax-2 and N-myc	Chen et al. (2008)
rats	Overexpressed *AT_2R*	Inhibition of cell proliferation in the outer adrenal cortex	Peters et al. (2012)

(*continued on next page*)

Table 1 (*continued*)

Biological System	Modulated AT_2R	Biological effect	Reference
mice	AT_2R ↓	Accelerated nephropathy in type I diabetes	Chang et al. (2011)
mice	AT_2R ↓	Podocyte dysfunction and loss; enhanced focal semental glomeruloschlerosis; glomerular fibrosis; albuminuria	Liao et al. (2021); Liao et al. (2022)
mesangial cells	Overexpressed AT_2R	Protective effects of AT_2R after treatment with macrophage conditioned medium	Pawluczyk and Harris (2012)
rats	Overexpressed AT_2R	Protection of renal tubule mitochondria in diabetes	Micakovic et al. (2018)
mesenchymal stem cells	AT_2R ↓	Accelerated adipogenesis	Matsushita et al. (2016)
rats	Epigenetically repressed AT_2R	β cell dysfunction and glucose intolerance	Kou et al. (2020)
mice	AT_2R ↓	Anxiety-like behavior; lower body temperature; lower pain threshold, decreased level of brain beta endorphins	Okuyama et al. (1999); Watanabe et al. (1999); Sakagawa et al. (2000)
prostate cancer cells; xenografts in mice	Overexpres-sed AT_2R	Apoptosis; inhibition of tumor growth	Li et al. (2009); Li et al. (2016)
rat insuloma cells	Overexpressed AT_2R	Apoptosis; impaired insulin secretion	Liu et al. (2015)
human prostate cancer cells	Overexpressed AT_2R	Up-regulation of 6 apoptosis-related genes	Pei et al. (2014)
bladder cancer cells	Overexpres-sion of AT_2R	Apoptosis and inhibition of angiogenesis	Pei et al. (2017)
mice	mesenchymal stem cells overexpressing AT_2R	Increased cell migration to injured lung	Xu et al. (2018)

RAS, multiple pathophysiological processes wherein RAS is involved, complex biological systems for functional testing and a possible lack of specificity in ligand-receptor interaction.

2.1. Activities of PD123319

AT2 receptor activation opposes AT1 receptor actions, but also exerts effects beyond inhibitory cross-talk with AT1 receptor signaling. This blurred the original picture of a clear difference between AT1 receptor stimulation potentially leading to unwanted effects and AT2 receptor stimulation balancing the stimulation of the AT1 receptor. Multiple studies with altered AT2 receptor expression consistently and firmly demonstrate beneficial roles of the AT2 receptor in various disorders including cardiovascular disease, nephropathy and metabolic disease (Table 1). The use of putative AT2 receptor antagonists seems to have important therapeutic effects in *in vitro* and *in vivo* experimental models of diseases including neuropatic pain, cystic fibrosis, neonatal lung injury, ischemic heart injury, nephropathy, colitis, glioblastoma multiforme (Table 2: Cohen et al., 2007; Darrah et al., 2019; Kilic et al., 2019; Moulder et al., 2004; Perryman et al., 2022; Smith et al., 2013a; Wagenaar et al., 2014; Zizzo et al., 2020). A set of structurally similar AT2 receptor antagonists, including PD123319, was initially used as analgesics (Table 2: Smith et al., 2013). One of these, EMA401, entered clinical trials for treating neuropathic pain. EMA400 produced analgesia in the chronic constriction injury of the sciatic nerve in a rat model of neuropathic pain already at a dose as low as 3 μg/kg. Receptor blockage usually requires excess of the antagonist over the (endogenous) agonist. To get a significant effect with an agonist temporary occupancy of only 5–20% receptors is sufficient, whereas with an antagonist occupancy of more than 50% of the receptors is needed (Muttenthaler et al., 2021). Furthermore, bolus administration of an agonist may have prolonged effects, whereas efficacy of antagonists requires constant blocking either by continuous infusion or by a very long $T_{1/2}$ of the antagonist. At higher doses, 1–10 mg/kg, ligands similar to EMA400, including EMA200 (PD123319) and EMA300, also acted as analgesics and therefore probably have the same mode of action as the 3 μg/kg dose of EMA400. Taken together, the bolus dose of 3 μg/kg appears to be much too low for an antagonist to be effective, but is compatible with an agonistic mode of action. This is supported by observations in AT2 receptor knockout mice which had a lower pain threshold and decreased level of brain beta endorphins (Table 1: Okuyama et al., 1999; Sakagawa et al., 2000; Watanabe et al., 1999). These findings indicate that the AT2 receptor plays a beneficial role in reducing pain perception. Hence, an AT2 receptor agonist may work as an analgesic, while an AT2 receptor antagonist would not. Taken together and in view of the biological activity at low doses, these data consistently indicate that PD1233319 and analogs probably act as agonists of the AT2 receptor when exerting analgesic effects.

In rat pups with hyperoxia-induced lung injury (Table 2: Wagenaar et al., 2014), a rat model for bronchopulmonary dysplasia, administration of PD123319 had therapeutic effects. At a low dose of 0.1 mg/kg/d, PD123319 attenuated cardiopulmonary injury by reducing pulmonary inflammation and fibrosis and prevented pulmonary arterial hypertension-induced right ventricle hypertrophy (Wagenaar et al., 2014). These effects were very similar to those obtained with AT2

Table 2
Overview of therapeutic effects in selected experimental studies of Ang II type 2 receptor ligands.

Molecule	System	Dosing	Results	Reference
EMA400	Chronic constriction injury rat model of neuropathic pain	0.003–0.3 mg/kg, intraperitoneal injection bolus dose	Analgesia	Smith et al. (2013a)
EMA200 (PD123319)	Idem	1–10 mg/kg, intraperitoneal injection bolus dose	Analgesia	Smith et al. (2013a)
PD123319	Cystic fibrosis mice	2 mg/kg/d subcutaneous injection bolus dose	Restored lung function comparable to wild type	Darrah et al. (2019)
PD123319	Rat model of neonatal chronic lung and heart disease	0.1 mg/kg/d, subcutaneous injection bolus dose	Reduced pulmonary hypertension and right heart disease	Wagenaar et al. (2014)
PD123319	Isolated rat heart	20 mg/kg, intraperitoneal injection of bolus dose	Beneficial effects on ischemic injury and oxidative damage	Kilic et al. (2019)
PD123319	Radiation-nephropathy rat-model	Infusion of 15 mg/kg/d	Delayed nephropathy	Moulder et al. (2004)
PD123319	Rat model of colitis	0.3–10 mg/kg/d, intraperitoneal injection bolus dose	Reduced oxidative stress and reduced inflammation; inhibit NF-κB activation	Zizzo et al. (2020)
PD123319	AT2 receptor -expressing GBM cells	1–30 μM	Reduced proliferation	Perryman et al. (2022)
EMA401	AT2 receptor -expressing GBM cells	1–30 μM	Reduced proliferation	Perryman et al. (2022)

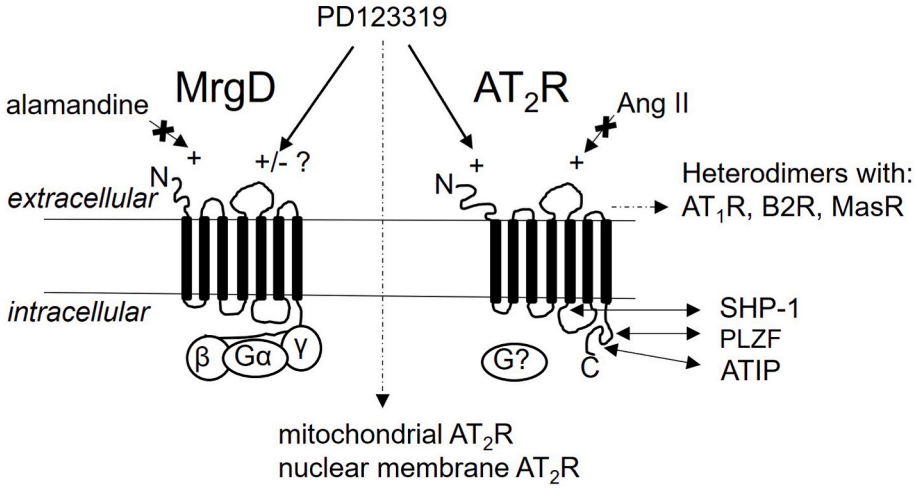

Fig. 3. PD123319 interacts with Mas-related receptor D and AT2 receptor.
PD123319, and low dose of EMA400 (Table 2), appears to stimulate AT2 receptor. PD123319 competes with alamandine for binding to Mas related G-protein coupled receptor D (MrgD) and with Ang II for binding to AT2 receptor (Lautner et al., 2013). Mas-related G-protein coupled receptor D signals via classical coupled G-proteins/arrestin recruitment, whereas AT2 receptor seems to signal via other effectors. AT2 receptor-adaptor proteins, promyelocytic leukemia zinc finger protein (PLZF) and AT2 receptor interacting protein (ATIP) which recruits the protein tyrosine phosphatase that contains an SH2 domain (SHP-1) are directly interacting with constitutively active AT2 receptor and may play a role in signaling (Reinemund, 2010). Erb-b2 receptor tyrosine kinase 3 (ErbB3) (Knowle et al., 2000), Connector enhancer of Ksr1 (CNK1) (Fritz and Radziwill, 2005), sodium-proton exchanger 6 (NHE6) (Pulakat et al., 2005), and tissue inhibitor of metalloproteinase 3 (TIMP3) (Kang et al., 2008) also interact with AT2 receptor. AT2 receptor can form heterodimers with AT1 receptor, bradykinin receptor (B2R) and Mas receptor (MasR).

receptor agonist LP2. Co-administration of AT2 receptor agonist LP2 and 0.5 or 2 mg/kg/d PD123319 abolished all beneficial effects on right ventricular hypertrophy in experimental neonatal cardiopulmonary disease. The therapeutic activity of PD123319 alone, which is similar to that of AT2 receptor agonist LP2, in combination with its relatively low dose, strongly suggests an agonistic mechanism.

Putative AT2 receptor antagonist EMA401 and PD123319 appear effective against glioblastoma (Perryman et al., 2022). PD123319 inhibits the proliferation of AT2 receptor-expressing cells in the absence of exogenous Ang II. Nevertheless, an AT2 receptor [Y]6-Ang II agonist does not cause proliferation of U87 and GBM96 cell-lines (Perryman et al., 2022). In contrast to the beneficial effects of the putative antagonists of the AT2 receptor in glioblastoma, LP2, an agonist of AT2 receptor, inhibits growth of cell line-derived xenografts of glioblastoma in mice at a dose as low as 0.2 μg/kg/d (Namsolleck et al., 2021b). Importantly, multiple studies with modulated AT2 receptor clearly demonstrate an anti-tumor role of AT2 receptor (Table 1: Li et al. (2009); Li et al. (2016); Liu et al., 2015; Pei et al. (2014); Pei et al. (2017). Hence, blocking AT2 receptor is not expected to exert anti-tumor activity, whereas stimulating AT2 receptor is. Therefore, AT2 receptor agonist activity of PD123319 and analogs may explain their anti-tumor activity.

Many studies on cardiovascular systems with modulated AT2 receptor expression provide a strong basis for beneficial roles of AT2 receptor stimulation (Table 1: Adachi et al., 2003; Akishita et al., 1999, 2000; Bove et al., 2004, 2005; Habashi et al., 2011; Hein et al., Hu et al., 2008, 1995; Ichihara et al., 2002; Ichiki et al., 1995; Li et al., 2003; Li et al., 2007; Metcalfe et al., 2004; Mirabito et al., 2014; Oishi et al., 2003; Qi et al., 2012; Tsutsumi et al., 1999; Wang et al., 2004; Wu et al., 2002; Zou et al., 2023). Low dose of PD123319 alone reduced right ventricle hypertrophy in a pup model of acute lung injury (Wagenaar et al., 2014). Of note, in a study on ischemia-reperfusion injury and oxidative damage in isolated rat heart PD123319 had even more beneficial effects than the well-known highly effective AT1 receptor antagonist losartan (Kilic et al., 2019), which could be well explained by agonistic effects via the AT2 receptor of PD123319. Taken together, all these data strongly indicate that the beneficial effects of AT2 receptor modulation by PD123319 may be caused by agonistic rather than antagonistic effects.

2.2 Stimulatory effect of PD123319 on mitochondria

In contrast to peptide ligands, which only bind to their receptor on the cell surface, small molecule drugs may translocate across the plasma membrane to bind the AT2 receptor on the membranes of organelles. This may explain the marked stimulation of mitochondrial respiration by PD123319 in Ang II-preconditioned rat hearts, resulting in activation of the respiratory chain and ATP production, thereby improving post-ischemic cardiac function (Escobales et al., 2019; Nuñez et al., 2018). Intracellular AT1 receptor- and AT2 receptor-linked mechanistic pathways of mitochondria-mediated cardioprotection by Ang II-preconditioning have been proposed (Escobales et al., 2019).

2.3. AT2 receptor ligand PD123319 may interact with other receptors

Putative AT2 receptor antagonist PD123319 has been reported to block some of the effects of Mas receptor agonist AVE-0991 and AT2 receptor/Mas receptor agonist Ang-(1–7) (Raffai et al., 2011). Further research needs to clarify these results. More conclusive data have been reported on the interaction of PD123319 with Mas-related G-protein coupled receptor D. The heptapeptide alamandine is either formed by decarboxylation of the aspartate residue of Ang-(1–7) or by ACE-2 action on Angiotensin A, a D1A analogue of Ang II (Lautner et al., 2013; Liu et al., 2018; Qaradakhi et al., 2016). PD123319 inhibits the vasorelaxant action of alamandine in the aorta by displacing the binding of alamandine from its receptor (Li et al., 2017). Even in the absence of the AT2 receptor in AT2 receptor knockout mice, PD123319 inhibited the vasorelaxant action of alamandine and its binding to Mas-related G-protein coupled receptor D-transfected Chinese hamster ovary cells (Lautner et al., 2013).

3. Discussion

The AT2 receptor is a highly important and special receptor which, at least in part, does not function via classical G-proteins or arrestin recruitment. Due to the lack of standard *in vitro* activity assays for this

receptor, the characterization of inhibitors/blockers and modulators is hampered. Allosteric sites have been reported for a wide range of G-protein coupled receptors (Congreve et al., 2017; Conn et al., 2014). As the AT2 receptor appears to adapt an active-like conformation with constitutive activity, one would expect the induction of a clearly non-active conformation as a result of the interaction with a true receptor blocker, that not only antagonizes receptor binding but also inhibits receptor-mediated signaling. Taken together, data on examples wherein putative AT2 receptor antagonists exert beneficial activities, even at 3 μg/kg for EMA400 (Table 2), indicate agonist activity. The data strongly suggest that a putative AT2 receptor antagonist, like PD123319, may act at low doses as agonist, consistent with the suggested agonist activity of PD123319 (Zhou et al., 1993). This is supported by the observations in many *in vitro* and *in vivo* experimental disease models, summarized in Table 1, that increased AT2 receptor expression results in beneficial pathophysiological effects and in which deletion/knockdown of the AT2 receptor results in adverse effects. At higher doses agonism or antagonism may depend on aspects such as signaling pathways, desensitization, cell type-dependent receptor internalization (Ferrão et al., 2017; Hein et al., 1997), receptor heterodimerization and the used system. In addition, PD123319 appears to interfere with Mas-related G-protein coupled receptor D accessibility and Mas-related G-protein coupled receptor D-mediated activity (Fig. 3).

4. Conclusion

While beneficial effects of molecules that are putative AT2 receptor antagonists may shed doubt on the therapeutic validity of stimulating AT2 receptor, elucidation of agonistic action of these putative AT2 receptor antagonists may restore therapeutic potential of specifically stimulating the AT2 receptor in several serious diseases. In conclusion, in the absence of unequivocal molecular mechanistic data, existing literature data urge for the utmost prudence in the interpretation of results obtained with PD123319 and similar AT2 receptor ligands.

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CRediT authorship contribution statement

Gerry T.M. Wagenaar: Conceptualization, Writing – review & editing. **Gert N. Moll:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

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