Vascular Effects of Current and Novel Antimigraine Drugs

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Vascular Effects of Current and Novel Antimigraine Drugs

Vasculaire effecten van huidige en nieuwe antimigraine geneesmiddelen

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En memoria de mis abuelos
Hugo (1927-2019) *y Martha* (1930-2018)

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PART I.

Chapter I.

Introduction

"All our knowledge *begins* with the senses...

Pathophysiology of migraine

Migraine is a highly disabling neurovascular disorder[1] and several theories have arisen regarding its pathophysiology[2]. Currently, migraine is considered a neurovascular disorder that involves activation of the trigeminovascular system[3]. This system comprises both peripheral and central projections, the former via the trigeminal ganglion that sends sensory fibers to the dura mater and the cranial vasculature[4] and the latter via the trigeminocervical complex that consists of the trigeminal nucleus caudalis and the upper two cervical divisions[5]. The activation of this system is considered to result in the release of calcitonin gene-related peptide (CGRP) from the sensory fibers, causing vasodilation of the cranial vasculature and nociceptive transmission[6]. In accordance with this, studies have shown that during a migraine attack, there is an increase in CGRP levels in plasma in the jugular vein[7,8], while treatment with triptans normalizes these levels[9]. Also, intravenous infusions of CGRP are known to provoke migraine-like attacks in migraine patients[10].

Treatment of migraine

Before the discovery of the fundamental role of CGRP in the pathophysiology and treatment of migraine, the main target for selective acutely acting antimigraine drugs was the serotoninergic signaling, with the triptans being the gold standard since the beginning of the 1990's[11]. The prophylactic treatment, however, consisted of medication not developed originally for migraine, but for other diseases, such as hypertension (HT), epilepsy and depression.

A decade later, and due to the important role of CGRP in migraine pathophysiology, CGRP receptor antagonists (gepants) were developed for the acute treatment of migraine and proved to be effective[12,13]. Unfortunately, pharmacokinetic limitations and hepatotoxicity cases did not allow the initial gepants to reach the market[14]. New gepants are currently in Phase II trials for the acute and prophylactic treatment of migraine, with no hepatotoxicity reported[15,16], nevertheless, the concerns about the hepatotoxicity reports led to the development of CGRP (receptor) antibodies for the prophylactic treatment of migraine[17-19], and so far, all clinical trials with these antibodies have shown promising results[20,21].

Migraine, CGRP and cardiovascular risk

CGRP is widely expressed throughout the body, participating not only in migraine pathophysiology, but also in several physiological processes and homeostatic responses during pathophysiological events. Therefore it is important to consider the possible side effects especially after long-term blockade of the CGRP pathway.

To begin with, sensory CGRPergic fibers have been described to innervate the coronary blood vessels as well as the myocardium[22-24]. Several studies have shown that CGRP plays an important role in the regulation of blood pressure and in the homeostatic responses during ischemic events, and it seems to act as a protective/compensatory mechanism during HT[25-31]. Moreover, CGRP is not only involved in peripheral mechanisms, but it also participates in the maintenance of cerebrovascular reactivity during chronic HT by increasing cerebral blood flow[32-35].

CGRP also seems to be involved in the vascular adaptations during pregnancy, as plasma levels increase through the gestation period, reaching their maximum during the last trimester and normalizing after delivery. However, in pre-eclampsia, a pregnancy disorder characterized by high blood pressure and proteinuria, CGRP levels are lower[36].

The role of CGRP as compensatory mechanism in ischemic events and blood pressure regulation poses a concern, especially as numerous studies have shown that migraine patients present an increased risk of hemorrhagic and ischemic stroke, with the risk being higher for women[37-42]. Moreover, a higher risk of myocardial infarction, coronary artery disease and altered arterial function

have also been described[43,44]. Unfortunately, the mechanisms behind these increases are not clear, but they are thought to involve genetic aspects and vascular dysfunction, amongst other factors.

One strategy to understand migraine pathophysiology and its relation with the increase in cardiovascular risk is the use of animal models of migraine, but current animal models, although useful, only represent certain features of this rather complex disorder. Another alternative is the use of models of monogenic disorders that are comorbid with migraine, such as "Autosomal dominant **R**etinal **V**asculopathy with **C**erebral **L**eukodystrophy" (RVCL)[45,46]. Interestingly, this vasculopathy, caused by a mutation in the *TREX1* gene, is associated with endothelial dysfunction[46], and almost two thirds of the patients present migraine without aura[45], providing an unique opportunity to study both the genetic and the vascular interactions in migraine pathophysiology.

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Chapter II.

Aims

Based on the questions posed in **Chapters I**, **III** and **VI**, the objective of this thesis was to investigate the vascular effects of current and novel antimigraine drugs. For this purpose, the following objectives were defined:

1. The gold standard for the acute treatment of migraine are the triptans. It has been shown that triptans cause vasoconstriction of the middle meningeal artery and, unfortunately, of the coronary arteries, due to their affinity for the 5-HT_{1B} receptors present in vascular smooth muscle cells. Therefore, novel serotonergic antimigraine drugs without vasoconstrictive properties and devoid of affinity for the 5-HT_{1B} receptor are needed. In **Chapter IV** we investigated the binding, functional activity and contractile responses of the selective 5-HT_{1F} receptor agonist lasmiditan, a novel antimigraine drug that is effective for the acute treatment of migraine.

2. Studies have shown that CGRP plays an important role in migraine pathophysiology. This has led to the development of antagonists (ubrogepant and atogepant) and an antibody (erenumab) against the CGRP receptor. We characterized the effect of ubrogepant, atogepant **(Chapter V)** and erenumab **(Chapter VIII)** in human cranial and coronary arteries to investigate the possible mechanism of their therapeutic effect, as well as their potential coronary side effects.

3. Before the arrival of the antibodies against CGRP or its receptor, prophylactic migraine treatment was not developed specifically for this disorder, but for hypertension, epilepsy or depression. Although these treatments are effective, their mechanism of action in migraine treatment unfortunately has not been studied in detail. Propranolol is one of the most widely prescribed drugs for prophylactic treatment of migraine, therefore we set out to investigate the effect of propranolol in the modulation of the trigeminovascular system in our human model of trigeminal nerve-mediated vasodilation **(Chapter IX)**. Since most of migraine patients are females and we have previously shown that trigeminovascular responses can be modulated by sex hormones, we stratified our data to see whether the responses between males and females were comparable.

4. Not all patients respond to treatments based on current targets. Based on the current knowledge of migraine pathophysiology, new drugs could act at receptors that are also activated by CGRP or that inhibit CGRP release. In **Chapter XI**, we investigated the role of imidazoline receptors in the inhibition produced by moxonidine and agmatine on the vasodepressor sensory CGRPergic outflow in pithed rats.

5. Migraine pathophysiology remains largely unknown. Current animal models, although useful, only represent certain features of this rather complex disorder. "Autosomal dominant **R**etinal **V**asculopathy with **C**erebral **L**eukodystrophy" (RVCL), caused by a mutation in the *TREX1* gene, is a vasculopathy that presents migraine as its earliest manifestation. Monogenic diseases such as RVCL provide an opportunity to study the genetic and vascular mechanisms involved in migraine pathophysiology. In **Chapter XII** we assessed whether RVCL-KI mice have features in line with the pathology seen in patients, such as a reduced life expectancy and a vascular phenotype (as assessed by functional vascular measurements and the induction of experimental stroke).

...*proceeds* then to the understanding...

PART II:

Acute treatment of migraine

Chapter III.

Is selective 5-HT$_{1F}$ receptor agonism an entity apart from that of the triptans in antimigraine therapy?

Based on: ***E Rubio-Beltrán****[*], A Labastida-Ramírez[*], CM Villalón, A MaassenVanDenBrink (2018) Pharmacology & Therapeutics; 186:88-97.*

[*]Both authors contributed equally

Abstract

Migraine is a neurovascular disorder that involves activation of the trigeminovascular system and cranial vasodilation mediated by release of calcitonin gene-related peptide (CGRP).

The gold standard for acute migraine treatment are the triptans, 5-$HT_{1B/1D/(1F)}$ receptor agonists. Their actions are thought to be mediated through activation of: (i) 5-HT_{1B} receptors in cranial blood vessels with subsequent cranial vasoconstriction; (ii) prejunctional 5-HT_{1D} receptors on trigeminal fibres that inhibit trigeminal CGRP release; and (iii) 5-$HT_{1B/1D/1F}$ receptors in central nervous system involved in (anti)nociceptive modulation. Unfortunately, coronary arteries also express 5-HT_{1B} receptors whose activation would produce coronary vasoconstriction; hence, triptans are contraindicated in patients with cardiovascular disease. In addition, since migraineurs have an increased cardiovascular risk, it is important to develop antimigraine drugs devoid of vascular (side) effects.

Ditans, here defined as selective 5-HT_{1F} receptor agonists, were developed on the basis that most of the triptans activate trigeminal 5-HT_{1F} receptors, which may explain part of the triptans' antimigraine action. Amongst the ditans, lasmiditan: (i) fails to constrict human coronary arteries; and (ii) is effective for the acute treatment of migraine in preliminary Phase III clinical trials. Admittedly, the exact site of action is still unknown, but lasmiditan possess a high lipophilicity, which suggests a direct action on the central descending antinociceptive pathways. Furthermore, since 5-HT_{1F} receptors are located on trigeminal fibres, they could modulate CGRP release.

This review will be focussed on the similarities and differences between the triptans and the ditans, their proposed sites of action, side effects and their cardiovascular risk profile.

Introduction

Migraine is a debilitating neurovascular disorder characterized by recurring unilateral pulsating headaches of moderate to severe intensity, associated with nausea, photophobia and/or phonophobia, lasting from 4 to 72 hours[1]. In the Global Burden of Disease Study, migraine was ranked as the third disabler in women, sixth disabler when taking both genders into account, and the most disabling of all neurological disorders, affecting approximately 15% of the world population[2], with a profound negative effect on the patient's quality of life[3]. Furthermore, this neurovascular disorder represents an economic loss of €20 billion in Europe every year[4]. Thus, migraine is a public health problem that affects both the individual and society.

Pathophysiology of migraine

Throughout the years, several theories regarding the pathophysiology of migraine have emerged[5]. In the late 1930's and early 1940's, Wolff's group described migraine as a disorder of vascular origin, with an intense extracranial vasodilation as the cause of migraine pain[6,7]. Decades later, Moskowitz introduced the neurogenic theory, where trigeminovascular axons from blood vessels of the dura mater released vasoactive peptides producing an sterile inflammatory response followed by pain[8]. Nowadays, migraine is considered as a neurovascular disorder that involves activation of the trigeminovascular system[9,10], presumably followed by vasodilation mainly mediated by the release of calcitonin gene-related peptide (CGRP), a neuropeptide present in perivascular sensory fibres[11,12].

Treatment of migraine

Despite the long history of migraine treatment, effective antimigraine drugs have been, until very recently, limited in number (for references see[13]). Basically, pharmacological treatment of migraine can be divided into prophylactic drugs, designed to reduce the frequency and severity of migraine attacks, and acutely acting drugs, aimed to reverse the attack once it has begun, including the

associated symptoms. The majority of migraine patients only need acute treatment, nevertheless, migraineurs that suffer from frequent attacks or have contraindications for the use of acutely acting drugs, are also prescribed prophylactic drugs[14]. The prophylactic treatment will not be discussed here as it is falls beyond the scope of the present review.

The acute treatment can be further subdivided in specific (ergot derivatives, triptans, gepants, "ditans"), and non-specific (nonsteroidal anti-inflammatory drugs, analgesics) antimigraine drugs[14,15]. While non-specific drugs aim to treat migraine as a general headache or other pain, specific treatment is developed based on the neurovascular basis of migraine. Thus, these drugs target the modulation of the trigeminovascular system, the CGRP-mediated vasodilation (i.e. extracranial vasoconstriction, inhibition of CGRP release, CGRP receptor antagonism) and/or the pain perception pathway, amongst others. On this basis, CGRP receptor antagonists (gepants) are a likely candidate for the acute treatment of migraine, and indeed, they were effective in clinical trials[12,16,17]; however, due to pharmacokinetic and/or hepatotoxic limitations none of the gepants have yet reached the market[18]. Currently, a potential concern with gepants includes the cardiovascular side effects when used chronically, considering the physiological protective role of CGRP in maintaining cardiovascular homeostasis in ischemic events (for further references see[19]). Several drugs targeting the CGRP receptor are presently under development for the acute and prophylactic treatment of migraine[19,20].

During the last 40 years, the target for selective antimigraine drugs has been the serotoninergic signalling. Long before the discovery of CGRP and its fundamental role in migraine, increased urinary and plasma levels of serotonin (5-hydroxytryptamine, 5-HT) and its metabolites were described[21,22]. Further studies showed that intravenous infusion of 5-HT was capable of aborting migraine attacks[23] and that antimigraine drugs like methysergide and ergotamine were acting on, amongst other receptors, 5-HT_1 receptors[24,25]. In this review, the main focus will be on the triptans, 5-$HT_{1B/1D/(1F)}$ receptor agonists, that are currently considered the gold standard for acute migraine treatment, and on the novel "ditans" (in this review defined as selective 5-HT_{1F} receptor agonists), not only developed based on the neurovascular origin of migraine, but also in view of the cardiovascular risk profile of migraine patients[26-34] .

Triptans

As mentioned in the above section, the role of serotoninergic neurotransmission in migraine led to the design of antimigraine drugs that targeted the 5-HT receptors[21-23]; however, the exact 5-HT receptors involved in the relief of migraine attacks were unknown. Indeed, intravenous infusion of 5-HT was able to abort migraine attacks, but considering that there are fourteen 5-HT receptors[35], and they were all activated, numerous side effects were observed[23]. After several studies using selective agonists and antagonists it was demonstrated that the therapeutic action of 5-HT was mediated by "5-HT_1-like receptors" that constricted cranial blood vessels[24,25,36], and the first triptan was developed: sumatriptan[37]. In the early 1990s, sumatriptan was officially introduced to the market[38]. In view of the low oral bioavailability and lipophilicity of sumatriptan[39], as well as the vast market potential, "second generation" triptans (zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan and avitriptan) were developed, with a chemical structure similar to sumatriptan (see Fig. 1), but with higher oral availability and lipophilicity (see Table 1), as well as a longer plasma half-life[35,40,41].

Mechanism of action

Initially, it was described that the therapeutic action of 5-HT on migraine was mediated by activation of "5-HT_1-like" receptors[36]. Years later, based on structural, transductional and operational criteria, these receptors were classified into 5-HT_{1B} and 5-HT_{1D} receptors (for further references see[42,43]).

Triptans are 5-$HT_{1B/1D}$ receptor agonists, and a grand majority of them are also $5HT_{1F}$ receptor agonists (see Table 2, Fig. 2). Sumatriptan, as previously mentioned, has low lipophilicity (see Table 1) and cannot cross the blood brain barrier (BBB). For a drug to be considered able to cross the BBB, it should have a distribution coefficient at physiological pH ($logD_{pH7.4}$) higher than -1[44,45]. Notably, second generation triptans were developed with higher lipophilicity[39] but their ability to cross the BBB is in controversy, since their reported $logD_{pH7.4}$ values are not consistent amongst studies (see Table 1). Furthermore, it is important to consider the possible interactions between triptans and BBB efflux transporters (*e.g.* P-glycoprotein) that limit the central actions of triptans, as it has been reported for eletriptan[46].

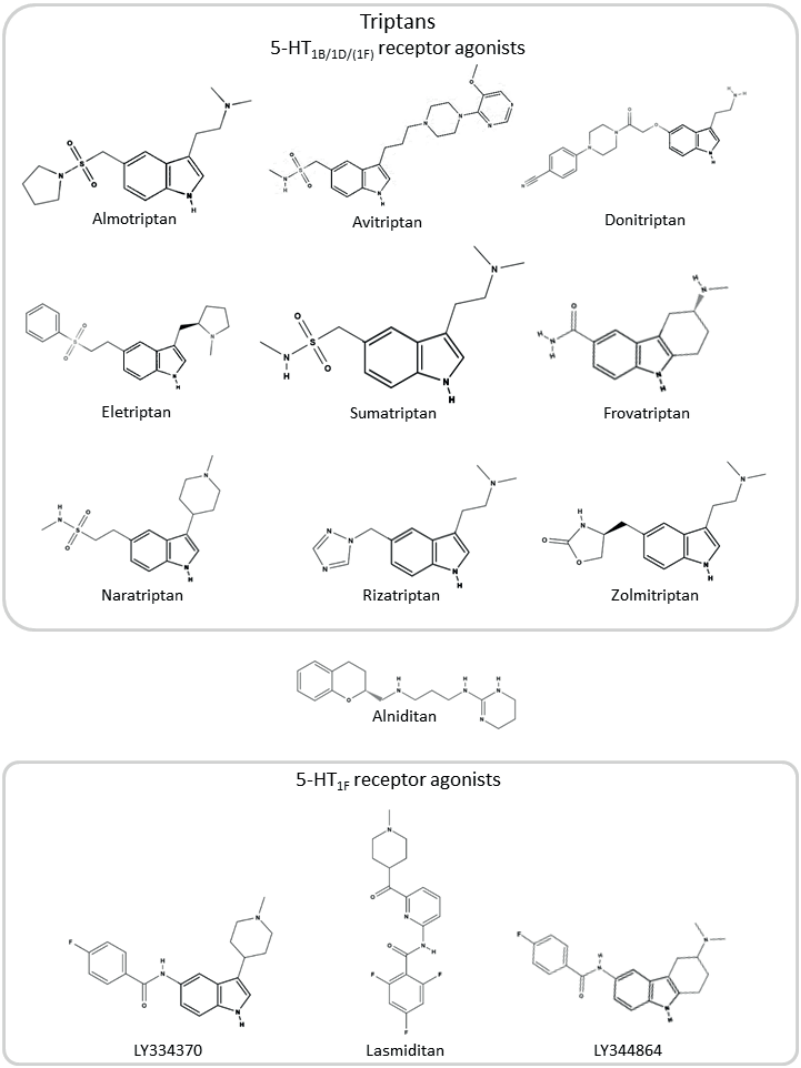

Fig. 1. Chemical structures of the triptans, alniditan and the selective 5-HT_{1F} receptor agonists. It is worth remarking the presence of the indole group in all the structures, with exception of lasmiditan and alniditan[35].

Table 1. Reported LogD$_{pH7.4}$ values for triptans. Compounds with values higher than -1 are considered to be able to cross the BBB.

	Sumatriptan	Zolmitriptan	Naratriptan	Rizatriptan	Almotriptan	Eletriptan	Frovatriptan
Fox[58]	-1.5	-1	-0.2	-0.7	ND	ND	-1
Ferrari, *et al*[59]	-1.3	-0.7	-0.2	-0.7	+0.35	+0.5	ND
Glennon and Dukat[60]	-1.7	-0.8	-1.2	-1.4	-0.5	+0.2	-2.1
Milton, *et al*[61]	ND	ND	ND	ND	ND	+1.1	ND
Pascual and Muñoz[62]	-1.3	-0.7	-0.2	-0.7	-2.1	+0.5	ND
Cheng, *et al*[63]	-1.4	-1.5	ND	-0.8	+0.4	+0.2	-1.9

ND. Not defined

Table 2. Summary of pEC$_{50}$ values of cAMP (5-HT$_1$ and 5-HT$_7$) and IP (5-HT$_2$) assays of individual antimigraine drugs at 5-HT receptors[64]. These values represent the negative logarithm of the molar concentration of these compounds at which 50% of their maximal response is exerted. *pEC$_{50}$ values correspond to [^{35}S]GTPγS assay.

	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1D}$*	5-ht$_{1E}$	5-HT$_{1F}$	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_7$
Ergotamine tartrate	9.78	9.94	9.43	5.95	5.97	9.25	8.72	7.09
Sumatriptan succinate	<5	7.32	8.30	5.99	8.03	<5	<5	5.22
Zolmitriptan	<5	7.87	9.51	8.18	8.00	<5	<5	6.28
Naratriptan hydrochloride	<5	8.05	8.80	7.75	8.38	<5	<5	<5
Rizatriptan benzoate	<5	7.08	8.11	7.34	6.54	<5	5.49	<5
Almotriptan malate	<5	7.08	7.75	<5	7.79	<5	5.20	<5
Eletriptan hydrobromide	<5	8.00	9.04	7.53	8.13	6.07	6.81	6.45
Frovatriptan racemate	<5	7.98	8.36	5.04	7.10	<5	<5	7.42
Donitriptan hydrochloride	5.94	9.96	9.51	<5	<5	8.10	7.61	5.23
Avitriptan fumarate	<5	8.57	9.27	5.52	7.09	6.91	6.41	5.38
Alniditan dihydrochloride	7.00	8.87	8.20	5.68	5.92	<5	7.15	6.32
Lasmiditan hemisuccinate	<5	<5	6.64	6.17	8.43	<5	<5	<5
LY334370 hydrochloride	5.84	6.52	6.92	7.53	9.08	<5	<5	<5
LY344864 hydrochloride	<5	<5	6.93	6.22	8.72	<5	<5	<5

Cardiovascular (side) effects

The initially proposed therapeutic action of triptans is through the selective vasoconstriction of cranial blood vessels due to the high expression of 5-HT$_{1B}$ receptors in this vasculature[36,47] in comparison with peripheral blood vessels[48]. In agreement with this, *in vitro* studies have shown that at therapeutic concentrations triptans contract the middle meningeal artery (MMA)[48]. Furthermore, magnetic resonance angiography studies demonstrated that migraine attacks are associated with dilation of the extra- and intracerebral arteries, ipsilateral to the headache side; and that contraction of dural (but not intracranial) arteries by triptans, is associated with headache relief[49], although it is worth mentioning that further results of the same group have been inconsistent[50-52] and it is still a matter of debate to what extent the vascular action of the triptans contributes to their therapeutic efficacy. Unfortunately, studies have consistently shown that triptans induce an increase in blood pressure[53] and contraction of coronary arteries[48,54-56], which is more pronounced in the distal than in the proximal portion of the human coronary artery[57].

Currently, although there is still a debate on whether the therapeutic action of triptans relays on their vasoconstrictive properties, it is clear that coronary vasoconstriction is a drug class effect of the triptans as 5-HT_{1B} receptor agonists[65]. Therefore, triptans are contraindicated in migraine patients with cardiovascular disease[66]. Additionally, it is important to consider that migraineurs are known to have an increased risk of haemorrhagic[29] and ischemic[26-28,30,31] stroke, with women presenting a higher risk[34]. Also, an altered arterial function[32], and a higher risk of myocardial infarction and coronary heart disease[33] have been described. Nonetheless, the use of triptans does not seem to increase the risk of stroke, myocardial infarction, cardiovascular death, ischemic heart disease or mortality[67-69]. However, taken together, their coronary vasoconstrictor potential justifies the contraindication of the triptans in patients with cardiovascular risk factors.

Neuronal effects

Although sumatriptan does not cross the BBB[59], it has long been speculated that during a migraine attack there would be a disruption of the BBB, which would enable the triptans, even those with a low lipophilicity, to exert a central effect. However, it has recently been demonstrated that there is no disruption of the BBB during a migraine attack[70,71], thus excluding a central action for the triptans, except for those with a high lipophilicity. Besides the potential role for blood vessels in the pathophysiology of migraines, this suggests that actions of the triptans can well be mediated through neuronal structures that are not protected by the BBB, as it is the case for the pituitary gland, choroid plexus and, most importantly, the trigeminal ganglion (TG)[72], a key structure in migraine pathophysiology. Accordingly, treatment with sumatriptan reduces CGRP plasma levels as migraine lessens[73]. More recently, sumatriptan was also shown to inhibit capsaicin-induced trigeminal CGRP release in healthy volunteers[74]. Furthermore, expression of prejunctional 5-HT_{1D} receptors has been described in trigeminovascular nociceptive neurons[47,75,76] which could suggest a role in the modulation of CGRP release, as well as plasma protein extravasation[77]. Remarkably, based on the prejunctional location of 5-HT_{1D} receptors in the TG, PNU-142633, a selective 5-HT_{1D} receptor agonist, was developed for the acute treatment of migraine, and showed a superior potency over sumatriptan for blocking plasma protein extravasation. Unfortunately, it was not effective in the acute treatment of migraine[78]. It is worth mentioning that PNU-142633 was developed based on the gorilla $5HT_{1D}$ receptor, which could explain its lack of efficacy, and thus a role for the 5-HT_{1D} receptor in the treatment of migraine cannot categorically be ruled out. Besides, it has been shown that activation of 5-HT_{1B}, but not 5-HT_{1D} receptors inhibits CGRP release in the pithed rat model[79], indicating that, while 5-HT_{1D} receptor activation may contribute to the therapeutic actions of triptans, it may not be their main site of action. Further studies are needed to completely elucidate the role of 5-HT_{1D} receptor activation in migraine treatment.

As discussed earlier, second generation triptans were developed with higher lipophilicity than sumatriptan[40,41]. Therefore, some of them may be able to cross the BBB[59,80]. Although the lipophilicity of triptans correlates with central side effects, it does not seem to be related to their efficacy[62], and there is no consistency in the lipophilicity reported in literature (see Table 2). Nevertheless, we cannot categorically exclude additional therapeutic actions mediated via activation of 5-$HT_{1B/1D/(1F)}$ receptors in the central nervous system by highly lipophilic triptans[818181]. In accordance with this, it has been shown that vasodilation of the canine external carotid artery induced by intracarotid administration of capsaicin, is inhibited by spinal (but not intravenous) administration of sumatriptan via activation of 5-HT_{1B} receptors; in contrast, intravenous administration of the highly lipophilic donitriptan inhibits the capsaicin-induced vasodilation, also mediated by activation of 5-HT_{1B} receptors[80,82].

Several studies have described 5-HT$_{1B}$ and (presynaptic) 5-HT$_{1D}$ receptors in the dorsal horn of the spinal cord (DHSC)[83], substantia nigra, globus pallidus[83,84], nucleus tractus solitarius (NTS), trigeminal nucleus caudalis (TNC)[47,83], periaqueductal grey area (PAG)[83,85], the ventroposteromedial nucleus of the thalamus[86], hypothalamic paraventricular nucleus (PVN)[87] and the rostral ventromedial medulla[88], structures previously associated with nociceptive and anti-nociceptive pathways[89-97]. Furthermore, it has been shown that spinal 5-HT$_{1B/1D/1F}$ receptors are involved in the serotonergic descending inhibitory pain system[98-102]. Of special interest, intravenous administration of naratriptan[103,104] results in inhibition of the spinal TNC. Also, the PAG, more specifically the ventrolateral division, is activated by afferents from the TG[105-107], and microinjection of naratriptan in this structure inhibits nociceptive dural responses[85]. Moreover, the PVN, that has been previously shown to participate in the endogenous modulation of pain[97,108] sends projections to the PAG[109] and the spinal trigeminal nucleus[87], and microinjection of naratriptan in this structure, attenuates dural-evoked trigeminovascular responses[87]. Thus, indeed highly lipophilic triptans could not only act through the vasoconstriction of extracranial vasculature and inhibition of the release of CGRP, but also through the activation of the descending inhibitory pain system and/or the inhibition of nociceptive transmission (see Fig. 3). Interestingly, most of these studies were performed using naratriptan, a highly lipophilic triptan that has a high affinity for the 5-HT$_{1F}$ receptor (see Table 2). Furthermore, although antagonists were used to confirm the role of 5-HT$_{1B/1D}$ receptors, there is no selective antagonist commercially available for the 5-HT$_{1F}$ receptor yet. Therefore, the involvement of this receptor in the neuronal actions of triptans can neither be confirmed nor excluded and requires further studies.

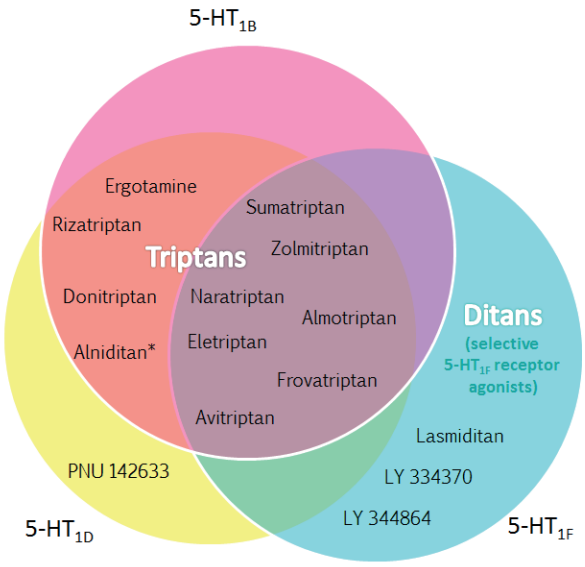

Fig. 2. Summary of agonist profiles of triptans, ditans (here considered as selective 5-HT$_{1F}$ receptor agonists) and other 5-HT receptor ligands, for 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{1F}$ receptors (pEC$_{50}$>7). *In view of the high affinity of alniditan for the 5-HT$_{1B/1D}$ receptors (pEC$_{50}$ cAMP 8.87 and 8.20, respectively), we classified it as a triptan, despite its generic name[64].

Ditans and the 5-HT$_{1F}$ receptor

Triptan monotherapy is ineffective for approximately 25% of migraineurs and in 40% of acute migraine attacks[110]. Several studies have tried to elucidate the lack of efficacy in some migraineurs, but were unable to explain why some patients are responders to the triptans while others are nonresponders. Whereas a difference in efficacy could be due to pharmacokinetic factors for some of the oral triptans, pharmacokinetics did not seem to be responsible for differences in efficacy in response to subcutaneous sumatriptan[111], nor polymorphisms in the 5-HT$_{1B}$ and 5-HT$_{1F}$ receptor genes were able to explain differences in clinical responses to sumatriptan[112-114]. Moreover, as previously discussed, migraine patients are known to have an increased cardiovascular risk; therefore, it is important to develop novel effective antimigraine drugs that are devoid of cardiovascular side effects.

Several triptans bind to the 5-HT$_{1F}$ receptor (see Table 2). Also, as will be discussed later, 5HT$_{1F}$ receptors are expressed in several structures associated with migraine pathophysiology and with the (neuronal) therapeutic actions of triptans. This led to the development of selective 5-HT$_{1F}$ receptor agonists as possible option for migraine treatment. Several selective 5-HT$_{1F}$ receptor agonists have been developed (see Fig. 1), including LY344864, an aminocarbazole, LY334370, a 4-(3-indolyl)piperidine[60] and lasmiditan (COL-144, LY573144), a piridinoyl-piperidine[115]. While the selective 5-HT$_{1F}$ receptor agonists were referred to as SSOFRAs (Selective Serotonin One F Receptor Agonists) for some time, in the last years the term "ditan" has been accepted as a synonymous of selective 5-HT$_{1F}$ receptor agonist[116]. In this context, it is important to consider alniditan, a 5-HT$_{1A/1B/1D}$ receptor agonist with only low 5-HT$_{1F}$ receptor affinity (see Table 2), suggesting that the suffix "ditan" is merely to distinguish novel acutely acting (5-HT$_1$ receptor agonists) antimigraine drugs from triptans, without any structural (see Fig. 2) and/or pharmacological criteria (see Table 2). As the following paragraph is only focused on 5-HT$_{1F}$ receptor agonists, alniditan will not be further discussed when ditans are mentioned.

Location

The human 5-HT$_{1F}$ receptor was first identified and cloned in 1993[117]. It has been described in the TG[75,118,119], globus pallidus, NTS, substantia nigra, PAG, DHSC[83], caudate nucleus[120], caudate putamen, nucleus accumbens[121], and the TNC[83,120,122,123]. Interestingly, it has also been shown to be expressed on cerebrovascular tissues[118] as well as peripheral arteries[54].

Preclinical studies

Selective 5-HT$_{1F}$ receptor agonists were developed as a novel therapeutic option for migraineurs, including those with increased cardiovascular risk. Therefore, the main concern was to study the (lack of) vascular effects, as well as their efficacy on predictive models of migraine treatment.

Cardiovascular (side) effects

An established *in vitro* model used to analyze potential contraction of human arteries is the contraction of the rabbit saphenous vein[124]. LY334370[125,126], LY344864[126,127] and lasmiditan[115] lacked vasoconstrictor effects in this model. Furthermore, in two *in vivo* studies in dogs, intracarotid administration of LY344864 did not affect carotid blood flow[128], and responses to continuous intravenous infusions of lasmiditan, in escalating cumulative doses, failed to constrict the coronary and carotid arteries; sumatriptan, on the other hand, constricted both arteries at clinically relevant doses[129]. Furthermore, in *in vitro* studies in human mammary and coronary arteries, lasmiditan was also devoid of vasoconstrictor properties, while sumatriptan was shown to contract already at subtherapeutic concentrations[64,129]. It is worth mentioning that in this study, also a threshold stimulation with the thromboxane A$_2$ analogue U46619 was performed to potentially "unmask"

vasoconstrictor responses in the internal mammary artery; notably, sumatriptan contracted at even lower concentrations, while lasmiditan failed to produce vasoconstriction. Similarly, pharmacological activation of 5-HT$_{1F}$ receptors failed to produce vasoconstriction in human cerebral and MMA[130,131].

Neuronal effects

A number of studies have shown that intravenous administration of the selective 5-HT$_{1F}$ receptor agonists LY334370[125], LY344864[132] and lasmiditan[115] inhibits protein plasma extravasation in the dura mater. However, it is important to consider that the selective 5HT$_{1D}$ receptor agonist PNU-142633 also showed a high potency for inhibiting protein plasma extravasation, but was not effective in the acute treatment of migraine[78]. Similar failures in the acute treatment of migraine were observed with endothelin receptor antagonists[133] and the highly potent inhibitor of protein plasma extravasation CP 122,288[134]. Therefore, it seems likely that 5-HT$_{1F}$ receptor agonists act through additional pathways, for instance, modulation of the trigeminovascular system, inhibition of the CGRP-mediated vasodilation and/or modulation of the pain perception pathway (see Fig. 3). In accordance with these potential mechanisms, activation of 5-HT$_{1F}$ receptors has been shown to inhibit the activation of second order neurons in the TNC in mice[135], rats[115,136-138] and cats[139], suggesting a modulation of the trigeminovascular system. Moreover, an *in vitro* study with LY344864 showed an inhibition of CGRP release in rat dura mater, but not in TNC or TG[122]. In contrast, a recent study showed that lasmiditan inhibits CGRP release in mouse dura mater, TG and TNC[140], although only supratherapeutic concentrations were studied. Therefore, more *in vitro* and *in vivo* studies (e.g. closed cranial window model) are required to confirm 5-HT$_{1F}$ receptor involvement in the inhibition of CGRP release. Nonetheless, in the pithed rat model it has been shown that activation of 5-HT$_{1F}$ receptors inhibits the release of CGRP from perivascular nerve fibres[141].

Furthermore, the expression of 5-HT$_{1F}$ receptors in the globus pallidus, NTS, the DHSC[83], caudate nucleus[120], putamen and the nucleus accumbens[121], structures associated with (anti)nociceptive pathways[89,91,93,94,142,143], suggests a possible role for 5-HT$_{1F}$ receptor activation in the modulation of nociceptive impulses.

Clinical studies

Twenty years have passed since the development of LY334370[136]; nowadays, two more 5-HT$_{1F}$ receptor agonists have been synthesized, namely, LY344864[132] and lasmiditan[115]. Only LY334370 and lasmiditan have reached clinical trials.

LY334370

The prototype for the 5-HT$_{1F}$ receptor agonists reached phase II of clinical trials[144], and the results were favourable for the acute treatment of migraine, as sustained headache response rates were higher on 60 mg (37%) and 200 mg (52%) compared to placebo (8%; $p<0.001$). However, further trials were halted due to observed liver damage in beagle dogs after treatment for longer than one month[145]. It is worth mentioning that liver damage was not reported in other species, discarding hepatotoxicity as a drug class effect.

Lasmiditan

Considered as the most promising of the 5-HT$_{1F}$ receptor agonists, lasmiditan differs from LY334370 and LY344864, as it does not possess the indole group (see Fig. 1).

Five phase I trials have been completed[146-148] for intravenous and oral formulations for safety, bioavailability, tolerability and pharmacokinetic studies. In 2007, a phase II study for the intravenous formulation was conducted[149], and later on, in 2009 for the oral formulation[150]. In both cases, lasmiditan was shown to be safe and effective in the acute treatment of migraine. Phase III trials

are ongoing, with preliminary statements showing positive results as the percentage of patients pain free at two hours was higher for 100 mg (28%) and 200 mg (32%), compared to placebo (15%; p<0.001[151]). It is worth mentioning, that the first phase III trial ("SAMURAI", NCT02439320) included a majority of migraineurs (80%) that had cardiovascular conditions or cardiovascular risk factors (*i.e.* obesity, hypertension, hyperlipidemia), the main group of patients that would benefit from the lack of vasoconstrictive properties. Two more phase III trials ("SPARTAN", NCT02605174: "GLADIATOR", NCT02565186) are under way and are aimed to compare different doses of lasmiditan, and to evaluate the safety and efficacy of long term use, respectively. Recently, preliminary results from SPARTAN have been released and showed that at two hours following the first dose of lasmiditan, the percentage of pain-free patients was statistically significantly higher for 50 mg (28%, p=0.003); 100 mg (31%, p<0.001) and 200 mg (38%, p<0.001) compared to placebo (21%)[152]. Only patients that received lasmiditan in previous trials are allowed to be included in the GLADIATOR trial. It is worth mentioning that no direct comparison has been performed between triptans and lasmiditan in clinical studies. Future phase III trials with a triptan as an active comparator would allow a better evaluation of their efficacy.

Conclusions

Triptans are 5-$HT_{1B/1D/(1F)}$ receptors agonists and are considered as the gold standard for acute migraine treatment that have been proven effective. Unfortunately, they are contraindicated in patients with cardiovascular diseases due to their vasoconstrictor (side) effects[55,65]. Furthermore, triptans are not effective in 25% of migraine patients[110]; thus, it is important to develop new antimigraine drugs that are cardiovascularly completely safe and at least equally effective. The vasoconstrictor properties of triptans are thought to be mediated via activation of 5-HT_{1B} receptors in blood vessels; this has led, in view of the contraindications in patients with cardiovascular pathologies, to the development of antimigraine drugs targeting the 5-HT_{1D} and 5-HT_{1F} receptors. While 5-HT_{1D} receptor activation was not effective in the acute treatment of migraine[78], the 5HT_{1F} receptor agonists have shown to be effective[149,150]. Furthermore, predictive preclinical models of migraine have shown that lasmiditan does not cause vasoconstriction and that its antimigraine effects are likely mediated via neural modulation[115,129,138,140]. Thus, 5-HT_{1F} receptor agonists may provide migraine patients with another type of specific acutely acting antimigraine drug, with a cardiovascular safety advantage over the triptans, and with a mechanism of action that is likely to be, at least partly, different from that of the triptans.

Based on the lack of vasoconstrictive properties and its presumably neuronal mode of action, 5-HT_{1F} receptor agonists can be considered as an entity apart from that of the triptans in antimigraine therapy.

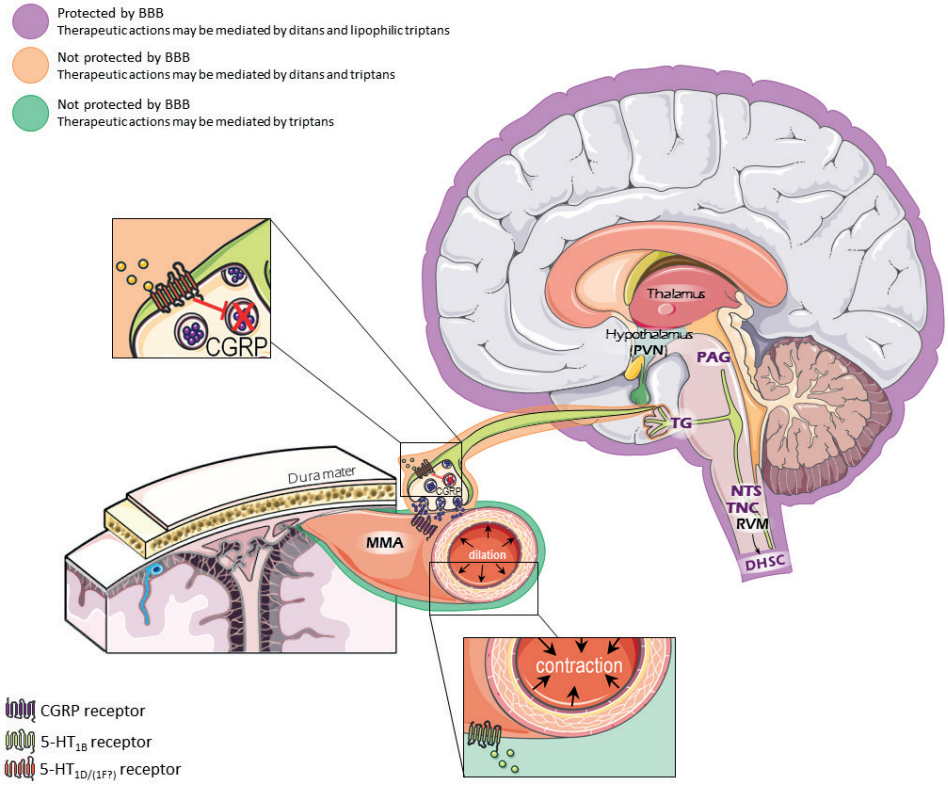

Fig. 3. Structures associated with migraine pathophysiology and/or treatment. The proposed therapeutic action of triptans is through the selective vasoconstriction of the MMA (green), as well as the inhibition of CGRP release from the sensory fibres and the modulation of the TG, since they are not protected by the BBB, where also 5-HT$_{1F}$ receptors have been described (orange). Highly lipophilic triptans could also act on the ventroposteromedial nucleus of the thalamus, PVN, PAG, LC, NTS, TNC, RVM and the (DH) SC, where 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors have been described and whose activation could also modulate the activity of the TG and/or the (anti)nociception pathways (purple). Furthermore, 5-HT$_{1F}$ receptors have also been reported in PAG, TG, TNC and the (DH)SC, which suggests a role in the modulation of the trigeminal responses as well as possible action on the (anti)nociceptive pathways (**BOLD** letters). BBB: blood brain barrier; CGRP: calcitonin-like related peptide; DHSC: dorsal horn of the spinal cord; MMA: middle meningeal artery; NTS: nucleus tractus solitarius; PAG: periaqueductal grey area; PVN: paraventricular nucleus of the hypothalamus; RVM: rostral ventromedial medulla; TG: trigeminal ganglion; TNC: trigeminal nucleus caudalis.

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Chapter IV.

Characterization of binding, functional activity and contractile responses of the selective 5 HT_{1F} receptor agonist lasmiditan

Based on: ***E Rubio-Beltrán,*** *A Labastida-Ramírez, KA Haanes, A vd Bogaerdt, AJJC Bogers, E Zanelli, L Meeus, AHJ Danser, MR Gralinski, PB Senese, KW Johnson, J Kovalchin, CM Villalón and A MaassenVanDenBrink (2019) British Journal of Pharmacology; In Press*

Abstract

Background and purpose. Triptans are 5-$HT_{1B/1D}$ receptor agonists (that also display 5-HT_{1F} receptor affinity) with antimigraine action, contraindicated in patients with coronary artery disease due to their vasoconstrictor properties. Conversely, lasmiditan was developed as an antimigraine 5-HT_{1F} receptor agonist. To assess the selectivity and cardiovascular effects of lasmiditan, we investigated the binding, functional activity and in vitro/in vivo vascular effects of lasmiditan, and compared it to sumatriptan.

Experimental approach. Binding and second messenger activity assays of lasmiditan and other serotoninergic agonists were performed for human 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E}, 5-HT_{1P}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{7} receptors, and the results were correlated with their potency to constrict human isolated coronary arteries (HCA). Furthermore, concentration-response curves to lasmiditan and sumatriptan were performed in proximal and distal HCA, internal mammary and middle meningeal arteries. Finally, anesthetized female Beagle dogs received intravenous infusions of lasmiditan or sumatriptan in escalating cumulative doses, and carotid and coronary artery diameters were measured.

Key results. Lasmiditan showed high selectivity for 5-HT_{1F} receptors. Moreover, the functional potency of the analyzed compounds to inhibit cAMP increase through 5-HT_{1B} receptor activation positively correlated with their potency to contract HCA. In human isolated arteries, sumatriptan, but not lasmiditan, induced contractions. Likewise, in vivo, sumatriptan decreased coronary and carotid artery diameters at clinically relevant doses, while lasmiditan was devoid of vasoconstrictor activity at all doses tested.

Conclusions and implications. Lasmiditan is a selective 5-HT_{1F} receptor agonist devoid of vasoconstrictor activity. This may represent a cardiovascular safety advantage when compared to the triptans.

Introduction

Migraine is a neurologic disease characterized by throbbing unilateral headaches of moderate to severe intensity, accompanied by nausea, vomiting, photophopbia and/or phonophobia (Headache Classification Committee of the International Headache Society, 2018). It has an estimated prevalence of 15% in the global population, with women being three times more affected than men[1,2].

Currently, the specific therapies for acute antimigraine treatment are the triptans, selective 5-$HT_{1B/1D}$ receptor agonists that also display varying levels of 5-HT_{1F} receptor affinity. Unfortunately, not all patients respond to triptans [3] and they are contraindicated in patients with cardiovascular disease, due to their contractile properties via activation of 5-HT_{1B} receptors in coronary arteries[4-7]. Therefore, there is a need for novel antimigraine drugs for the patients that are not respondent to the current available treatments, but also, for those patients with cardiovascular disease.

Based on results from preclinical studies, the 5-HT_{1F} receptor agonist, lasmiditan, was developed for acute antimigraine treatment[8,9] and Phase III trials showed positive results[10]. Considering the increased cardiovascular risk of migraine patients[1,11-13], and the presence of 5-HT_{1F} receptors in the vasculature[14-16], it is important to determine whether lasmiditan lacks affinity for *human* 5-HT_{1B} receptors and whether activation of 5-HT_{1F} receptors will result in vasoconstrictive responses. On this basis, the aim of this study was to investigate the pharmacological properties of lasmiditan, and in particular: (i) to assess the selectivity and functional activity of lasmiditan, triptans and other 5-HT receptor ligands on various *human* 5-HT receptors; (ii) to analyze its potential to induce vasoconstriction in *in vitro* (human isolated proximal and distal coronary, internal mammary and middle meningeal arteries) and *in vivo* (carotid and coronary artery diameters in anesthetized dogs) preclinical models; and (iii) to compare our findings with lasmiditan to those obtained with sumatriptan, one of the most prescribed triptans to treat acute migraine.

We hypothesise that, unlike sumatriptan, lasmiditan selectively activates the *human* 5-HT_{1F} receptor and does not induce vasoconstriction in the above *in vitro* (including human coronary arteries) and *in vivo* vascular models.

Materials and methods

Cell membrane preparation

CHO-K1 cells expressing the human recombinant 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B} or 5-HT_7 receptors, were grown prior to the test in media without antibiotic at Ogeda S.A (Gosselies, Belgium). Cells were prepared using a protocol from Ogeda. In brief, cells were harvested by scraping from the culture vessels in ice-cold Ca^{2+}- and Mg^{2+}-free Phosphate-buffered saline (PBS). The cells were then centrifuged for 10 min at 5,000 x g and 4°C and the pellets were suspended in buffer A (15 mM Tris-HCl pH 7.5; 2 mM $MgCl_2$; 0.3 mM EDTA; 1 mM EGTA) and homogenized in a glass-glass homogenizer. The crude membrane fraction was collected by 2 consecutive centrifugation steps at 35,000 x g and 4°C for 30 min separated by a washing step in buffer A. The final membrane pellet was suspended in buffer B (75 mM Tris-HCl pH 7.5; 12.5 mM $MgCl_2$; 0.3 mM EDTA; 1 mM EGTA; 250 mM sucrose) and flash-frozen in liquid nitrogen. Protein content was determined by the BCA method (Interchim, UP40840A).

Radioligand binding competition assay

Competition binding was performed in duplicate in the wells of a 96-well plate containing binding buffer (optimized for each receptor), cells membrane extracts (approximately 20,000 cells distributed in the 96-well plate), radiotracer and test agonist. Nonspecific binding was determined by co-incubation with 200-fold excess of competitor. Cells were incubated and exposed to varying concentrations (1 pM to 10 µM) of a range of displacer agonists (see below). The samples were incubated in a final volume of 0.1 mL and then filtered over Unifilter plates (Perkin Elmer, Massachusetts, United States) pre-treated for 2 hours to limit tracer nonspecific binding. Filters were washed five times with 0.5 mL of ice-cold washing buffer (tris 50 mM pH 7.4) and 50µL of Microscint 20 (Perkin Elmer, Massachusetts, United States) were added to each filter. The plates were incubated 15 min at room temperature on an orbital shaker and the counted with a TopCount™ (Perkin Elmer, Massachusetts, United States) for 1 min per well.

cAMP HTRF assay for G_i coupled receptors

Concentration-response curves were performed in parallel with the agonists. For agonist tests, 12 µl of cells were mixed with 6 µl of the test compound (at increasing concentrations) and 6µl of forskolin, then incubated 30 min at room temperature. After addition of the lysis buffer and 1 h incubation, cAMP concentrations were estimated according to the manufacturer specification with the HTRF kit (Cisbio International, Codelet, France). In brief, increasing concentrations of agonists were added to stably transfected cells in buffer in an Optiplate (PerkinElmer Life Sciences, Massachusetts, United States). The plates were incubated, and cells were then lysed by the addition of HTRF reagents (cAMP-Cryptate and anti-cAMP-d_2 reagents) and diluted in lysis buffer, followed by incubation at room temperature. As 5-HT_{1B} receptors have been reported in naïve CHO cells[18], we also tested 5-carboxamidotryptamine (5-CT, the reference agonist for the 5-HT_{1B} receptor), in CHO cells transfected with a non-5-HT, G protein-coupled receptor.

cAMP HTRF assay for G_s coupled receptors

Concentration-response curves were performed in parallel. For agonist tests, 12 µl of cells were mixed with 12 µl of the test compound at increasing concentrations and then incubated 30 min at room temperature. After addition of the lysis buffer and 60 min incubation, cAMP concentrations

were estimated, according to the manufacturer specification, with the HTRF kit (Cisbio International, Codelet, France). Briefly, increasing concentrations of agonists were added to stably transfected cells in buffer in an Optiplate (PerkinElmer Life Sciences, Massachusetts, United States). The plates were incubated, and cells were then lysed by the addition of HTRF reagents (cAMP-Cryptate and anti-cAMP-d_2 reagents) and diluted in lysis buffer, followed by incubation at room temperature.

IPOne HTRF assay

The assay was performed on adherent cells. For agonist testing, the medium was removed and 20 µl of assay buffer plus 20 µl of the studied agonist or the reference agonist were added in each well. The plate was incubated for 60 min at 37°C with 5% CO_2. IP_1-D2 reagent and anti-IP_1 cryptate reagents were dispensed in the wells and IP_1 concentrations were then measured following the manufacturer instructions (IPOne HTRF assay kit; Cisbio International, Codolet, France). In brief, increasing concentrations of agonists were added to stably transfected cells in buffer in an Optiplate (PerkinElmer Life Sciences, Massachusetts, United States). The plates were incubated, and cells were then lysed by the addition of HTRF reagents (IP_1-D2 reagent and anti-IP_1 cryptate reagents) and diluted in lysis buffer, followed by incubation at room temperature.

GTPγ^{35}[S] assay

For agonist testing, membrane extracts expressing the receptor of interest was mixed with GDP. In parallel, $GTP_\gamma[^{35}S]$ was mixed with the beads just before starting the reaction. The following reagents were successively added in the wells of an Optiplate (Perkin Elmer, Massachusetts, United States): 50 µl of reference ligand, 10 µl of assay buffer, 20 µl of the cells:GDP mix, and 20 µl of the $GTP_\gamma[^{35}S]$:beads mix. The plate was incubated for 60 min, then centrifuged and counted with a PerkinElmer TopCount™ reader.

Agonists tested

Serotonin (5-hydroxytryptamine; 5-HT), 5-CT, ergotamine, sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan, avitriptan, alniditan, lasmiditan, LY334370 and LY344864 were tested. The radioligands and reference compounds used for the radioligand and second messenger studies are specified in Suppl. Tables 1 and 2.

Human isolated arteries collection

Coronary arteries

Coronary arteries were obtained from six "heart beating" organ donors (three males and three females; 48-62 years), who died of non-cardiac disorders less than 24 h before the tissue was taken to the laboratory. The hearts were provided by the Heart Valve Bank Beverwijk Bank (nowadays ETB-BISLIFE Tissue Bank) at that time still located in Rotterdam, from Dutch post-mortem donors, after donor mediation via Bio Implant Services/Eurotransplant Foundation (Leiden, The Netherlands), following removal of the aortic and pulmonary valves for homograft valve transplantation. Immediately after circulatory arrest, the hearts were stored at 4°C in a sterile organ protecting solution and were brought to the laboratory within the first 24 hours of death. After arrival at the laboratory, the right proximal (internal diameter 3–5 mm) and distal (internal diameter 0.5–1 mm) portions of the coronary artery were dissected and placed in a cold, oxygenated (95% O2/5% CO2) Krebs buffer solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 8.3 mM glucose; pH 7.4.

Internal mammary arteries

Internal mammary arteries (internal diameter 2–3 mm) were obtained peri-operatively from eighteen patients (sixteen males and two females; 51–80 years) undergoing coronary bypass

surgery. The tissue was immediately placed in a sterile organ-protecting solution and was brought to the laboratory within 15 min. Subsequently, the artery was cleaned of connective tissue and placed in a cold, oxygenated Krebs buffer solution (for composition, see above).

Middle meningeal arteries

Middle meningeal arteries (internal diameter 0.5–1.5 mm) were obtained from the dura mater of six patients (two males and four females; 12-68 years) who underwent neurosurgery. The dura mater, together with a small piece of the meningeal artery, was collected in a sterile organ-protecting solution and immediately transported to the laboratory. The dura mater and connective tissue were dissected and the artery was placed in a cold, oxygenated Krebs solution of the following composition: 119 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_2$ and 11.1 mM glucose; pH 7.4.

All arteries were used on the same day or stored overnight and used the following day for functional experiments. The studies on coronary arteries were approved by the Scientific Advisory Board of the Rotterdam Heart Valve Bank. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, approved the study protocols with regard to mammary arteries and middle meningeal arteries.

Isometric tension measurements

Proximal coronary arteries were cut into segments of 2–4 mm length, excluding distinct, macroscopically visible atherosclerotic lesions. The segments were mounted on stainless steel hooks in 15 mL organ baths filled with oxygenated Krebs buffer solution at 37°C. After equilibration for at least 30 min and a wash every 15 min, the vessel segments were stretched to a stable tension of about 15 mN, with the optimal pretension as determined earlier[5]. Changes in tissue tension were measured with an isometric force transducer (Harvard, South Natick, MA, U.S.A.) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

The distal coronary, internal mammary and middle meningeal arteries were cut into circular 1-2 mm long segments and mounted in Mulvany myographs (Danish Myo Technology, Aarhus, Denmark) between two parallel small stainless-steel wires (40 µm). All the baths were filled with warm Krebs buffer and aerated with carbogen. The tension was normalized to 90% of I_{100} for all segments, the diameter when transmural pressure equals 100 mm Hg[19]. Data were recorded using a LabChart data acquisition system (AD Instruments Ltd, Oxford, UK).

Experimental protocols

A paired parallel set up (*i.e.* all compounds were tested in different segments obtained from the same artery) was used. Initially, all segments were exposed to 30 mM KCl to 'prime' the tissue for stable contractions. After washout, the tissue was exposed to 100 mM KCl to determine the maximal contractile response. After further washout, a concentration-response curve to vehicle, sumatriptan or lasmiditan was constructed, using whole logarithmic steps from 1 nM up to 10 µM. After finishing the curve and washing several times until reaching equilibrium, the functional integrity of the endothelium was verified by observing relaxation to substance P (10 nM; coronary and meningeal arteries) or bradykinin (1 µM; mammary arteries), after precontraction with thromboxane A_2 analogue U46619 (10-100 nM)[4,5].

Furthermore, in the internal mammary arteries, a concentration-response curve to lasmiditan and sumatriptan was also constructed after adding threshold concentrations of U46619 (i.e. concentrations eliciting a contraction of ~10% of 100 mM KCl response, determined in quarter logarithmic steps), used to unmask contractile properties of some agonists in the presence of an increased tension, as previously described[20]. These contractile responses were evaluated post-hoc

in the absence (relaxation to bradykinin <18%) or presence (relaxation to bradykinin >18%) of functional endothelium; for this, endothelial function data was divided in percentiles, where values below the 50th percentile were considered without endothelium, and above the 50th percentile were considered with endothelium. Also, segments were preincubated with clinically relevant concentrations of sumatriptan (0.3 μM) or lasmiditan (1 μM), and followed by a concentration-response curve to lasmiditan or sumatriptan, respectively, to evaluate the possible interactions (i.e. augmented vasoconstriction) between agonists. The clinically relevant concentration of sumatriptan was calculated as previously described[5]; in the case of lasmiditan, it was estimated based on the C_{max} observed in humans following a 100 mg dose (0.25 μM)[21].

Correlation between binding (pK$_i$) and the contractile potency of lasmiditan and other triptans

We related previous[6,22-24] and current data obtained (see Results) to the potency of these compounds to contract the human isolated coronary artery. In case a compound failed to contract human coronary artery, a fixed pEC_{50} value of 5 was set. Our pK_i values used for this correlation are in agreement with those previously published in the literature (Suppl. Table 4, Suppl. Fig. 1).

Animal preparations

Although *in vitro* experiments with human isolated arteries provide invaluable information on vasoconstrictive responses in specific vascular beds, to discard hemodynamic changes after systemic administration of novel experimental therapeutic compounds an in vivo model is necessary. The beagle dog is a well-accepted species that has been in use for several years to predict human cardiovascular responses to novel experimental therapeutic compounds[25]. Therefore, a total of eighteen adult female Beagle dogs (*Canis familiaris*) were selected from the CorDynamics, Inc. animal colony. These animals were obtained from Marshall BioResources (North Rose, N.Y., U.S.A.). Upon receipt at the Biologic Resources Laboratory (BRL) of the University of Illinois-Chicago, dogs were examined by a BRL veterinary personnel to ensure acceptable health status. Veterinary care was provided by the veterinarians and staff employed by the BRL. Dogs were acclimatized for at least 7 days prior to use, and were pair-housed in runs (meeting the size requirement set forth by the USDA Animal Welfare Act) with various cage-enrichment devices. Room temperature set at 18-27°C, humidity at 30–70%, and fluorescent lights timed to give a 12 hour-light and 12 hour-dark cycle. Harlan Certified Canine food (25% Protein Diet #2025C, Harlan Teklad, Madison, WI) was fed daily (500 grams per day) and water was freely available in their runs. At the day of their terminal experiment, the animals were 10.0 to 11.5 months old, and their body weights ranged from 5.4 to 7.9 kg. Body weights were measured twice (approximately one week between measurements) prior to each animal's terminal procedure. Dogs were fasted for 16-18 hours prior to dosing.

All experimental protocols were approved and conducted by CorDynamics in compliance with the US FDA Good Laboratory Practice guidelines (21 CFR Part 58), the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, the Office of Laboratory Animal Welfare, ARRIVE guidelines for reporting experiments involving animals[26]; and the current guidance on experimental design and analysis for the British Journal of Pharmacology[27]. For more specific details on design and statistical analysis, see below the sections entitled: sample size calculation, randomization and blinding; and data presentation and statistical evaluation.

General methods

Dogs were dosed with morphine subcutaneously (s.c., 1 mg·kg^{-1}) approximately 10-20 min prior to administration of propofol anaesthesia i.v. (5-6 mg·kg^{-1}) to allow tracheal intubation. They were placed on a ventilator with isoflurane delivered at 1-2% in oxygen to maintain anaesthesia

throughout the experiment and s.c. morphine (0.5 mg·kg^{-1}) was administered approximately every two hours while under anaesthesia. The local anaesthetic bupivacaine was infiltrated into the incision sites. A continuous 0.9% NaCl solution for injection drip (approximately 10 mL·kg^{-1}·hour^{-1}) was maintained until the start of dosing at which time it was discontinued. Dogs were placed on a heating pad set to maintain the animal's body temperature at approximately 37ºC and their body temperature was monitored throughout the experiment by placing a rectal temperature probe. Additionally, surface ECG leads were placed for anaesthesia monitoring throughout the experimental protocol.

A mid-lateral neck incision was made and the left common carotid artery was exposed. A Transonic Systems Inc. (Ithaca, N.Y., U.S.A.) blood flow probe and two Sonometric Corporation (London, Ontario, Canada) crystals for arterial dimensional analysis were affixed to the artery. A left lateral thoracotomy (6th intercostal space) was performed and the left circumflex coronary (LCX) artery was exposed. A Transonic Systems Inc. blood flow probe and two Sonometric Corporation crystals for arterial dimensional analysis were affixed to the artery. A solid-state high-fidelity pressure catheter (Millar Inc., Houston, TX, U.S.A.) for measurement of arterial pressure (mean, MAP; systolic, SAP; and diastolic, DAP) and heart rate was inserted into a femoral artery and secured in place with silk suture. An indwelling catheter was placed into the femoral vein for collecting blood (2 mL) prior to the start of dosing and at the end of each 20 min infusion period (see experimental protocol) for bioanalytical analysis.

Experimental protocol

Upon completion of the general instrumentation, a 15 min equilibrium period was allowed for a stable hemodynamic condition. Baseline values (defined as the average of the three 5 min values at the aforementioned 15 min) of MAP, heart rate, and carotid and left circumflex coronary artery diameter and flow were determined. Subsequently, the 18 dogs were randomly assigned into three groups (n=6 each), which received vehicle (saline), lasmiditan (0.03, 0.13, 1.13, 4.13 and 11.13 mg·kg^{-1}) or sumatriptan (0.03-11.3 mg·kg^{-1}), respectively. All treatments were filtered through a 0.2 µM membrane and administered i.v. in the escalating cumulative doses mentioned above. Dose-intervals amongst different treatments were administered each 20 min. At the end of the experiment, dogs were euthanized while under anaesthesia via a barbiturate overdose.

Sample size calculation, randomization and blinding

Sample size calculation. The animal sample size (n=6 each group) was calculated by CorDynamics based on their previous studies[28]. For the *in vitro* studies, we based the experimental number (n=5-7 each group) on previous studies from our group[5,29].

Randomization. For the *in vitro* experiments, all artery segments were cut into rings and randomly assigned to a bath, then the treatment group was designed by using a table of random numbers. For the *in vivo* experiments, the animals initially divided in sets (n=6 each group as described above), were randomly assigned to study groups by CorDynamics staff.

Blinding. For the radioligand and second messenger assays, the analyst was not blinded to the compounds but to the research hypothesis. For the vascular *in vitro* experiments, values were calculated using the dose-response auto-analyze selection feature of LabChart. During the analysis, the investigator was unaware of which concentration response curve was being analyzed. The *in vivo* experimental values (*i.e.* the changes in MAP or artery diameter) in each group of animals were simultaneously obtained by at least two different CorDynamics investigators, with at least one of the investigators blinded.

Data presentation and statistical analysis

All data in the text and Fig.s are presented as the mean±SEM from (n) experiments, as shown in the Fig. legends. Data and statistical analysis comply with the recommendations on analysis and experimental design in pharmacology[27].

Radioligand binding and second messenger activity

Reference compounds were tested at several concentrations in duplicate to obtain a concentration-response curve, and an estimated pEC_{50} (negative logarithm of the concentration eliciting 50% of the maximal contractile response, *i.e.* E_{max}) or pIC_{50} value (negative logarithm of the concentration that displaced 50% of the radioligand) was calculated using XLFit (IDBS, Guildford, United Kingdom). Additionally, the reference values obtained were compared to historical values obtained from the same receptor and used to validate the experimental session. A session was considered as valid only if the reference value was found to be within a 0.5 log interval from the historical value, for assays where historical values (determined in at least 5 experiments) were available[30-32]. For the new assays developed in this study (*i.e.* 5-ht_{1E} receptor), the two independent pIC_{50} determined must be concordant with a 1 log unit interval for the assays to be validated. When less than 50% inhibition of binding or second messenger activation was obtained at 10 µM a pIC_{50}/pEC_{50} of "<5" was set.

Human isolated arteries experiments

For the human vessels *in vitro* studies, concentration-response curves were analyzed using GraphPad software (GraphPad Software Inc., San Diego, CA, U.S.A.) to determine pEC_{50} values as previously reported[5]. When a plateau in the concentration-response curve was not reached, the response observed with the highest concentration used (*i.e.* 100 µM) was considered as E_{max}. Differences between pEC_{50} and E_{max} values of the compounds were evaluated with Tukey's test, once an analysis of variance (ANOVA) for paired data had revealed that the samples represented different populations. Values of $P<0.05$ were considered to indicate significant differences.

In vivo *studies*

In the *in vivo* studies, each hemodynamic parameter was analyzed with a repeated measure analysis of covariance (RANCOVA) for changes from baseline at time intervals of 5, 10, 15, and 20 min for each of the 5 dose levels. The model factored the treatment (TRT), the time after dose (TIME), and the interaction of time after dose with treatment (TRT*TIME). The SAS® procedure PROC MIXED was used for analysis with TIME as the repeated effect and ANIMAL as the subject. The covariance between errors from the same animal at different time points was selected based on the corrected Akaike's Information Criterion from selected covariance structures of VC, AR(1), UN, and CS. Non-monotonic dose-responses were evaluated. Within the framework of the RANCOVA, comparisons were made for vehicle *vs*. lasmiditan-treated animals and for vehicle *vs*. sumatriptan-treated animals. If TRT*TIME was significant, the comparisons were conducted for each time interval using an analysis of covariance (ANCOVA) model with an effect for treatment and baseline as a covariate. If only the TRT effect was significant, the comparison was conducted across the pooled time intervals for the overall phase only. These non-monotonic treatment group comparisons were conducted at the $p=0.01$ significance level. Baseline data was analyzed with an ANOVA for each time interval. Factors in the model included treatment (TRT). All statistical analyses were conducted with SAS® version 9.2. After the database lock, post-hoc analyses for coronary artery diameter and carotid artery diameter (primary endpoints) at the clinically relevant time interval 20 min (completion of dose administration) for each cumulated dose (0.03-11.13 $mg{\cdot}kg^{-1}$) were performed for comparisons between sumatriptan and vehicle. A significance level of $p\leq0.025$ was used for the RANCOVA using Bonferroni correction of two tests.

Compounds

The compounds used in the present study (obtained from the sources indicated) were: 5-HT hydrochloride, naratriptan hydrochloride, almotriptan malate, avitriptan fumarate and sumatriptan succinate (Sigma Chemical Co., St. Louis, MO, U.S.A.); lasmiditan hemisuccinate (Eli Lilly & Co., Indianapolis, IN, U.S.A.); 5-CT maleate, sumatriptan succinate, zolmitriptan, rizatriptan benzoate, eletriptan hydrobromide, donitriptan hydrochloride, LY334370 hydrochloride and LY344864 hydrochloride (Tocris Bioscience Co., Park Ellisville, MO, U.S.A.); ergotamine tartrate (TEVA Pharmaceutical Industries Ltd., Petach Tivka, Israel) and alniditan salt (kind gift of Janssen Pharmaceutica, Beerse, Belgium).

All compounds were dissolved in distilled water or physiological saline for the *in vitro* and *in vivo* studies, respectively. These vehicles had no effect on the baseline MAP values or artery diameter (not shown). Fresh solutions were prepared for each experiment. The doses mentioned in this text refer to the free base of substances.

Results

Pharmacological characterization of lasmiditan

As shown in Table 1, radioligand studies revealed that lasmiditan selectively binds to the human 5-HT_{1F} receptor. On the other hand, the triptans almotriptan, avitriptan, eletriptan, frovatriptan, naratriptan, sumatriptan and zolmitriptan showed affinity for 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors; while alniditan, donitriptan, ergotamine and rizatriptan had affinity for 5-HT_{1B} and 5-HT_{1D} receptors. Most importantly, when analyzing their second messenger activity, we observed that ergotamine is an agonist of the 5-$HT_{1A/B/D}$, 5-$HT_{2A/B}$ and 5-HT_{7} receptors (but not 5-HT_{1F} receptor). Similar as above, sumatriptan, zolmitriptan, naratriptan, almotriptan, eletriptan, frovatriptan and avitriptan are agonists of the 5-$HT_{1B/1D/1F}$ receptors. Lasmiditan, as well as LY344864, displayed a high potency only for the 5-HT_{1F} receptor (Table 2). In Fig.1, the agonistic profile of the different antimigraine drugs tested on the 5-$HT_{1B/D/F}$ receptors (*i.e.* relevant for migraine therapy), are represented. When comparing our results with those previously published, our values are in agreement with those in the literature (see Suppl. Tables 4-5, Suppl. Fig. 1). Moreover, no functional responses to 5-CT were observed in the CHO cells transfected with an unrelated G protein-coupled receptor (Suppl. Fig. 2).

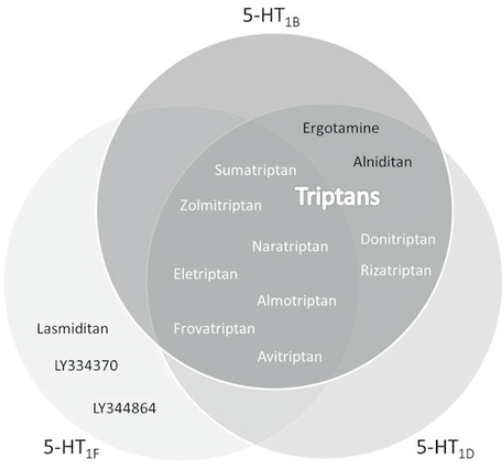

Fig. 1. Summary of the agonist profiles (pEC_{50} >7) of the antimigraine drugs tested on the 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors. Redrawn from[37].

Table 1. Summary of pIC_{50} (negative logarithm of the molar concentration of these compounds at which 50% of the radioligand is displaced) and pK_i (negative logarithm of the molar concentration of the dissociation constant) values of individual antimigraine drugs at 5-HT receptors. The lesser than symbol (<) indicates that less than 50% inhibition of binding was obtained at 10 μM. The radioligands used and their concentrations are described in Suppl. Table 1.

Agonist	5-HT_{1A}		5-HT_{1B}		5-HT_{1D}		5-ht_{1E}		5-HT_{1F}		5-HT_{2A}		5-HT_{2B}		5-HT_{7}	
	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i
Ergotamine tartrate	9.19	9.70	8.87	9.34	8.63	9.31	6.08	6.39	6.71	7.13	7.62	8.14	7.73	7.94	7.13	7.23
Sumatriptan succinate	6.63	7.14	7.81	8.29	8.31	9.00	5.42	5.72	7.13	7.55	<5	<5	<5	<5	6.10	6.19
Zolmitriptan	7.28	7.79	8.85	9.33	9.28	9.97	7.51	7.81	7.13	7.55	<5	<5	<5	<5	6.97	7.06
Naratriptan hydrochloride	7.31	7.82	8.75	9.22	8.62	9.30	7.83	8.13	8.33	8.75	<5	<5	<5	5.08	5.84	5.93
Rizatriptan benzoate	6.81	7.32	7.51	7.99	8.15	8.83	6.48	6.78	6.4	6.82	<5	<5	5.30	5.51	<5	<5
Almotriptan malate	6.23	6.73	7.97	8.45	7.57	8.26	<5	<5	7.15	7.57	<5	<5	<5	<5	6.36	6.46
Eletriptan hydrobromide	8.20	8.71	8.80	9.28	9.31	9.99	6.91	7.21	7.35	7.77	5.42	5.94	6.14	6.35	6.61	6.70
Frovatriptan racemate	6.83	7.34	8.09	8.57	8.10	8.78	<5	5.18	6.50	6.92	<5	<5	<5	<5	6.88	6.97
Donitriptan hydrochloride	7.42	7.93	9.29	9.77	9.18	9.86	5.47	5.77	<5	5.18	5.83	6.35	5.88	6.09	6.12	6.21
Avitriptan fumarate	7.20	7.71	8.32	8.80	8.42	9.11	5.15	5.45	6.69	7.11	5.11	5.63	5.73	5.94	6.03	6.12
Alniditan dihydrochloride	8.81	9.32	8.93	9.41	8.66	9.35	5.98	6.28	6.02	6.44	<5	5.43	6.67	6.88	7.16	7.26
Lasmiditan hemisuccinate	5.88	6.39	5.54	6.02	5.62	6.31	5.54	5.84	8.09	8.51	<5	<5	5.01	5.22	<5	<5
LY334370 hydrochloride	7.98	8.49	6.74	7.21	6.24	6.92	6.83	7.13	9.03	9.45	5.11	5.63	5.98	6.19	5.66	5.75
LY344864 hydrochloride	6.12	6.63	6.13	6.61	5.83	6.52	6.05	6.35	8.38	8.80	5.11	5.63	5.31	5.52	5.69	5.78

Table 2. Summary of pEC_{50} values of cAMP (5-$HT_{1A/1B/1E/1F}$ and 5-HT_7), GTPγS (5-$HT_{1A/1B/1D/1E/1F}$) and IP (5-HT_2) assays of individual antimigraine drugs at 5-HT receptors. These values represent the negative logarithm of the molar concentration of these compounds at which 50% of their maximal response is exerted. The lesser than symbol (<) indicates that less than 50% response was obtained at 10 μM. The reference compounds used and their concentrations are described in Suppl. Table 2.

Agonist	5-HT_{1A}		5-HT_{1B}		5-HT_{1D}	5-ht_{1E}		5-HT_{1F}		5-HT_{2A}	5-HT_{2B}	5-HT_{7}
	cAMP	GTPγS	cAMP	GTPγS	GTPγS	cAMP	GTPγS	cAMP	GTPγS	IP	IP	cAMP
Ergotamine tartrate	9.78	9.63	9.94	9.52	9.43	5.95	5.74	5.97	6.30	9.25	8.72	7.09
Sumatriptan succinate	<5	<5	7.32	7.91	8.30	5.99	5.79	8.03	6.80	<5	<5	5.22
Zolmitriptan	<5	5.52	7.87	8.42	9.51	8.18	7.81	8.00	6.67	<5	<5	6.28
Naratriptan hydrochloride	<5	6.52	8.05	8.86	8.80	7.75	8.17	8.38	8.05	<5	<5	<5
Rizatriptan benzoate	<5	<5	7.08	7.56	8.11	7.34	6.90	6.54	5.91	<5	5.49	<5
Almotriptan malate	<5	5.48	7.08	7.85	7.75	<5	<5	7.79	6.90	<5	5.20	<5
Eletriptan hydrobromide	5.74	6.38	8.00	8.09	9.04	7.53	6.90	8.13	6.88	6.07	6.81	6.45
Frovatriptan racemate	<5	6.12	7.98	8.14	8.36	5.04	<5	7.10	6.35	<5	<5	7.42
Donitriptan hydrochloride	5.94	6.74	9.96	9.52	9.51	<5	<5	<5	<5	8.10	7.61	5.23
Avitriptan fumarate	<5	6.19	8.57	8.68	9.27	5.52	<5	7.09	6.05	6.91	6.41	5.38
Alniditan dihydrochloride	7.00	7.29	8.87	8.90	8.20	5.68	5.21	5.92	5.17	<5	7.15	6.32
Lasmiditan hemisuccinate	<5	<5	<5	<5	6.64	6.17	5.34	8.43	7.80	<5	<5	<5
LY334370 hydrochloride	5.84	6.96	6.52	5.80	6.92	7.53	6.95	9.08	9.38	<5	<5	<5
LY344864 hydrochloride	<5	<5	<5	5.82	6.93	6.22	6.12	8.72	7.85	<5	<5	<5

Human isolated arteries

In the human isolated coronary arteries, sumatriptan induced significant contractions in a concentration dependent manner in the proximal (E_{max} 39±12%, pEC_{50} 6.4±0.2; n=6) and distal (E_{max} 59±41%, pEC_{50} 6.02±0.2; n=6) coronary portions, even at clinically relevant concentrations (Fig. 2). In contrast, lasmiditan was devoid of any significant contractile effects in both coronary portions, even at its highest concentration. Moreover, Fig. 3 shows the effects of lasmiditan and sumatriptan on internal mammary arteries in the absence and presence of endothelium. Sumatriptan induced concentration-dependent contractions in segments with (E_{max} 46±18%, pEC_{50} 6.07±0.07; n=5) and without functional endothelium (E_{max} 31±12%, pEC_{50} 5.6±0.94; n=7). After precontraction with threshold concentrations of U46619, sumatriptan also produced concentration-dependent contractions in the presence (E_{max} 63±19%, pEC_{50} 6.83±0.05, n=5) and absence (E_{max} 59±16%, pEC_{50} 6.02±0.59; n=7) of functional endothelium. In contrast, lasmiditan was devoid of any significant contractile effects in internal mammary arteries, even after a modest precontraction with U46619.

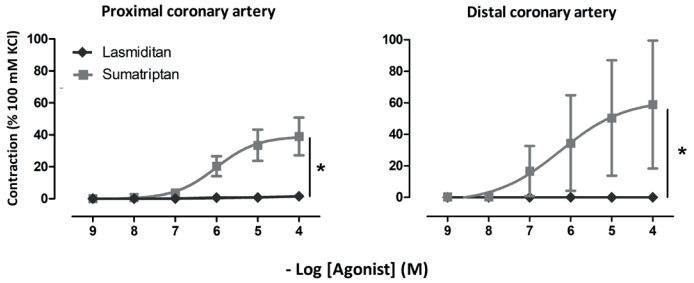

Fig. 2. Contractile responses to lasmiditan and sumatriptan (1 nM – 100 μM) in the human isolated proximal (left) and distal (right) coronary arteries; *P<0.05; n=6 each.

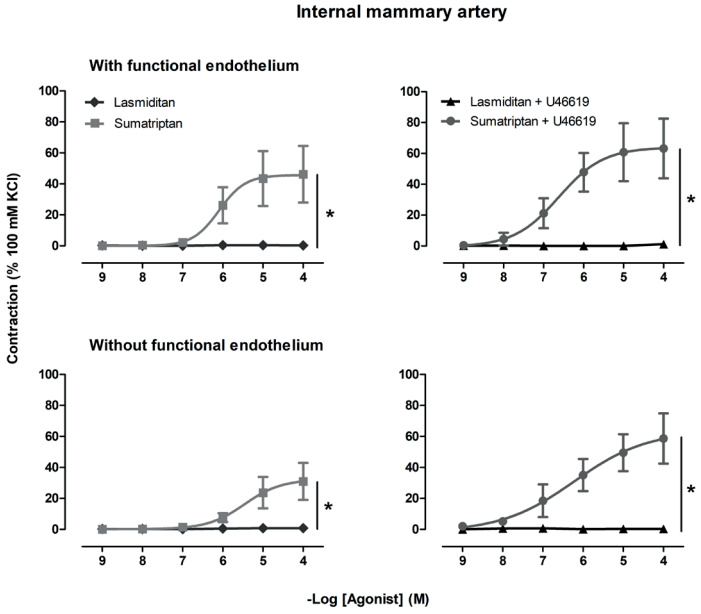

Fig. 3. Contractile responses to sumatriptan and lasmiditan (1 nM–100 μM) in the absence (left) and presence (right) of a threshold precontraction with U46619 (1–10 nM) in human isolated internal mammary arteries with (upper panel, n=5) and without (lower panel, n=7) functional endothelium; *P<0.05.

In middle meningeal arteries, sumatriptan induced significant concentration-dependent contractions (E_{max} 73±13%, pEC_{50} 6.32±0.15; n=6), whereas lasmiditan did not induce any significant contraction at all concentrations tested (E_{max} 0±0%, Fig. 4).

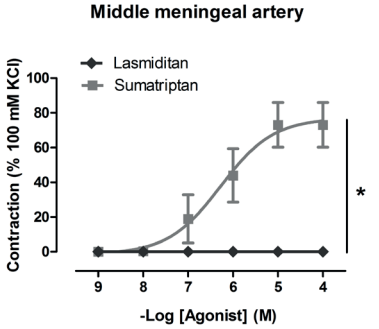

Fig. 4. Contractile responses to sumatriptan and lasmiditan (1 nM – 100 μM) in the human isolated middle meningeal arteries; *P<0.05; n=6 each.

Interaction experiments

As shown in Fig. 5, in human isolated internal mammary arteries, after preincubation with lasmiditan (1 μM), no changes in the contractile responses to sumatriptan were observed when compared to the concentration-response curve to sumatriptan alone (E_{max} 59±16%, pEC_{50} 5.34±0.1 *vs*. E_{max} 51±19%, pEC_{50} 5.71±0.7; n=5 each). In addition, the highest concentration of lasmiditan produced a non-significant vasodilation when preincubated with sumatriptan's clinically relevant concentration (0.3 μM) when compared to the concentration-response curve to lasmiditan without sumatriptan (E_{max} -4.8±5.95% *vs*. E_{max} 0±0% respectively; n=5 each).

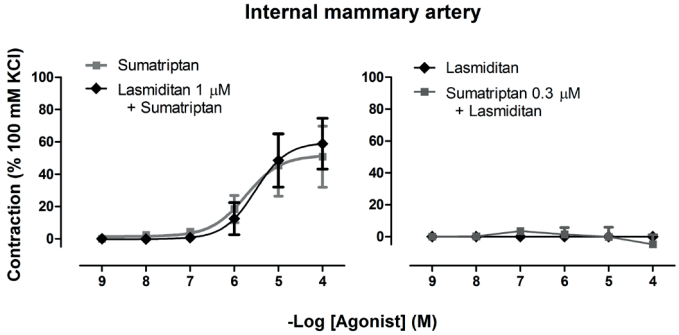

Fig. 5. Contractile responses to sumatriptan and lasmiditan (1 nM – 100 μM) in the internal mammary artery, after preincubation with the clinically relevant concentration of sumatriptan (0.3 μM) or lasmiditan (1 μM), and followed by a concentration-response curve to lasmiditan or sumatriptan, respectively (n=6 each).

Correlation between binding (pK_i) and the contractile potency of lasmiditan and other triptans

As shown in Fig. 6, the potency of the compounds tested to contract the human isolated coronary artery was positively correlated with their potency to bind the 5-HT_{1B} receptor, whereas it was negatively correlated for the 5-HT_{1F} receptor. This was also observed when correlating the pEC_{50} values obtained in our second messenger assays and the contractile potency of the compounds tested in the human coronary arteries (Suppl. Fig. 3).

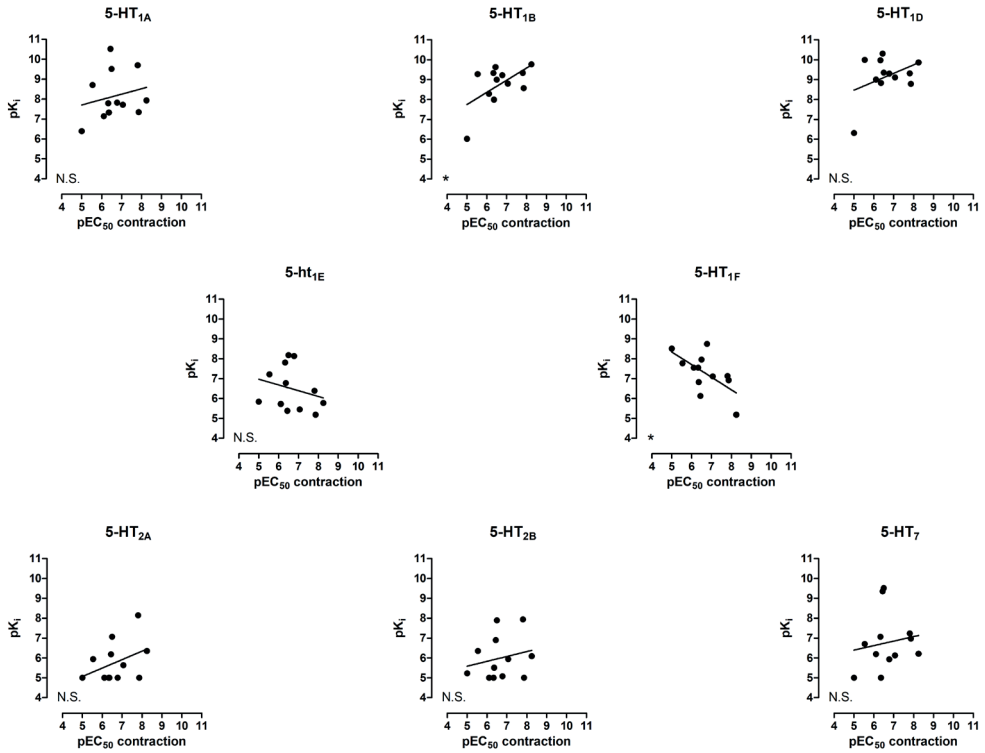

Fig. 6. Correlation between the pK_i values obtained in our study and the contractile potency of lasmiditan, triptans (sumatriptan, zolmitriptan, naratriptan, rizatriptan, eletriptan, frovatriptan, donitriptan, avitriptan) and other 5-HT receptors ligands (ergotamine, alniditan, 5HT, 5-carboxamidotryptamine) in human isolated coronary arteries; N.S., non-significant; $^{*}P<0.05$.

In vivo *studies*

In anaesthetized dogs, a directly proportional relationship was observed between lasmiditan and sumatriptan cumulative i.v. doses and their plasma concentrations; these latter values were used for validating the concentrations used in the *in vitro* studies (data not shown). Moreover, as shown in Fig. 7, changes in carotid artery diameter were not statistically significant in the lasmiditan-treated group as compared to the time-matched vehicle control group. In contrast, as expected, sumatriptan induced dose-dependent decreases in carotid artery diameter, although these effects were statistically significant only at the doses of 0.13 mg·kg^{-1} (clinically relevant) and 11.13 mg·kg^{-1} (Fig. 7). In the LCX coronary artery, lasmiditan failed to induce any statistically significant change in diameter at any dose. Conversely, statistically significant decreases in the LCX coronary artery diameter were observed at all doses in sumatriptan-treated animals as compared to the time-matched vehicle control animals, even at the lowest dose of 0.03 mg·kg^{-1}, which already corresponds to a clinically relevant dose.

Carotid blood flow was not significantly different after vehicle or clinically relevant doses of lasmiditan (0.03-1.13 mg·kg^{-1}). Lasmiditan decreased carotid blood flow significantly, but only after the supratherapeutic cumulative doses of 4.13 mg·kg^{-1} and 11.13 mg·kg^{-1}. In contrast, sumatriptan elicited a statistically significant rapid, dose-dependent, decrease in carotid blood flow at all doses tested. Regarding coronary blood flow, the administration of vehicle, lasmiditan or sumatriptan did

not elicit any statistically significant changes (data not shown). Heart rate was stable over the course of the study and no significant changes were observed in the lasmiditan or vehicle groups. In the sumatriptan–treated group, cumulative doses of 4.13 and 11.13 mg·kg^{-1} elicited dose-dependent decreases in heart rate which were statistically significant, with a peak decrease at 90 min of 16.5±6 bpm (data not shown). MAP, SAP and DAP showed no significant changes in either sumatriptan or lasmiditan-treated groups as compared to the time-matched vehicle group at cumulative doses of up to 4.13 mg·kg^{-1}. At higher doses, both lasmiditan and sumatriptan-treated groups showed a dose-dependent trend to decrease MAP, SAP and DAP; however, these changes were not statistically significant (Fig. 7).

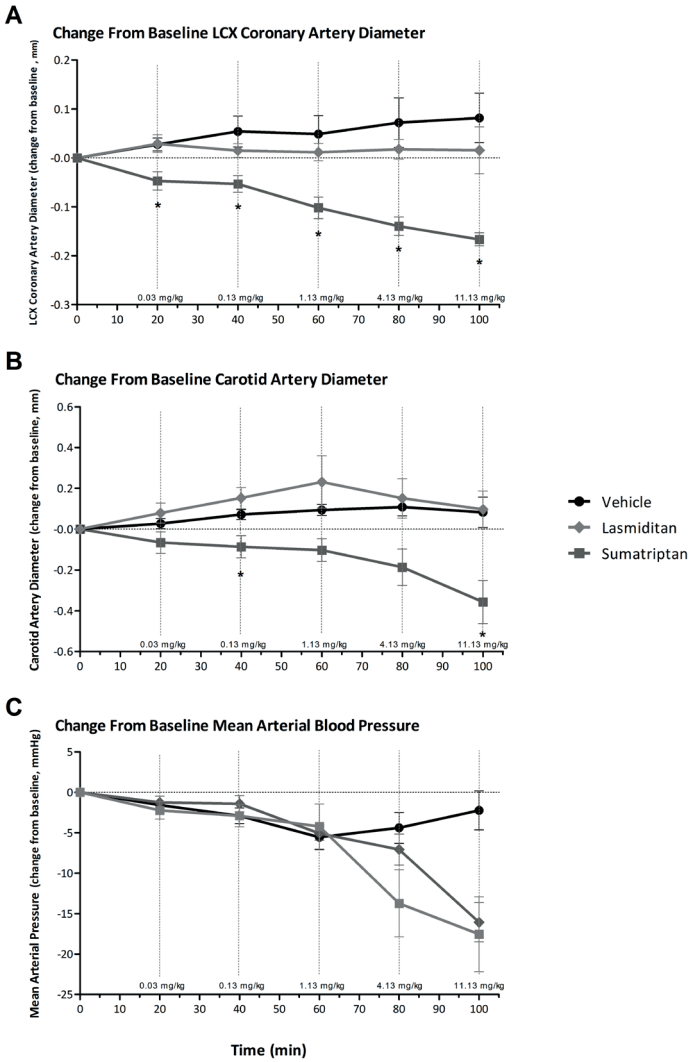

Fig. 7. Changes in the left circumflex (LCX) coronary artery diameter (A), carotid artery diameter (B) and mean arterial blood pressure (C) after the continuous infusion of lasmiditan and sumatriptan (0.03-11.13 mg·kg^{-1} each) or the corresponding infusion volumes of vehicle in female beagle dogs (n=6 each). *P<0.025 vehicle *vs.* sumatriptan post hoc analysis.

Discussion and conclusions

The current study was designed to investigate the selectivity and vasoconstrictor profile of lasmiditan, which belongs to a novel class of acute antimigraine drugs, the ditans. According to its binding and functional activity, it was confirmed that lasmiditan is a highly selective agonist of the 5-HT_{1F} receptor. Moreover, since lasmiditan was developed based on the premise that coronary vasoconstriction is a side effect of the triptans attributed to 5-HT_{1B} receptors, we studied the vasoconstrictor potential of 5-HT_{1F} agonism in two different vascular models and compared our *in vitro* and *in vivo* results to those obtained with sumatriptan, since it is the 'gold standard' triptan for acute antimigraine treatment. This allowed us to compare the results from the current study with results obtained earlier. In accordance with our previous work[4], sumatriptan induced a concentration-dependent contraction in human isolated coronary arteries, which tended to be larger in distal than in proximal coronary artery segments. This contraction was apparent at clinically relevant concentrations, and is most likely due to activation of 5-HT_{1B} receptors in vascular smooth muscle[4]. In contrast, lasmiditan did not induce a contraction at concentrations up to 100 μM (≥ 100x the clinically relevant concentrations) in either proximal or distal coronary arteries. Although moderate to intense expression of mRNA encoding the 5-HT_{1F} receptor in human coronary arteries has been described[16], presence of mRNA does not necessarily mean protein expression, which may well be the case. Thus, the physiological role of this receptor in blood vessels remains to be determined.

Subsequently, we performed more in-depth experiments in the internal mammary artery, where we studied the influence of endothelial functional quality, and the effects of a precontraction induced by the thromboxane A_2 analogue U46619, since such a precontraction is known to 'unmask' or augment contractions to other ligands, such as sumatriptan[20]. As in the coronary artery, sumatriptan contracted the internal mammary artery, similarly in both segments with and without functionally active endothelium. In accordance with earlier observations[20], the contractions to sumatriptan were augmented in the presence of U46619. In contrast, lasmiditan did not induce any contraction in the absence or presence of U46619 in either vessel segments with or without endothelium. Interestingly, in the rabbit saphenous vein, precontraction with $PGF_{2\alpha}$ unmasked a contractile response to the 5-HT_{1F} receptor agonists, LY334370 and LY344864, but only after high concentrations (>10 μM), and therefore likely due to activation of vascular 5-HT_{1B} receptors[33]. Hence, the absence of contractile responses with high concentrations of lasmiditan, even in precontracted vessels, is surprising, given the difference in affinity between sumatriptan and lasmiditan to the 5-HT_{1B} receptor. However, binding affinity does not always correlate with second messenger activation and biological response[34]. Therefore, while our radioligand studies (Table 1) showed a ~100-fold binding difference to the 5-HT_{1B} receptor between sumatriptan (pIC_{50}=7.81) and lasmiditan (pIC_{50}=5.54), our cAMP assays (Table 2) showed that the functional potency (pEC_{50}) of sumatriptan was 7.32 and lasmiditan was <5. As we could not determine the precise pEC_{50} value of lasmiditan, the potency difference between both compounds could be larger than 1000 fold and thus, explain the complete absence of vasoconstrictive responses even at supra-therapeutic concentrations such as 100 μM. This could represent a cardiovascular safety advantage over its triptan predecessors.

Additionally, as contraction of the meningeal artery is thought to contribute to the antimigraine effects of the triptans[35-37], but is not a class effect of all anti-migraine drugs (e.g. gepants), we investigated the contractions to sumatriptan and lasmiditan in human meningeal arteries. In accordance to the previously described craniovascular selectivity of the triptans[22, 38], contractions to sumatriptan were larger in this dural artery than those in the proximal coronary artery. However, as

we have also previously shown, contractions to sumatriptan in distal coronary artery (and internal mammary artery) were not significantly different from those in meningeal artery[4]. In contrast, lasmiditan was devoid of vascular effects in this cranial vessel. Therefore, the efficacy of lasmiditan as acute migraine treatment may be due to inhibition of CGRP release from perivascular fibres or direct central (antinociceptive) modulation[37].

Our binding studies showed that, as mentioned previously, most of the triptans available in the market, namely, almotriptan, frovatriptan, naratriptan, sumatriptan and zolmitriptan are also agonists of the 5-HT_{1F} receptor (Fig. 1). Furthermore, the correlation between binding and the contractile potency of the compounds tested revealed that the potency of the agonists to contract the HCA positively correlated to their potency to bind the 5-HT_{1B} receptor, whereas it negatively correlated for the 5-HT_{1F} receptor (Fig. 6) and this was also observed when correlating the contractile potency and second messenger activation (Suppl. Fig. 3). Moreover, when analyzing the correlation between second messenger activation by 5-HT_{1B} *vs*. 5-HT_{1F} receptors, also a negative correlation was observed (Supplementary Fig. 4). These results, together with our *in vitro* data suggest that, although the mRNA of both receptor subtypes has been described in human vasculature[4,14-16,22,23,39], only activation of the 5-HT_{1B} receptor will result in vasoconstriction of, at least, coronary, mammary and meningeal arteries, whereas activation of the 5- HT_{1F} receptor will not. This could suggest that 5-HT_{1F} receptors in human vasculature are not functional or, that 5-HT_{1F} receptor mRNA is not translated to protein.

When considering the acute hemodynamic effect of sumatriptan in humans, it is well-known that after subcutaneous administration, there are vasopressor responses in the systemic arterial circulation and coronary artery vasoconstriction[40]. Although we observed carotid and coronary vasoconstriction in anesthetized dogs, there were no significant increases in blood pressure, as previously reported in this animal model[41]. In fact, after high doses of sumatriptan a non-significant tendency to decrease blood pressure and significant decreases in heart rate were observed, most probably due to inhibition of vascular and cardiac sympathetic outflows via the stimulation of prejunctional 5-$HT_{1B/1D}$ receptors on perivascular[42] and cardiac[43,44] sympathetic nerves. Lasmiditan only showed a trend to decrease blood pressure at the highest (supratherapeutic dose) which, based on the affinity of lasmiditan (see Table 1), could be due to a non-selective activation of prejunctional 5-HT_{1D} receptors and subsequent inhibition of sympathetic perivascular nerves[42]. Admittedly, this has not been shown directly in dogs but in pithed Wistar rats and, in patients, no changes in blood pressure have been observed[45,46]. Further experiments, falling beyond the scope of the present study, would be required to shed more light on the mechanisms behind these responses, which are only observed at non clinically relevant doses.

In summary, our *in vitro* and *in vivo* results indicate that lasmiditan is devoid of contractile properties in isolated human and anesthetized dog arteries, respectively. This might be of particular relevance in migraine patients who have a high risk of developing cardiovascular disease, such as subjects with hemiplegic migraine, prolonged migraine with aura, or with established cardiovascular disease. Clearly, further studies are needed to evaluate the safety of lasmiditan in these specific patient populations and its effectiveness compared with triptans. Finally, clinical trials have shown that lasmiditan is effective for migraine treatment[10], suggesting a mechanism of action (partially) different to that of the triptans[27].

In conclusion, our data support our initial hypothesis that lasmiditan is a high-affinity and highly selective agonist for the *human* 5-HT_{1F} receptor that is devoid of contractile properties in human isolated blood vessels and in anesthetized canines.

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Supplementary material

Suppl. Table 1. Reference tracers (concentration, nM), and reference competitors used for radioligand binding competition assays for the different receptors studied. *Historical pIC_{50} values obtained at Ogeda S.A. (now Epics Therapeutics S.A., Gosselies, Belgium). Values represent mean values±SEM of a stated number of averaged technical duplicates (n).

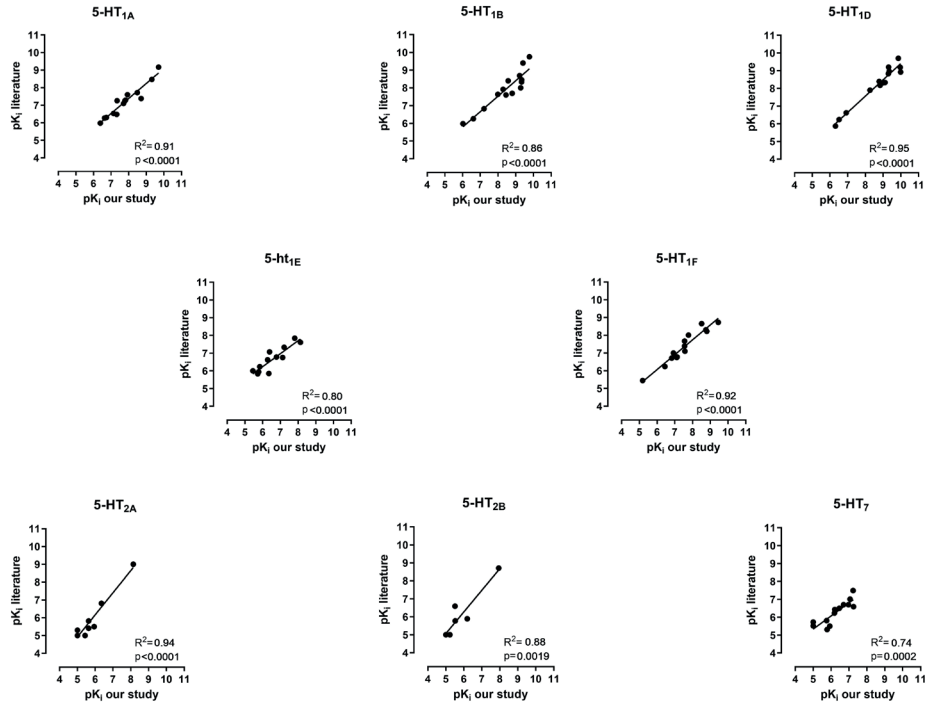
Receptor	Reference tracer	Assay concentration (nM)	Reference competitor	Historical* pIC_{50} (reference competitor)	Estimated pIC_{50} (reference competitor)	pK_i (reference competitor)
5-HT_{1A}	[^{3}H]-8-OH-DPAT	0.39	5-HT hydrochloride	8.83±0.04 (23)	9.00±0.06 (2)	9.51±0.06 (2)
5-HT_{1B}	[^{3}H]-5-CT	0.60	5-HT hydrochloride	8.42±0.10 (8)	8.61±0.19 (2)	9.09±0.19 (2)
5-HT_{1D}	[^{3}H]-5-CT	0.50	5-HT hydrochloride	8.35±0.08 (7)	8.67±0.18 (2)	9.35±0.18 (2)
5-ht_{1E}	[^{3}H]-LSD	14.0	BRL-54443	8.49±0.09 (5)	8.44±0.07 (3)	8.74±0.07 (3)
5-HT_{1F}	[^{3}H]-LSD	8.00	BRL-54443	8.59±0.17 (5)	8.54±0.12 (3)	8.96±0.12 (3)
5-HT_{2A}	[^{3}H]-Ketanserin	1.48	Ketanserin	8.22±0.18 (6)	8.17±0.04 (2)	8.69±0.04 (2)
5-HT_{2B}	[^{3}H]-Mesulergin	1.00	5-HT hydrochloride	7.67±0.09 (8)	7.68±0.05 (3)	7.89±0.05 (3)
5-HT_7	[^{3}H]-LSD	1.00	5-CT maleate	9.28±0.05 (13)	9.42±0.17 (3)	9.51±0.17 (3)

Suppl. Table 2. Reference agonists for second messenger activation assays of cAMP (5-$HT_{1A/B/E/F}$ and 5-HT_7), GTPγS (5-HT_{1D}) and IP (5-$HT_{2A/B}$). *Historical pEC_{50} values obtained at Ogeda S.A. (now Epics Therapeutics S.A., Gosselies, Belgium). Values represent mean values±SEM of a stated number of averaged technical duplicates (n).

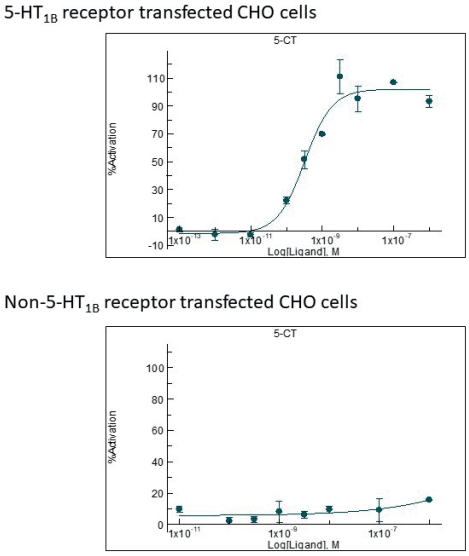
Receptor	Reference agonist	Historical* pEC_{50} cAMP/IP/ GTPγS	Estimated pEC_{50} cAMP/ IP/ GTPγS
5-HT_{1A}	5-CT maleate	9.18±0.05 (35)	9.02±0.17 (4)
5-HT_{1B}	5-CT maleate	8.78±0.07 (23)	8.80±0.01 (2)
5-HT_{1D}	5-CT maleate	9.30±0.05 (21)	9.26±0.13 (3)
5-ht_{1E}	5-HT hydrochloride	9.00±0.16 (2)	8.61±0.05 (3)
5-HT_{1F}	5-HT hydrochloride	8.94±0.15 (7)	8.69±0.16 (3)
5-HT_{2A}	α-Me-5-HT	8.68±0.05 (31)	8.33±0.06 (2)
5-HT_{2B}	α-Me-5-HT	9.70±0.09 (28)	9.63±0.08 (2)
5-HT_7	5-CT maleate	9.59±0.03 (45)	9.62±0.01 (3)

Supplementary Table 3. Summary of pEC_{50} values of vasoconstriction of the human coronary artery. These values represent the negative logarithm of the molar concentration of these compounds at which 50% of their maximal response was exerted. When a compound was devoid of vasoconstrictor activity, a pEC_{50} of 5 was set.

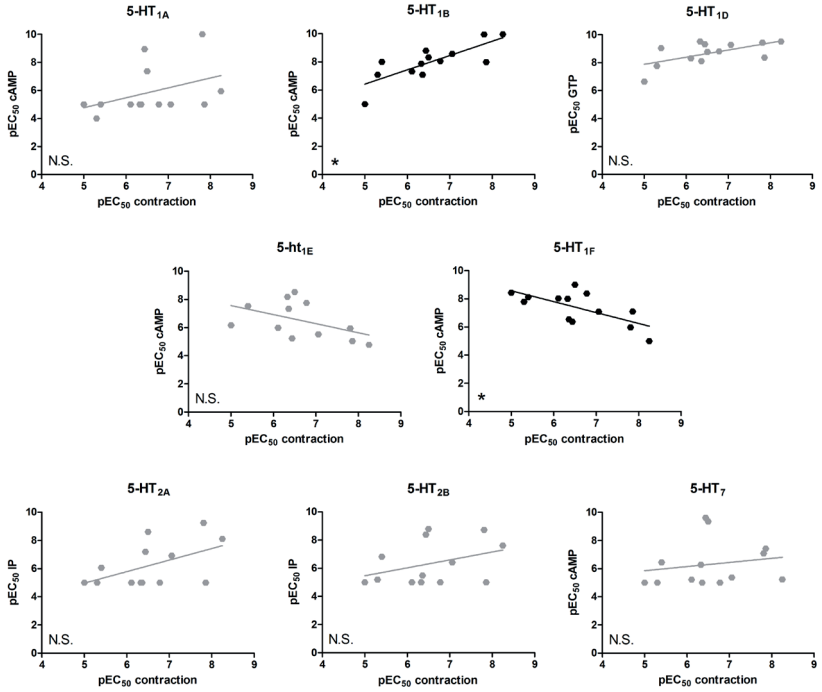
Agonist	pEC_{50}	Reference
5-HT hydrochloride	6.50	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998; Parsons et al., 1998)
5-CT maleate	6.44	(MaassenVanDenBrink, Reekers, Bax & Saxena, 2000)
Ergotamine tartrate	7.81	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Sumatriptan succinate	6.11	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Zolmitriptan	6.33	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Naratriptan hydrochloride	6.78	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Rizatriptan benzoate	6.36	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Eletriptan hydrobromide	5.54	(van den Broek et al., 2000)
Frovatriptan Racemate	7.86	(Parsons et al., 1998)
Donitriptan hydrochloride	8.25	(van den Broek et al., 2002)
Avitriptan fumarate	7.06	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998; Saxena et al., 1997)
Lasmiditan hemisuccinate	5.00	



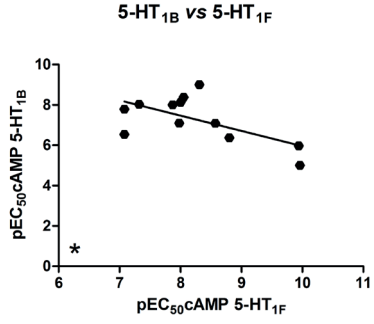
Suppl. Fig. 1. Correlation between the pK_i values obtained from literature and the pK_i values obtained in our study for lasmiditan, triptans (sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan, avitriptan) and other 5-HT receptors ligands (ergotamine, alniditan, 5-HT, 5-carboxamidotryptamine). For references see Suppl. Table 5.



Suppl. Fig. 2. Functional responses (cAMP assay) to 5-CT in CHO cells transfected with $5\text{-}HT_{1B}$ receptor (upper) and in CHO cells transfected with an unrelated G protein-coupled receptor.



Suppl. Fig. 3. Correlation between second messenger activation (*i.e.* cAMP, IP) and the contractile potency of lasmiditan, triptans (sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan, avitriptan) and other 5-HT receptors ligands (ergotamine, 5HT, 5-CT) in human isolated coronary arteries; N.S., non-significant; *P<0.05.



Suppl. Fig. 4. Correlation between the second messenger activation of 5-HT_{1B} receptor *vs* 5-HT_{1F} receptor by lasmiditan, triptans (sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan, avitriptan) and other 5-HT receptors ligands (ergotamine, 5HT, 5-carboxamidotryptamine) in human isolated coronary arteries; *P<0.05.

PART III:

Prophylactic treatment of migraine

Chapter VI.

Blocking CGRP in migraine patients – a review of pros and cons

Based on: M Deen, E Correnti*, K Kamm*, T Kelderman*, L Papetti*,* ***E Rubio-Beltrán*,*** *S Vigneri*, L Edvinsson*, A MaassenVanDenBrink*. On behalf of the European Headache Federation School of Advanced Studies (2017) Journal of Headache and Pain; 18:96.*

**Authors contributed equally*

Abstract

Migraine is the most prevalent neurological disorder worldwide and it has immense socioeconomic impact. Currently, preventative treatment options for migraine include drugs developed for diseases other than migraine such as hypertension, depression and epilepsy. During the last decade, however, blocking calcitonin gene-related peptide (CGRP) has emerged as a possible mechanism for prevention of migraine attacks. CGRP has been shown to be released during migraine attacks and it may play a causative role in induction of migraine attacks. Here, we review the pros and cons of blocking CGRP in migraine patients. To date, two different classes of drugs blocking CGRP have been developed: small molecule CGRP receptor antagonists (gepants), and monoclonal antibodies, targeting either CGRP or the CGRP receptor. Several trials have been conducted to test the efficacy and safety of these drugs. In general, a superior efficacy compared to placebo has been shown, especially with regards to the antibodies. In addition, the efficacy is in line with other currently used prophylactic treatments. The drugs have also been well tolerated, except for some of the gepants, which induced a transient increase in transaminases. Thus, blocking CGRP in migraine patients is seemingly both efficient and well tolerated. However, CGRP and its receptor are abundantly present in both the vasculature, and in the peripheral and central nervous system, and are involved in several physiological processes. Therefore, blocking CGRP may pose a risk in subjects with comorbidities such as cardiovascular diseases. In addition, long-term effects are still unknown. Evidence from animal studies suggests that blocking CGRP may induce constipation, affect the homeostatic functions of the pituitary hormones or attenuate wound healing. However, these effects have so far not been reported in human studies. In conclusion, this review suggests that, based on current knowledge, the pros of blocking CGRP in migraine patients exceed the cons.

Introduction

Migraine is a highly prevalent and disabling disorder for which treatment options are still inadequate. The underlying pathophysiology is largely unknown, but calcitonin gene-related peptide (CGRP) most likely plays an important role. The first time CGRP was hypothesized to be involved in migraine was in 1985[1]. This hypothesis was later supported by the finding of CGRP release during acute migraine attacks and the subsequent demonstration of normalization of CGRP levels in migraine patients after efficacious sumatriptan treatment[2]. In animal studies, triptans also inhibit the release of CGRP[3]. Evidence for a causative role of CGRP in migraine came from a study showing that intravenous provocation with CGRP induces migraine-like attacks in migraine patients[4]. This led to focus on this peptide and its receptor as a possible target for new migraine therapies.

CGRP and its receptor are expressed in both the peripheral and the central nervous system (CNS), including the trigeminovascular pathways. More than 30 years ago CGRP was demonstrated in trigeminal ganglion (TG) pseudounipolar neurons[5]. These neurons connect cranial structures to the central nervous system at the lower brainstem, caudal part of the trigeminal nucleus caudalis and upper spinal cord at C1-C2[6]. In the peripheral trigeminovascular system, as well as in the TG, CGRP is located in about 50% of the neurons and in unmyelinated Cfibers, whereas the CGRP receptor elements are expressed in about 40% of the TG neurons and in myelinated Aδ-fibers, which connect the PNS with the CNS[7,8]. In humans, CGRP is present in two isoforms, αCGRP and CGRP, where αCGRP is most abundantly found in primary spinal afferent from sensory ganglia, whereas βCGRP is mainly found in the enteric nervous system[6]. The CGRP receptor consists of three subunits: receptor activity-modifying protein 1 (RAMP1), calcitonin-like receptor (CLR) and receptor component protein (RCP)[9]. As well as playing a role in cranial nociception[10], CGRP is a potent general arterial vasodilator. At peripheral synapses, CGRP is released from trigeminal terminals results in vasodilation via CGRP

receptors on the smooth muscle cells of meningeal and cerebral blood vessels[8,11]. CGRP and its receptor are also located in the cardiovascular system where they are assumed to exert a protective role[9,12].

The first designer drug able to competitively block the effect of CGRP was olcegepant[13]. This nonpeptide CGRP-receptor antagonist showed high efficacy but had a low oral bioavailability[14]. This led, however, to the synthesis of several other small molecule CGRP receptor antagonists. This class was later called the gepants. Though promising with regards to efficacy, further development of some of the gepants was discontinued due to liver toxicity upon repeated exposure[15]. Encouraged by the efficacy of blocking CGRP for the treatment of migraine, monoclonal antibodies able to block either CGRP or its receptor were developed and tested in several preclinical modalities[16,17]. The antibodies are designer drugs that are highly specific for the target but about 500 times the size of gepants or triptans[6]. They have been designed for prophylactic use in frequent episodic and chronic migraine. In this review, we will discuss the pros and cons of blocking CGRP in migraine patients. We will review the efficacy and safety of already tested drugs and compare it to the efficacy and safety of topiramate, a widely-used migraine prophylactic. Additionally, we will review the possible consequences of blocking CGRP based on findings from animal studies. Lastly, we will discuss other concerns such as long-term use and cost of the treatment.

Efficacy of CGRP (receptor) blockade: Evidence from double-blind, placebo-controlled trials

In 2004, a proof-of-concept study showed that intravenous olcegepant was effective in the acute treatment of migraine[18]. Since then, five other gepants have been tested for the acute treatment of migraine[19-31]. Fig. 1 provides an overview of the efficacy data for these agents. All gepants were significantly better than placebo at achieving their primary outcome at adequate doses: pain freedom or relief at 2 hours. Only one study, a study on safety in coronary patients, could not demonstrate difference in pain freedom at 2 hours after telcagepant; however, only 165 of the planned 400 patients were included, reducing the statistical power of this study[27].

Five of these studies also included a comparison to a triptan[19,21,26,29,30]. In one of these studies, telcagepant showed a numerically higher efficacy than rizatriptan with regards to sustained pain relief[29]. In other trials, the efficacy of telcagepant, BI44370 and rimegepant showed no significant difference to that of zolmitriptan (5 mg), eletriptan (40 mg) and sumatriptan (100 mg), respectively[21,26,30]. In one large study, assessing the long-term safety of telcagepant, 19820 attacks were treated with telcagepant and 10981 attacks with rizatriptan. For two endpoints, pain freedom and pain relief at 2 hours, rizatriptan was superior compared to telcagepant (OR < 1 in favor of rizatriptan. OR (95% CI): 0.58 (0.45, 0.75) and 0.70 (0.55, 0.89), respectively). For all other pre-specified efficacy outcome measurements, no difference was found between the efficacy of telcagepant and rizatriptan at 2 hours[19].

Telcagepant has also been tested as prophylactic treatment of episodic migraine[25,28]. The first of these studies was terminated early due to adverse events and the pre-specified analyses could not be performed. However, post-hoc analysis showed telcagepant to be effective at four weeks in reducing migraine days[25]. In the second study, in a population of patients with perimenstrual migraine, administration of telcagepant in the perimenstrual period did not result in a significant reduction in mean monthly headache days, which was the primary endpoint[28]. There was a reduction of mean monthly on-drug headache days, but the reliability of this analysis is questionable, since no correction for multiple comparisons was done.

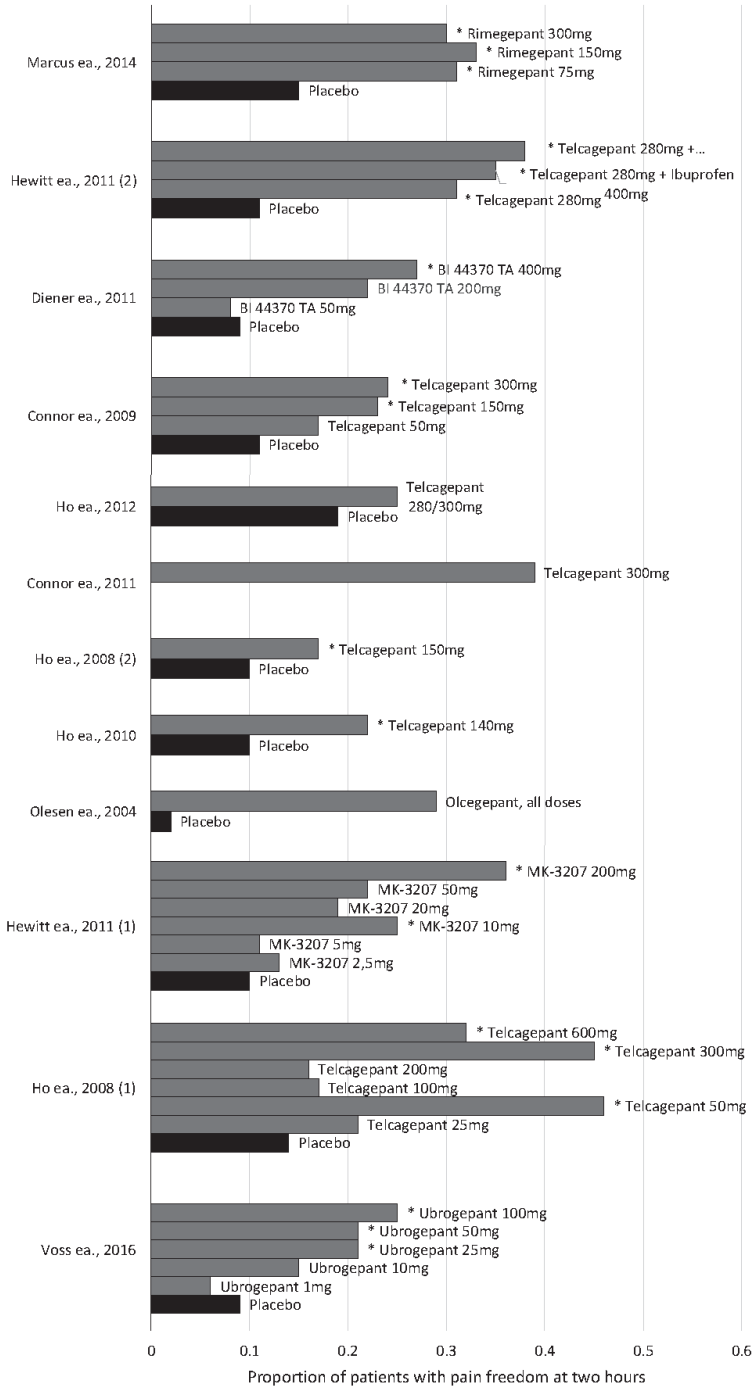


Fig. 1. Efficacy of gepants in the acute treatment of migraine. Bars indicated with * represents statistically significant values compared to placebo ($p<0.05$).

Antibodies against CGRP or the CGRP receptor have been tested as prophylactic treatment of episodic and chronic migraine. To date, four agents have been studied in six clinical studies[32-37]. Fig. 2 provides an overview of the efficacy data of the studies where reduction in migraine days was the primary endpoint. All monoclonal antibodies showed a significant reduction in their primary endpoint, either mean change from baseline in monthly migraine days (5 studies) or mean change in headache hours from baseline (1 study). These agents had an additional reduction over placebo of between 1 and 2.8 migraine days per month. In the study in chronic migraine, where change in headache hours was the primary outcome (data not included in the Fig.), the additional reduction over placebo was 22.7 and 30.4 hours per month for the two doses tested[37]. Interestingly, in one study, 11 of the 67 (16%) patients who had 5 to 14 migraine days per month at baseline, experienced no migraine days during the 12 week study period, versus no patients in the placebo group[34]. In another study, 31 of 98 (32%) patients reporting 4 to 15 migraine days at baseline had a 100% response (defined as a 28-day migraine free period over the 12-week treatment period). In the placebo group, only 18 of 104 (17%) of placebo patients had a 100% response[35]. No other studies reported on the 100% responder rate. Although these data seem interesting, they come from post-hoc analyses and so their significance remains unclear. The data from these 20 studies provides robust and consistent evidence for a crucial role of CGRP in migraine pathophysiology and a high efficacy of blocking CGRP as a prophylactic treatment.

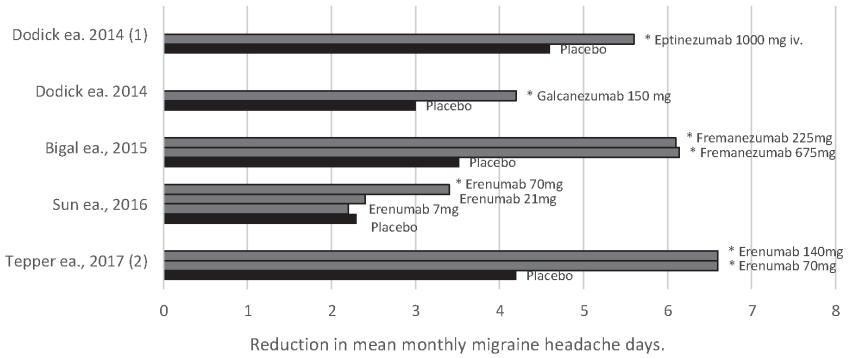


Fig. 2. Efficacy of monoclonal antibodies in the preventive treatment of migraine. Bars indicated with * represents statistically significant values compared to placebo ($p < 0.05$). (1) had change in mean monthly migraine days from baseline to weeks 5-8 as the primary endpoint. All other studies had change in monthly migraine days from baseline to weeks 9-12 of the 12-week double-blind treatment phase as the primary endpoint. In (1) the drug/placebo was administered intravenously. In all other studies, the drug/placebo were given subcutaneously. (2) is on chronic migraine patients. All other studies are on episodic migraine patients.

Is blocking CGRP as or more efficient than current preventative treatments?

Current preventative treatment options for migraine include antihypertensive drugs, antidepressants and antiepileptic medication. In contrast to CGRP (receptor) blockers, these have all been developed for diseases other than migraine and it is estimated that less than 50% of patients on prophylactics experience a 50% reduction in their monthly attack frequency[38].

Topiramate was proven efficient as a preventative treatment of episodic migraine after positive results from three randomized, multi-center, placebo-controlled studies. Thus, topiramate is currently recommended as a level A medication for prevention of episodic migraine with established efficacy (≥ 2 Class I trials) in the 2012 AHS/AAN guidelines[38]. Here, we review so far

published data from Phase III studies of the monoclonal antibodies[39-42] in relation to pivotal studies on topiramate[43-47] in episodic and chronic migraine. In three phase III studies, including over 1500 patients, topiramate 100 mg/d significantly reduced the number of monthly migraine days compared to placebo (reduction of monthly migraine days about -1.8 to -2.6 for topiramate vs. -1.0 to -1.3 for placebo). The ≥50% responder rates were also significantly higher for topiramate (37-54% vs. 22-23%, respectively)[44-46]. In the so far available data from Phase III studies of CGRP (receptor) antibodies, blocking of CGRP showed a similar efficacy with a reduction of monthly migraine days from baseline of -2.9 (verum) vs. -1.8 (placebo) for erenumab (AMG 334)[42]; -4.3 (300mg)/-3.9(100mg) vs. -3.2 (placebo) for eptinezumab (ALD-403)[40]; -4.0 (120mg)/-3.8 (240mg) vs. -2.15 (placebo) for galcanezumab (LY2951742)[39] and -3.7 (225mg monthly)/-3.4 (675mg quarterly) vs. -2.2 (placebo) for fremanezumab (TEV-48125)[41]. The ≥50% responder rates were also significantly higher than for placebo and similar, albeit a little higher, to those of topiramate, ranging from 56.3% to 62.3% (≥50% responder rates: eptinezumab: 56.3% (300mg)/49.8% (100mg) vs. 37.4% (placebo)[40]; galcanezumab: 62.3% (120mg)/60.9% (120mg) vs. 38.6% (placebo)[39]).

Topiramate has also proven efficacious in patients with chronic migraine[47]. In two randomized, placebo-controlled, double-blinded studies with 387 subjects with a daily dose of 100mg or 50-200mg topiramate showed a significant reduction in monthly migraine days compared to placebo (-6.4±5.8 vs. -4.7±6.1 and -3.5±6.3 vs. 0.2±4.7). The ≥50% responder rate was also significantly higher for topiramate (22% vs. 0% for placebo)[47]. In line with this, blocking of CGRP significantly reduced the number of monthly headache migraine days in 1113 chronic migraine patients with an average of 19.4 headache days (-4.8(120mg)/-4.6(240mg) vs. -2.7(placebo)). Likewise, the ≥50% responder rate was significantly higher for the active drug compared to placebo (27.6% (120mg)/27.5% (240mg vs. 15.4%)[48].

Another important aspect of medication is the incidence and severity of adverse events. Compared to topiramate, adverse events reported from CGRP trials were generally mild and less frequent. Upper respiratory tract infection/nasopharyngitis, and injection-site pain have so far been the most frequent reported adverse events[39-42] (see below for more details). In contrast, reported adverse events of topiramate, such as taste disturbance, weight loss, anorexia, fatigue, memory problems and paresthesia were more common in the active groups than in the placebo groups.

Safety issues regarding blocking of CGRP – are there any?

Evidence from clinical studies

Even though the knowledge of the presence and function of CGRP in the CNS is sparse, the function in both the peripheral and enteric nervous system is well established and CGRP is expressed widely throughout both systems. Thus, a wide variety of possible adverse events could be anticipated when blocking CGRP. However, reported adverse events after blocking of CGRP have in general been mild to moderate and the incidences have been low.

Among the first CGRP receptor antagonists under trial, intravenous olcegepant caused mild to moderate adverse events such as paresthesia, nausea, headache, dry mouth and unspecific vision disturbances in a minority of patients[18]. However, more serious adverse events were reported with telcagepant and MK-3207, which caused liver toxicity with transient increase of transaminases in a small group of included subjects (n=13 for telcagepant) upon repeated doses. This lead to discontinuation of the trial program for these molecules[15,25]. Other non-peptide CGRP receptor antagonists such as BI44370TA, BMS-927711, and, most recently, MK-1602 have also been tested. For all three molecules adverse events were mild to moderate and the incidence was low and similar to the placebo group[21,30,31]. No liver toxicity was reported for these drugs, and the gepant program is thus still ongoing.

More recently, great attention has been given to the development and testing of monoclonal antibodies (mABs) targeting circulating CGRP or its receptors. Most importantly, none of these drugs show liver toxicity. This is in line with the theoretical probability of mABs causing liver toxicity, which is very low, since metabolism of mABs do not result in production of toxic metabolites[49]. In addition, despite the potentially harmful inhibition of vasodilation due to CGRP inhibition, no cardiovascular concerns have been disclosed with any of these drugs[50]. In trials, eptinezumab, galcanezumab and fremanezumab, monoclonal antibodies which all target CGRP, showed variable percentages of adverse events, which in line with the gepants, were mild to moderate (e.g. upper respiratory or urinary tract infection, fatigue, back pain, arthralgia, nausea and vomiting). Erenumab, which binds to the CGRP receptor, was also safe and well tolerated in a phase 2 trial[32].

In line with the poor chance of both the non-peptide CGRP receptor antagonists and the antibodies crossing the blood-brain barrier (BBB)[51], no central side effects have been reported so far. Therefore, although crossing of the BBB is likely to occur to some extent, telcagepant has been detected in primates cerebrospinal fluid, suggesting its presence in the CNS[52], a central effect, and side effect, of these drugs seems unlikely.

Do preclinical studies give reason to be concerned about side effects?

CGRP is an ubiquitous peptide that is not only involved in migraine, but also in several physiological processes[12] and in homeostatic responses during pathophysiological conditions (Fig. 3)[9,12]. As such, it is vital to consider the possible side effects caused by the non-selective blockade of α and βCGRP with the CGRP (receptor)-antibodies. As discussed in the previous section, the adverse events of the Phase II trials were mild[32-37], but it should be noted that the duration of these trials is not sufficient to see the long-term effects of continuingly blocking CGRP or its receptor.

In the cardiovascular system, CGRP is present in nerve fibers that innervate blood vessels[53] and the heart[54,55], and participates in the regulation of blood pressure[12,55-57]. Furthermore, it has also been described to have a role in the maintenance of (cardio)vascular homeostasis during ischemic events[9] and in tissue remodeling in pulmonary hypertension[58]. This protective role raises a concern, since migraine patients present an increased cardiovascular risk[59,60]. This topic was recently reviewed elsewhere[9]. Hence, it is important to consider preexisting cardiovascular risk factors in patients (i.e. family history, tobacco exposure, obesity) to prevent a possible cardiovascular event.

Although CGRP participates in inflammatory processes[61-63], it has also been associated with facilitation of wound healing[64]. This is thought to be mediated through its ability to promote keratinocytes proliferation[65], enhance revascularization[66], reduce expression of TNF-α and attenuate macrophage infiltration[67]. A consequence of blocking CGRP could thus be alterations in wound healing and increased inflammatory responses in skin injuries at the site of injection for the antibodies. However, this is a theoretical risk which has so far not been observed in clinical trials.

The antibodies against CGRP are not selective for αCGRP but also block βCGRP. The gastrointestinal tract is highly innervated by βCGRPergic fibers from the enteric nervous system[68,69]. In fact, animal studies with antibodies against CGRP showed extensive mucosal damage[70,71], suggesting a role of CGRP in maintaining the mucosal integrity of the gastrointestinal tract. Blocking this could thus contribute to inflammatory bowel disease. Gastrointestinal motility is also considered to be modulated by CGRP, and administration of this peptide induces a dose-dependent biphasic response[72], which could lead to episodes of diarrhea or constipation. Furthermore, studies with CGRP KO mice have suggested CGRP agonists as a possible treatment for ulcer healing[73]; therefore, monitoring of gastrointestinal complications (i.e. ulcers, constipation) is recommended, even though 12 week studies have not reported these.

Finally, since, as mentioned, it is unlikely that the antibodies cross the BBB, and that the BBB penetration is changed during migraine attacks[74,75], it is important to consider the structures from CNS that are not protected by the BBB. Recent studies have demonstrated that the TG, together with the pituitary, are outside the BBB[76]. An effect on the TG could thus, partly, explain the therapeutic effect of the antibodies. However, CGRP and its receptor are also expressed in the anterior pituitary, suggesting a possible involvement in the regulation of hypothalamo-pituitary tract functions[77]. The exact involvement is still unknown, and further studies are needed to determine the long-term effects of blocking CGRP on the homeostatic functions of the pituitary hormones.

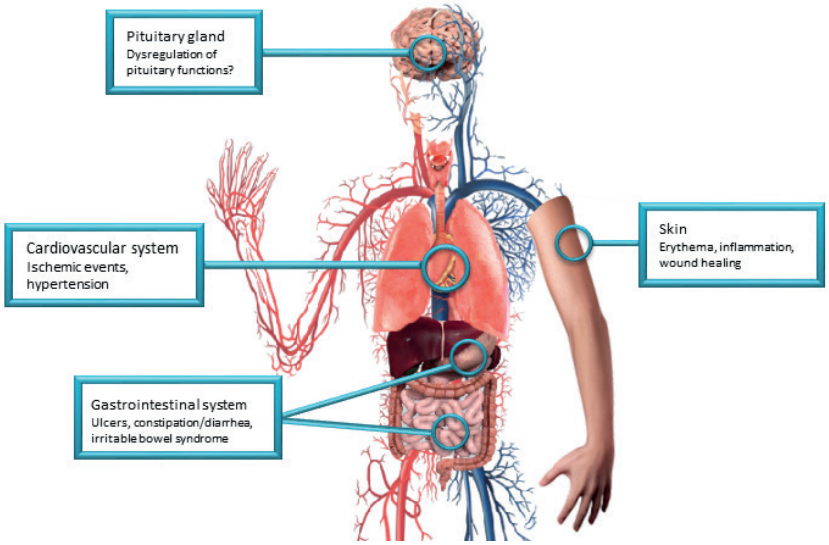


Fig. 3. Possible side effects after long-term exposure to CGRP (receptor)-antibodies. An overview of the organ systems where CGRP and the receptor are present and possible side effects that could be caused by the non-selective blockade of α- and β-CGRP with the CGRP (receptor)-antibodies

Other considerations

Even though blocking of CGRP seems to be an efficacious and safe preventative treatment of migraine, there are many other aspects to consider with regards to the pros and cons of blocking CGRP in migraine patients.

Firstly, the administration of the newly developed monoclonal antibodies is either intravenous or subcutaneous. This could potentially cause complications at the injection site, and common adverse events in those treated with fremanezumab, galcanezumab and erenumab were indeed mild injection-site pain, pruritus and erythema[78]. A disadvantage of the intravenous administration route is the need of it being administered by a medical doctor. This not only increases the placebo response in clinical trials, but does also require for the patient to spend time visiting the clinic – increasing the risk of pathologization of the patient. However, the monthly administration, which is feasible due to the long half-lives of the medication, could improve adherence and compliance to medication, which is a common problem in treating migraine[79,80]. Additionally, the CGRP antibodies seem to show a low risk for drug-drug interactions and hepatotoxicity since they are metabolized by degradation into peptides and single amino acids[81], which could be important for patients using multiple medications.

Secondly, as mentioned, the long-term risks of blocking CGRP are still unknown. Even though the absence of liver toxicity or other abnormalities in routine blood testing is in support of no or low long-term risks[78], studies testing the cardiovascular safety of the long-term blockade are warranted in order to answer the numerous questions on the possibility of higher risk in cardio- and cerebrovascular compromised patients. For example, it is unknown whether blocking CGRP could potentially transform transient mild cerebral ischemia into a full-blown brain infarct[9] and whether these risks are higher in women[9,82]. To investigate these aspects, future studies should include patients with preexisting cardiovascular conditions.

Thirdly, the exact site of action of blocking CGRP is still partly unknown and CGRP could exert its effects on receptors distinct from the CGRP receptor[9]. Recently it was put forward that CGRP may act on the amylin receptor in TG[83] as well as in human coronary arteries[84]. If this is the case, this could pose an additional – unknown – potential risk of wiping out CGRP. We can also only guess whether patients not benefitting from receptor blockade would benefit from blockage of the peptide itself. Future studies should investigate how to differentiate responders from non-responders.

Lastly, a disadvantage when using antibodies is the risk of development of antibodies against the drug[15]. Indeed, antidrug antibodies were detected with all four antibodies[78], but these did not seem to affect efficacy[32]. However, long-term studies are needed to investigate whether, at long term, neutralizing antidrug antibodies will pose a problem for efficacy and safety of blocking CGRP with monoclonal antibodies. Finally, it is well known that antibody treatment is costly and the price of the drugs has to be taken into consideration when deciding whether to use CGRP antibodies as a prophylactic treatment and which patient groups to treat.

Conclusion

Here, we have reviewed the pros and cons of blocking CGRP in migraine patients. In favor of using blocking of CGRP as a treatment of migraine, is that, based on evidence from clinical trials, whether using small molecule receptor antagonists or antibodies, the treatment is efficacious. Additionally, the liver toxicity induced by some of the gepants is not present with the antibodies, which are well tolerated. Lastly, in contrast to current prophylactic treatments, the drugs are developed specifically for migraine, based on findings from human migraine studies. Thus, the drugs may exert a more direct effect on migraine specific pathways than previously used prophylactic drugs. In addition, this provides hope and encouragement for further research into the pathophysiological mechanisms of migraine and potentially the discovery of other migraine specific therapeutic targets.

Speaking against chronically blocking CGRP, the long-term effects, particularly regarding the cardiovascular risks, are still unknown as well as the exact mode of action of the antibodies. In addition, development of neutralizing antidrug antibodies may, with time, affect the efficacy of the antibodies. Lastly, as with all antibody therapies, CGRP antibodies have the problem of being costly. However, taking into consideration the enormous socioeconomically burden that migraine is[85], the price may be well payed off.

In conclusion, based on current knowledge, we believe that the benefits of blocking CGRP, including the perspectives of improving the lives of those suffering from frequent headaches, seems to be greater than the disadvantages.

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Chapter VII.

Is CGRP receptor blockade cardiovascularly safe? Appropriate studies are needed

*Based on: A Maassen van den Brink, **E Rubio-Beltrán**, D Duncker, CM Villalón (2018) Headache; 58:11257-1258*

With great interest, we read the publication by Depre and colleagues[1] describing that inhibition of the canonical CGRP receptor does not seem to worsen myocardial ischemia contrary to theoretical concerns[2]. In a randomized, double-blind, placebo-controlled study the authors did not find evidence for an adverse effect of the CGRP receptor antibody erenumab on exercise time during a treadmill test in patients with stable angina. Although we certainly appreciate the endeavor to address this important issue, we are concerned that the study population, the study design, and the interpretation of the results do not allow for such a reassuring conclusion.

While the authors rightly indicate that it is currently not clear to which extent CGRP is relevant in maintaining blood flow in case of myocardial and cerebral ischemia, we do not agree with their argument stating that "the concentrations of exogenous CGRP required to increase total exercise time or protect against myocardial ischemia far exceed the endogenous physiological levels of CGRP that are released during a response to ischemia". This is because it is not the systemic plasma concentration that is relevant in this perspective, but the actual concentration of CGRP at the *neuro-vascular junction*, where CGRP is released. Obviously, the plasma concentration is likely to be several log units lower than the junctional concentration due to dilution and hydrolysis. Further, we feel that the argument that erenumab does not contract the human isolated coronary artery per se[3] does not add to the introduction, since the question that should be answered here is whether inhibition of the actions of CGRP is potentially harmful in myocardial ischemia.

More importantly, the patients included in this study suffered from stable angina pectoris, which often is caused by a stenosis of the epicardial conducting portions of the coronary artery. As we pointed out earlier[2], the importance of CGRP in the proximal, epicardial portions of the coronary artery bed seems limited, while CGRP is a highly effective vasodilator in the intramyocardial, smaller (distal) sections of the coronary artery bed. Thus, it is unfortunate that a patient population with, most likely, mainly diseased proximal coronary arteries, was chosen for this study, despite the advantage of a clear-cut definition of these patients. Although stable angina pectoris due to epicardial stenosis may occur in both men and women, this is typically considered a "male" form of cardiac pathology[4], as illustrated by the fact that 78% of patients included in the current study were male. In contrast, in females, who are the majority of migraine sufferers and thus also the majority of the population likely to use erenumab or related drugs in future, coronary artery disease often presents as diffuse atherosclerosis, without an angiographically detectable stenosis[4-7]. These observations indicate that coronary microvascular dysfunction plays a more important role in angina pectoris in female patients and that blocking the effects of CGRP in female patients may have different effects than in male patients.

An essential concern of this study is based on pharmacokinetic and pharmacodynamic considerations. The authors rightly indicate that plasma concentrations obtained 30 minutes after intravenous infusion of 140-mg erenumab (the time interval until the start of the treadmill test) will provide "a substantial margin over concentrations achieved by subcutaneous administration of 140 mg". In contrast, their claim that "the use of 140 mg intravenous dose of erenumab ensured rapid and robust blockade of the CGRP receptor" is not substantiated by any evidence. It should be taken into account that, before a receptor blocking antibody can effectively occupy the receptor where it is binding to, the antibody should first have access to the receptor biophase. In this case, erenumab was infused intravenously and thus reached the blood vessel wall from the luminal side. The CGRP receptor is located in the smooth muscle wall[5], thus it may take several hours before the receptor was reached by erenumab at sufficiently high concentrations to induce an effective blockade of the CGRP receptor, especially given the large molecular size (150 kDa) of erenumab. This is well beyond the time the treadmill test had finished. A way to verify whether blockade has been achieved (at

least in skin blood vessels) is by assessing blockade of capsaicin-induced increases in dermal blood flow. The earliest time point for such measurements that has been published, to the best of our knowledge, for erenumab is 2 days after intravenous administration[8], which is about 100-fold longer than the period applied in the current study. Thus, we feel that evidence for significant blockade of the canonical CGRP receptor should have been provided to substantiate the statement that CGRP receptor blockade was reached during the treadmill study, since otherwise the interpretation of the current study is questionable.

Taken together, we would politely urge the authors to provide evidence for the fact that vascular CGRP receptor blockade has been achieved 30 minutes after intravenous infusion of erenumab, since this is a crucial part of the study. Further, we plead for cardiovascular safety studies on patients and/or experimental animals with microvascular disease, as such a group may better represent the patients at cardiovascular risk after the use of erenumab. Even if the antibodies against CGRP or its receptor would not increase the risk for cardiovascular ischemia, there will be cases of patients with ischemic complaints, even without a causal relationship. Appropriate studies in relevant subjects may avoid sudden distress, such as happened with the triptans in the past.

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Chapter VIII.

Characterization of vasodilatory responses in the presence of the CGRP receptor antibody erenumab in human isolated arteries

Based on: ***E Rubio-Beltrán****, A Labastida-Ramírez, KA Haanes, A vd Bogaerdt, AJJC Bogers, C Dirven, AHJ Danser, C Xu, J Snellman and A MaassenVanDenBrink (2019) Cephalalgia; In Press.*

Abstract

Background: Migraine is associated with activation of the trigeminovascular system, release of calcitonin gene-related peptide (CGRP) and dilation of dural arteries. Novel treatments target CGRP or its receptor, which are present in all vascular beds, raising cardiovascular concerns. Erenumab is a human CGRP-receptor antibody approved for the prophylactic treatment of migraine.

Methods: We characterized the relaxant responses to CGRP in the absence and presence of erenumab (1 μM) in isolated human middle meningeal (HMMA), internal mammary (HIMA) and (proximal and distal) coronary arteries (HCA). Furthermore, in HIMA from cardiovascularly-compromised patients, we assessed the pharmacological specificity of erenumab by investigating whether the vasodilatory responses to acetylcholine, sodium nitroprusside, pituitary adenylate cyclase activating polypeptide-38 (PACAP), vasoactive intestinal peptide (VIP) and nicardipine, along with the vasoconstrictor responses to dihydroergotamine, were modified by erenumab.

Results: CGRP induced concentration-dependent vasodilatory responses in all vessels studied that were significantly antagonized by erenumab. In HIMA from cardiovascularly-compromised patients, the responses to acetylcholine, sodium nitroprusside, PACAP, VIP, nicardipine and dihydroergotamine were unaffected by erenumab.

Conclusion: Erenumab inhibits CGRP-induced vasodilatory responses in HMMA, HIMA and HCA. Moreover, erenumab shows functional specificity as no interaction was observed with the relaxant responses to several vasodilators, nor the dihydroergotamine-dependent vasoconstrictor responses.

Introduction

Migraine is a highly disabling neurovascular disorder and its pathophysiology remains elusive. However, it has been associated with an activation of the trigeminovascular system, release of calcitonin gene-related peptide (CGRP) and increase in middle meningeal artery circumference specific to the head pain side[1]. Based on the involvement of CGRP in the pain signalling pathway of migraine, small molecule-CGRP receptor antagonists (gepants) were developed for the treatment of migraine. The first gepants did not reach the market, due to hepatotoxicity cases and pharmacokinetic problems[2]. Although novel gepants are currently being developed, with no reported toxicity so far[3], the most recent approach for CGRP blockade consists of the antibodies against CGRP (eptinezumab, fremanezumab, galcanezumab) or its receptor (erenumab). They have all shown to be effective for the prophylactic treatment of migraine and are either approved or likely soon to be approved for commercialization[4].

While the development of antibodies directed against the CGRP pathway (*i.e.,* antibodies against CGRP or the CGRP receptor) represents a milestone in migraine treatment, it is important to also consider the implications of peripheral CGRP receptor blockade. To begin with, CGRP fibres innervate blood vessels, and are thought to contribute in the homeostatic responses to ischemic events[5-7]. This raises some concerns, especially as migraine patients present increased cardiovascular risk[8,9]. Indeed, all the antibodies have been reported to be well tolerated even in subjects exposed longer that one year, with no cardiovascular events reported in the clinical trials that were considered to be related to CGRP pathway blockade[10]. Moreover, a study explored the effect of erenumab on exercise time during a treadmill test in mainly male patients with stable angina, and no changes were observed[11], although no evidence was provided for CGRP receptor blockade to be established already at the time of the treadmill test[12]. In addition, previous studies have also shown that the vasodilatory role of CGRP in the coronary arteries is more prominent in the distal portion when compared to the proximal portion[13]. While males are more prone than females to present ischemic events in the proximal portion of the coronary artery, females are more prone than men to present

myocardial ischemic events in the distal portion of the coronary artery[14]. As the vast majority of migraine patients are female, it is important to study the effects of erenumab in the distal portion of the coronary arteries. Also, for migraine patients with established cardiovascular disease, it is important to investigate whether blockade of the CGRP pathway could worsen their disease[6]. Thus, appropriate *in vitro* and *in vivo* studies are needed to assess the vascular safety of blocking the CGRP pathway.

The aim of this study was to investigate the inhibition of the vasodilatory responses to CGRP by erenumab in human isolated meningeal artery (HMMA), one of the proposed sites of therapeutic action. But also, in view of theoretical cardiovascular safety concerns, in human isolated proximal and distal coronary arteries (HCA), and in internal mammary arteries (HIMA) from cardiovascularly compromised patients undergoing coronary artery bypass grafting surgery. Furthermore, in HIMA, we studied the functional specificity of erenumab by comparing the relaxant responses to several vasodilators in the absence and presence of erenumab, namely: (i) acetylcholine, coupled to endothelium-dependent, nitric oxide-cGMP signalling; (ii) sodium nitroprusside, coupled to endothelium-independent, nitric oxide-cGMP signalling; (iii) pituitary adenylate cyclase activating peptide-38 (PACAP) and vasoactive intestinal peptide (VIP), peptides of interest for migraine pathophysiology that are coupled to an adenylate cyclase-cAMP signalling pathway; and (iv) nicardipine, a calcium channel blocker, prescribed for the treatment of hypertension that may also be used in migraine. Finally, as migraine patients under prophylactic treatment could still use acute antimigraine medication, the vasoconstrictor responses to dihydroergotamine (DHE) in absence and presence of erenumab were also analyzed, to discard a possible augmentation of the contractile responses due to the inhibition of the CGRP-mediated vasodilation.

Methods

Human isolated arteries collection

Middle meningeal arteries

Segments of HMMA (internal diameter 0.5–1.5 mm) were obtained from six patients (two male and four female, 49±8 years old) who underwent neurosurgical procedures requiring a trepanation of the skull. The HMMA, attached to the *dura mater*, was collected in a sterile organ-protecting solution and immediately transported to the laboratory to be dissected and subsequently placed in a cold, oxygenated Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_2$ 25 and glucose 11.1; pH 7.4.

Coronary arteries

Coronary arteries were obtained from six "heart beating" organ donors (two male and four female; 52±5 years old), who died of non-cardiac disorders. The hearts were provided by the Heart Valve Bank Beverwijk (at that time still located in Rotterdam) from Dutch post-mortem donors, after donor mediation by The Dutch Transplantation Foundation (Leiden, The Netherlands), following removal of the aortic and pulmonary valves for homograft valve transplantation. All donors gave permission for research. Immediately after circulatory arrest, the hearts were stored at 4°C in a sterile organ protecting solution and were brought to the laboratory within 24 hours of death. After arrival, the right proximal (internal diameter 3–5 mm) and distal (internal diameter 0.5–1 mm) portions of the HCA were dissected and placed in a cold, oxygenated with carbogen (95% O_2/5% CO_2) Krebs buffer solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. The studies on coronary arteries were approved by the Scientific Advisory Board of the Rotterdam Heart Valve Bank.

Internal mammary arteries

Segments of HIMA (internal diameter 2–3 mm) were obtained perioperatively from 10 male patients (72±2 years old) undergoing coronary artery bypass surgery. After completion of the coronary bypass procedure, the remaining segment of HIMA was immediately brought to the laboratory. Connective tissue was removed from the segment and the tissue was kept in cold Krebs solution (for composition see above), aerated with carbogen. All vessels were used on the same day or stored overnight and used the following day for functional experiments. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, approved the study protocols with regard to mammary arteries and middle meningeal arteries.

Isometric tension measurements

Proximal HCAs were cut into segments of 2–4 mm length, excluding macroscopically visible atherosclerotic lesions. Segments were mounted on stainless steel hooks in 15-mL organ baths filled with oxygenated Krebs buffer solution at 37°C. After 30 min of stabilization, the vessel segments were stretched to a tension of about 15 mN, as described earlier by our group[13]. Changes in tension were measured with an isometric force transducer (Harvard, South Natick, MA, U.S.A.) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

The HMMA, distal HCA and HIMA were cut into circular 1-2 mm long segments and mounted in Mulvany myographs (Danish Myo Technology, Aarhus, Denmark) between two parallel small stainless-steel wires (40 µm Ø). Baths were filled with oxygenated Krebs buffer (37°C) and their tension was normalized to 90% of I_{100} for all segments (the diameter when transmural pressure equals 100 mm Hg) as previously reported[15]. Data was recorded using a LabChart data acquisition system (AD Instruments Ltd, Oxford, UK).

Experimental protocols

A paired parallel set up (*i.e.* experiments with and without erenumab were performed in different segments obtained from the same artery) was used. Initially, all segments were exposed to 30 mM KCl to 'prime' the tissue for stable contractions. After washout, the tissue was exposed to 100 mM KCl to determine the reference contractile response. All segments were pre-contracted with 30 mM KCl after being incubated with vehicle or erenumab (1 µM)[16] for 15 min. After 15 min of precontraction (*i.e.*, after a total incubation time of 30 min for erenumab), a concentration response curve to human αCGRP (0.1 nM-1 µM, half logarithmic steps) was performed.

Additionally, in HIMA, after segments were pre-contracted (30 mM KCl) and incubated with vehicle or erenumab (1 µM), concentration response curves to acetylcholine (0.1 nM-3 µM, half logarithmic steps), sodium nitroprusside (1 nM-10 µM, half logarithmic steps), PACAP (0.1 nM-1 µM, whole logarithmic steps), VIP (0.1 nM-1 µM, whole logarithmic steps), or nicardipine (1 nM-30 µM, whole logarithmic steps) were performed. Also, in vessels incubated 30 min with vehicle or erenumab (1 µM), a concentration response curve to DHE (1 nM-100 µM, whole logarithmic steps) was performed.

Finally, at the end of each experiment, i.e., after construction of a concentration response curve, and washing out, the functional integrity of the endothelium was verified by observing relaxation to bradykinin (1 µM, HIMA) or substance P (10 nM, HMMA and HCA) after precontraction with the thromboxane A_2 analogue U46619 (10 nM) in every individual vessel segment.

Statistical analysis

Vasodilatory responses were expressed as percentage of the precontraction induced by 30 mM KCl. For contractile responses, the values were expressed as percentage of the contraction induced by

100 mM KCl. Curves covering the full sigmoidal range were analyzed by means of a computerized curve fitting technique to obtain pEC_{50} (negative log of the molar concentration of an agonist needed to reach half of its maximal effect) and E_{max} (maximal response) values. If E_{max} was not reached, the contraction or relaxation obtained at the highest concentration of agonist was considered as E_{max}, except for CGRP in the presence of erenumab, where the respective control E_{max} (i.e., in the absence of erenumab) was used as E_{max} for fitting. The blocking potency of erenumab in each tissue was estimated by calculating EC_{50} ratios and plotting a Schild plot [17] and constraining the slope to unity to obtain the apparent pK_b values. All data are presented as mean±S.E.M. Significant differences in pEC_{50} and E_{max} between control and erenumab groups were examined with a paired t test. Differences between tissues were analyzed by a one-way analysis of variance (ANOVA), followed by Tukey's post hoc analysis. P values of 0.05 or less were assumed to denote significant changes.

Compounds used

Erenumab and vehicle were kindly provided by Amgen (Thousand Oaks, CA, U.S.A.). Acetylcholine chloride, sodium nitroprusside, nicardipine hydrochloride, dihydroergotamine mesylate and U46619 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human α-CGRP, PACAP and VIP were obtained from PolyPeptide (Strasbourg, France).

Results

Responses to 100 mM and 30 mM KCl resulted in a mean contraction of 19±4 mN and 17±3.1 mN, respectively.

Effect of erenumab on the vasodilatory responses to CGRP in human isolated middle meningeal arteries

In HMMA (Fig. 1), the vasodilatory responses to CGRP were significantly shifted in the presence of 1 μM erenumab (control: pEC_{50} 8.56±0.16 *vs.* erenumab: pEC_{50} 6.51±0.19; n=6 each; t(5)=16.74, p<0.0001). The apparent pK_b value was 8.05±0.12. No significant changes were observed in the maximal responses to CGRP (control: E_{max} 64±11% *vs.* erenumab E_{max}: 56±10%; n=6 each; t(5)=2.11, p=0.088). Verification of endothelial function resulted in a mean dilation of 83±3% of the precontraction.

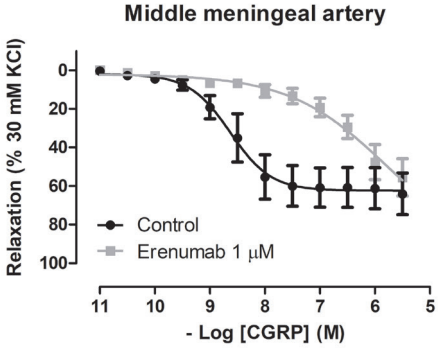

Fig. 1. Relaxant responses to CGRP in the middle meningeal artery in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=6.

Effect of erenumab on the vasodilatory responses to CGRP in human isolated coronary arteries

Relaxant responses to CGRP in the proximal HCA (pEC_{50}<6.54; n=4), were inhibited in the presence of 1 μM erenumab (pEC_{50}<5.24; n=4), with no significant change in the response obtained at the highest

concentration (control: E_{max} 36±6% vs. erenumab: E_{max} 21±3%; t(3)=1.86, p=0.15). Due to the limited effect of CGRP on the proximal portion of the HCA, no apparent pK_b value was calculated. In the distal HCA, a significant shift was observed in the vasodilatory responses to CGRP (control: pEC_{50} 9.04±0.18 vs. erenumab: pEC_{50} 6.81±0.18; n=6 each; t(5)=13.46, p<0.0001), with no change in the maximal relaxation (control: E_{max} 85±5% vs. erenumab: E_{max} 83±6%; n=6 each; t(5)=0.88, p=0.42; Fig. 2) and an apparent pKb value of 8.22±0.17. A significant difference was observed between the maximal relaxation to CGRP in proximal and distal HCA (E_{max} proximal: 36±6% vs. E_{max} distal: 85±5%; t(8)=4.95, p=0.001). Endothelial function analysis resulted in a mean dilation of 12 ± 3% of the precontraction in proximal HCA and of 89 ± 4% in distal HCA.

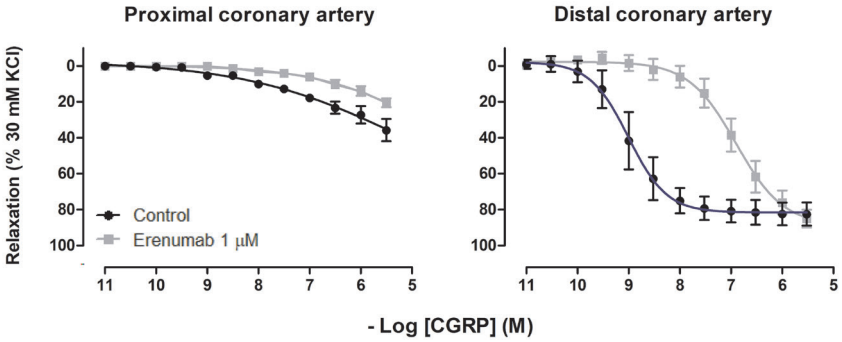

Fig. 2. Relaxant responses to CGRP in the proximal (left) and distal (right) coronary arteries in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=4-6.

Effect of erenumab on the vasodilatory responses to CGRP in human isolated internal mammary artery

In HIMA, the vasodilatory responses to CGRP were also significantly inhibited by erenumab (control: pEC_{50} 7.83±0.34 *vs.* erenumab: pEC_{50} 5.94±0.37; n=8 each; t(7)=4.18, p=0.004), with an apparent pK_b value of 7.85±0.46, and no significant change in the maximal response was observed (control: E_{max} 32±12% vs. erenumab: 24±10%; n=8 each; t(7)=1.44, p=0.19; Fig. 3). Verification of endothelium function resulted in a dilation of 6±3% of the precontraction. Precontraction with 30 mM KCl was not modified by erenumab (control: 154±47% *vs.* erenumab 141±22% of the contraction to 100 mM KCl; t(7)=0.47, p=0.66; data not shown).

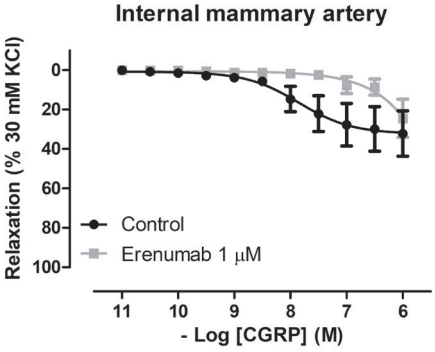

Fig. 3. Relaxant responses to CGRP in the human isolated internal mammary artery in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=8.

Effect of erenumab on non-CGRP induced vasodilatory responses in human isolated mammary artery

The relaxant responses to the different vasodilators studied were not modified in the presence of erenumab (Table 1, Fig. 4)

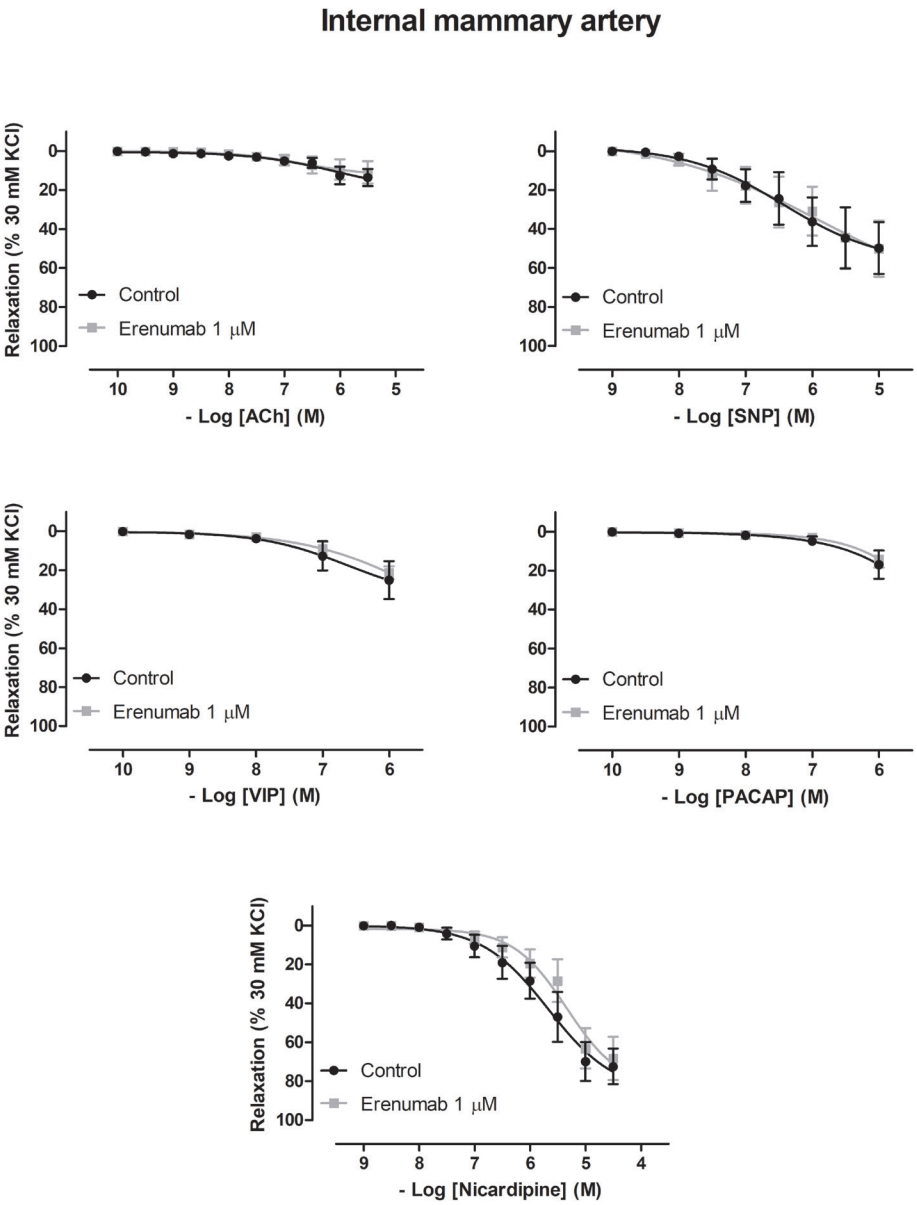

Fig. 4. Vasodilatory responses to acetylcholine, sodium nitroprusside, VIP, PACAP and nicardipine in the human isolated internal mammary artery in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=7 each.

Table 1. Vasodilatory responses to acetylcholine, sodium nitroprusside, VIP, PACAP and nicardipine in the HIMA in the absence and presence of erenumab; n=7 each.

Agonist	pEC_{50}			E_{max} (%)		
	Control	Erenumab	P value	Control	Erenumab	P value
Acetylcholine	7.42±0.96	6.22±0.45	0.66	14±4	11±6	0.57
Sodium nitroprusside	6.31±0.29	6.44±0.44	0.98	50±13	50±14	0.97
VIP	6.83±0.11	7.05±0.45	0.29	25±10	21±3	0.69
PACAP	6.78±0.01	7.00±0.21	0.28	17±7	14±4	0.63
Nicardipine	5.77±0.24	5.49±0.06	0.23	72±9	68±11	0.49

E_{max}: maximal response; PACAP: pituitary adenylate cyclase activating polypeptide-38; pEC_{50}: negative log of the molar concentration of an agonist needed to reach half of its maximal effect; VIP: vasoactive intestinal peptide

Effect of erenumab on the contractile responses to dihydroergotamine

Similar to above, contractile responses to DHE (pEC_{50} control: 6.78±0.40 *vs.* pEC_{50} erenumab: 6.65±0.40, t(6)=0.41, p=0.72; E_{max} control: 11±6% *vs.* E_{max} erenumab: 17±12%, t(6)=0.66, p=0.53; n=7 each) were unaffected by the presence of erenumab (Fig. 5).

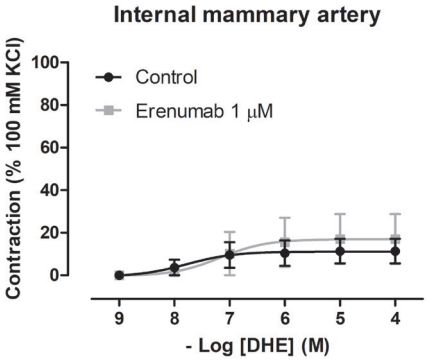

Fig. 5. Contractile responses dihydroergotamine (DHE) in the human isolated internal mammary artery in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=7 each.

Comparison of the responses to CGRP in human middle meningeal, coronary and mammary arteries

The vasodilatory responses to CGRP were more potent in the distal portion of the HCA when compared to the HIMA (pEC_{50} 9.04±0.18 *vs.* pEC_{50} 7.83±0.34, respectively; F(2,17)=5.471, $p_{apparent}$=0.01). No significant differences were observed in the potency between HMMA and HIMA (pEC_{50} 8.56±0.16 *vs.* pEC_{50} 7.83±0.34, respectively; F(2,17)=5.471, $p_{apparent}$=0.15), nor between HMMA and the distal portion of the HCA (pEC_{50}: 8.56±0.16 *vs.* pEC_{50}: 9.04±0.18, respectively; F(2,17)=5.471, $p_{apparent}$=0.47).

When comparing the E_{max}, the vasodilatory responses to CGRP were more pronounced in the distal portion of the HCA, when compared to the HIMA (E_{max} distal HCA: 85±5% *vs.* E_{max} HIMA: 32±12%; F(3,20)=6.302, $p_{apparent}$=0.004). No significant differences were observed between the maximal responses to CGRP in HMMA and HIMA (E_{max} HMMA: 64±11% *vs.* E_{max} HIMA: 32±12%; F(3,20)=6.302, $p_{apparent}$=0.112) nor HMMA and distal HCA (E_{max} HMMA: 64±11% *vs.* E_{max} distal HCA: 85±5%; F(3,20)=6.302, $p_{apparent}$=0.47). Also, no significant differences were observed between the maximal vasodilatory response to CGRP in proximal HCA and HIMA (E_{max} proximal HCA: 36±6% *vs.* E_{max} HIMA: 32±12%; F(3,20) = 6.302, $p_{apparent}$=0.991) nor proximal HCA and HMMA (E_{max} proximal HCA: 36±6% *vs.* E_{max} HMMA: 64±11%; F(3,20)=6.302, $p_{apparent}$=0.311)

Finally, no significant difference was observed in the potency of erenumab to antagonize the responses to CGRP amongst the tissues studied (p=0.73; pK_b HMMA: 8.05±0.12 *vs.* pK_b HCA: 8.22±0.17, F(2,17)=0.3106, $p_{apparent=}$0.94; pK_b HMMA: 8.05±0.12 *vs.* pK_b HIMA: 7.85±0.46, F(2,17)=0.3106, $p_{apparent=}$0.91; pK_b HCA: 8.22±0.17 *vs.* pK_b HIMA: 7.85±0.46, F(2,17)=0.3106, $p_{apparent}$=0.72).

Discussion

In this study the inhibition of the CGRP-vasodilatory responses by erenumab was examined in human isolated arteries. We used a supratherapeutic concentration of erenumab (1 μM)[16] to allow a clear analysis of its effects on CGRP as well as other vasoactive substances of interest.

Firstly, we investigated the effect of erenumab on the vasorelaxant responses to CGRP in HMMA, with our results showing a significant shift of the concentration response curve to CGRP in the presence of erenumab. As antibodies are considered to have a BBB permeability of < 0.1%[18] and erenumab has been shown to be effective for the prophylactic treatment of migraine[19,20], it is considered that the mechanisms of action of erenumab are peripheral, with one of them being possibly inhibition of the CGRP-mediated vasodilation of the dural arteries[1]. While erenumab has no vasoconstrictive properties *per se*[21], its success as prophylactic treatment may well be (partly) by effectively preventing the vasodilatory responses to CGRP in the HMMA, associated with the onset of migraine attacks. In accordance with this, human provocation studies have shown dilation of the HMMA on the headache side at migraine onset, and headache relief after vasoconstriction of the HMMA by sumatriptan[1,22]. Certainly, these studies have been performed during exogenously provoked migraine-like attacks and a magnetic resonance angiography study of the intracranial and extracranial arteries in patients with spontaneous migraine attacks failed to show extracranial arterial dilatation[23]. However, as previously addressed by our group[24], in the latter study authors could not exclude dilatation of dural branches of the HMMA, as those small branches could not be analyzed due to technical limitations.

Due to the theoretical cardiovascular concerns of blocking the actions of CGRP[6,25], especially since migraine patients have an increased cardiovascular risk[8,9], we further studied the effect of erenumab in proximal and distal HCA (Fig. 2). Although it has been shown that erenumab does not contract the HCA[21], it is important to consider the risks of the blockade of the cardioprotective vasodilation by CGRP[7]. In our study, and in accordance with previous work[13,26,27], the vasodilatory responses to CGRP in the distal portion of the HCA were significantly more pronounced than in the proximal portion of the HCA and HIMA. Moreover, in the presence of erenumab, a significant shift was observed in both portions of the HCA, that seemed to be more pronounced in the distal portion. This reinforces the importance of appropriate vascular safety studies in migraine patients, with especial emphasis in female patients that are more prone to present ischemic events in the distal portion of the coronary arterial bed, where the role of CGRP in cardioprotection seems to be more significant[7,12,14].

Furthermore, we analyzed the effect of erenumab on isolated arteries from cardiovascularly compromised patients. For this, we obtained HIMA peri-operatively from coronary artery bypass surgery patients, most of them suffering from atherosclerotic disease that is usually more prominent in the proximal HCA and more common in men as previously mentioned, thus all our experiments were performed in HIMAs obtained from male subjects. However, while the responses to CGRP are more pronounced in the distal coronary artery (where women are more prone to present ischemic events), as previously mentioned, when a patient undergoes coronary bypass surgery, a portion of HIMA is grafted and thus gets incorporated in the proximal coronary arterial bed, making it a relevant tissue to study the characteristics of the CGRP-mediated vasodilatory responses and the effects of erenumab. Based on the limited relaxation to bradykinin that was analyzed in every individual HIMA segment, and in accordance with the small vasodilatory responses to acetylcholine (Fig. 4), functional endothelial quality was limited in these coronary artery bypass grafts, probably associated with the endothelial dysfunction associated with cardiovascular disease[28]. When analyzing the responses to CGRP, a concentration-dependent vasodilation was observed, which was significantly antagonized in the presence of erenumab, with no change in the maximal response. The E_{max} of CGRP in the HIMA was significantly lower when compared to the distal portion of the HCA and not significantly different when compared to the proximal HCA (Figs. 2-3), suggesting a similar role for CGRP in HIMA and proximal HCA. The vasodilatory peptides VIP and PACAP, currently considered possible therapeutic targets for migraine[29], do not seem to play an important role in HIMA vasodilation as their maximal response was rather low. Most importantly, erenumab did not modify the responses to acetylcholine and sodium nitroprusside (nitric oxide-cGMP signalling), nor the vasorelaxant responses to PACAP and VIP (adenylate cyclase-cAMP signalling). Even though the cardiovascular safety concerns are theoretical and trials have not reported cardiovascular events that were considered to be related to inhibition of the CGRP pathway[10], the vasodilatory responses to acetylcholine, VIP and PACAP in our study were limited. Therefore, further studies should address the vasodilatory pathways involved in ischemic conditions after long-term blockade of the CGRP pathway. Nonetheless, erenumab did not modify the vasodilatory responses to nicardipine, an antihypertensive given to cardiovascularly compromised patients (Fig. 4), and did not augment the vasoconstrictor responses to 30 mM KCl or DHE, an acute acting antimigraine drug that could be taken concomitantly with erenumab (Fig. 5). Similar results have previously been reported in HCA with sumatriptan[21], which is of great importance for patients under ergot (or triptan) treatment.

Finally, as the efficacy and potency of the CGRP-dependent vasodilatory responses differ amongst arteries[13], CGRP receptor blockade by erenumab could also present differential responses depending on the vessel studied; however, erenumab had a similar potency (pK_b) across the distal HCA (8.22±0.17), HMMA (8.05±0.12) and HIMA (7.85±0.46). When comparing these results to the gepants, similar pK_b values were obtained previously by our group for telcagepant in distal HCA and HMMA (8.43±0.24 and 8.03±0.16, respectively)[13,26]. Interestingly, olcegepant was more potent in HMMA (pK_b: 10.59±0.54) than in the distal portion of the HCA, with pK_b values ranging from 8.41±0.26 to 9.29±0.34, depending on the concentration studied[30,31]. While we do not know the reason for this discrepancy, it may well be caused by an underlying heterogeneity in CGRP receptors that could be targeted by olcegepant[30,32,33], whereas erenumab only acts at the canonical CGRP receptor[34], thus, receptors other than the CGRP receptor to which CGRP may still bind (*e.g.* amylin 1 receptor), may compensate for blockade of the CGRP receptor. Conversely, in the case of the antibodies directed against CGRP, peptides other than CGRP that may also bind to the CGRP receptor, may exert compensatory effects. Further studies should address whether there are clinically relevant differences (*i.e.* in efficacy or cardiovascular safety), between the prophylactic treatment with the antibodies directed against CGRP and against the CGRP receptor.

Our results, taken together, show a differential response profile to CGRP in human isolated arteries, being more potent in the distal portion of the HCA, when compared to the proximal portion and the HMMA and HIMA. Moreover, erenumab significantly inhibits CGRP-mediated vasodilation *in vitro* and does not interact with responses to other vasodilatory or contractile agents of interest.

Conclusion

In conclusion, erenumab is a potent inhibitor of the vasodilatory responses to CGRP in HMMA, HCA and HIMA. While the prominent role of CGRP in distal coronary artery warrants further safety studies, particularly in women, it is important to point out that erenumab does not interact with vasodilatory responses to other vasodilators, nor with the contractions to DHE.

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PART IV:

New therapeutic targets and pathophysiology of migraine

Chapter X.

PACAP38 and PAC_1 receptor blockade: a new target for headache?

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Abstract

Pituitary adenylate cyclase activating polypeptide-38 (PACAP38) is a widely distributed neuropeptide involved in neuroprotection, neurodevelopment, nociception and inflammation. Moreover, PACAP38 is a potent inducer of migraine-like attacks, but the mechanism behind this has not been fully elucidated.

Migraine is a neurovascular disorder, recognized as the second most disabling disease. Nevertheless, the antibodies targeting calcitonin gene-related peptide (CGRP) or its receptor are the only prophylactic treatment developed specifically for migraine. These antibodies have displayed positive results in clinical trials, but are not effective for all patients; therefore, new pharmacological targets need to be identified.

Due to the ability of PACAP38 to induce migraine-like attacks, its location in structures previously associated with migraine pathophysiology and the 100-fold selectivity for PAC_1 receptor when compared to VIP, new attention has been drawn to this pathway and its potential role as a novel target for migraine treatment. In accordance with this, antibodies against PACAP38 (ALD 1910) and PAC_1 receptor (AMG 301) are being developed, with AMG 301 already in Phase II clinical trials. No results have been published so far, but in preclinical studies, AMG 301 has shown responses comparable to those observed with triptans. If these antibodies prove to be effective for the treatment of migraine, several considerations should be addressed, for instance, the potential side effects of long-term blocking of the PACAP (receptor) pathway. Moreover, it is important to investigate whether these antibodies will indeed represent a therapeutic advantage for the patients that do not respond the CGRP (receptor)-antibodies.

In conclusion, the data presented in this review indicate that PACAP38 and PAC_1 receptor blockade are promising migraine therapies, but results from clinical trials are needed in order to confirm their efficacy and side effect profile.

Discovery of PACAP

The description of the pituitary adenylate cyclase activating polypeptide-38 (PACAP38) was made by Arimura and his team in 1989, following the extraction of the peptide from more than 4000 samples of ovine hypothalamus. After the isolation, its characterization showed that it was formed by 38 amino acids, with a 68% homology with the vasoactive intestinal peptide (VIP), described almost twenty years earlier[1]. Subsequently, the peptide was synthesized and shown to activate adenylyl cyclase (AC) in cultures of rat pituitary cells, thereby obtaining its name as pituitary adenylate cyclase activating polypeptide. A year later, a fragment of PACAP38 with similar AC activation profile was isolated. This was formed by 27 amino acids and thus named PACAP27[2]. That same year, cloning of cDNA from ovine PACAP38 revealed that the amino acid sequence of the mature human PACAP38 was identical to that of the ovine. In addition, later studies showed that it was identical in all mammals[3], suggesting that it has been conserved during evolution.

This review will give an overview of PACAP, its complex signaling pathway, the role PACAP and its receptors have in physiological conditions and their involvement in some disorders, with special focus on migraine. Moreover, the preclinical results of PACAP (receptor) blockade in migraine models, the side effects that could be expected in clinical trials, and the considerations that must be taken if PACAP (receptor)-antibodies are effective for migraine treatment will be discussed.

Pharmacology

PACAP belongs to a wider group of peptides called the VIP/glucagon/growth hormone releasing factor/secretin superfamily. The ADCYAP1gene, located on chromosome 18, encodes PACAP;

initially, a proprotein is expressed, and later processed to form a 38 amino acid peptide (PACAP38) with a cleavage-amidation site that can generate a 27-residue-amidated fragment (PACAP27). In mammals, the most prevalent form is PACAP38[4], therefore, in this review PACAP38 will be referred as PACAP unless stated otherwise.

Three PACAP receptors have been described: $VPAC_1$, $VPAC_2$ and PAC_1, all coupled to G-proteins (Fig. 1). $VPAC_1$ and $VPAC_2$ receptors present equal affinity for PACAP and VIP and their activation stimulates AC. On the other hand, PAC_1 receptor is 100 times more selective for PACAP and presents a complex signaling pathway[4].

Alternative splicing of the PAC_1 receptor gene results in several isoforms. These receptor variants are characterized by shorter extracellular domains (PAC_1*short*, PAC_1*veryshort*), different inserts in an intracellular loop important for G-protein interaction (PAC_1*null*, PAC_1*hip*, PAC_1*hop1*, PAC_1*hop2*, PAC_1*hiphop1*, PAC_1*hiphop2*) and/or discrete sequences located in transmembrane domains II and IV (PAC_1*TM4*)[5-8]. Of relevance, in humans, twelve homologues have been reported[7,9-11], and have been reviewed elsewhere[12,13]. For each splice variant, PACAP38 and PACAP27 present similar affinity and potency for AC and phospholipase C (PLC) stimulation, but different efficacy (i.e. maximal effect) of PLC responses[14,15]. Although in several processes the activation of AC or PLC can result in similar "stimulatory" responses, in smooth muscle cells (e.g. blood vessels), activation of AC leads to vasodilation, whereas PLC activation results in vasoconstriction. This plays an important role in disorders such as migraine, where expression of a PAC_1 receptor isoform with a lower PLC efficacy could favor AC stimulation, thus facilitating vasodilatory responses in cranial blood vessels[16,17].

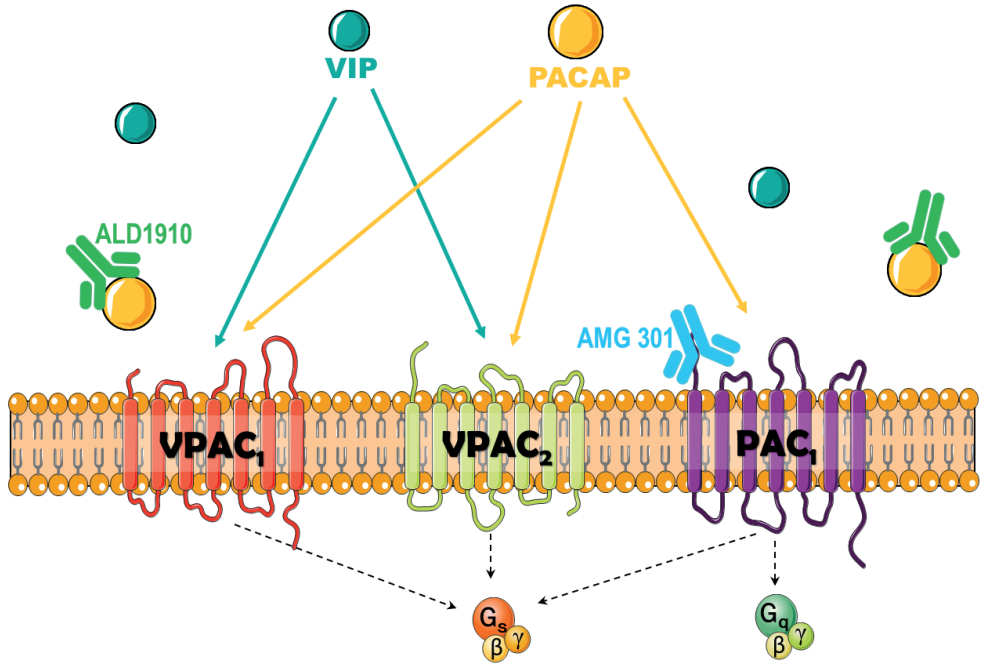

Fig. 1. PACAP receptors. Three receptors to PACAP have been described: $VPAC_1$, $VPAC_2$ and PAC_1. VIP and PACAP show similar affinity for $VPAC_1$ and $VPAC_2$, whereas PACAP is 100-fold more selective for PAC_1 receptor. The antibodies developed for prophylactic migraine treatment bind either to PACAP (PACAP38, ALD1910) or to the PAC_1 receptor (AMG 301).

To study PAC_1 receptor-mediated responses, selective agonists and antagonists are used. Currently, one selective agonist has been described, maxadilan[18,19] and three antagonists M65, Max.d.4 and PACAP6-38[20]. However, no study has investigated whether such compounds are selective for one PAC_1 receptor variant, or whether they bind to all isoforms. Moreover, PACAP6-38 also binds to the $VPAC_2$ receptor, and, together with M65, have been shown to behave as agonists of the PAC_1 receptor in certain tissues[21,22]. Hence, novel selective pharmacological tools are needed to characterize PAC_1 receptor-mediated responses. Indeed, an antibody against PAC_1 receptor, such as AMG 301, could be useful for characterization; however, it is yet not clear if this antibody is selective for one specific variant. If the antibody would be selective for one of the splice variants, this may affect its therapeutic potential, in particular if there are different splice variants expressed in different human populations. On the other hand, different splice variants might hypothetically offer the possibility of designing a drug that would selectively affect the PAC_1 receptor in the trigeminovascular system, while not affecting PAC_1 receptors at other sites in the body, thus reducing its potential side effects.

Physiological roles of PACAP and PAC_1 receptor

Preclinical studies have shown that PACAP and PAC_1 receptors are widely distributed, both centrally and peripherally. It is therefore not surprising that it is described as a (neuro)hormone, neurotransmitter, neuromodulator, neurotrophic factor and immunomodulator[13]. As the PAC_1 receptor is currently under investigation for migraine treatment, only the distribution of this receptor will be reviewed, while the distribution of $VPAC_{1/2}$ receptors is reviewed extensively elsewhere[13,23,24].

PACAP/PAC_1 receptor in the central nervous system

PACAP fibers and PAC_1 receptors are widely expressed throughout the central nervous system (CNS) with the highest density of both in the hypothalamus and supraoptic nucleus[25-31]. In accordance with this, PAC_1 receptor activation has been associated with release of vasopressin and regulation of drinking behavior[32,33], decrease of food intake[34-36], modulation of the sleep/wake cycle[37,38], clock gene expression[38], melatonin synthesis stimulation[39], sexual maturation[40,41], stress and sexual behavior[41,42], learning[43], pain processing[44] and psychomotor responsiveness[45] .

Of special interest for migraine, both PACAP fibers and the PAC_1 receptor are present in the paraventricular nucleus of the hypothalamus, the ventrolateral periaqueductal gray, the locus coeruleus, the solitary nucleus, the trigeminal nucleus caudalis (TNC) and the trigeminal ganglion (TG). These structures have all been associated with nociception and/or migraine pathophysiology[23,46-49].

PACAP/PAC_1 receptor in the periphery

Peripherally, PACAP fibers and/or cell bodies have been described in acrosome caps of primary spermatocytes, mature spermatids, in the testis, epithelial cells from epididymal tubules, the ovaries, mammary glands, in stromal stem cells and terminal placental villi, and the amount of PACAP mRNA increases with the progression of pregnancy[50-52]. Similarly, PAC_1 receptors have been described in spermatids, the penile corpus cavernosum, the ovaries, the chorionic vessels and in stromal and decidual cells of the placenta[51,53-55]. Considering the presence of PACAP and PAC_1 receptors also in hypothalamus and pituitary, an important role in modulation of the hypothalamo-pituitary-gonadal axis is suggested.

PACAP fibers and cell bodies are also found in the adrenal gland, pancreas, epithelium and smooth muscle cells of the urinary tract, the bladder, urethra, larynx, lungs, gastrointestinal smooth muscle cells, duodenal mucosa, thymus, spleen and innervating vascular smooth muscle cells[23,26,56-67]. PAC_1 receptors have been described in the adrenal medulla, pancreas, liver, lungs, enterochromaffin-like cells, thymus and vascular smooth muscle cells[47,56,62,67-70].

Due to their vast distribution peripherally, PACAP and the PAC_1 receptor are involved in a variety of physiological processes, such as regulation of adrenaline release[71], stimulation of adipocyte thermogenesis[72], lipid metabolism[73], metabolic stress adaptation[74], glucose and energy homeostasis[75], renin production[76,77] and inflammatory responses[78]. Furthermore, PACAP and the PAC_1 receptor have a crucial role in the long-term maintenance of neurogenic vasodilation in the periphery and in the homeostatic responses to cerebral, retinal, cardiac, hepatic, intestinal and renal ischemic events[79-88]. This topic has been extensively reviewed elsewhere[89].

PACAP and PAC_1 receptor in pathophysiological conditions

Besides being involved in several physiological processes, PACAP is thought to contribute to the pathophysiology of several conditions.

PACAP has been associated with regulation of inflammatory processes. In an arthritis model, $PACAP^{-/-}$ mice showed absence of arthritic hyperalgesia and reduction of joint swelling, vascular leakage and inflammatory cell accumulation. In the late phase of the disease, immune cell function and bone neoformation were increased[90]. In rheumatoid arthritis, the vasodilatory effects of PACAP through activation of the PAC_1 receptor facilitated plasma leakage, edema formation, and leukocyte migration[91,92]. Furthermore, $PACAP^{-/-}$ mice developed more severe inflammation and tumors in a model of colitis[78]. In preclinical models, upregulation of PACAP and its receptors in micturition pathways contributed to the development of urinary bladder dysfunction, including symptoms of increased voiding frequency and pelvic pain[58], suggesting a role in low urinary tract dysfunction. In the nervous system, studies demonstrated anxiogenic actions of PACAP and the possibility of blocking anxiety-related behaviors with PAC_1 receptor antagonists[93-95]. In patients with post-traumatic stress disorder (PTSD), blood levels of PACAP correlated with severity of stress-related symptoms[96], and in females, a single nucleotide polymorphism in the estrogen response element of the PAC_1 receptor gene is predictive of PTSD diagnosis[97].

Furthermore, PACAP plays a complex role in pain transmission. At the peripheral sensory nerve terminals, pro- and anti-nociceptive effects are observed; while in CNS, central sensitization, increase of neuronal excitation and induction of chronic pain have been described[98]. In an acute somatic and visceral inflammatory model, PACAP decreased pain transmission; however, after application in the spinal cord, a transient induction of analgesia was followed by long-lasting algesia[99]. Moreover, injection of PACAP into the paraventricular nucleus of hypothalamus increased the activity of the TNC, an effect which was inhibited by the PAC_1 receptor antagonist[48]. Although it has been shown that PACAP is transported through the blood-brain barrier (BBB) actively, it is rapidly degraded or returned by efflux pumps[100]. Thus, a direct central action of peripheral PACAP is unlikely.

Although the role of PACAP in pain processing remains elusive, clinical data strongly suggest the involvement of PACAP in the pathophysiology of migraine and cluster headache (CH). Recent evidence of a correlation between a genetic variant of the PAC_1 receptor gene (ADCYAP1R1) and susceptibility to CH was demonstrated[103]. Another study identified a relationship between altered PACAP levels in peripheral blood and different types of headache[104]. Further, two studies reported low interictal plasma levels of PACAP in migraine and CH when compared to controls[105,106]. Particularly, a detailed analysis of PACAP mRNA expression in peripheral blood mononuclear cells detected a significantly lower level of PACAP in migraine patients compared to healthy controls, with no significant differences revealed between the control group and tension-type headache, CH or medication overuse headache groups. Interestingly, PACAP increased ictally in jugular or cubital blood of migraine[105,107,108] and CH patients[93,106], and levels decreased as headache ameliorated after sumatriptan administration[108]. Finally, when administered to migraine patients, PACAP induced an

instant headache in 90% of patients, which was later followed by a delayed headache similar to a migraine-like attack in two thirds of the subjects[109]. This has led to study the role of PACAP in migraine pathophysiology as will be discussed in the next section.

PACAP in migraine pathophysiology

The use and development of experimental animal and human models of headache, migraine in particular, have provided invaluable insight into the pathophysiological mechanisms underlying headache disorders[110,111]. To investigate the molecular mechanisms behind the headache-inducing effects of PACAP, a number of animal studies have been conducted. Additionally, several human studies have been performed, some of these in combination with imaging techniques. In the following sections, both human and animal studies investigating the headache-related effects of PACAP will be reviewed.

Human studies

The headache-inducing effect of PACAP was first reported in a study on cerebral blood flow in healthy volunteers, where 10 out of 11 participants reported mild to moderate headache after PACAP infusion[112]. A double-blind, randomized, placebo-controlled, crossover study later showed that 12 out of 12 healthy subjects and 11 out of 12 migraine patients reported headache after intravenous infusion of PACAP, compared to two and three, respectively, after placebo[109]. Further, two healthy subjects and one migraine patient reported a migraine-like attack within 2 h after infusion, whereas six migraine patients reported a migraine-like attack within 6 h after infusion. This study also found dilation of middle cerebral artery (MCA) and the superficial temporal artery after PACAP infusion.

The role of vasodilation in PACAP induced headache was further explored in a magnetic resonance angiography (MRA) study in healthy volunteers[113]. Eight out of nine participants reported an immediate headache and 100% reported a delayed headache after PACAP infusion. Further, over a 5 h period PACAP induced a sustained dilation of the *extracranial* middle meningeal artery (MMA) but no change in intracerebral MCA. Collectively, these studies support the notion that PACAP induces headache via sustained vasodilation. In another MRA study, PACAP infusion induced headache in 91% of included migraine patients, and 73% reported migraine-like attacks compared to 82% and 18%, respectively, after VIP administration. Further, PACAP induced a long-lasting (>2 h) dilation of extracranial arteries, whereas the dilation caused by VIP normalized after 2 h. This further underlines prolonged vasodilation as the migraine inducing mechanism of PACAP[114]. Interestingly, in an *in vitro* study neither PACAP nor VIP were potent in inducing vasodilation of the human MMA[115].

In a resting-state magnetic resonance study, infusion of PACAP affected connectivity in the salience, the default mode and the sensorimotor network during migraine attacks. VIP had no effect on these networks[116]. Another study in migraine patients reproduced the induction of migraine-like attacks in 72% of patients and showed that PACAP induced premonitory symptoms in 48% of patients compared to 9% after CGRP[117], suggesting an effect on central PAC_1 receptors. However, PACAP is rapidly degraded or transported back after actively crossing the BBB[100]; therefore, the premonitory symptoms could be mediated via activation of a central structure that is not protected by the BBB. Lastly, increased blood markers of hypothalamic activation, neuronal damage and peptide release from parasympathetic and sensory perivascular nerve fibers were found during PACAP-induced migraine-like attacks[118].

The human studies point out PACAP as a key player in migraine pathophysiology[102]. As VIP does not induce migraine-like attacks, it is assumed that PACAP's actions are mediated by PAC_1 receptor activation. However, it is still too early to rule out $VPAC_{1/2}$ receptors as additional potential antimigraine targets, since no studies in humans have been performed with antagonists. Further, the short half-life of VIP (2 min[119]), could be the cause of its lack of migraine-inducing effects.

Animal studies

To characterize the exact receptor involved in PACAP-mediated actions, the vasodilatory effect of PACAP was elucidated in animal studies showing that both VIP, PACAP38 and PACAP27 induce vasodilation of the rat MMA *in vivo*[120,121]. Interestingly, this effect was blocked by $VPAC_1$ antagonists in the former[120] and $VPAC_2$ antagonists in the latter[121]. Both studies found no effect of PAC_1 antagonists on vasodilation. In contrast, an *ex vivo* study found that PAC_1 antagonists reversed the PACAP-induced vasodilation in the rat MMA[17]. As mentioned previously, PAC_1 receptor antagonists have shown agonistic behavior and affinity for $VPAC_2$ receptors. This could explain the contradictory results observed in the rat MMA vasodilation studies. Therefore, different methods must be used, in order to elucidate the receptors involved in migraine pathophysiology. For example, in a *in vivo* model of chronic migraine, induced by recurrent chemical dural stimulation, PAC_1 receptor mRNA was shown to be increased in the TG, but not in the TNC, and no significant differences were found in the expression of the $VPAC_1$ and $VPAC_2$ receptors[122]. Moreover, in an *in vivo* rat model, intravenous administration of AMG 301, the PAC_1 receptor antibody, inhibited evoked nociceptive activity in the trigemino-cervical complex, and the results were comparable to the inhibition observed with sumatriptan[123].

In addition to sustained vasodilation, mast cell degranulation has also been suggested as one of the headache inducing mechanisms of PACAP. This hypothesis is based on findings from animal studies showing that PACAP degranulates mast cells from the rat dura mater[124]. Further, PACAP-induced delayed vasodilation of the rat MMA is attenuated in mast cell depleted rats[125]. Interestingly VIP did not result in mast cell release of histamine from the dura[126].

Collectively, the animal studies confirm that PACAP induces vasodilation and suggest that this effect might be mediated through degranulation of mast cells. Also, recent results show that these effects are most likely exerted through activation of the PAC_1 receptor. Due to the contradictory results, further studies are warranted to confirm this.

PACAP (receptor) blockade as a therapeutic target

As shown above, PACAP seems to play an important role in migraine pathophysiology. Although the exact receptor involved has not yet been elucidated, some studies indicate that the PAC_1 receptor is the most important[17,48,113,117,122,123]. Therefore, both PACAP and PAC_1 receptor have been suggested as novel targets for migraine treatment and possibly a new therapeutic option for patients who do not respond to CGRP (receptor) blocking drugs. Although both neuropeptides co-localize in the trigeminal ganglion[49], and could share some biological cascades, the PACAP-induced migraine attacks indicate an independent role of PACAP in the genesis of migraine.

In this light, the interest from pharmaceutical companies for blocking the PACAP/PAC_1 receptor pathway has increased. There are two therapeutic approaches to inhibit PACAP: (i) PAC_1 receptor antagonists or antibodies directed against this receptor; or (ii) antibodies directed against the peptide PACAP[102]. Since PAC_1 receptor antagonists have been reported to act as agonists depending on the tissue (see Pharmacology), the antibodies seem a better option for blocking this receptor.

Currently, a phase 2a, randomized, double blind, placebo-controlled study is underway to evaluate the efficacy and safety of a PAC_1 receptor antibody (AMG 301) in subjects with chronic or episodic migraine (Clinical trials identifier: NCT03238781). Unfortunately, no preliminary results have been published so far. Preclinical studies are also evaluating a monoclonal antibody (ALD1910) targeting PACAP38 for its potential in the treatment of migraine patients who have an inadequate response to therapeutics directed at CGRP or its receptor[128].

Potential side effects of PACAP/PAC$_1$ receptor blockade

Indeed, the possibility of a new therapeutic target for prophylactic migraine treatment is exciting; however, it is important to consider that PACAP and PAC$_1$ receptor participate in numerous physiological processes (see Fig. 2). As antibodies are not likely to cross the BBB, only the possible side effects regarding peripheral blockade of PACAP and PAC$_1$ receptor will be discussed.

As PACAP and PAC$_1$ receptor are expressed throughout the components of the hypothalamo-pituitary-gonadal axis[50-52], and the pituitary gland is not protected by the BBB, a dysregulation of the functions of this axis could be a concern. Also, the immune system has been described to be regulated by activation of PAC$_1$ receptor[61]. This, together with its participation in the modulation of inflammatory processes, could result in alterations in the immune response and increased production of pro-inflammatory cytokines[78,129]. In accordance with this, in a mouse model of colitis, PACAP-deficient mice developed a more severe disease[78].

Blocking PACAP might also alter the response to metabolic stress. Studies with PACAP-deficient mice have shown a more profound and longer lasting insulin-induced hypoglycemia and a reduction in glucose-stimulated insulin secretion[74,75]. Moreover, PACAP-deficient mice had hepatic microvesicular steatosis, intracellular fat accumulation in muscle and skeletal muscle and depletion of subcutaneous white fat[73].

Furthermore, PACAP and PAC$_1$ receptor participate in vasodilatory responses, renin release and regulation of cardiovascular function[77,115,125]. Although the density of VPAC$_{1/2}$ and PAC$_1$ receptors in coronary artery is less than that in cranial MMA[115], arguing for a limited role in cardiac ischemia, a protective role in ischemic events has been described. Thus, considering the increased cardiovascular risk that migraine patients present[130-133], careful monitoring of patients with preexisting cardiovascular risk factors is advised. However, similar concerns have been raised with the CGRP (receptor)-antibodies[134,135], with no cardiovascular adverse events reported in the clinical trials[136].

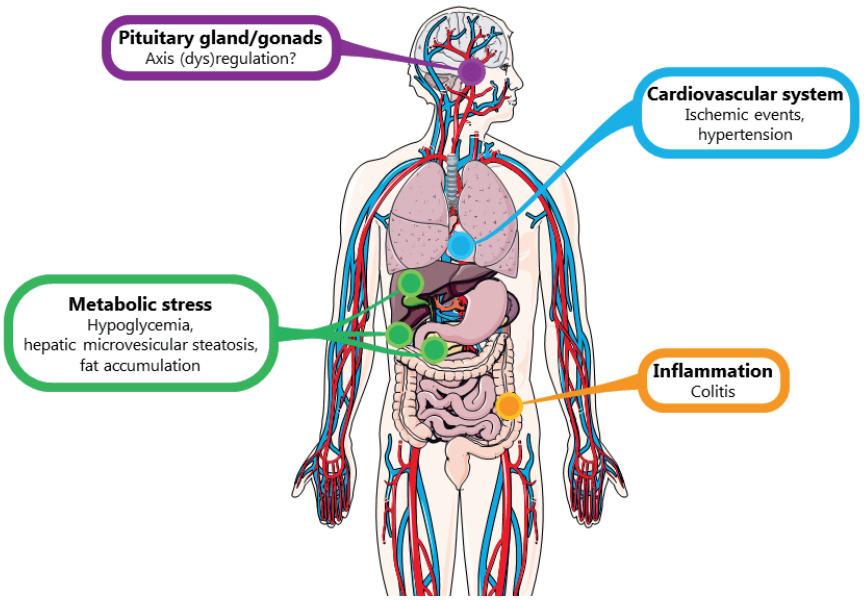

Fig. 2. Possible side effects after long-term exposure to PACAP (receptor)-antibodies. An overview of the organ systems where PACAP and PAC$_1$ receptor are present and the possible side effects that could be observed.

Further considerations

If the antibodies against the PAC_1 receptor prove to be effective for the prophylactic treatment of migraine, some concerns should be addressed. Firstly, as previously discussed, it is important to consider the possible side effects of long-term blocking of PACAP/PAC_1 receptor, with emphasis on the cardiovascular system, as migraine patients present a higher cardiovascular risk. Therefore, safety studies in patients with cardiovascular disease are needed. Moreover, the administration route of the antibody against the PAC_1 receptor is subcutaneous, thus erythema, pruritus and mild pain in the injection site could be expected, as it has been observed with the CGRP (receptor) – antibodies[137]. Nevertheless, the monthly administration represents an advantage for treatment adherence.

It will also be important to define if PAC_1 receptor antibodies will really represent a therapeutic advantage for the patients that are not responding to the CGRP (receptor)-antibodies. Since studies have shown that PACAP and CGRP co-localize in structures relevant for migraine pathophysiology (e.g. trigeminal ganglion)[49], PACAP blockade may only be effective for the same patients to whom CGRP blockade is already effective. If a distinction can be made between patient groups this would also shed light on the pathophysiology of migraine, as it could distinguish between CGRP-associated or PACAP-associated migraine patients. Moreover, the PAC_1 receptor sequence that is recognized by the antibody has not been disclosed, thus, the variants of the receptor to which the antibody binds are not known. If revealed, it would be interesting to study whether certain receptor isoforms predispose patients to present migraine, or whether the treatment will only be effective in patients with those isoforms.

Finally, as mentioned previously, it is still too early to rule out $VPAC_{1/2}$ receptors as therapeutic targets for migraine treatment. Therefore, ALD1910, the antibody against PACAP38, currently undergoing preclinical studies[128], broadens the therapeutic options for migraine treatment. However, further safety studies should be addressed, as blocking PACAP38 would inhibit the actions of three different receptors, increasing the possibilities of adverse side effects.

Conclusion

The possible role of PACAP/PAC_1 receptor blockade as migraine treatment has been reviewed. All three PACAP receptors have been described in TG, TNC and (dural) arteries, structures previously related to migraine pathophysiology[47,49]. Indeed, infusion of PACAP is able to induce migraine-like attacks[109]. Moreover, interictally, low plasma levels of PACAP have been described[105], while during a migraine attack, PACAP increases in jugular and cubital blood[105,108] and decreases as headache ameliorates after sumatriptan administration[108].

Clinical studies have shown that infusion of VIP does not induce migraine-like headaches[114], therefore, it is considered that the possible receptor involved in PACAP actions is PAC_1 receptor, as VIP has affinity for $VPAC_1$ and $VPAC_2$ receptors. Although this could be attributed to pharmacokinetic aspects (i.e. half-life), rather than pharmacodynamic. Pharmacological characterization in preclinical studies has given contradictory results, indicating a complex pharmacology of the PAC_1 receptor[21,22]. However, a recent *in vivo* study showed that intravenous infusion of PAC_1 receptor antibody, inhibited evoked nociceptive activity in the trigemino-cervical complex in rats, and these results were comparable to the inhibition observed with sumatriptan[123]. These results have led to the development of antibodies against PACAP (ALD1910) and PAC_1 receptor (AMG 301) for migraine treatment.

In conclusion, the data presented in this review indicate that PACAP and PAC_1 receptor blockade are promising migraine therapies but results from clinical trials are needed in order to confirm their efficacy and their side effects profile.

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Chapter XI.

Pharmacological analysis of the inhibition produced by moxonidine and agmatine on the vasodepressor sensory CGRPergic outflow in pithed rats

Based on: ***E Rubio-Beltrán****, A Labastida-Ramírez, O Hernández-Abreu, A MaassenVanDenBrink, CM Villalón (2017) European Journal of Pharmacology; 812:97-103.*

Abstract

Calcitonin gene-related peptide (CGRP) plays a role in several (patho)physiological functions, and modulation of its release is considered a therapeutic target. In this respect, electrical spinal (T_9-T_{12}) stimulation of the perivascular sensory outflow in pithed rats produces vasodepressor responses mediated by CGRP release. This study investigated the role of imidazoline I_1 and I_2 receptors in the inhibition by moxonidine and agmatine of these vasodepressor responses. Male Wistar pithed rats (pretreated i.v. with 25 mg/kg gallamine and 2 mg/kg·min hexamethonium) received i.v. continuous infusions of methoxamine (20 µg/kg·min) followed by physiological saline (0.02 ml/min), moxonidine (1, 3, 10 or 30 µg/kg·min) or agmatine (1000 or 3000 µg/kg·min). Under these conditions, electrical stimulation (0.56-5.6 Hz; 50 V; 2 ms) of the spinal cord (T_9-T_{12}) produced frequency-dependent vasodepressor responses which were: (i) unchanged during saline infusion; and (ii) inhibited during the above infusions of moxonidine or agmatine. Moreover, using i.v. administrations, the inhibition by 3 µg/kg·min moxonidine or 3000 µg/kg·min agmatine (which failed to inhibit the vasodepressor responses by αCGRP; 0.1-1 µg/kg) was: (i) unaltered after saline (1 ml/kg), rauwolscine (300 µg/kg; α_2-adrenoceptor antagonist) or BU224 (300 µg/kg; imidazoline I_2 receptor antagonist); and (ii) reversed after AGN 192403 (3000 µg/kg; imidazoline I_1 receptor antagonist). This reversion was relatively more pronounced after AGN 192403 plus rauwolscine. These blocking doses of antagonists lacked any effects on the electrically-induced vasodepressor responses. Therefore, the inhibition of the vasodepressor sensory CGRPergic outflow by moxonidine and agmatine is mainly mediated by prejunctional imidazoline I_1 receptors on perivascular sensory nerves.

Introduction

Blood pressure is determined by the interaction of neural, humoral and local mechanisms. The neural modulation of the vascular tone is mainly mediated by activation of sympathetic (noradrenergic) and sensory (non-adrenergic non-cholinergic) fibres[1]. In turn, perivascular sensory fibres may release (depending on the vascular bed) different neuromediators, including adenosine triphosphate, vasoactive intestinal peptide, neuropeptide Y, substance P, nitric oxide and calcitonin gene-related peptide (CGRP)[2-5]. Of these neuromediators, CGRP has been shown to play an important role in the modulation of vascular tone, as well as in the pathogenesis of several diseases/disorders, such as migraine, diabetes, arthritis and obesity[6].

Due to the (patho)physiological importance of CGRP, it is crucial to elucidate the mechanisms associated with its release. For example, in the pithed rat model, electrical spinal (T_9-T_{12}) stimulation of the perivascular sensory outflow results in vasodepressor responses mainly mediated by CGRP release, as these responses are blocked by the CGRP receptor antagonists $CGRP_{8-37}$[7] or olcegepant[8]. This vasodepressor sensory CGRPergic outflow is modulated by activation of prejunctional $\alpha_{2A/2C}$-adrenoceptors[9], serotonin $5\text{-}HT_{1B/1D/1F/7}$[10,11], dopamine D_2-like receptors[12] and histamine H_3 receptors[13]. Additional evidence suggests that imidazoline receptors can also modulate neurotransmitter release[14,15]. In the pithed rat model, Cobos-Puc *et al.*[16] have demonstrated that activation of prejunctional imidazoline I_1 receptors inhibits the sympathetic vasopressor outflow. Furthermore, Villalón *et al.*[9] have shown that clonidine, an α_2-adrenoceptor and imidazoline I_1 receptor agonist, inhibits the vasodepressor sensory CGRPergic outflow by activation of $\alpha_{2A/2C}$-adrenoceptors; however, this study could not exclude the possible role of imidazoline receptors.

On this basis, the present study was designed to investigate if moxonidine (imidazoline I_1/α_2-adrenoceptor agonist) and agmatine (endogenous ligand of imidazoline receptors) inhibit the vasodepressor sensory CGRPergic outflow, and the receptors involved in this inhibition by using selective antagonists (see Table 1).

Table 1. Affinity constants (pK_i) of the different drugs considered in this study. Receptor binding affinities (pK_i) of the drugs considered in the present study at α_2 adrenoceptors, imidazoline I_1 and I_2 receptors. Data taken from the following references: [1][Ernsberger and Haxhiu, 1997]; [2][Atlas, 1994] ; [3][Li et al., 1994] ; [4][Hieble and Ruffolo] ; [5][Munk et al., 1996]; [6] [Flamez et al., 1997] . N.D. stands for "not determined".

Agonists	α_2	I_1	I_2
Moxonidine	7.1[1]	8.6[1]	5.0[1]
Agmatine	5.4[2]	6.15[3]	6.0[3]
Antagonists			
Rauwolscine	8.0[4]	4.0[1]	N.D.
AGN 192403	4.6[5]	7.4[5]	N.D.
BU224	4.8[6]	5.7[6]	8.6[6]

Materials and methods

Ethical approval of the study protocol

The experimental protocol of this study was approved by our Institutional Ethics Committee (CICUAL Cinvestav; permission protocol number 507-12) and followed the regulations established by the Mexican Official Norm (NOM-062-ZOO-1999) in accordance with the guide for the Care and Use of Laboratory Animals in the U.S.A.[23], the ARRIVE guidelines for reporting experiments in animals[24] and the Legislation for the protection of animals used for scientific purposes Directive 2010/63/EU[25].

General methods

Experiments were carried out in 135 male Wistar normotensive rats (300-350 g). The animals were maintained at a 12/12h light-dark cycle in a special room at a constant temperature (22±2°C) and humidity (50%), with food and water freely available in their home cages. After anaesthesia with sodium pentobarbital (60 mg/kg, i.p.) and cannulation of the trachea, the rats were pithed as reported earlier [9]. This procedure consists in inserting a stainless steel rod through the orbit and *foramen magnum* and into the vertebral *foramen* to avoid the influence of the central nervous system. Immediately afterwards, the animals were artificially ventilated with room air using an Ugo Basile pump model 7025 (56 strokes/min; stroke volume: 20 ml/kg). After bilateral cervical vagotomy, catheters were placed in: (i) the left and right femoral veins for the continuous infusions of methoxamine and the agonists (moxonidine, agmatine or vehicle), respectively; (ii) the left jugular vein for the continuous infusion of hexamethonium; and (iii) the right jugular vein, for the bolus injections of gallamine, αCGRP or the antagonists. Then, the left carotid artery was connected to a Grass pressure transducer (P23 XL), for the recording of blood pressure. Both heart rate (measured with a 7P4F tachograph) and blood pressure were recorded simultaneously by a model 7D Grass polygraph (Grass Instrument Co., Quincy, MA, U.S.A.). At this point, the 135 rats were divided into two main sets to study the effects of i.v. infusions of either the vehicle (physiological saline), moxonidine or agmatine, on the vasodepressor responses induced by either: (i) electrical stimulation of the vasodepressor sensory CGRPergic outflow (set 1, n=115); or (ii) i.v. bolus of αCGRP (set 2, n=20). The dose-response curves (D–R curves) and vasodepressor stimulus-response curves (S–R curves) elicited by α-CGRP and electrical stimulation, respectively, were completed in about 50 min, with no changes in resting blood pressure. The electrical stimuli (0.56, 1, 1.8, 3.1 and 5.6 Hz), as well as the i.v. doses of αCGRP (0.1, 0.18, 0.31, 0.56 and 1 µg/kg), were given using a sequential schedule at 5–10 min intervals as previously reported [9,16]. The body temperature of each pithed rat was maintained at 37 °C by a lamp and monitored with a rectal thermometer.

Experimental protocols

After the animals had been in a stable hemodynamic condition for at least 20 min, baseline values of diastolic blood pressure (a more accurate indicator of peripheral vascular resistance) and heart rate were determined. Subsequently, the following experimental protocols were applied.

Electrical stimulation of the vasodepressor sensory CGRPergic outflow

In the first set or rats (n=115), the pithing rod was replaced by an electrode enamelled except for 1.5 cm length 9 cm from the tip, placing the uncovered segment at the thoracic segments T_9–T_{12} of the spinal cord to allow selective stimulation of the vasodepressor sensory CGRPergic outflow[7,9,16]. Before electrical stimulation, the animals received (i.v.): (i) gallamine (25 mg/kg) to avoid the electrically-induced muscular twitching; (ii) 10 min later, a continuous infusion of hexamethonium (2 mg/kg·min) to block the vasopressor responses resulting from stimulation of the preganglionic sympathetic outflow; and (iii) 10 min later, a continuous infusion of methoxamine (20 μg/kg·min) to increase diastolic blood pressure at a value of, at least, 135 mm Hg[9]. Then, this set of rats was divided into 4 groups (n=40, 25, 25 and 25).

The first group (n=40) was subdivided into four subgroups that received an i.v. continuous infusion of: (i) nothing (control; n=5); (ii) 0.02 ml/min physiological saline (n=5); (iii) moxonidine (1, 3, 10 or 30 μg/kg·min; n=5 each); or (iv) agmatine (1000 or 3000 μg/kg·min; n=5 each). Twenty min later, the perivascular sensory CGRPergic outflow was electrically stimulated during the above treatments to elicit vasodepressor responses by applying 10-s trains of monophasic rectangular pulses (2 ms, 50 V) at 0.56, 1, 1.8, 3.1 and 5.6 Hz. When diastolic blood pressure returned to baseline levels, the next frequency was applied. This procedure was performed until the S-R curve had been completed.

The second group (n=25) received an i.v. continuous infusion of 0.02 ml/min saline and, 10 min later, it was subdivided into 5 subgroups (n=5 each) that were treated with i.v. bolus injections of: (i) 1 ml/kg saline; (ii) 300 μg/kg rauwolscine (α_2-adrenoceptor antagonist); (iii) 3000 μg/kg AGN 192403 (imidazoline I_1 receptor antagonist); (iv) 300 μg/kg BU224 (imidazoline I_2 receptor antagonist); and (v) 300 μg/kg rauwolscine+3000 μg/kg AGN 192403. Ten min later, a S-R curve was elicited as described above, to analyze the effect *per se* of each of these antagonists.

The third group (n=25) received an i.v. continuous infusion of 3 μg/kg·min moxonidine; 10 min later, it was subdivided into 5 subgroups (n=5 each) that received i.v. bolus injections of: (i) 1 ml/kg saline; (ii) 300 μg/kg rauwolscine; (iii) 3000 μg/kg AGN 192403; (iv) 300 μg/kg BU224; and (v) 300 μg/kg rauwolscine+3000 μg/kg AGN 192403. Ten min later, a S–R curve was elicited.

The fourth group (n=25) received an i.v. continuous infusion of 3000 μg/kg·min agmatine. As previously, 10 min later, was subdivided into 5 subgroups (n=5 each) that were administered i.v. bolus injections of: (i) 1 ml/kg saline; (ii) 300 μg/kg rauwolscine; (iii) 3000 μg/kg AGN 192403; (iv) 300 μg/kg BU224; and (v) 300 μg/kg rauwolscine+3000 μg/kg AGN 192403. Ten min later, a S–R curve was elicited.

The interval between the different frequencies of stimulation depended on the duration of the resulting vasodepressor responses (5–10 min), as we waited until diastolic blood pressure had returned to baseline values. It is important to highlight that tachyphylaxis of the vasodepressor responses was observed when eliciting a second S–R curve; as a result, only one S–R curve was performed per animal, as previously reported[9,12].

Administration of exogenous αCGRP

The second set of rats (n=20) was pithed as previously described, but the pithing rod was not replaced with an electrode since electrical stimuli were not applied. Consequently, the pithing rod was left throughout the experiment and the administration of both gallamine and hexamethonium was omitted[9,12]. Subsequently, an i.v. continuous infusion of 20 μg/kg·min methoxamine was administered. Ten min later (with diastolic blood pressure maintained at values of, at least, 135 mmHg), this set was divided into four groups (n=5 each) that received i.v. continuous infusions of: (i) nothing (control group); (ii) 0.02 ml/min physiological saline; (iii) 3 μg/kg·min moxonidine; and (iv) 3000 μg/kg·min agmatine. Twenty min later, the vasodepressor responses by i.v. bolus injections of αCGRP (0.1, 0.18, 0.31, 0.56 and 1 μg/kg) were determined during the continuous infusion of the agonists.

Other procedures applied to the experimental protocols

Hexamethonium, methoxamine, moxonidine, agmatine and saline were infused at a rate of 0.02 ml/min by a WPI model sp100i pump (World Precision Instruments Inc., Sarasota, FL, U.S.A.). The continuous infusion of methoxamine (20 μg/kg·min) increased diastolic blood pressure in all cases. The interval between the different frequencies of stimulation or doses of α-CGRP depended on the duration of the resulting vasodepressor responses (5-10 min), as we waited until diastolic blood pressure had returned to baseline values.

Compounds

The compounds used in this investigation were: gallamine triethiodide, hexamethonium chloride, methoxamine hydrochloride, rat α-CGRP, moxonidine hydrochloride, agmatine sulphate, rauwolscine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); (+/-)-2-endo-Amino-3-exo-isopropylbicyclo[2.2.1]heptane hydrochloride (AGN 192403), 2-(4,5-Dihydroimidazol-2-yl) quinoline hydrochloride (BU224 hydrochloride; purchased from Tocris Bioscience, Ellisville, MO, U.S.A.). All compounds were dissolved in physiological saline. The doses of the above compounds refer to the free base of substances, except in the case of gallamine and hexamethonium, where they refer to the corresponding salts.

Data presentation and statistical evaluation

All data in the text and Fig.s are presented as mean±S.E.M. The changes in baseline values of diastolic blood pressure before and during the continuous i.v. infusion of saline, moxonidine or agmatine were compared with a paired Student's *t*-test. Furthermore, the changes in diastolic blood pressure produced by i.v. bolus of αCGRP or electrical stimulation during saline, moxonidine and agmatine-infused animals were determined and expressed as percentage of change from baseline at the steady state effect of methoxamine. The difference between the changes in diastolic blood pressure induced by electrical stimulation or exogenous α-CGRP within the different subgroups of animals was compared with a two-way analysis of variance. Such analysis was followed, if applicable, by the Student-Newman-Keul's *post hoc* test. Statistical significance was accepted at $P<0.05$.

Results

Systemic haemodynamic variables

The baseline values of diastolic blood pressure and heart rate in the 135 rats were 55±4 mm Hg and 237±8 beats/min, respectively. These values did not change ($P>0.05$) after gallamine or

hexamethonium in the rats receiving electrical stimulation (n=115; data not shown). Twenty min after starting the infusion of methoxamine, diastolic blood pressure was significantly increased in all cases (179±8 mm Hg; n=115). The values of diastolic blood pressure and heart rate in the different subgroups before, immediately after and 10 min after i.v. saline or antagonists during methoxamine infusion were not significantly different (data not shown).

Vasodepressor responses elicited by electrical stimulation of the sensory CGRPergic outflow or exogenous α-CGRP

During the continuous infusion of methoxamine, the responses to electrical stimulation or to i.v. bolus of α-CGRP were immediate and resulted in frequency- or dose- dependent decreases in diastolic blood pressure as previously reported [9,10]. In all cases, the vasodepressor responses were considered to be due to selective vasodepressor stimulation (Fig. 1), as only minor and inconsistent effects in heart rate were observed, as previously shown by Villalón *et al*.[9].

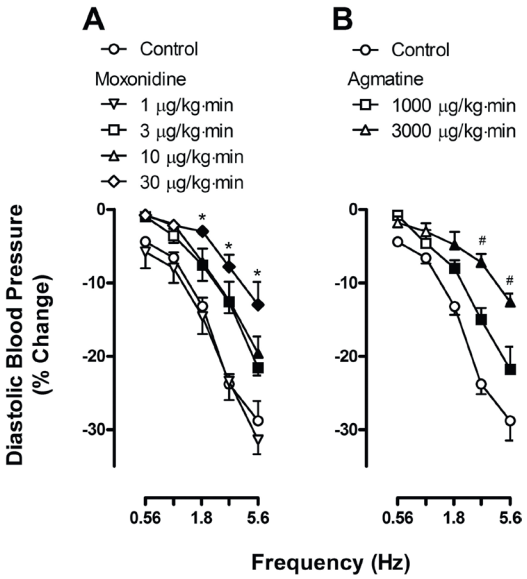

Fig. 1. Effect of continuous i.v. infusions of moxonidine (1, 3, 10 or 30 μg/kg·min) or agmatine (1000 or 3000 μg/kg·min) on the vasodepressor responses induced by electrical stimulation of the sensory CGRPergic outflow. Full symbols represent significant differences *vs* control (P<0.05); *P<0.05 comparing 30 μg/kg·min moxonidine *vs* 3 μg/kg·min moxonidine and *vs* 10μg/kg·min moxonidine; #P<0.05 comparing 3000 μg/kg·min agmatine *vs* 1000 μg/kg·min agmatine. Note that there was no significant difference between the inhibition produced by 3 and 10 μg/kg·min moxonidine.

Effect of i.v. continuous infusions of moxonidine or agmatine on the vasodepressor responses induced by electrical stimulation of the sensory CGRPergic outflow

Fig. 1A shows the inhibition of the vasodepressor responses induced by electrical stimulation of the sensory CGRPergic outflow during i.v. continuous infusions of moxonidine (1, 3, 10 and 30 μg/kg·min). This inhibition, significant as from 3 μg/kg·min moxonidine, was dose-dependent at 10 and 30μg/kg·min moxonidine. On the basis of these results, and considering its selectivity (see Table 1), the dose of 3 μg/kg·min moxonidine was chosen for further pharmacological analysis.

Moreover, the i.v. continuous infusions of agmatine (1000 and 3000 µg/kg·min) dose-dependently inhibited the electrically-induced vasodepressor responses (Fig. 1B). This inhibition was significant at 1.8, 3.1 and 5.6 Hz in all cases (P<0.05).

Effects of compounds per se on the electrically-induced vasodepressor responses

Fig. 2 illustrates that i.v. bolus injections of 1 ml/kg saline (vehicle; Fig. 2A), 300 µg/kg rauwolscine (Fig. 2B), 3000 µg/kg AGN 192403 (Fig. 2C), the combination of 300 µg/kg rauwolscine+3000 µg/kg AGN 192403 (Fig. 2D) or 300 µg/kg BU224 (Fig. 2E) produced no significant effects (P>0.05) on the electrically-induced vasodepressor responses.

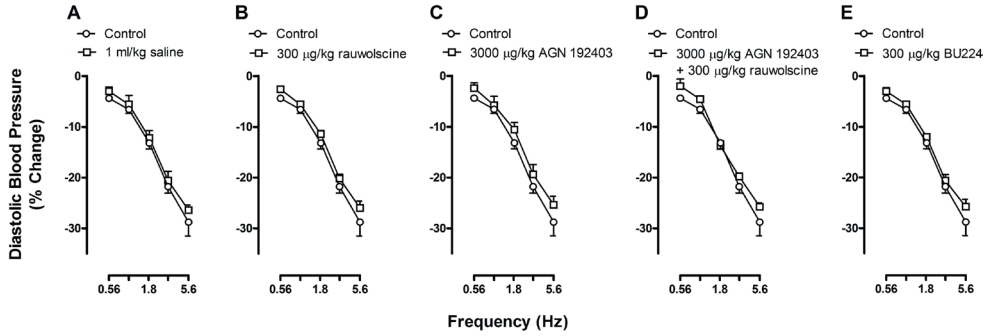

Fig. 2. Effect of i.v. bolus injections of 1 ml/kg saline, 300 µg/kg rauwolscine, 3000 µg/kg AGN192403, 3000 g/kg AGN 192403+300 µg/kg rauwolscine or 300 µg/kg BU224 on the electrically-induced vasodepressor responses. Empty symbols represent no significant difference *vs* control (P>0.05).

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Effect of vehicle or antagonists on the electrically-induced vasodepressor responses during the i.v. continuous infusions of moxonidine

The inhibition by the continuous infusion of 3 µg/kg·min moxonidine on the vasodepressor sensory CGRPergic outflow: (i) was not significantly modified (P>0.05) by the i.v. administration of 1 ml/kg saline (Fig. 3A), 300 µg/kg rauwolscine (Fig. 3B) or 300 µg/kg BU224 (Fig. 3E); and (ii) was significantly (P<0.05) attenuated by the i.v. administration of 3000 µg/kg AGN 192403 (Fig. 3C) or 3000 µg/kg AGN 192403+300 µg/kg rauwolscine (Fig. 3D).

Effect of vehicle or antagonists on the electrically-induced vasodepressor responses during the i.v. continuous infusions of agmatine

The inhibition produced by 3000 µg/kg·min agmatine: (i) was not significantly modified (P>0.05) after the i.v. bolus administration of 1 ml/kg saline (Fig. 3F), 300 µg/kg rauwolscine (Fig. 3G) or 300 µg/kg BU224 (Fig. 3J); and (ii) was reversed by the i.v. administration of 3000 µg/kg AGN 192403 or the combination of 3000 µg/kg AGN 192403+300 µg/kg rauwolscine (Fig. 3H and 3I; P<0.05).

Effect of i.v. continuous infusions of moxonidine or agmatine on the vasodepressor responses induced by i.v. bolus injections of αCGRP

The continuous infusion of: (i) 0.02 ml/min saline (Fig. 4A); (ii) 3 µg/kg·min moxonidine (Fig. 4B); or (iii) 3000 µg/kg·min agmatine (Fig. 4C) did not modify the vasodepressor responses induced by i.v. bolus of αCGRP (P>0.05).

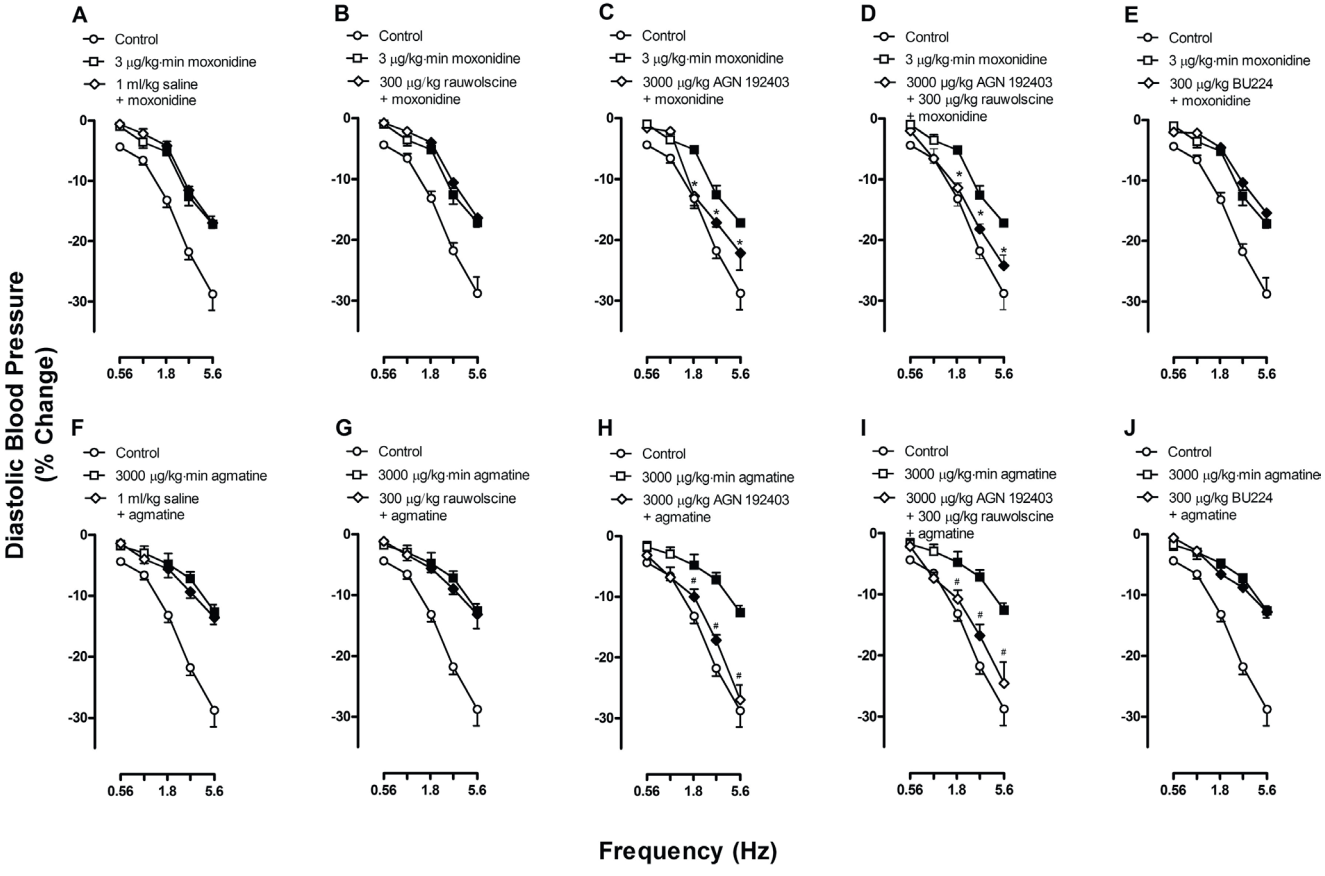

Fig. 3. Effect of i.v. injections of 1 ml/kg saline, 300 μg/kg rauwolscine, 3000 μg/kg AGN 192403, 3000 μg/kg AGN 192403+300 μg/kg rauwolscine or 300 μg/kg BU224 on the inhibition by 3 μg/kg·min moxonidine (upper panel) or 3000 μg/kg·min agmatine (lower panel). Full symbols represent significant differences *vs* control ($P<0.05$); *$P<0.05$ comparing 3 μg/kg·min moxonidine *vs* 3000 μg/kg AGN 192403+moxonidine and *vs* 3000 μg/kg AGN 192403+300 μg/kg rauwolscine+moxonidine; #$P<0.05$ comparing 3000 μg/kg·min agmatine *vs* 3000 μg/kg AGN 192403+agmatine and *vs* 3000 μg/kg AGN 192403+300 μg/kg rauwolscine+agmatine.

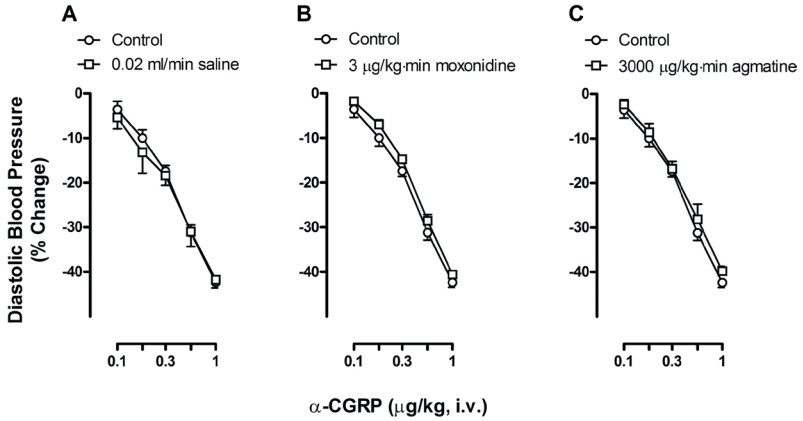

Fig. 4. Effect of i.v. continuous infusions of 0.02 ml/min saline (vehicle), 3 μg/kg·min moxonidine or 3000 μg/kg·min agmatine on the vasodepressor responses induced by i.v. bolus injections of αCGRP. Empty symbols represent no significant differences *vs* control (P>0.05).

Discussion

General

The pithed rat is an experimental model useful for determining the cardiovascular effects of different drugs at the peripheral nervous system (sympathetic and sensory). The nature of this model allows us to discard the influence of the central nervous system, as previously established[9,16]. It is worth mentioning that, although we did not measure sensory nerve activity directly, the electrically-induced neurotransmitter release (i.e. CGRP) was estimated indirectly by the measurement of the evoked vasodepressor responses[9]. Our study shows that the vasodepressor sensory CGRPergic outflow was inhibited by the continuous infusion of moxonidine and agmatine (Fig. 1). These inhibitory responses are mainly mediated by imidazoline I_1 receptors in view that they were: (i) significantly blocked by the imidazoline I_1 receptor antagonist AGN 192403 (Fig. 3); and (ii) resistant to blockade by the α_2-adrenoceptor antagonist rauwolscine or the imidazoline I_2 receptor antagonist BU224, which display very low affinity for imidazoline I_1 receptors (see Table 1). Moreover, the responses to moxonidine and agmatine are considered sensory-inhibitory, since they inhibited the vasodepressor responses to electrical sensory stimulation without affecting those to i.v. bolus injections of exogenous α-CGRP (Fig. 4).

Haemodynamic changes

Our group has previously shown that, in order to observe vasodepressor responses in pithed rats, diastolic blood pressure has to be initially raised and maintained above 135 mm Hg by a continuous i.v. infusion of the α_1-adrenoceptor agonist, methoxamine[9]. Moreover, α_2-adrenoceptors, as well as the imidazoline I_1 and I_2 receptors, do not seem to play a physiological role in the modulation of the vasodepressor sensory CGRPergic outflow as the electrically-induced vasodepressor responses were not significantly affected by i.v. administration of rauwolscine, AGN 192403 or BU224 at doses previously shown to block their respective receptors in the pithed rat model[9,16].

Role of imidazoline I_1 receptors in the inhibition of the vasodepressor sensory CGRPergic outflow by moxonidine and agmatine

Imidazoline I_1 receptors have been shown to inhibit the cardioaccelerator sympathetic outflow[16], but no studies have thus far analyzed whether these receptors also modulate the vasodepressor

sensory CGRPergic outflow. Our experimental approach in pithed rats shows that moxonidine and agmatine inhibited the vasodepressor responses induced by electrical stimulation (Fig. 1) without affecting those by exogenous CGRP (Fig. 4). This finding implies a prejunctional inhibition of the vasodepressor sensory CGRPergic outflow, particularly at the higher stimulation frequencies (1.8, 3.1 and 5.6 Hz). Moreover, considering the binding selectivity of the compounds used (see Table 1), our results suggest a predominant role of the imidazoline I_1 receptors since the inhibitory responses to moxonidine and agmatine were markedly (but not completely) reversed by blocking doses of AGN 192403 (Fig. 3C and 3H).

Pharmacological evidence for the exclusion of the role of α_2-adrenoceptors and imidazoline I_2 receptors

Our findings additionally allow us to exclude the role of α_2-adrenoceptors and imidazoline I_2 receptors. In this respect, since the inhibitory responses to moxonidine and agmatine were resistant to blockade by rauwolscine (Figs. 3B and 3G), an α_2-adrenoceptor antagonist with very low affinity for imidazoline I_1 receptors (see Table 1), the role of α_2-adrenoceptors can be excluded. Nevertheless, in view that moxonidine displays affinity for α_2-adrenoceptors and imidazoline I_1 receptors (Table 1), it was reasonable to assume that the small (though significant) inhibition remaining after AGN 192403 could involve α_2-adrenoceptors. If this were the case, then the combination AGN 192403 plus rauwolscine should have abolished this remaining inhibition. However, the blockade of this response by the above combination (Figs. 3D and 3I) was practically identical to that produced by AGN 192403 alone (Fig. 3C and 3H). Accordingly, the inhibition remaining after AGN 192403 is most likely mediated by novel receptors (probably coupled to $G_{i/o}$) that warrant further experiments falling beyond the scope of the present study.

Finally, the fact that the inhibitory responses to moxonidine and agmatine were resistant to blockade by BU224 (Figs. 3E and 3J), an imidazoline I_2 receptor antagonist with very low affinity for α_2-adrenoceptors and imidazoline I_1 receptors (Table 1), rules out imidazoline I_2 receptors, as previously shown in other experimental models[26].

Potential clinical relevance

As a final point of reflexion, it is important to highlight the potential clinical relevance of the present findings. Indeed, the pharmacological modulation of perivascular CGRP release is of most interest due to the established antimigraine efficacy of the novel CGRP receptor antagonists (i.e. the gepants), with the downside of hepatotoxic side effects and pharmacokinetic limitations[6,27], as well as the potential for cardiovascular side effects[28]. For example, the antimigraine drug olcegepant (a potent CGRP receptor antagonist[29]), has recently been shown to block the vasodepressor sensory CGRPergic outflow and to potentiate the vasopressor sympathetic outflow[8], an effect that might result in an increased vascular resistance and, consequently, in a prohypertensive action. Moreover, the triptans (acute antimigraine 5-$HT_{1B/1D}$ receptor agonists) are capable of inhibiting the trigeminal release of CGRP[30] and also the vasodepressor sensory CGRPergic outflow[10]. On this basis, it is not unreasonable to postulate imidazoline I_1 receptors as a therapeutic target for the development of novel antimigraine drugs with no prohypertensive action. For this purpose, moxonidine (an established central antihypertensive agents[31]), could be explored in other models predictive of antimigraine action, such as the inhibition of neurogenic dural vasodilatation induced by periarterial electrical stimulation in rats, using intravital microscopy[32]. Furthermore, there are other pathologies such as arthritis, obesity and diabetes where the role of CGRP is less clear[6], but its modulation by imidazoline receptors could also be a possible therapeutic target.

Conclusion

Our results suggest that the inhibition of the vasodepressor sensory CGRPergic outflow by 3 µg/kg·min moxonidine and 3000 µg/kg·min agmatine in pithed rats is mainly mediated by a prejunctional activation of imidazoline I_1 receptors on perivascular sensory nerves.

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PART V:

Summary and general conclusions

Chapter XIII.

Summarizing discussion and future perspectives

...and *ends* with reason.

There is nothing higher than reason."

Migraine is a highly prevalent and complex neurovascular disorder[1]. It has been shown that during migraine, (dysfunctional) activation of the trigeminovascular system leads to cranial vasodilation mediated by the release of calcitonin gene-related peptide (CGRP)[2]. Despite the high prevalence of migraine, current treatment options are not effective for all patients. Therefore, a need for novel acute and prophylactic antimigraine drugs exists.

In **Chapter III** we reviewed a new class of acutely acting antimigraine drugs, "ditans", and compared them to the current gold standard for the acute treatment, the triptans. Triptans are 5-$HT_{1B/1D/(1F)}$ receptor agonists that in the past already have been shown to inhibit the release of CGRP from trigeminal fibers via 5-HT_{1D} receptor activation and to constrict cranial and coronary arteries via 5-HT_{1B} receptor activation[3-6]. Due to the coronary vasoconstriction, they are contraindicated in patients with cardiovascular disease[7]; however, it is important to also consider that migraineurs have an increased cardiovascular risk[8,9], therefore it is important to develop antimigraine drugs devoid of vascular effects. On the other hand, lasmiditan was developed as a selective 5-HT_{1F} receptor agonist, which has been shown to be effective for the acute treatment of migraine and with no reported vasoconstrictive properties, suggesting no vascular (side) effects. To corroborate the specificity and vascular profile of lasmiditan, in **Chapter IV**, we set out to investigate its pharmacological properties, in particular, to assess the selectivity, second messenger activity and its potential to induce vasoconstriction *in vitro* (in human isolated coronary, internal mammary and middle meningeal arteries) and *in vivo* (carotid and coronary artery diameters in anesthetized dogs). We compared our results to the responses obtained with sumatriptan, the most commonly prescribed triptan. Our results showed that lasmiditan is a selective agonist of the *human* 5-HT_{1F} receptor that is devoid of contractile properties *in vitro* and *in vivo*. The latter may represent a cardiovascular safety advantage when compared to the triptans.

Due to the important role of CGRP in migraine pathophysiology small-molecule antagonists against the CGRP receptor (gepants) were developed. Unfortunately, due to pharmacokinetic issues and hepatotoxicity reports[10], their development was temporarily halted. In the recent years, novel gepants have been developed and have been shown to be effective for the acute (ubrogepant) and prophylactic (atogepant) treatment of migraine[11]. In **Chapter V**, we analyzed the effects of ubrogepant and atogepant on the relaxations induced by CGRP in human isolated middle meningeal, cerebral and coronary arteries, as well as their antagonistic profile. As triptans are contraindicated in patients with cardiovascular disease due to their contractile properties, we also studied the effects *per se* in proximal and distal coronary arteries and compared them to the responses elicited by zolmitriptan. Our results showed that both gepants antagonized the CGRP-induced relaxations in all vessels studied, being more potent in the cranial arteries when compared to the inhibition observed in human coronary arteries. This property may be of clinical relevance and an advantage for these antagonists, since they seem to be less potent where the antagonism of CGRP-induced vasodilation is not desired. Moreover, atogepant was shown to be more potent in inhibiting the CGRP-mediated vasodilatory responses in all vessels tested. The analysis of the antagonistic profile in human coronary arteries showed that ubrogepant presents a competitive profile and, interestingly, atogepant a non-competitive one. Furthermore, while zolmitriptan elicited concentration-dependent contractions, neither of the gepants had significant vasoconstrictor responses in human coronary arteries.

As the initial trials with gepants (namely, telcagepant)[10] for the acute and prophylactic treatment of migraine were halted due to hepatotoxicity reports, a new class of prophylactic antimigraine drugs was developed: the antibodies against CGRP (eptinezumab, fremanezumab, galcanezumab) or its receptor (erenumab). This represents a milestone in prophylactic treatment, as previous

preventive treatments were not originally developed for migraine but were only later shown to be effective in this disorder. Nevertheless, the antibodies against CGRP (receptor) raise a concern, as CGRP is an ubiquitous peptide, not only involved in migraine pathophysiology, but also in physiological/homeostatic processes[12]. In light of this, in **Chapter VI**, we discussed the pros and cons of long-term CGRP blockade in migraine patients. In support of this therapeutic approach, clinical trials have shown that this treatment is efficacious, with results that seem comparable to the currently used prophylactic drugs. Furthermore, no major adverse effects related to CGRP (receptor) blockade have been reported. Additionally, the liver toxicity induced by some of the gepants is not present with the antibodies, which are well tolerated. On the other hand, as previously mentioned, there are concerns about the long-term effects, as CGRP and its receptor are abundantly present in both the vasculature, and in the peripheral and central nervous system, and are involved in several physiological processes and in the homeostatic response to ischemic events. Therefore, blocking CGRP may pose a risk in subjects with comorbidities such as cardiovascular diseases. In addition, evidence from animal studies suggests that CGRP blockade may induce constipation, affect the homeostatic functions of the pituitary hormones and/or attenuate wound healing, though, none of these effects have so far been reported in clinical trials. Taking into consideration the abovementioned aspects, the pros of blocking CGRP in migraine patients seem to exceed the cons, but adequate (cardiovascular) safety studies are required.

In line with the cardiovascular concerns, Depre *et al.*[13], recently addressed this matter by evaluating the effect of erenumab (CGRP receptor antibody) on exercise time during a treadmill test in patients with stable angina, concluding that the inhibition of the canonical CGRP receptor does not seem to worsen myocardial ischemia, contrary to theoretical concerns. Nevertheless, in **Chapter VII**, we commented on this study, as we considered that the design, the chosen patient population and the interpretation of the results do not entirely support such conclusions. To begin with, the authors of the study stated that the concentrations of exogenous CGRP required to increase total exercise time or protect against myocardial ischemia far exceed the endogenous physiological levels of CGRP that are released during a response to ischemia. This is not completely correct, as it is not the *systemic* plasma concentration that is relevant, but the concentration of CGRP at the *neuro-vascular junction*, where CGRP is released. Furthermore, the authors quoted the lack of vasoconstrictive properties of erenumab[14] as supporting argument of their conclusion, when the main concern is whether inhibition of the (vasodilatory) actions of CGRP is detrimental during an ischemic event. Regarding the study population, the patients included (78% male) suffered from stable angina pectoris, a typically considered "male" form of cardiac pathology[15], often caused by stenosis of the epicardial conducting portions of the coronary artery, where the importance of CGRP is limited. In contrast, in females, coronary artery disease often presents without an angiographically detectable stenosis but as diffuse atherosclerosis in the intramyocardial, smaller (distal) sections of the coronary artery bed[15-18] where the role of CGRP vasodilation seems more pronounced. As the majority of migraine sufferers are females, and therefore represent the main patient population under treatment with the CGRP (receptor) antibodies, the study failed to represent a relevant population. Moreover, the pharmacokinetic and pharmacodynamic considerations of this study were not adequate, since the plasma concentrations obtained 30 minutes after intravenous infusion could not ensure blockade of the CGRP receptor because it may well have taken several hours before the drug reached sufficiently high levels at the level of the receptor (located in the smooth muscle wall[16]), able to induce an effective blockade. Unfortunately, the authors did not confirm the blockade of the CGRP receptor and in previous studies the earliest time point shown for such blockade is 2 days after intravenous administration[19]. Overall, the study lacked to provide

evidence of effective CGRP receptor blockade and did not represent the patients that will benefit the most from the use of erenumab (*i.e.* females with microvascular disease) and might potentially be at the highest risk. As migraine patients have an increased cardiovascular risk, there will be cases of patients with ischemic complaints, even without a causal relationship. In fact, in the most recent interim analysis of the open-label extension study of erenumab, a patient died of what researchers called an "arteriosclerosis event"[20]. Therefore, appropriate studies in relevant study populations are needed to avoid sudden distress, such as happened with the triptans in the past.

Considering the abovementioned concerns, in **Chapter VIII** we characterized the relaxant responses to CGRP *in vitro,* in the absence and presence of erenumab, the human CGRP-receptor antibody approved for the prophylactic treatment of migraine, in isolated human middle meningeal, internal mammary of cardiovascularly compromised patients undergoing bypass surgery and (proximal and distal) coronary arteries. Furthermore, in mammary arteries, we also assessed whether the vasodilatory responses to acetylcholine, sodium nitroprusside, pituitary adenylate cyclase activating polypeptide-38 (PACAP), vasoactive intestinal peptide (VIP) and nicardipine, as well as the vasoconstrictor responses to dihydroergotamine (DHE), were modified by erenumab. Our results showed that the CGRP-mediated vasodilatory responses were significantly antagonized in the presence of erenumab with no significant difference in potency among tissues, contrary to what we observed with the gepants in **Chapter V**. Moreover, in mammary arteries, erenumab did not affect the responses to the other vasoactive compounds, suggesting functional specificity.

Even though the development of the antibodies against CGRP or its receptor for the prophylactic treatment of migraine are a major breakthrough, for the past decades the preventive treatment has consisted of drugs initially developed for other diseases, such as hypertension, epilepsy and depression. Although these drugs have been proven to be effective[21], their exact mechanism of action has not been described. As the trigeminovascular system is currently the main target for migraine treatment, in **Chapter IX**, we set out to investigate whether propranolol, one of the most widely prescribed drugs for the prophylactic treatment of migraine, modulates the activation of the trigeminovascular system. For this purpose, we investigated the effect of propranolol on the rise of dermal blood flow (DBF) of the forehead skin (innervated by the trigeminal nerve) by capsaicin application and electrical stimulation before and after placebo and propranolol in a randomized, double-blind, cross-over study, including healthy females on contraceptives and males. Additionally, we correlated our results with data from a Dutch prescription database by analyzing the change in triptan use after propranolol prescription in a population similar to our DBF study subjects. Our results showed that the DBF responses to capsaicin were attenuated after propranolol, but not after placebo. Interestingly, when we stratified by sex, no changes in the DBF responses to capsaicin were observed in females after propranolol, whereas a significant decrease remained present in males. DBF responses to electrical stimulation were not modified in any of the cases. Furthermore, when comparing the change in triptan use after propranolol, a more pronounced decrease was observed in male patients than in female patients on contraceptives. Our results suggest that propranolol modulates the trigeminovascular system activation in a sex-dependent manner, as in female subjects, that unfortunately represent the great majority of migraine patients, no significant DBF inhibition was observed after propranolol (80 mg) and in our retrospective study, a seemingly lower decrease in triptan use was observed in female patients.

As the understanding of migraine pathophysiology increases, novel therapeutic targets are proposed. In **Chapter X**, we reviewed one of the most recently proposed targets: the pituitary adenylate cyclase activating polypeptide-38 (PACAP) and one of its receptors, the PAC_1 receptor. PACAP is a neuropeptide described to be involved in neuroprotection, neurodevelopment,

nociception and inflammation. Interestingly, PACAP and its receptors are present in the different components of the trigeminovascular system, and intravenous infusion of PACAP induces migraine-like attacks. This led to the development of antibodies against PACAP (ALD 1910) and also against the receptor considered to be the most likely involved in the pathophysiology of migraine, the PAC_1 receptor (AMG 301), with the latter drug already being in Phase II clinical trials. No results have been published so far, but preclinical studies with AMG 301 have shown positive results comparable to those observed with triptans. However, as previously discussed with the CGRP (receptor)-antibodies, if these antibodies prove to be effective for the treatment of migraine, several considerations must be addressed, such as the potential side effects of long-term blocking of the PACAP (receptor) pathway and whether these antibodies will really represent a therapeutic advantage for the patients that do not respond to the CGRP (receptor)-antibodies.

Since inhibition of CGRP release is one of the proposed mechanisms of action of triptans[22] and imidazoline receptors have been described to inhibit neurotransmitter release[23,24], in **Chapter XI** we investigated whether moxonidine, an imidazoline I_1/α_2-adrenoceptor agonist, and agmatine, the endogenous ligand of the imidazoline receptors, inhibit the vasodepressor sensory CGRPergic outflow in pithed rats, and we further characterized the receptors involved. Our results showed that the infusion of moxonidine or agmatine inhibited the vasodepressor responses induced by stimulation of the sensory CGRPergic outflow, but not the responses to i.v. bolus of CGRP. Moreover, the inhibition of the vasodepressor responses was reversed after administration of the imidazoline I_1 receptor antagonist, and relatively more pronounced after administration of the combination of the imidazoline I_1 receptor antagonist plus the $\alpha_2$adrenoceptor antagonist. Therefore, the inhibition of the vasodepressor sensory CGRPergic outflow by moxonidine and agmatine is mainly mediated by prejunctional imidazoline I_1 receptors on perivascular sensory nerves and could represent a therapeutic target for migraine treatment.

Despite the major therapeutic advances in the last decades with the triptans and, more recently, with the novel ditans, gepants and the antibodies against CGRP or its receptor, not all migraine patients respond to treatment, and thus, new therapeutic targets are needed. For this, we need to understand migraine pathophysiology, as it still remains largely unknown. Animal models have contributed greatly to our current knowledge, but only represent certain features of this rather complex disorder. There are, however, monogenic diseases such as Autosomal dominant Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL, a vasculopathy caused by a mutation in the *TREX1* gene that presents migraine as the earliest manifestation), that provide an opportunity to study the genetic and vascular mechanisms involved in migraine pathophysiology. In **Chapter XII**, we assessed whether a new mouse model of RVCL (RVCL-KI), has features in line with the pathology seen in patients, such as a reduced life expectancy and a vascular phenotype (as assessed by functional vascular measurements and induction of experimental stroke). Our results showed that, in line with the phenotype in patients, mutant mice showed increased mortality, signs of abnormal vascular function and increased sensitivity to experimental stroke, suggesting that this transgenic mouse model can be instrumental to study the mechanisms underlying RVCL, as well as to further understand the pathophysiology of migraine.

Future perspectives

In *Chapter IV*, we demonstrated that lasmiditan is a selective agonist of the *human* 5-HT_{1F} receptor, devoid of contractile properties *in vitro* and *in vivo*. Currently, the exact mechanism(s) of action of lasmiditan remains elusive. As the therapeutic efficacy of triptans for the acute treatment of migraine is believed to be mediated via vasoconstriction of the trigeminal-innervated vasculature

and inhibition of CGRP release from the trigeminal fibers[22], future studies should now assess whether lasmiditan modulates the activation of the trigeminovascular system. For this, our human model of trigeminal nerve-mediated vasodilation is an excellent option, as the results could be compared to the results obtained previously by our group in healthy volunteers with sumatriptan[25]. Furthermore, the response to lasmiditan could also be studied in migraine patients, to analyze whether in their case, modulation of the trigeminovascular system is altered.

As mentioned above, our *human* model of trigeminal nerve-mediated vasodilation is an excellent option to study modulation of the trigeminovascular system *in vivo*. In *Chapter XI* we showed that moxonidine inhibits the CGRPergic outflow in pithed rats by activation of prejunctional (imidazoline I_1) receptors; therefore, it would be of great interest to further confirm our results by analyzing the effect of moxonidine in our human model of trigeminovascular activation. This could reinforce moxonidine (and the I_1 imidazoline receptors), as a therapeutic option for migraine treatment. Moreover, in this thesis, we characterized the inhibition of the CGRP-induced relaxations in human cranial and coronary arteries by ubrogepant, atogepant (*Chapter V*) and erenumab (*Chapter VIII*). Further studies could evaluate in our human model the modulation of the trigeminovascular system by both gepants and erenumab and assess whether the responses differ depending on the type of drug (gepants *vs.* antibodies), the intended type of treatment (acute *vs.* prophylactic) or whether there are no differences at all. Especially as at the moment, the only rationale behind choosing atogepant and erenumab for the prophylactic and ubrogepant for the acute treatment of migraine seems to be their half-life.

Besides investigating the mechanisms underlying the therapeutic efficacy of ubrogepant, atogepant and erenumab, future well-designed studies should evaluate the cardiovascular safety of these drugs. In the case of the gepants, experiments can be performed in a porcine *in vivo* model of ischemia-reperfusion, where one might assess cardiac function and infarct size in the absence and presence of cumulative doses of ubrogepant and, more importantly, atogepant (developed for the prophylactic treatment). In the case of erenumab, studies cannot be performed in animal models as it is a selective antibody against the *human* CGRP receptor, unless an antibody with affinity for the porcine (or rat/mice) receptor is developed. Alternatively, we could evaluate the effect of erenumab in a human model of microvascular function. Our group has previously assessed microvascular function with local thermal hyperemia (LTH) of the skin, measured with a laser Doppler flow imager[26] in healthy volunteers. Prospective studies should assess microvascular function before and after erenumab in a population representative of migraine patients, taking into consideration the pharmacokinetics of erenumab.

Our group has previously evaluated the vasodilatory responses to PACAP in human meningeal and coronary arteries[27], however, due to the complex pharmacology of the $VPAC_{1/2}$ and PAC_1 receptors[28], the exact receptor mediating the vasodilatory responses remained unclear. As discussed in *Chapter X*, if clinical trials show that the novel antibody against the PAC_1 receptor (AMG 301) is effective for the prophylactic treatment of migraine, this would suggest a peripheral site of action. Therefore, studies should evaluate whether the observed vasodilatory responses to PACAP in human meningeal and coronary arteries are inhibited in the presence of AMG 301. Otherwise, it would mean that the most likely site of action of the antibodies against the PAC_1 receptor is the trigeminal ganglion rather than the dural vasculature.

Finally, CGRP does not only bind to the canonical CGRP receptor, but also to the adrenomedullin (AM_1, AM_2) and amylin (AMY_1, AMY_3) receptors. Similarly, adrenomedullin and amylin can bind to the canonical CGRP receptor[29]. In migraine patients under erenumab treatment, CGRP may stimulate the amylin receptors described in the trigeminovascular system[30], reducing its efficacy. Conversely,

the amylin receptor has been proposed as the 'second' CGRP receptor previously reported by our group in the human coronary artery[31,32], which may represent a cardiovascular safety benefit for erenumab. Future studies should evaluate the vasodilatory responses to adrenomedullin and amylin in human isolated meningeal and coronary arteries in the presence of erenumab and whether these responses differ between meningeal and coronary arteries.

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Nederlandse samenvatting

Migraine is een complexe neurovasculaire aandoening met een hoge prevalentie[1]. Het is aangetoond dat, tijdens een migraine aanval, (dysfunctionele) activatie van het trigeminovasculaire systeem tot craniale vasodilatatie leidt, opgewekt door de afgifte van calcitonin gene-related peptide (CGRP)[2]. Ondanks de hoge prevalentie van migraine zijn de huidige behandelopties niet bij alle patiënten effectief. Daarom bestaat er een behoefte aan de ontwikkeling van nieuwe acute en profylactische antimigraine middelen.

In **hoofdstuk III** geven we een overzicht van een nieuwe klasse acuut-werkende antimigraine middelen, "ditanen", en vergeleken deze met de huidige gouden standaard voor de acute behandeling, de triptanen. Triptanen zijn 5-$HT_{1B/1D/(1F)}$ receptor agonisten waarvan in het verleden al aangetoond is dat ze de afgifte van CGRP in trigeminale vezels remmen door middel van 5-HT_{1D} receptor activatie en craniële en coronaire arteriënvernauwen door middel van 5-HT_{1B} receptor activatie[3-6]. Vanwege de coronaire vasoconstrictie worden deze middelen gecontra-indiceerd bij patiënten met cardiovasculaire aandoeningen[7]. Het is belangrijk om ook rekening te houden met het feit dat migraineurs een verhoogd risico op cardiovasculaire aandoeningen hebben[8, 9]. Om deze reden is het belangrijk dat antimigraine middelen zonder vasculaire effecten ontwikkeld worden. Lasmiditan is ontwikkeld als een selectieve 5-HT_{1F} receptor agonist, hetgeen aangetoond effectief was bij de acute behandeling van migraine en zonder vermelde vasoconstrictieve eigenschappen, wat erop wijst dat lasmiditan geen vasculaire (bij)werkingen heeft. Om de specificiteit en het vasculair profiel van lasmiditan te onderzoeken, hebben we in **hoofdstuk IV** de farmacologische eigenschappen van dit middel onderzocht. We hebben de selectiviteit en second messenger activiteit bestudeerd, net als de potentie om *in vitro* vasoconstrictie te induceren (in de humane geïsoleerde coronair arterie, de a. mammaria interna en de a. meningea media), net als *in vivo* (diameters van de a. carotis en de coronair arterie in honden onder anesthesie). We vergeleken onze resultaten met die verkregen met sumatriptan, de triptaan die het vaakst voorgeschreven wordt. Onze resultaten toonden aan dat lasmiditan een selectieve agonist van de *humane* 5-HT_{1F} receptor is, die zowel *in vitro* als *in vivo* geen vaatvernauwende eigenschappen heeft. Dit laatste vormt mogelijk een cardiovasculair veiligheidsvoordeel ten opzichte van de triptanen.

Gezien de belangrijke rol van CGRP in de pathofysiologie van migraine zijn kleine molecuul antagonisten tegen de CGRP receptor (gepanten) ontwikkeld. Helaas is hun ontwikkeling tijdelijk stopgezet wegens farmacokinetische en hepatotoxische tekortkomingen[10]. Recent zijn nieuwe gepanten ontwikkeld en deze zijn aangetoond effectief voor de acute (ubrogepant) en profylactische (atogepant) behandeling van migraine[11]. In **hoofdstuk V** analyseerden we de effecten van ubrogepant en atogepant op de door CGRP veroorzaakte relaxaties in de humane geïsoleerde a. meningea media, cerebrale en coronaire arteriën, evenals hun antagonistische profiel. Aangezien triptanen gecontraïndiceerd zijn bij patiënten met hart- en vaatziekten vanwege hun contractiele eigenschappen hebben we ook de effecten *per se* bestudeerd in proximale en distale coronair arteriën en deze vergeleken met de contractiele effecten van zolmitriptan. Onze resultaten lieten zien dat beide gepanten de CGRP-geïnduceerde relaxaties in alle bestudeerde bloedvaten remden, waarbij de remming potenter was in de craniële arteriën in vergelijking met die in humane kransslagaders. Deze eigenschap is mogelijk klinisch relevant , aangezien een voordeel van deze antagonisten is dat deze minder sterk werkzaam lijken te zijn op plaatsen waar de remming van CGRP-geïnduceerde vasodilatatie niet wenselijk is. Bovendien toonde atogepant zich meer potent in het remmen van de CGRP-gemedieerde vasodilatoire response in alle geteste bloedvaten. De analyse van het antagonistische profiel in humane kransslagaders laat zien dat ubrogepant zich profileert als een competitieve antagonist, terwijl atogepant opmerkelijk genoeg een non-competitieve antagonist

lijkt. Terwijl zolmitriptan dosis-afhankelijke contracties teweeg bracht, gaf geen van de gepanten na toediening een significant vaatvernauwende respons in humane kransslagaders.

Aangezien de eerste studies met gepanten (namelijk telcagepant)[10] voor de acute en profylactische behandeling van migraine stopgezet waren wegens gerapporteerde hepatotoxicitet, werd een nieuwe klasse profylactische antimigrainemiddelen ontwikkeld: de antlichamen tegen CGRP (eptinezumab, fremanezumab, galcanezumab) of de CGRP receptor (erenumab). Dit is een mijlpaal in de profylactische behandeling van migraine, aangezien eerdere preventieve behandelingen niet specifiek voor migraine ontwikkeld waren, maar later pas effectief bleken te zijn voor de behandeling van deze aandoening. Desondanks zijn er zorgen over de antilichamen tegen de CGRP (receptor), omdat CGRP een alom aanwezig peptide is dat niet alleen betrokken is bij de pathofysiologie migraine, maar ook bij fysiologische/homeostatische processen[12]. In het licht hiervan bespreken we in **hoofdstuk VI** de voor- en nadelen van lange-termijn CGRP remming bij migraine patiënten. In het voordeel van deze therapeutische aanpak spreken klinische studies waarin werd aangetoond dat deze behandeling effectief is, met resultaten die vergelijkbaar lijken te zijn met de profylactische middelen die op dit moment gebruikt worden. Bovendien zijn er geen grote schadelijk effecten rondom CGRP (receptor) remming gevonden. Daarnaast induceren de antilichamen niet de levertoxiciteit die bij sommige gepanten optreedt. Aan de andere kant zijn er, zoals eerder genoemd, zorgen over de lange-termijn effecten, gezien het feit dat CGRP en de CGRP receptor in overvloed aanwezig zijn in zowel de vasculatuur als in het perifere en centrale zenuwstelsel, en dit peptide betrokken is bij meerdere fysiologische processen en in de homeostatische respons op ischemische gebeurtenissen. Om deze reden kan de remming van CGRP een risico vormen bij patiënten met comorbiditeiten zoals cardiovasculaire ziekten. Bovendien wijzen dierstudies uit dat CGRP remming kan leiden tot constipatie, een negatieve invloed heeft op de homeostatische functies van de hypofyse hormonen en/of wondheling afzwakt, alhoewel geen van deze effecten tot dusver gevonden worden bij klinische studies. De voorgenoemde zaken in ogenschouw nemend, lijken er meer voordelen aan aan CGRP remming bij migraine patiënten verbonden te zijn dan nadelen, maar adequate (cardiovasculaire) veiligheidsstudies zijn noodzakelijk.

In lijn met de cardiovasculaire bezwaren, zijn Depre *et al.*[13] recentelijk op deze kwestie ingegaan met een evaluatie van het effect van erenumab (CGRP receptor antilichaam) op de trainingstijd tijdens een inspanningstest bij patiënten met stabiele angina pectoris. Hieruit kwam naar voren dat de remming van de canonieke CGRP receptor de myocard ischemie niet lijkt te verergeren, in tegenstelling tot de theoretische bezwaren. Desalniettemin geven wij in **hoofdstuk VII** commentaar op deze studie, aangezien wij vinden dat de opzet, de gekozen patiëntenpopulatie en de interpretatie van de resultaten dit soort conclusies niet geheel ondersteunen. Om te beginnen beweren de auteurs van de studie dat de concentraties van exogeen CGRP die nodig zijn om de totale trainingstijd te verhogen of om te beschermen tegen myocard ischeme veel hoger zijn dan de endogene fysiologische CGRP spiegels die afgegeven worden tijdens een respons op ischemie. Dit is niet geheel juist, aangezien het niet de *systemische* plasma concentratie is die relevant is, maar de concentratie van CGRP bij de *neurovasculaire verbinding*, waarbij CGRP vrijkomt. Voorts halen de auteurs het gebrek aan vasoconstrictieve eigenschappen van erenumab[14] aan als ondersteunend bewijs voor hun conclusie, terwijl het belangrijkste probleem is of remming van de (vasodilatoire) werking van CGRP tijdens een ischemische gebeurtenis nadelig is. Wat de studiepopulatie betreft: er waren patiënten geïncludeerd (78% mannelijk) die leden aan stabiele angina pectoris, wat als een typisch "mannelijke" vorm van cardiale pathologie[15] beschouwd wordt, welke veelal veroorzaakt wordt door stenose in de epicardiale, geleidende delen van de kransslagader, waar het belang van

CGRP beperkt is. In tegenstelling tot mannen openbaren coronaire hartziekten zich bij vrouwen vaak zonder een angiografisch meetbare stenose, maar als diffuse atherosclerose in de intra-myocardiale, kleinere (distale) secties van het coronaire vaatbed[15-18] waar de rol van CGRP vasodilatatie veel groter lijkt te zijn. Gezien het feit dat de meerderheid van de patiënten die aan migraine ljiden vrouw is, en daarom de belangrijkste patiëntenpopulatie is die onder behandeling zal staan van de CGRP (receptor) antilichamen, werd in de bovengenoemde studie niet de relevante populatie onderzocht. Daarnaast waren de farmacokinetische en farmacodynamische redeneringen in deze studie niet adequaat, aangezien de plasma concentraties, die 30 minuten na intraveneuze infusie verkregen zijn, geen remming van de CGRP receptor kunnen garanderen omdat het een paar uur kan duren voordat het middel een voldoende hoge concentratie bereikt heeft op het niveau van de receptor (die zich in de gladde spiercel wand[16] bevindt), om zo een effectieve remming teweeg te brengen. Helaas hebben de auteurs de remming van de CGRP receptor niet bevestigd, en in eerdere studies werd een dergelijke remming op zijn vroegst 2 dagen na intraveneuze toedieningvastgesteld[19]. Samengevatwerd in de studie geen bewijs voor effectieve CGRP receptor remming geleverd en omvatte de studie niet de groep patiënten die het meeste baat zouden hebben bij het gebruik van erenumab (d.w.z. vrouwen, veelal met microvasculaire aandoeningen) en wellicht het grootste risico lopen bij gebruik van het middel. Aangezien migraine patiënten een verhoogd risico op hart- en vaatziekten hebben, zullen er gevallen van patiënten met ischemische klachten zijn, zelfs zonder een causaal verband. Er is zelfs in de meest recente interim-analyse van de open-label extensiestudie van erenumab een patiënt overleden aan wat onderzoekers een "manifestatie van arteriosclerose" noemden[20]. Daarom zijn passende studies in relevante studiepopulaties noodzakelijk om plotselinge problemen, zoals in het verleden met triptanen, te voorkomen.

De voorgenoemde bedenkingen in ogenschouw nemend, typeren we in *hoofdstuk VIII* de relaxerende respons op CGRP *in vitro,* in de aan- en afwezigheid van erenumab, het humane CGRP-receptor antilichaam geregistreerd voor de profylactische behandeling van migraine. We hebben de studie uitgevoerd in de humanegeïsoleerde a. meningea media, a. mammaria interna van cardiovasculair gecompromitteerde patiënten die een bypass operatie ondergingen en (proximale en distale) coronair arteriën. Bovendien hebben we in de a. mammaria interna onderzocht of de vaatverwijdende respons op acetylcholine, natrium nitroprusside, pituitary adenylate cyclase activating polypeptide-38 (PACAP), vasoactive intestinal peptide (VIP) en nicardipine, evenals de vaatvernauwende respons op dihydroergotamine (DHE), beïnvloed werden door erenumab. Onze resultaten lieten zien dat de CGRP-gemedieerde vaatverwijdende responsen significant afnemen in de aanwezigheid van erenumab, waarbij geen significant verschil in werkzaamheid is opgemerkt tussen de verschillende weefsels, in tegenstelling tot wat we bij de gepanten zagen in *hoofdstuk V.* In de a. mammaria interna liet erenumab bovendien geen effect zien op de responsen op de andere vasoactieve stoffen, wat wijst op een functionele specificiteit.

Waar de ontwikkeling van de antilichamen tegen CGRP of de CGRP receptor voor de profylactische behandeling van migraine een enorme doorbraak markeren, bestond de preventieve behandeling in de afgelopen decennia voornamelijk uit middelen die in eerste instantie ontwikkeld waren voor andere ziekten, zoals hypertensie, epilepsie en depressie. Ofschoon deze middelen bewezen effectief zijn[21], is hun exacte werkingswijze nog niet bekend. Aangezien het trigeminovasculaire systeem op dit moment het voornaamste doelwit is bij de behandeling van migraine, onderzoeken we in *hoofdstuk IX* of propranolol, één van de meest voorgeschreven middelen voor de profylactische behandeling van migraine, de activatie van het trigeminovasculaire systeem reguleert. Voor dit doel onderzochten we het effect van propranolol op de verhoging van de doorbloeding van de huid (dermal blood flow, DBF) in het voorhoofd (geprikkeld door de n. trigeminus) door

de toediening van capsaïcine en electrische stimulatie voor en na toediening van placebo en propranolol in een gerandomiseerde dubbelblinde cross-over studie bij gezonde vrouwen die contraceptie gebruiken en bij mannen. Daarnaast correleerden we onze resultaten met data uit een Nederlandse doktersreceptendatabase door de verandering in triptaangebruik na voorschrijven van propranolol te analyseren in een populatie overeenkomstig met onze DBF proefpersonen. Onze resultaten toonden aan dat de DBF respons op capsaïcine na toediening van de propranolol werd afgezwakt, maar niet na toediening van de placebo. Interessant genoeg zagen we dat, wanneer we de proefpersonen op sekse indeelden, er bij vrouwen geen veranderingen in de DBF respons op capsaïcine was na toediening van propranolol, terwijl bij mannen een significante afname aanwezig bleef. In geen van de gevallen trad een verandering in DBF respons op bij electrische stimulatie. Bij een vergelijking van de verandering in triptaangebruik na propranolol zagen we bovendien een sterkere afname bij mannelijke patiënten dan bij vrouwelijke patiënten die contraceptie gebruiken. Onze resultaten wijzen erop dat propranolol de trigeminovasculaire systeem activatie op een sekse-afhankelijke wijze reguleert, aangezien bij vrouwelijke proefpersonen, die het overgrote deel van de migraine patiënten vertegenwoordigen, geen significante DBF remming werd gezien na toediening van propranolol (80 mg). In onze retrospectieve studie werd een ogenschijnlijk lagere afname van triptaangebruik gevonden bij vrouwelijke patiënten.

Met het toenemen van het begrip van de pathofysiologie van migraine worden nieuwe therapeutische aangrijpingspunten gepostuleerd. In *hoofdstuk X* beoordelen we één van de meest recentelijk voorgestelde doelen: pituitary adenylaat cyclase activerend polypeptide-38 (PACAP) en één van de receptoren, de PAC_1 receptor. PACAP is een neuropeptide waarvan de literatuur stelt dat dit betrokken is bij neuroprotectie, neuro-ontwikkeling, nociceptie en ontstekingen. Het is interessant dat PACAP en de bijbehorende receptoren aanwezig zijn in de verschillende componenten van het trigeminovasculaire systeem, en dat intraveneuze infusie van PACAP migraine-achtige aanvallen teweegbrengt. Dit leidde tot de ontwikkeling van antilichamen tegen PACAP (ALD 1910) en ook tegen de receptor die het meest verdacht wordt van betrokkenheid bij de pathofysiologie van migraine, de PAC_1 receptor (AMG 301). AMG 301 bevindt zich reeds in Fase II klinische studies. Er zijn tot dusver nog geen resultaten van de klinische studies gepubliceerd, maar pre-klinische studies met AMG 301 hebben positieve resultaten laten zien die vergelijkbaar zijn met de resultaten met triptanen. Echter, zoals eerder opgemerkt in het kader van de CGRP (receptor)-antilichamen, zijn er ook voor de remming van PACAP verschillende zaken die eerst in overweging genomen moeten worden, zoals de mogelijke bijwerkingen bij langdurige remming van de PACAP (receptor) route en of deze antilichamen daadwerkelijk therapeutische voordelen bieden bij de groep patiënten die niet reageert op de CGRP (receptor)-antilichamen.

Aangezien remming van de CGRP afgifte één van de voorgestelde werkingsmechanismen is van de triptanen[22] en imidazoline receptoren volgens de literatuur afgifte van neurotransmitters remmen[23,24], hebben we in *hoofdstuk XI* onderzocht of moxonidine, een imidazoline I_1/α_2-adrenoceptor agonist, en agmatine, de endogene liganden van de imidazoline receptoren, de vasodepressor sensorische CGRP uitstroom in verdoofde ratten onderdrukken. Voorts hebben we de betrokken receptoren gekarakteriseerd. Uit onze resultaten blijkt dat de infusie van moxonidine of agmatine de vasodepressor responsen, opgewekt door stimulatie van de sensorische CGRP uitstroom, onderdrukten, maar niet de respons op een intraveneus toegediende bolus met CGRP. Daarnaast werd de onderdrukking van de vasodepressor responsen ongedaan gemaakt na toediening van een imidazoline I_1 receptor antagonist, en werd deze relatief verhoogd na toediening van de combinatie van de imidazoline I_1 receptor antagonist plus de α_2-adrenoceptor antagonist. We concluderen dat de onderdrukking van de vasodepressor sensorische CGRP uitstroom door moxonidine en agmatine

hoofdzakelijk gemedieerd wordt door prejunctionele imidazoline I_1 receptoren op perivasculaire sensorische zenuwen, dit zou een therapeutisch doel voor de behandeling van migraine kunnen zijn.

Ondanks de grote therapeutische vooruitgang in de afgelopen decennia met de triptanen en, meer recentelijk, met de nieuwe ditanen, gepanten en de antilichamen tegen CGRP of de CGRP receptor, reageren niet alle migraine patiënten op de behandeling en zijn nieuwe therapeutische doelen nodig. Hiervoor is een beter begrip van de pathofysiologie van migraine noodzakelijk, aangezien deze nog steeds grotendeels onbekend is. Diermodellen hebben veel bijgedragen aan onze huidige kennis, maar vertegenwoordigen slechts bepaalde kenmerken van deze redelijk complexe ziekte. Er bestaan echter monogenische ziekten zoals Autosomaal dominante Retinale Vasculopathie met Cerebrale Leukodystrophie (RVCL, een vasculopathie veroorzaakt door een mutatie in het TREX1 gen, waarbij migraine één van de vroegste manifestaties is), die mogelijkheden bieden om de genetische en vasculaire mechanismen die betrokken zijn bij de pathofysiologie van migraine nader te onderzoeken. In *hoofdstuk XII* bekeken we of een nieuw muismodel van RVCL (RVCL-KI) kenmerken heeft die parallellen vertonen met de pathologie die bij patiënten gezien wordt, zoals een verkorte levensverwachting en een vasculair fenotype (beoordeeld door middel van functionele vasculaire metingen en het induceren van een experimenteel herseninfarct). Onze resultaten toonden aan dat, overeenkomstig het fenotype in patiënten, mutante muizen een verhoogd sterftecijfer hadden, tekenen van abnormale vasculaire functie vertoonden en een verhoogde gevoeligheid hadden voor een experimenteel herseninfarct, wat erop wijst dat dit transgene muismodel kan bijdragen aan de bestudering van de onderliggende mechanismen bij RVCL, alsook voor het beter doorgronden van de pathofysiologie van migraine.

Toekomstperspectief

In *hoofdstuk IV* toonden we aan dat lasmiditan een selectieve agonist van de *humane* 5-HT_{1F} receptor is, zonder vaatvernauwende eigenschappen *in vitro* en *in vivo*. Momenteel is het precieze werkingsmechanisme van lasmiditan nog onbekend. Aangezien de therapeutische effictiviteit van triptanen voor de acute behandeling van migraine lijkt te worden gemedieerd door vasoconstrictie in de trigeminale-geïnnerveerde vasculatuur en remming van de CGRP afgifte vanuit de trigeminale vezels[22], zouden toekomstige studies moeten uitwijzen of lasmiditan de activatie van het trigeminovasculaire systeem reguleert. Hiervoor is ons humane model voor trigeminale zenuw-gemedieerde vasodilatatie een zeer geschikte optie, daar in dat geval de resultaten vergeleken kunnen worden met de eerdere resultaten bij onze groep gezonde vrijwilligers met sumatriptan[25]. Bovendien kan de respons op lasmiditan ook bestudeerd worden bij migraine patiënten, om te analyseren of in hun geval regulatie van het trigeminovasculaire systeem verandert.

Zoals eerder genoemd, is ons *humane* model van trigeminale zenuw-gemedieerde vasodilatatie een zeer geschikte manier om de regulatie van het trigeminovasculaire systeem *in vivo* te onderzoeken. In *hoofdstuk XI* lieten we zien dat moxonidine de CGRP-uitstroom remt in verdoofde ratten door activatie van prejunctionele (imidazoline I_1) receptoren. Om deze reden zou het erg interessant zijn onze resultaten verder te bevestigen door middel van een analyse van het effect van moxonidine in ons humane model voor trigeminovasculaire activatie. Dit versterkt mogelijk de positie van moxonidine (en de I_1 imidazoline receptoren) als een therapeutische optie voor de behandeling van migraine. Bovendien hebben wij in dit proefschrift de remming van de CGRP-geïnduceerde relaxaties in humane craniële arteriën en kransslagaders met ubrogepant, atogepant (*hoofdstuk V*) en erenumab (*hoofdstuk VIII*) getypeerd. Nader onderzoek in ons humane model is nodig om de regulatie van het trigeminovasculaire systeem door zowel gepanten als erenumab te evalueren en om vast te stellen of de responsen anders zijn afhankelijk van het type middel

(gepanten *vs.* antilichamen), het bedoelde behandeltype (acuut *vs.* profylactisch) of dat er wellicht helemaal geen verschillen optreden. Dit is zeker interessent omdat op dit moment de halfwaardetijd de enige gedachte achter de keuze tussen atogepant en erenumab voor de prophylactische en ubrogepant voor de acute behandeling van migraine lijkt te zijn.

Naast het onderzoek naar de onderliggende mechanismen van de therapeutische effectiviteit van ubrogepant, atogepant en erenumab, zouden toekomstige, goed opgezette studies dienen te bepalen wat de cardiovasculaire veiligheid van deze middelen is. In het geval van de gepanten, kunnen experimenten gedaan worden in een varkens *in vivo* model van ischemie en reperfusie, waarin gekeken kan worden naar de hartfunctie en infarctgrootte in de aan- en afwezigheid van cumulatieve doses ubrogepant en, nog belangrijker, atogepant (ontwikkeld voor de profylactische behandeling). In het geval van erenumab, kunnen studies niet in diermodellen worden uitgevoerd omdat het een selectief antilichaam tegen de *humane* CGRP receptor betreft, tenzij een antilichaam met affiniteit voor het varkens- (of rat-/muis-) receptor ontwikkeld wordt. Als alternatief zou gekeken kunnen worden naar het effect van erenumab in een human model voor microvasculaire functie. Onze groep heeft eerder de microvasculaire functie onderzocht met lokale thermale hyperemie (LTH) van de huid, gemeten met een laser Doppler flow imager[26] in gezonde vrijwilligers. Toekomstige studies zouden de microvasculaire functie voor en na toediening van erenumab dienen te onderzoeken in een populatie die representatief is voor migraine patiënten, rekening houdend met de farmacokinetische eigenschappen van erenumab.

Onze groep heeft eerder onderzoek gedaan naar de vaatverwijdende responsen op PACAP in humane meningeale en coronaire arteriën[27], maar door de complexe farmacologie van de $VPAC_{1/2}$ en PAC_1 receptoren[28] blijft het onduidelijk welke receptor precies verantwoordelijk is voor het opwekken van de vaatverwijdende respons. Zoals besproken in *hoofdstuk X* zou, indien kllinische studies aantonen dat het nieuwe antilichaam tegen de PAC_1 receptor (AMG 301) effectief is voor de profylactische behandeling van migraine, dit wijzen op een perifere plaats van werking. Daarom dienen nadere studies na te gaan of de geobserveerde vaatverwijdende responses op PACAP in humane meningeale en coronaire arteriën geremd worden in de aanwezigheid van AMG 301. Anders zou dit betekenen dat de meest waarschijnlijke plaats van werking van de antilichamen tegen de PAC_1 receptor de trigeminale ganglion is in plaats van de durale vasculatuur.

Tot slot bindt CGRP zich niet alleen aan de canonieke CGRP receptor, maar ook aan de adrenomedulline (AM_1, AM_2) en amyline (AMY_1, AMY_3) receptoren. Evenzo kunnen adrenomedulline en amyline zich binden aan de canonieke CGRP receptor[29]. Bij migraine patiënten die behandeld worden met erenumab kan CGRP de amyline receptoren stimuleren, zoals beschreven in het trigeminovasculaire systeem[30], hetgeeen de effectiviteit vermindert. Daartegenover wordt de amyline receptor voorgesteld als de 'tweede' CGRP receptor die eerder werd gerapporteerd door onze groep in de humane coronair arterie[31, 32], hetgeen kan betekenen dat er een cardiovasculair veiligheidsvoordeel bestaat voor erenumab. Toekomstige studies dienen de vaatverwijdende responses op adrenomedulline en amyline in humane geïsolateerde meningeale en coronaire arteriën in de aanwezigheid van erenumab nader te onderzoeken en vast te stellen of er verschillen zijn in de respons tussen meningeale en coronaire arteriën.

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In 2007 she started her studies in Chemical Pharmaceutical Biology at the Autonomous University of Querétaro. In 2011 she initated her Bachelor internship at the Department of Pain and Epilepsy of the National Institute of Neurobiology under the supervision of Prof. dr. Miguel Condés-Lara. After completing her Bachelor, she joined the Master degree program in Neuropharmacology and Experimental Therapeutics in the Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV) in Mexico City, under the supervision of Prof. dr. Carlos M. Villalón. In 2015 she started her PhD training at the Department of Internal Medicine, Division of Vascular Medicine and Pharmacology of Erasmus MC under the supervision of dr. Antoinette Maassen van den Brink.

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PhD Portfolio

Name PhD Student:	Amada Eloísa Rubio-Beltrán
PhD period:	2015-2019
Erasmus MC Department:	Department of Internal Medicine, Division of Vascular Medicine and Pharmacology
Promotor:	Prof. dr. A. H. J. Danser
Supervisor:	Dr. A. Maassen van den Brink
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)

In-depth Courses (13.7 ECTS)

2018	*Homeostatic regulation and migraine, a view from the bench*, London, England
	iHead Meeting, The International Headache Society, London, England
	Experimental Neurophysiology: Theory and Practice, Amsterdam, The Netherlands
2017	*Headache research for young scientists*, Vancouver, Canada
	1st School of Advanced Studies of the European Headache Federation, Rome, Italy
2016	Laboratory animal science, Erasmus MC, Rotterdam, The Netherlands
2015-2019	*COEUR Courses and Research Seminars*, Erasmus MC, Rotterdam, The Netherlands

Teaching activities (6 ECTS)

2015-2019	Pharmacology practical courses, scientific internship of Junior Med School students, Autonomic Nervous system practicum
2018	Supervising Master's thesis of a Biomedical Science's student
	Supervising Master's thesis of a NIHES student
2016-2017	Supervising Bachelor's thesis of Psychobiology students

Presentations (5.7 ECTS)

2019	19th Congress of the International Headache Society, Dublin, Ireland. *Characterization of the effects of the CGRP receptor antagonists, atogepant and ubrogepant, on isolated human coronary, cerebral, and middle meningeal arteries* (E-poster).

61th Annual Scientific Meeting of the American Headache Society, Philadelphia, United States. *Characterization of the effects of the CGRP receptor antagonists, atogepant and ubrogepant, on isolated human coronary, cerebral, and middle meningeal arteries* (Oral presentation).

13th European Headache Federation Congress, Athens, Greece. *Characterization of the effects of the CGRP receptor antagonists, atogepant and ubrogepant, on isolated human coronary, cerebral, and middle meningeal arteries* (Oral presentation).

2018

12th European Headache Federation Congress, Florence, Italy. *Effect of propranolol in a non-invasive human model of trigeminovascular activation* (Poster presentation).

12th European Headache Federation Congress, Florence, Italy. *PACAP38 and PAC_1 receptor blockade: a new target for headache?* (Oral presentation).

17th Biennial Migraine Trust International Symposium, London, England. *Pharmacological selectivity of inhibition of CGRP-induced relaxations by erenumab studied in human isolated internal mammary artery* (E- poster presentation).

4th School of Advanced Studies of the European Headache Federation. *Blocking CGRP in migraine patients, pros and cons* (Oral presentation).

28th ADMA Annual Meeting, Zwolle, The Netherlands. *Effect of propranolol in a non-invasive human model of trigeminovascular activation* (Oral presentation).

NVF Spring Meeting, Utrecht, The Netherlands. *Functional activity of antimigraine drugs at 5-HT receptors. Coronary artery contraction correlation* (Poster presentation).

Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Oral presentation).

Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium. *Disturbed Prelamin-A processing in VSMC causes aortic root dilation and aging-resembling macrovascular motor dysfunction* (Poster presentation).

2017

11th European Headache Federation Congress, Rome, Italy. *Blocking CGRP in migraine patients, pros and cons* (Oral presentation).

18th Congress of the International Headache Society, Vancouver, Canada. *In vitro characterization of agonist binding and functional activity at a panel of serotonin receptor subtypes for lasmiditan, triptans and other 5-HT receptor ligands and activity relationships for contraction of human isolated coronary artery* (Poster presentation).

18th Congress of the International Headache Society, Vancouver, Canada. *Pharmacological analysis of the inhibitory effects produced by moxonidine and agmatine on the sensory vasodepressor CGRPergic outflow in pithed rats* (Poster presentation).

27th ADMA Annual Meeting, Norwich, England. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Oral presentation).

FIGON Dutch Medicine Days 2017, Ede, The Netherlands. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Poster presentation).

Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Poster presentation).

2016 FIGON Dutch Medicine Days 2016, Ede, The Netherlands. *Effects of AMG 334 on human isolated coronary artery* (Poster presentation).

5th European Headache and Migraine Trust International Congress – EHMTIC, Glasgow, Scotland. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Poster presentation).

5th European Headache and Migraine Trust International Congress – EHMTIC, Glasgow, Scotland. *Effects of AMG 334 on human isolated coronary artery* (Poster presentation).

NVF Spring Meeting, Groningen, The Netherlands. *Pharmacological analysis of the inhibitory effects produced by moxonidine and agmatine on the sensory vasodepressor CGRPergic outflow in pithed rats* (Poster presentation).

Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium. *Pharmacological analysis of the inhibitory effects produced by moxonidine and agmatine on the sensory vasodepressor CGRPergic outflow in pithed rats* (Poster presentation).

2015 FIGON Dutch Medicine Days 2016, Ede, The Netherlands. *Pharmacological analysis of the inhibitory effects produced by moxonidine and agmatine on the sensory vasodepressor CGRPergic outflow in pithed rats* (Poster presentation).

International conferences (8.1 ECTS)

2019 19th Congress of the International Headache Society, Dublin, Ireland.

61th Annual Scientific Meeting of the American Headache Society, Philadelphia, United States.

13th European Headache Federation Congress, Athens, Greece.

2018 12th European Headache Federation Congress, Florence, Italy.

17th Biennial Migraine Trust International Symposium, London, England.

28th ADMA Annual Meeting, Zwolle, The Netherlands.

2017 11th European Headache Federation Congress, Rome, Italy.

18th Congress of the International Headache Society, Vancouver, Canada.

27th ADMA Annual Meeting, Norwich, England.

2016 5th European Headache and Migraine Trust International Congress – EHMTIC, Glasgow, Scotland.

26th ADMA Annual Meeting, Dordrecht, The Netherlands.

Grants & awards

Fellowship of the International Headache Society for the International Headache Academy (iHEAD) 2019 (Dublin, 2-4 September).

Fellowship of the International Headache Society for the International Headache Academy (iHEAD) 2018 (London, 3-5 September).

Travel grant for the 28th ADMA Annual Meeting 2017 (Zwolle, 8-10 June).

IHS travel grant to attend the IHC 2017 (Vancouver, 7-10 September).

Travel grant for the 27th ADMA Annual Meeting 2017 (Norwich, 25-27 May).

Fellowship of the European Headache Federation for the 1st School of Advanced Studies 2017 (Rome, 7-9 April).

1st prize Poster competition with the poster "*Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries*" Wetenschapsdagen Interne Geneeskunde 2017 (Antwerpen, 12-13 January).

Abbreviations

5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine
AC	adenylyl cyclase
AMY_1	amylin type 1 receptor
ANCOVA	analysis of covariance
ANOVA	analysis of variance
BBB	blood brain barrier
CGRP	calcitonin gene-related peptide
CH	cluster headache
CLR	calcitonin-like receptor
CNS	central nervous system
DAP	diastolic arterial pressure
DBF	dermal blood flow
DHSC	dorsal horn of the spinal cord
E_{max}	maximal response
HCA	human coronary artery
HCxA	human cerebral artery
HMMA	human middle meningeal artery
LCX	left circumflex coronary artery
$logD_{pH7.4}$	distribution coefficient at physiological pH
mABs	monoclonal antibodies
MAP	mean arterial pressure
MCA	middle cerebral artery
MMA	middle meningeal artery
MRA	magnetic resonance angiography
ND	not defined
NTS	nucleus tractus solitarius
PACAP27	pituitary adenylate cyclase activating polypeptide-27
PACAP38	pituitary adenylate cyclase activating polypeptide-38
PAG	periaqueductal grey area
pEC_{50}	negative logarithm of the half maximal effective concentration
PLC	phospholipase C
PTSD	post-traumatic stress disorder
PVN	hypothalamic pararaventricular nucleus
RAMP1	receptor activity-modifying protein 1
RANCOVA	repeated measure analysis of covariance
RCP	receptor component protein
SAP	systolic arterial pressure
TG	trigeminal ganglion
TNC	trigeminal nucleus caudalis
VIP	vasoactive intestinal peptide

"All our knowledge *begins* with the senses,
proceeds then to the understanding,
and *ends* with reason.
There is nothing higher than reason."

Immanuel Kant
Critique of Pure Reason