Inhibition of full smooth muscle contraction in isolated human detrusor tissues by mirabegron is limited to off-target inhibition of neurogenic contractions

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Nonstandard abbreviations: BOO, bladder outlet obstruction; E_{f50} , frequency inducing half of the maximum EFS-induced contraction; EFS, electric field stimulation; OAB, overactive bladder

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Abstract

Mirabegron is used for treatment of storage symptoms in overactive bladder (OAB), caused by spontaneous bladder smooth muscle contractions. However, owing to limitations in available studies using human tissues central questions are still unresolved, including mechanisms underlying improvements by mirabegron and its anticontractile effects in the detrusor. Here, we assessed concentration-dependent mirabegron effects on contractions of human detrusor tissues in frequency-response curves and concentration-response curves for different cholinergic and non-cholinergic agonists. Detrusor tissues were sampled from patients undergoing radical cystectomy. Contractions were induced by electric field stimulation (EFS), and by cumulative concentrations of cholinergic agonists, endothelin-1 and the thromboxane A₂ analog U46619. EFS-induced contractions were inhibited using 10 μ M mirabegron, but not using 1 μ M. Inhibition by 10 μ M mirabegron was resistant to the β_3 -adrenergic antagonist L-748,337. Concentration-dependent contractions by carbachol were not inhibited by 1 μ M or 10 μ M mirabegron. Concentration response curves for methacholine were slightly right-shifted by 10 μ M, but not 1 μ M mirabegron. Concentration-dependent contractions by endothelin-1 or U46619 were not changed by mirabegron. In contrast, the muscarinic antagonist tolterodine right-shifted concentration response curves for carbachol and methacholine, and inhibited EFS-induced contractions. In conclusion, inhibition of neuogenic contractions in isolated detrusor tissues by mirabegron requires concentrations highly exceeding known plasma levels during standard dosing and the known K_i values for β_3 -adrenoceptors. Full contractions by cholinergic agonists, endothelin-1 and U46619 are not affected by therapeutic concentrations of mirabegron. Improvements of storage symptoms are most likely not imparted by inhibition of β_3 -adrenoceptors in the bladder wall itself.

Significance statement

Mirabegron is used for OAB treatment, but the underlying mechanisms are unclear and preclinical and clinical findings are controversial, due to limitations in available studies. Our findings suggest that inhibition of detrusor contractions by mirabegron is limited to neurogenic contractions, which requires unphysiologic concentrations and does not involve β_3 -adrenoceptors. Mechanisms accounting for improvements of OAB by mirabegron are located outside the urinary bladder.

Introduction

The β_3 -adrenoceptor agonist mirabegron is available for treatment of storage symptoms in overactive bladder (OAB) (Nambiar et al., 2018; Oelke et al., 2013). Storage symptoms in OAB are caused by spontaneous, exaggerated bladder smooth muscle contractions, leading to urgency and nocturia, or incontinence in advanced stages (Andersson and Arner, 2004). Under normal conditions, voiding is effectuated by cholinergic detrusor contractions, following muscarinic receptor activation on bladder smooth muscle cells after cholinergic neurotransmission (Andersson, 2011; Andersson and Arner, 2004). Muscarinic receptor antagonists represent the goldstandard for medical treatment of storage symptoms, but show limited efficacy with disproportional side effects, resulting in discontinuation rates up to 90% within twelve month following first prescription (Chancellor et al., 2013; Nambiar et al., 2018; Sexton et al., 2011; Soda et al., 2020). Mirabegron has been introduced as an alternative, and was initially believed to reduce symptoms by relaxation of bladder smooth muscle by activation of β_3 -adrenoceptors on bladder smooth muscle cells (Nambiar et al., 2018; Oelke et al., 2013).

Despite the clinical use of mirabegron, central questions still remain, regarding its actions, pharmacology and mechanisms accounting for its beneficial effects in OAB (Igawa et al., 2019; Michel, 2016). Incomplete understanding and controversial findings from *in vivo* and *in vitro* studies may be attributed to limitations of available studies addressing mirabegron effects in human bladder smooth muscle contraction (Dale et al., 2014). Thus, mirabegron-induced relaxation of human detrusor tissues *in vitro* required concentrations ranging much higher than affinities to β_3 -adrenoceptors and than plasma levels during standard dosing. EC₅₀ values for mirabegron-induced relaxation of precontracted human detrusor tissues ranged between 588 nM to 3.9 μ M (Svalo et al., 2013; Takasu et al., 2007), whereas a

binding constant of 2.5 nM for the β_3 -adrenoceptor, an EC_{50} value of 22.4 nM for mirabegron-induced cAMP production in β_3 -adrenoceptor-transfected cells, and maximum plasma concentrations of 137 nM during standard dosing have been reported (Krauwinkel et al., 2012; Takasu et al., 2007; Tasler et al., 2012). However, previous *in vitro* studies using human tissues focussed on mirabegron-induced relaxation of precontracted tissues, while studies addressing effects on concentration response curves for agonist-induced contractions are limited to one study applying 30 μ M mirabegron during carbachol-induced contractions. Generally, β -adrenergic effects on smooth muscle contractions critically depend on study design and species-dependent differences (Dale et al., 2014), what may account for unresolved questions in the context of mirabegron (Igawa et al., 2019; Michel, 2016).

Following its approval for OAB treatment, it became clear that the efficacy of mirabegron does not exceed that of anticholinergics (Nambiar et al., 2018). Apart from cholinergic contractions, bladder smooth muscle contractions are induced by non-cholinergic agonists, including endothelin-1 and thromboxane A_2 (Li et al., 2020b; Palea et al., 1998), and spontaneous detrusor contractions in OAB are most likely non-cholinergic and non-neurogenic (Akino et al., 2008; Kushida and Fry, 2015). Presuming that activation of β_3 -adrenoceptors by mirabegron in bladder smooth muscle cells results in cAMP-formation, inhibition of non-cholinergic contractions and thus, a higher efficacy compared to anticholinergics could be at least theoretically expected. In fact, however, effects of mirabegron on non-cholinergic contractions of human detrusor tissues have never been reported. Consequently, the specificity of mirabegron has been questioned and different mechanisms have been proposed to account for improvements of storage symptoms by mirabegron, including inhibition of cholinergic neurotransmission by mirabegron, antagonism of muscarinic receptors, and effects on afferent signaling or in the central nervous system (CNS), both interfering with the voiding reflex (Igawa et al., 2019; Michel, 2016). To date, it is

still unclear, which mechanism accounts for effects of mirabegron on storage symptoms (Igawa et al., 2019).

In face of the clinical relevance emerging from the use of mirabegron in medical treatment of storage symptoms together with unresolved questions, further attempts to understand its actions appear adequate. Here, we examined concentration-dependent effects of mirabegron on neurogenic contractions, and on contractions in concentration response curves for cholinergic and non-cholinergic agonists in human detrusor tissues.

Materials and methods

Human tissues

Tissues from the lateral bladder wall were sampled in 2020 and 2021 from 65 male patients undergoing radical cystectomy for bladder cancer. Our study was performed in compliance with the Declaration of Helsinki of the World Medical Association, and has been approved by the local ethics committee (Ludwig-Maximilians University, Munich, Germany). All patients provided informed consent. All samples were anonymized, so that all data obtained with these tissues were analyzed anonymously. Apart from the gender, no patients' data were collected, stored, or analyzed in the context of this study, and tissues were not assayed for any pathologies or other conditions. Macroscopic examination of bladders for tumor burden and sampling were performed by pathologists, approximately within 30 min after final removal of bladders from patients. Experiments were started not later than 60 min after sampling, i. e. approximately 90 min following surgical resection of bladders. For temporary storage and for transport, organs and tissues were placed in Custodiol® solution (Köhler, Bensheim, Germany). For macroscopic inspection and tissue sampling, bladders were opened by a longitudinal cut from the bladder outlet to the bladder dome. Subsequently, the bladder wall and intravesical surface were macroscopically checked for tumor infiltration. Provided that tumor burden in the bladder wall allowed sampling, tissues were taken from the inner lateral bladder wall and urothelial layers were resected from samples.

Organ bath

Detrusor tissues (3 x 3 x 6 mm) were mounted in chambers of an organ bath device (myograph 720M, Danish Myotechnology, Aarhus, Denmark). The device includes four chambers, each filled with 10 ml gassed (5% CO₂, 95% O₂) Krebs-Henseleit solution (pH 7.4, 37°C) containing 118 mM NaCl, 4.7 mM KCl, 2.55 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 7.5 mM glucose. Following mounting, tissues were stretched to a tension of 4.9 mN and left to equilibrate for 45 min. Typically, these pretensions decrease spontaneously in the early phase of this period. Therefore, tensions were restored three times during these 45 min, until the designated, stable resting tone of 4.9 mN was obtained within 45 min. Subsequently, contractions were induced by 80 mM KCl by addition of a 2 M stock solution, in order to assess highmolar KCl-induced contractions as a measure for content and condition of smooth muscle, and for later reference of agonist-induced and neurogenic contractions. Highmolar KCl induced biphasic responses, starting with a phasic contraction reaching a maximum within few minutes (used for normalization, as described below), followed by a decrease to a tonic, stable tension (Li et al., 2020b). When tensions started to decrease to the tonic phase, chambers were washed three times with Krebs-Henseleit solution to attain a stable resting tension, close to the first baseline before KCl (Li et al., 2020b). Thereafter, mirabegron (final concentration 1 µM or 10 µM), L-748,337 (final concentration 1 µM), tolterodine (final concentration 300 nM), or corresponding amounts of dimethylsulfoxide (DMSO) as control for mirabegron or tolterodine were added. Stock solutions of mirabegron, L-748,337 and tolterodine had concentrations of 10 mM. Cumulative concentration response curves for carbachol, methacholine (both 100 nM to 1 mM), endothelin-1 (0.01-3 µM) or U46619 (0.01-30 µM), or frequency response curves by electric field stimulation (EFS) (2-32 Hz) were constructed 30 min after addition of drugs or DMSO.

EFS imitates action potentials, resulting in bladder smooth muscle contractions by cholinergic neurotransmission within the tissues (Andersson and Arner, 2004; Li et al., 2020a). For EFS, tissues were mounted between two opposite platinum electrodes

connected to a CS4 generator (Danish Myotechnology, Denmark). Square pulses were applied with a voltage of 20 V and a duration of 1 ms, and with a train duration of 10 s. EFS-induced contractions were assessed at frequencies of 2, 4, 8, 16, and 32 Hz, with intervals of 30 s between trains of different frequencies.

In a single, independent experiment, all four chambers of one device were filled with tissues from the same bladder. Two of them were assessed with drug (mirabegron or tolterodine), and the two others with DMSO for controls. In experiments including L-748,337, L-748,337 was added to all four chambers, and mirabegron and DMSO were assigned as described above. Each mounted tissue was used for only one concentration/frequency response curve. For randomization, allocations of drug and control channels were changed between different experiments. Blinding was not feasible because experiments were performed by the same experimenters who weighed, dissolved and diluted the drugs. Independent experiments were repeated in indicated numbers (n), using tissues from n different patients, resulting in numbers of independent experiments as indicated for each series. Accordingly, control and drug groups were examined in the same experiments, and obtained using tissues from the same bladder in each series. Each experiment was based on double determination wherever this was possible. From a total of 65 single experiments, this was possible for control groups in 61 experiments, and for drugs (mirabegron, tolterodine) in 62 experiments.

EFS- and agonist-induced contractions are reported as % of 80 mM KCl-induced contractions, calculated by referring maximum contractions after each frequency or after each agonist concentration to the maximum of the phasic contraction after 80 mM KCl. Normalization to KCl may correct differences in tissue composition or smooth muscle content, individual variations between patients, and other heterogeneities between different bladders, e. g. resulting from different pathologic backgrounds. Values of the maximum contraction level at a given agonist concentration or given frequency was assessed,

irrespective of the contraction pattern (see supplementary figures 1 and 2). In fact, contraction patterns differed between agonists. At most concentrations and in most tissues, contractions induced by application of carbachol or methacholine at given concentrations were composed of a rapid but transient peak contraction (“phasic contraction”), typically followed by a decrease to a stable tension (“tonic contraction”) (supplementary fig. 1). The next concentration was applied shortly after the maximum of phasic contractions was reached, i. e. when the decline to the tonic contractions was obviously beginning (see supplementary fig. 1). Thus, maximum contractions were observed shortly (i. e., within 2 min) after agonist application. In contrast, phasic and tonic contractions induced by endothelin-1 or U46619 could not be distinguished at most concentrations and in most tissues, resulting in contractions with a sigmoidal shape and a maximum plateau contraction at the end (supplementary fig. 2). In these cases, the next concentration was added directly when the plateau was reached and if further, substantial increases were not expected. Contractions in concentration and frequency response curves describe the maximum contraction levels at each concentration and frequency, in control and drug groups.

Typically, the overall maximum contraction within a concentration response curve for carbachol, methacholine or endothelin-1 did not occur at the highest concentration, but at one of the medial concentrations (in the “middle” of curves). Apart from tolterodine groups, tensions became lower at highest concentrations, in particular at 300 μ M and 1 mM of cholinergic agonists. In these parts of concentration response curves (“downhill” parts), small contractions may still occur in some samples, but tension remain lower than previous tension levels (see supplementary fig. 1). However, these concentrations were included to concentration response curves, in order to realize antagonistic effects. Statistical analyses of concentration response curves were performed by including all concentrations and downhill parts (as described below). If required (and as described below in detail), curve fitting was performed without downhill parts, while presentation of concentration response curves in

diagrams is limited to the most relevant parts (i. e., uphill portions) in concentration response curves for cholinergic agonists for reasons of clearest illustration.

EC₅₀ values for contractile agonists, frequencies (f) inducing half of the maximum EFS-induced contraction (E_{f50}), and E_{max} values were calculated by curve fitting using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA), and analyzed as described below. Curve fitting was performed separately for each single experiment, resulting in separate values for each independent experiment. Frequency and concentration response curves were fitted without predefined borders for top, bottom, or EC₅₀ values, by ordinary fit, without weighting, and without choosing automatic outlier elimination by non-linear regression. As recommended in the “GraphPad Curve Fitting Guide” (GraphPad Software Inc., San Diego, CA, USA), values were checked for plausibility and calculations were repeated with adapted settings as follows, if error messages occurred. Thus, the highest frequency (32 Hz) was excluded in three control groups of three EFS experiments, as their inclusion resulted in implausible results or values were flagged as “ambiguous” by the software. From concentration response curves with cholinergic agonists, uphill parts of curves were used for curve fitting, as curves could not be converged if downhill portions were included. The same applies for the control group of one experiment with endothelin-1, while no values had to be excluded for curve fitting of concentration response curves with U46619. Notably, these procedures apply only to curve fitting, while all concentrations were included to statistical analyses performed for concentration response curves. In the series addressing effects of 1 μM on methacholine-induced contractions, curve fitting revealed highly ambiguous EC₅₀ values (5 mM with solvent, 1 mM with mirabegron), which could not be corrected by adjusting curve fitting, so that these values were omitted from presentation in the scatter plot.

Drugs and nomenclature

Mirabegron (2-Amino-N-[4-[2-[[[(2R)-2-hydroxy-2-phenylethyl]amino]ethyl]phenyl]-4-thiazoleacetamide) is an adrenoceptor ligand, which is available as a β_3 -adrenoceptor agonist for drug treatment of storage symptoms in OAB (Alexander et al., 2021; Nambiar et al., 2018). Its affinity is highest for β_3 -adrenoceptors, ranging in nanomolar concentrations (see introduction and discussion for details), while its specificity has been questioned (Michel, 2016). L-748,337 (N-[[3-[(2S)-2-Hydroxy-3-[[2-[4-[(phenylsulfonyl)amino]phenyl]ethyl]amino]propoxy]phenyl]methyl]-acetamide) is a β -adrenoceptor antagonist, with K_i values of 4 nM for β_3 -, 204 nM for β_2 - and 390 nM for β_1 -adrenoceptors, respectively (Alexander et al., 2021; Candelore et al., 1999). Tolterodine L-tartrate (2-[(1R)-3-[Bis(1-methylethyl)amino]-1-phenylpropyl]-4-methylphenol (2R,3R)-2,3-dihydroxybutanedioate) is a muscarinic receptor antagonist, with K_i values of 4-6.3 nM at cloned human M2 receptors, and 3.2-12.6 nM at cloned human M3 receptors, and is available for treatment of storage symptoms in OAB (Alexander et al., 2021; Michel, 2008; Nambiar et al., 2018; Oelke et al., 2013). For all three compounds, stock solutions (10 mM) were prepared with DMSO, and aliquots were stored at -20 °C until being used in experiments.

Carbachol (carbamoylcholin) and methacholine (acetyl- β -methylcholin) are muscarinic acetylcholine receptor agonists (Alexander et al., 2021). Aqueous stock solutions (10 mM) and dilutions were freshly prepared for each experiment. U46619 ((Z)-7-[(1S,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid) is a thromboxane A_2 receptor agonist and was dissolved in ethanol (Alexander et al., 2021). Thromboxane A_2 itself is highly unstable, so that U46619 is commonly used as a thromboxane A_2 receptor agonist (Li et al., 2020b). Stock solutions (10 mM) were stored at -80°C before being used in experiments. Aqueous stock solutions of endothelin-1 (0.4 mM) were stored at -20°C as small aliquots, to avoid repeated freezing and thawing. Carbachol

and metacholine were purchased from Sigma (Munich, Germany). Mirabegron and tolterodine tartrate were purchased from Cayman (Ann Arbor, MI, USA). L-748,337 was purchased from Tocris (Bristol, UK). U46619 and endothelin-1 were purchased from Enzo Life Sciences (Lörrach, Germany).

Statistical analyses

Data in frequency and concentration response curves are means $\pm$ standard deviation (SD). $E_{\max}$, E_{f50} and EC_{50} values are presented as single values from all independent experiments together with means in scatter plots. Effect sizes become obvious from frequency and concentration response curves. In addition, relevant effects (i. e., pointing to obvious differences) are reported as mean differences (MD) with 95% confidence intervals (CIs) in the text, which were calculated by setting values in controls to 100% in each single experiment, expressing values of drug groups as % of the corresponding control value, and finally calculating the difference in each single experiment. This normalization was limited to the text, where it may illustrate effect sizes better than expression of absolute contractions, which are shown in figures. Calculation of MDs with 95% CIs was performed using GraphPad Prism 6. However, these values were not analyzed by statistical tests, as this is generally disadvised for values resulting from normalization to controls and in order to use p values sparingly, as recommended by recent guidelines (Curtis et al., 2015; Michel et al., 2020). Comparison of whole concentration/frequency response curves was performed by one-way analysis of variance (ANOVA), while contractions at single frequencies and single concentrations were compared by two-way ANOVA, both using the SPSS® version 20 (IBM SPSS Statistics, IBM Corporation, Armonk, New York, USA). Post-hoc tests were only performed if F was significant and there was no variance of inhomogeneity. $E_{\max}$, E_{f50} , and EC_{50} values were compared by a paired Student's t-test, using GraphPad Prism 6. P values <0.05 were considered significant.

Our study and its analyses have exploratory character, and were not designed to test a statistical or pre-specified null hypothesis. Indeed, typical characteristics of hypothesis-testing studies are lacking, including a defined hypothesis to be tested, a preset study plan based on biometric calculation of groups sizes, or blinding. Consequently, p values reported here are not hypothesis-testing but descriptive, and the focus was on reporting of effect sizes but not of p values (Michel et al., 2020). Accordingly, p values are limited to figures, while no p values are reported in the text, and p values ≥ 0.05 are not shown. Consequently, data sets shown without p values are without significant differences. Minimum numbers of independent experiments and group sizes in each series were pre-planned as $n=5$, to allow statistical analyses. Thus, values were extracted from experiments and analyzed after five experiments of a series. Initially, we intended to continue a series, if interim analysis after five independent experiments revealed p values ≥ 0.05 , but suggested that an effect may occur. Discontinuation was intended, if lacking effects were obvious in concentration response curves, or if p values were already <0.05 in whole frequency/concentration response curves or at single frequencies or agonist concentrations. This approach is possible in explorative, but not in hypothesis-testing studies designed to test a pre-specified statistical null-hypothesis (Michel et al., 2020), and as flexible group sizes were recommended for data showing large variations (Curtis et al., 2018; Curtis et al., 2015). However, all series in this study provided conclusive results after five independent experiments. Consequently, each group subjected to statistical analyses were based on five independent experiments and included tissues from five patients. No experiments were excluded from analyses, and no data were excluded with the required exceptions in curve fitting and for construction of diagrams as described above.

Results

Effects of mirabegron on EFS-induced contractions

EFS (2-32 Hz) induced frequency-dependent contractions of human detrusor tissues (fig. 1). EFS-induced contractions were inhibited by 10 μ M mirabegron, but not by 1 μ M mirabegron (fig. 1A, B). Inhibition by 10 μ M mirabegron occurred at all examined frequencies (fig. 1B). If contractions under 10 μ M mirabegron were normalized to corresponding controls in each single experiment, contractions were reduced by 69% [54 to 85] at 2 Hz, by 46% [11 to 82] at 4 Hz, by 51% [35 to 68] at 16 Hz, by 59% [45 to 72] at 16 Hz, and by 40% [12 to 67] at 32 Hz by 10 μ M mirabegron. $E_{\max}$ values calculated by curve fitting were reduced from 161% [127 to 195] of KCl-induced contraction in controls to 75% [56 to 94] of KCl-induced contraction by 10 μ M mirabegron (MD -85 percentage points [-130 to -42]). Ef_{50} values were not changed by mirabegron (fig. 1B).

The inhibitory effect of 10 μ M mirabegron persisted, if the β_3 -adrenergic antagonist L-748,337 (1 μ M) was added to controls (i. e., L-748,337 + DMSO as control for mirabegron) and together with mirabegron (i. e., L-748,337 + 10 μ M mirabegron) (fig. 1C). If contractions under 10 μ M mirabegron + 1 μ M L-748,337 were normalized to corresponding controls (i. e., L-748,337 + DMSO), contractions were reduced by 40% [-50 to 129] at 2 Hz, by 54% [-8 to 115] at 4 Hz, by 61% [13 to 109] at 16 Hz, by 68% [29 to 108] at 16 Hz, and by 67% [30 to 104] at 32 Hz by 10 μ M mirabegron. $E_{\max}$ values calculated by curve fitting were reduced from 135% [63 to 207] of KCl-induced contraction after application of L-748,337 + DMSO to 45% [-7 to 97] of KCl-induced contraction after application of 10 μ M mirabegron + L-748,337 (MD -90 percentage points [-150 to -30]). Ef_{50} values were not changed by mirabegron (fig. 1C).

Effects of mirabegron on carbachol-induced contractions

Carbachol (0.1-100 μ M) induced concentration-dependent contractions of human detrusor tissues, which remained unchanged by mirabegron, applied using concentrations of 1 μ M (fig. 2A) and 10 μ M (fig. 2B). None of both concentrations affected concentration response curves for carbachol, or $E_{\max}$ and EC_{50} values for carbachol, calculated by curve fitting (fig. 2A, B).

Effects of mirabegron on methacholine-induced contractions

Methacholine (0.1-100 μ M) induced concentration-dependent contractions of human detrusor tissues, which remained unchanged by 1 μ M mirabegron (fig. 3A). 10 μ M of mirabegron slightly right-shifted concentration response curves for methacholine (fig. 3B). The rightshift included slight (but significant) inhibition of contractions at submaximum methacholine concentrations, and recovery at high agonist concentrations (fig. 3B). If contractions at each methacholine concentration under 10 μ M mirabegron were normalized to corresponding controls, contractions were reduced by 42% [20 to 65] at 0.3 μ M methacholine, by 30% [16 to 45] at 1 μ M methacholine, and by 25% [7 to 43] at 3 μ M methacholine, but were similar between control and mirabegron groups at methacholine concentrations of 10-100 μ M (fig. 3B). $E_{\max}$ values for methacholine remained unchanged by 10 μ M mirabegron (fig. 3B). EC_{50} values for methacholine were increased by trend by mirabegron, resulting in EC_{50} values of 517 nM [-550 nM to 1.5 μ M] in controls and 992 nM [-701 nM to 2.7 μ M] under 10 μ M mirabegron.

Concentration response curves for methacholine-induced contractions were similar, if L-748,337 was added to controls (without mirabegron) and together with mirabegron (10 μ M) (fig. 3C). However, EC_{50} values for methacholine were again increased by trend under 10 μ M mirabegron in the presence of L-748,337, resulting in EC_{50} values of 191 nM [-5 nM to 388 nM] in controls and 711 nM [-96 nM to 1.5 μ M] under 10 μ M mirabegron. E_{max} values for methacholine-induced contractions remained unchanged (fig. 3C).

Effects of mirabegron on endothelin-1-induced contractions

Endothelin (0.01-3 μ M) induced concentration-dependent contractions of human detrusor tissues, which remained unchanged by mirabegron (10 μ M) (fig. 4A). Mirabegron neither affected concentration response curves or E_{max} values for endothelin-1 (fig. 4A). EC_{50} values for endothelin-1 were increased by trend by mirabegron, resulting in EC_{50} values of 9 nM [3 to 15] in controls and 54 nM [-47 to 156 nM] under 10 μ M mirabegron, but without reaching significance and without an obvious rightshift in concentration response curves (fig. 4A).

Effects of mirabegron on U46619-induced contractions

U46619 (0.01-30 μ M) induced concentration-dependent contractions of human detrusor tissues, which remained unchanged by mirabegron (10 μ M) (fig. 4B). Mirabegron neither affected concentration response curves or E_{max} values for U46619 (fig. 4B). EC_{50} values for U46619 were decreased by trend by mirabegron, resulting in EC_{50} values of 38 nM [-47 to 123] in controls and 12 nM [1 to 22 nM] under 10 μ M mirabegron without reaching significance or that a shift was observed in concentration response curves (fig. 4B).

Effects of tolterodine on EFS-induced and cholinergic contractions

EFS-induced contractions (2-32 Hz) of human detrusor tissues were inhibited by 300 nM tolterodine (fig. 5A). Inhibition by 300 nM tolterodine occurred at all examined frequencies (fig. 5A). If contractions under 300 nM tolterodine were normalized to corresponding controls, contractions were inhibited by 50% [9 to 92] at 2 Hz, by 60% [36 to 84] at 4 Hz, by 69% [51 to 86] at 8 Hz, by 71% [57 to 84] at 16 Hz, and by 72% [62 to 81] at 32 Hz by 300 nM tolterodine. $E_{\max}$ values were reduced from 152% [101 to 202] of KCl-induced contraction in controls to 41% [29 to 53] of KCl-induced contraction by 300 nM tolterodine (MD -111 percentage points [-156 to -65]). E_{f50} values were not changed by tolterodine (fig. 5A).

Concentration response curves for carbachol- and methacholine-induced contractions of human detrusor tissues were shifted to the right by 300 nM tolterodine, resulting in unchanged $E_{\max}$ values but increased EC_{50} values for both cholinergic agonists (fig. 5B, C). Compared to controls, contractions were reduced at carbachol concentrations of 0.3 to 10 μ M, but fully recovered at carbachol concentrations of 30 μ M to 1 mM under tolterodine (fig. 5B). EC_{50} values for carbachol calculated by curve fitting increased from 652 nM [-317 nM to 1.6 μ M] after application of DMSO to 29 μ M [-20 to 77] after application of 300 nM tolterodine (MD 28 μ M [-20 to 75]). $E_{\max}$ values for carbachol-induced contractions calculated by curve fitting amounted to 99% [58 to 140] of KCl-induced contraction in controls, and to 96% [69 to 123] of KCl-induced contraction with 300 nM tolterodine (MD -3 percentage points [-28 to 21]). Compared to controls, contractions were reduced at methacholine concentrations of 0.3 to 30 μ M, but fully recovered at methacholine concentrations of 100 μ M to 1 mM under tolterodine (fig. 5C). EC_{50} values for methacholine calculated by curve fitting increased from 184 nM [-31 to 400] after application of DMSO to 83 μ M [-35 to 201] after application of 300 nM tolterodine (MD 83 μ M [-35 to 200]). $E_{\max}$ values for methacholine-induced contractions calculated by curve fitting amounted to 92% [69 to 114] of KCl-induced contraction in

controls, and to 96% [25 to 167] of KCl-induced contraction with 300 nM tolterodine (MD 4 percentage points [-58 to 67]).

Discussion

Despite the clinical use of mirabegron in OAB, mechanisms underlying its benefits are still unclear. Unresolved issues and controversial findings may be related to limitations in study design, which may critically determine β -adrenergic smooth muscle relaxations (Dale et al., 2014). Previous studies focussed on mirabegron-induced relaxation of precontracted detrusor tissues, or applied high concentrations of mirabegron, exceeding known K_i values for β_3 -adrenoceptors and expected plasma concentrations. Our present findings, obtained by a different experimental design suggest that inhibition of human detrusor contractions by mirabegron *in vitro* is limited to neurogenic contractions, which occurs independently from β_3 -adrenoceptors, while full agonist-induced contractions in concentration response curves are not affected (fig. 6).

Other than previous studies, mostly addressing mirabegron-induced relaxation of precontracted bladder tissues or exploring a concentration of 30 μ M, we assessed effects of 1 μ M and 10 μ M mirabegron in frequency and concentration response curves. EFS-induced contractions in our experiments were inhibited by 10 μ M mirabegron, but not 1 μ M mirabegron. From radioligand binding assays performed in cells transfected with human β -adrenoceptors, K_i values of 2.5 nM for β_3 -, 383 nM for β_1 -, and 977 nM for β_2 -adrenoceptors were reported (Tasler et al., 2012). Similarly, mirabegron induced generation of cAMP with an EC_{50} of 22.4 nM in cells transfected with human β_3 -adrenoceptors (Takasu et al., 2007). The recommended daily standard dosage is 50 mg, resulting in maximum plasma levels of 137 nM in men (Krauwinkel et al., 2012). Although urine concentrations of mirabegron between 1.6-8.2 μ M were estimated (Yamada et al., 2021), it is unknown whether these occur within the bladder wall. Taking known plasma levels, K_i values and effective concentrations *in vitro* into account, it may be excluded that mirabegron improves storage

symptoms by activation of β_3 -adrenoceptors on bladder smooth muscle cells. While the inhibition of EFS-induced contractions by 10 μ M obviously represents a local mechanism, it is probably mediated by off-target effects. Lacking activation of β_3 -adrenoceptors on smooth muscle cells, and lacking cAMP formation by β_3 -adrenoceptors are confirmed by unchanged non-cholinergic contractions in our study. Finally, effects on EFS were resistant to L-748,337, suggesting that they occurred independently from β_3 -adrenoceptors. Considering known K_i values of mirabegron for β_3 -adrenoceptors, full saturation should be expected using our concentrations, which was, however, not confirmed by binding experiments in our study.

Our results contrast previous findings suggesting inhibition of cholinergic contractions, which were, however, obtained by fundamentally different protocols. Apart from a small rightshift of methacholine curves, we did not observe effects on cholinergic contractions. In previous studies, mirabegron was applied after stable carbachol-induced precontractions were attained. However, these occurred after strong initial carbachol-induced peak contractions decreased to lower steady state (pre-)contractions. Obviously, these pretensions amounted to only 27-44% of initial carbachol-induced peak contractions (equivalent to 46-55% of KCl-induced contractions, while maximum peak contractions by carbachol amounted to 113-183% of KCl) (Svalo et al., 2013). Under these conditions, EC_{50} values for mirabegron-induced relaxation of carbachol-precontracted tissues amounted to 588 nM, 912 nM and 3.89 μ M in normal detrusor tissues, in tissues from patients with bladder outlet obstruction (BOO) and from patients with BOO and detrusor overactivity, respectively, with maximum relaxations of 28-36% at 30 μ M (Svalo et al., 2013). Extrapolated to our design, where we assessed peak contractions in concentration response curves, this may represent an inhibition around 15%, which is close to our findings with methacholine. In another study, relaxation of human bladder strips was started 60 min after precontraction with carbachol, resulting in an EC_{50} of 780 nM and in a maximum relaxation of 89.4% at 10 μ M mirabegron (Takasu et al., 2007). Low steady state contractions (e. g. 60 min after carbachol application)

are physiologically less relevant than immediate peak contractions, considering that bladder emptying relies on cholinergic contractions and occurs within seconds to minutes after voiding initiation. To exclude that lacking mirabegron effects in our study were artefacts, we examined effects of the muscarinic receptor antagonist tolterodine, which provided expectable results.

In another study, concentration response curves for carbachol were created with solvent or 30 μM mirabegron, resulting in a rightshift of carbachol curves by mirabegron (Maki et al., 2019). Rightshifts in that and our study may result from antagonism of muscarinic receptors. Mirabegron binding to muscarinic receptors has been described for the M2 subtype, with a K_i value of 2.1 μM (Dehvari et al., 2018; FDA, 2012). Acknowledging both a presumed activation of β_3 -adrenoceptors plus antagonism of muscarinic receptors, a recent study calculated two EC_{50} values for mirabegron-induced relaxation of carbachol-precontracted rat detrusor tissues, using a biphasic calculation model and pointing to EC_{50} values of 87 nM and 10.7 μM (Yamada et al., 2021). In the same study, mirabegron replaced a radiolabelled muscarinic antagonist in rat bladder tissues with an IC_{50} of 2.4 μM . Obviously, antagonism of muscarinic receptors by mirabegron requires micromolar concentrations, and is therefore unlikely to occur *in vivo* with nanomolar plasma concentrations.

Generally, the comparability of β -adrenergic relaxations and their modulation by ligands may be limited between human and non-human detrusor tissues (Dale et al., 2014). Species-dependent differences in β -adrenergic detrusor relaxation may be imparted by divergent expression patterns of receptor subtypes, and by differences in ligand-receptor interactions (Cernecka et al., 2015; Dale et al., 2014). β -Adrenergic relaxation of human detrusor tissues has been widely attributed to β_3 , whereas β -adrenergic relaxation of detrusor tissues from mice is predominantly mediated by β_2 (Propping et al., 2016; Propping et al., 2015; Wuest et al., 2009). Data are available from wildtype and β_2 -adrenoceptor knockout mice, but

obviously not from β_3 -adrenoceptor knockout mice. In addition to expression patterns, binding pockets differ between human and rodent β_3 -adrenoceptors, resulting in different potencies of β_3 -agonists (Cernecka et al., 2015).

Other β_3 -adrenergic agonists have been examined in the context of human detrusor relaxation as well. KUC-7322 relaxed KCl-precontracted human detrusor tissues, without affecting concentration response curves for carbachol, even using 10 μ M KUC-7322, what is fully in line with our findings (Igawa et al., 2012). In most, if not all other studies using human detrusor tissues, effects of β_3 -agonists were studied after precontraction, i. e. under similar conditions as described above for mirabegron. Examined β_3 -agonists included solabegron, BRL-37,344, CPG 12177, CL316243 and ZD-7114, which induced relaxations of similar extent as mirabegron (Badawi et al., 2007; Biers et al., 2006; Rouget et al., 2014; Takeda et al., 1999; Tyagi et al., 2009; Yamanishi et al., 2006). As the specificity for some of these agonists is limited (BRL-37,344, CL316243) and off-targets are known for high concentrations of others (Michel, 2020), truly β_3 -mediated effects are rather small and unspecific actions at higher concentrations may not be excluded.

We assume that inhibition of EFS-induced contractions by 10 μ M mirabegron in our study was caused by off-target inhibition of contractile neurotransmission. Inhibition of EFS-induced acetylcholine release in human detrusor tissues by mirabegron has been repeatedly demonstrated, with an EC_{50} value of 129 nM (D'Agostino et al., 2015), with reductions of acetylcholine release between 30-55% using 0.01-1 μ M mirabegron (Silva et al., 2019), or with a decrease of 57% by 100 nM mirabegron, which was sensitive to a β_3 -adrenoceptor antagonist (Silva et al., 2017). The limited degree of reduced neurotransmission (<60%) may be insufficient to translate to motoric effects, explaining why 1 μ M mirabegron failed to inhibit EFS-induced contractions in our experiments. To the best of our knowledge, only one study demonstrated inhibition of EFS-induced contractions of human detrusor tissues by

mirabegron, using a concentration of 100 nM (D'Agostino et al., 2015). However, a conclusive comparison to our study is impeded by lacking information about study design and experimental details, and as tissues were stored over night.

Several previously proposed mechanisms can be precluded to account for improvements of storage symptoms by mirabegron *in vivo* (Igawa et al., 2019), including detrusor relaxation by β_3 -adrenoceptors on bladder smooth muscle cells, inhibition of cholinergic neurotransmission in the bladder wall, or antagonism of muscarinic receptors (fig. 6). Our findings support previously proposed concepts that improvements by mirabegron are based on effects on afferent signaling or in the CNS (fig. 6). In rat models, mirabegron improved storage symptoms by inhibition of mechosensitive, afferent signaling by A δ fibers in the bladder wall (Aizawa et al., 2012; Aizawa et al., 2015; Sadananda et al., 2013). Mechanosensitive signals emerge with increasing pressure and bladder filling, and are transduced to the CNS to induce bladder emptying under normal conditions, but may be caused by spontaneous, local microcontractions in the bladder wall, in particular in OAB (Drake et al., 2005; Vahabi and Drake, 2015). Consequently, inhibition of mechanoafferent signaling was proposed to account for decreases of frequency by mirabegron (Aizawa et al., 2012; Aizawa et al., 2015; Sadananda et al., 2013). Notably, these effects occurred using doses close to human standard doses, or at nanomolar concentrations in intravesical instillation (Aizawa et al., 2012; Aizawa et al., 2015; Sadananda et al., 2013). Alternatively, it has been suggested that mirabegron improves storage symptoms by mechanisms located to the sacral spinal cord, where afferent and efferent signals in the voiding reflex are processed (Fullhase et al., 2011).

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Author contribution statement

Participated in research design: Stief, Hennenberg. Conducted experiments: Huang, Tamalunas, Strittmatter, Stief, Hennenberg. Contributed new reagents or analytic tools: n. a. Performed data analysis: Huang, Waidelich, Hennenberg. Wrote or contributed to writing of the manuscript: Huang, Stief, Hennenberg.

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Footnotes

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Legends to figures

Figure 1: Mirabegron effects on EFS-induced contractions of human detrusor tissues. Contractions of bladder tissues from the lateral bladder wall and without urothelium were induced by EFS in an organ bath, 30 min after adding 1 μ M mirabegron (**A**), 10 μ M mirabegron (**B**), or a corresponding volume of DMSO (controls), which was used as solvent for mirabegron, and of 1 μ M L-748,337 in addition to solvent and mirabegron (i. e. to all chambers) in (**C**). Shown are data from experiments using tissues from n=5 patients in each panel. For each experiment, tissue from one patient was allocated to the control and mirabegron group. Data are means with standard deviation in frequency response curves (# p<0.05 for control vs. mirabegron, and p value for whole groups), and $E_{\max}$ and E_{f50} values from curve fitting of all single experiments in scatter plots.

Figure 2: Mirabegron effects on carbachol-induced contractions of human detrusor tissues. Contractions of bladder tissues from the lateral bladder wall and without urothelium were induced by cumulative concentrations of carbachol in an organ bath, 30 min after adding mirabegron at concentrations of 1 μ M (**A**) or 10 μ M (**B**) or a corresponding volume of DMSO (controls), which was used as solvent for mirabegron. Shown are data from experiments using tissues from n=5 patients in each panel. For each experiment, tissue from one patient was allocated to the control and mirabegron group. Data are means with standard deviation in concentration response curves, and $E_{\max}$ and EC_{50} values from curve fitting of all single experiments in scatter plots.

Figure 3: Mirabegron effects on methacholine-induced contractions of human detrusor tissues. Contractions of bladder tissues from the lateral bladder wall and without urothelium were induced by cumulative concentrations of methacholine in an organ bath, 30 min after

adding 1 μ M mirabegron **(A)**, 10 μ M mirabegron **(B)** or a corresponding volume of DMSO (controls), which was used as solvent for mirabegron, and of 1 μ M L-748,337 in addition to solvent and mirabegron (i. e. to all chambers) in **(C)**. Shown are data from experiments using tissues from n=5 patients in each panel. For each experiment, tissue from one patient was allocated to the control and mirabegron group. Data are means with standard deviation in concentration response curves (p values for whole groups), and $E_{\max}$ and EC_{50} values from curve fitting of all single experiments in scatter plots. An exception is one experiment in **(A)**, where curve fitting revealed EC_{50} values of 5 mM with solvent and 1 mM with mirabegron, which could not be corrected by adjusting curve fitting, so that these values were omitted from presentation and statistical analysis.

Figure 4: Mirabegron effects on non-cholinergic contractions of human detrusor tissues. Contractions of bladder tissues from the lateral bladder wall and without urothelium were induced by cumulative concentrations of endothelin-1 **(A)** or U46619 **(B)** in an organ bath, 30 min after adding mirabegron (10 μ M) or a corresponding volume of DMSO (controls), which was used as solvent for mirabegron. Shown are data from experiments using tissues from n=5 patients in each panel. For each experiment, tissue from one patient was allocated to the control and mirabegron group. Data are means with standard deviation in concentration response curves, and $E_{\max}$ and EC_{50} values from curve fitting of all single experiments in scatter plots.

Figure 5: Tolterodine effects on EFS-induced and cholinergic contractions of human detrusor tissues. Contractions of bladder tissues from the lateral bladder wall and without urothelium were induced by EFS **(A)**, cumulative concentrations of carbachol **(B)** or methacholine **(C)** in an organ bath, 30 min after adding tolterodine (300 nM) or a corresponding volume of DMSO (controls), which was used as solvent for tolterodine. Shown are data from experiments using tissues from n=5 patients in each panel. For each

experiment, tissue from one patient was allocated to the control and tolterodine group. Data are means with standard deviation in frequency and concentration response curves (# $p < 0.05$ for control vs. tolterodine, and p values for whole groups), and $E_{\max}$, E_{50} and EC_{50} values from curve fitting of all single experiments in scatter plots.

Figure 6: Effects and targets of mirabegron on detrusor contraction *in vitro* (A) and on storage symptoms *in vivo* (B). *In vitro*, inhibition of smooth muscle contractions in human bladder tissues by mirabegron requires concentrations highly exceeding the known plasma concentrations (max. 137 nM in men) or the known K_i values for β_3 -adrenoceptors (2.5 nM). Inhibition of EFS-induced contractions by mirabegron is probably caused by inhibition of cholinergic neurotransmission, while rightshifts of concentration response curves for cholinergic agonists may result from antagonism of muscarinic receptors (A). Both effects represent off-target effects and do not occur *in vivo*, as they require mirabegron concentrations of 10 μ M or higher. Concentrations of 1 μ M or less did not affect agonist- or EFS-induced contractions in this and in another study. *In vitro*, effects of nanomolar concentrations are limited to small relaxations of precontracted tissues, which are physiologically not relevant. Consequently, several previously proposed mechanisms can not account for improvements of storage symptoms by mirabegron *in vivo*, including detrusor relaxation by β_3 -adrenoceptors on bladder smooth muscle cells, prejunctional inhibition of cholinergic neurotransmission in the bladder wall, or inhibition of detrusor contractions by antagonism of muscarinic receptors. Rather, improvements by mirabegron may be caused by activation of β_3 -adrenoceptors (β_3 -AR) in afferent fibers or in the central nervous system (CNS) (B), which are involved in the voiding reflex and have been previously proposed based on findings using standard dosing *in vivo* or nanomolar concentrations in intravesical instillation in rodent models.

A **B** **C**

Figure 1

A

B

Figure 2

A **B** **C**

Figure 3

A **B**

Figure 4

A **B** **C**

Figure 5

A **B**

Figure 6