Unique receptor dissociation kinetics of the novel endothelin receptor antagonist macitentan

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INTRODUCTION

- Pulmonary arterial hypertension (PAH) is caused by progressive pulmonary artery remodelling and constriction,[1,2] and a central pathogenic role of endothelin-1 (ET-1) in PAH has been demonstrated in several clinical trials evaluating different ERAs.[3]
- ET-1 responses are mediated via two G protein-coupled receptors (GPCR), ET_A and ET_B.[4,5] In pulmonary arterial smooth muscle cells (PASMC), ET receptors couple to the Gq pathway and increase inositol-trisphosphate (IP_3) and diacylglycerol (DAG) levels. These cause a biphasic calcium response resulting in sustained elevation of intracellular calcium levels.[3,6] Elevated calcium levels promote cytoskeletal contraction and cell proliferation and thereby mediate vasoconstriction and vascular remodelling.

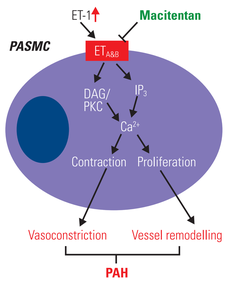

Figure 1. Endothelin-1 signalling in pulmonary arterial smooth muscle cells (PASMC) and its contribution to PAH. PKC, protein kinase C.

- Two ERAs, bosentan and ambrisentan, are currently approved for PAH treatment. Macitentan is a novel and potent dual ERA[7] that has recently concluded a Phase III morbidity/mortality clinical trial in PAH.[8]

AIMS

- Association and dissociation rates of GPCR antagonists can influence their pharmacological activity *in vivo*.[9] We used second messenger assays in normal human PASMC to characterise the inhibitory potency and receptor inhibition kinetics of macitentan versus the approved ERAs bosentan and ambrisentan.

METHODS

Intracellular calcium release measurements using CHO-ET_A and CHO-ET_B cells

- Within the Fluorescent Imaging Plate Reader (FLIPR® Tetra, Molecular Devices), cells were incubated with ERA dilutions, stimulated with ET-1 and peak relative fluorescence units (RFU) were converted to IC_{50} values. K_b values were calculated via the Cheng-Prusoff equation $[K_b=IC_{50}/(1+[ET\text{-}1_{stim}]/EC_{50_ET\text{-}1})]$.[10]

Intracellular calcium release measurements using normal human PASMC

- PASMC (TCS Cellworks [ZHC-3116]) were incubated with ERA dilutions and stimulated with ET-1. Peak RFU were converted to IC_{50} values, and K_b values were calculated.[10]
- For the determination of ERA receptor occupancy half-lives ($ROt_{1/2}$), cells were incubated with ERA dilutions and then either immediately stimulated with ET-1 or washed. At different times after washing, cells were stimulated with ET-1, and K_b values were calculated from the peak RFU. For $ROt_{1/2}$ estimation, the earliest time point with a K_b significantly ($p<0.05$, one-way ANOVA) shifted versus the K_{b0min} was used in the equation $ROt_{1/2}=t_x/[\log_2 (K_{bxmin}/K_{b0min})]$, where x = time point of first significant K_b change.
- For the determination of ERA effects on sustained calcium release, PASMC were incubated with ERA dilutions and stimulated with ET-1 dilutions. Sustained calcium release was quantified from FLIPR traces as area under the curve (AUC) between 3 min and 23 min after ET-1 addition.

Inositol-1-phosphate (IP_1) measurements

- PASMC were incubated with ERA dilutions and ET-1 dilutions were added for 20 min or 90 min. Cells were processed (IP-One HTRF® kit, Cisbio, #62IPAPEC). IP_1 concentrations calculated from the 665 nm/620 nm emission ratios (excitation 337 nm; PHERAstar, BMG labtech). Schild K_b values (90 min ET-1) were generated by Gaddum/Schild non-linear regression (GraphPadPrism).

RESULTS

ET-1-induced calcium signalling in PASMC is mainly driven by ET_A receptors

- The good correlation of antagonistic potencies determined in normal human PASMC and CHO-ET_A cells demonstrated that ET_A receptors are the main contributors to ET-1-induced calcium release in PASMC.

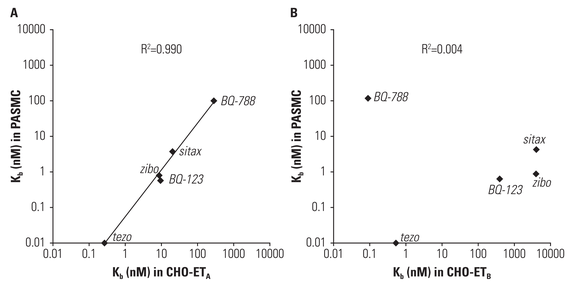

Figure 2. Contribution of endothelin receptor subtypes to calcium signalling in normal human PASMC: PASMC or CHO-ET_A or CHO-ET_B pre-incubated with reference ERA dilutions (120 min) were stimulated with ET-1 (EC_{50}–EC_{70}). Correlation of K_b values obtained in PASMC versus CHO-ET_A cells (A) or versus CHO-ET_B cells (B). Geometric means, n=3–8.

Macitentan displays slow apparent association with ET receptors in PASMC

- Bosentan, ambrisentan and macitentan displayed steady-state (120 min pre-incubation) antagonistic potencies of K_b = 1.1 nM, 0.12 nM and 0.14 nM.
- Macitentan showed a 6.3-fold increase in potency over time indicating a slow apparent association with ET receptors in PASMC.
- The majority of other ERAs displayed fast apparent association kinetics.

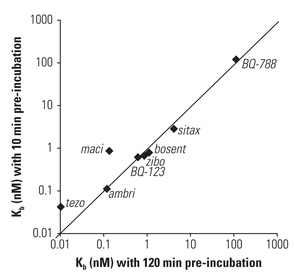

***Table 1.** Mean K_b values in calcium release assays using human PASMC with different pre-incubation times (geometric means, n=6).*

	K_b (nM) 10 min	K_b (nM) 120 min	K_b ratio
Ambrisentan	0.11	0.12	1.0
Bosentan	0.79	1.1	0.7
BQ-123	0.61	0.62	1.0
BQ-788	121	117	1.0
Macitentan	0.86	0.14	6.3
Sitaxentan	2.9	4.2	0.7
Tezosentan	0.042	0.010	4.1
Zibotentan	0.65	0.87	0.7

Figure 3. Antagonistic potency and apparent association kinetics of different ERAs using calcium release assays in PASMC: cells pre-incubated with ERA dilutions (10 min or 120 min) were stimulated with ET-1 (EC_{50}–EC_{70}). Correlation of the K_b values at 10 min versus 120 min pre-incubation. Geometric means, n=6.

Macitentan displays slow receptor dissociation kinetics and a long receptor occupancy half-life in PASMC

- Macitentan displayed slow receptor dissociation after washout compared to ambrisentan and bosentan. Calculated $ROt_{1/2}$ were 17 min (macitentan), 40 sec (ambrisentan) and 70 sec (bosentan).

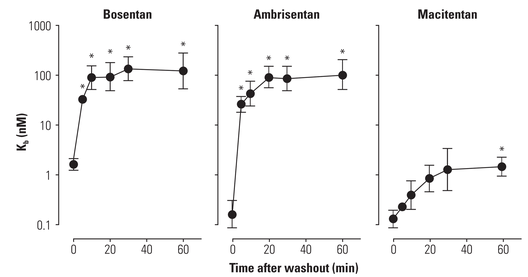

*Figure 4. Dissociation kinetics of ambrisentan, bosentan and macitentan determined by calcium release assays in PASMC: cells pre-incubated with ERA dilutions (120 min) were either directly stimulated with ET-1 (EC_{50}–EC_{70}) or subjected to compound washout followed by ET-1 stimulation at different time points. Shown are the time-dependent changes in K_b after washout (geometric means ± SEM, n=2–4). * K_b values after washout significantly different ($p<0.05$) from the K_b at 0 min (one-way ANOVA, Dunnett's post test).*

Slow receptor dissociation renders macitentan insurmountable in IP_1 assays using short ET-1 stimulation times in PASMC

- Bosentan and ambrisentan behaved as surmountable antagonists of ET-1-induced IP_1 accumulation. In contrast, macitentan behaved as an insurmountable antagonist after 20 min of ET-1 stimulation.
- Upon prolongation of the ET-1 stimulation time to 90 min, also macitentan behaved as a surmountable antagonist, revealing its competitive binding mode. Mean Schild K_b values (n=3) were 16 nM (bosentan), 1.6 nM (ambrisentan) and 1.4 nM (macitentan).

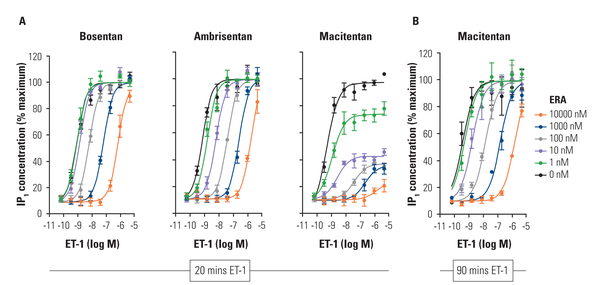

Figure 5. Effect of bosentan, ambrisentan and macitentan on ET-1-induced IP_1 accumulation: PASMC pre-incubated with ERA dilutions (120 min) were stimulated with ET-1 dilutions. After 20 min (A) or 90 min (B), the cellular IP_1 content (stable degradation product of IP_3) was determined. (A) Average values ± SEM (n=4). (B) Representative experiment of n=3 ± SD of duplicates.

Macitentan is an insurmountable antagonist of ET-1-induced sustained elevation of intracellular calcium concentrations in PASMC

- Bosentan and ambrisentan displayed surmountable antagonism of ET-1-induced sustained elevation of intracellular calcium concentrations in PASMC.
- In contrast, macitentan behaved as an insurmountable antagonist.

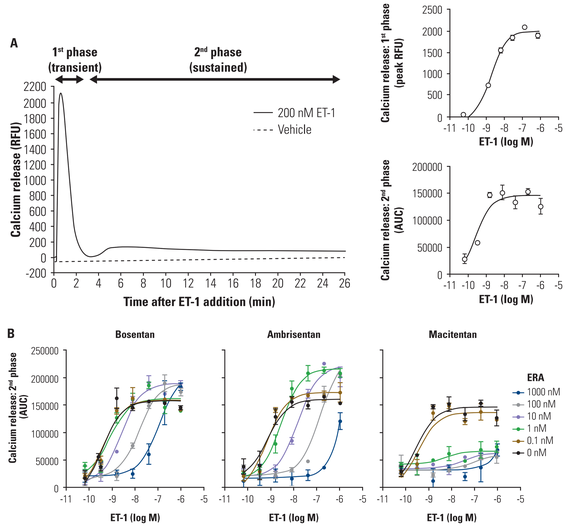

Figure 6. The biphasic intracellular calcium response to ET-1 and the effect of bosentan, ambrisentan and macitentan on sustained intracellular calcium elevation: (A) FLIPR fluorescence traces and concentration-response-curves of ET-1 for the transient (peak RFU) and the sustained (AUC) response phase. (B) PASMC pre-incubated with ERA dilutions (120 min) were stimulated with ET-1 dilutions. Effect of ERAs on the ET-1 concentration-response-curves of the sustained response phase. Representative experiment of n=3.

CONCLUSIONS

- Macitentan differed from the two currently approved ERAs through its slower receptor dissociation kinetics and insurmountable antagonism in non-equilibrium assays using human PASMC.
- Under conditions of locally fluctuating ET-1 levels *in vivo* (paracrine and autocrine secretion and rapid local degradation), macitentan has the potential to block ET-1-induced signalling more effectively than other ERAs, which show faster receptor dissociation.
- These qualities may contribute to a unique effectiveness of macitentan in diseases characterised by increased autocrine and paracrine ET-1 signalling such as PAH.

References

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Disclosures: J Gatfield, C Mueller Grandjean, T Sasse, B Steiner, P Steiner, M Iglarz, K Kauser, M Clozel and O Nayler are all employees of Actelion Pharmaceuticals Ltd.
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