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The Discovery of Macitentan – A Standard Medicinal Chemistry Program?

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Abstract: A plethora of properties are typically studied during a medicinal chemistry program and many of these parameters may shape the cascade of compound selection. Given the task to discover a molecule with a profile superior to that of the dual endothelin receptor antagonist bosentan, we tailored our compound profiling cascade to the specific properties that were not optimal in bosentan, namely *in vivo* efficacy and safety. Contrary to conventional thinking, we therefore focused on corresponding *in vivo* experiments. In the following, we highlight and illustrate some key learnings of our approach that led to the discovery of macitentan (**1**), an orally available potent dual endothelin receptor antagonist approved for the treatment of pulmonary arterial hypertension.

Keywords: Endothelin receptor antagonist · Macitentan · Medicinal chemistry · Pulmonary arterial hypertension

Martin H. Bolli *studied biology and chemistry at the Swiss Federal Institute of Technology in Zürich (ETHZ) and received a PhD from the University of Bern (Prof. Dr. C. J. Leumann). After postdoctoral studies in the groups of Prof. A. Eschenmoser (ETHZ and The Scripps Research Institutes, La Jolla, USA) and Prof. S. V. Ley (University of Cambridge, UK), he joined Actelion Pharmaceuticals Ltd. in 1998, where he first worked as a laboratory head and later as a group leader in medicinal chemistry. His team significantly contributed to the discovery of macitentan (**1**, Fig. 1), aprocitentan, ponesimod, and cenerimod. Upon the acquisition of Actelion by Janssen Pharmaceutical, a company of Johnson&Johnson, Dr. Bolli and his team joined the newly established company Idorsia Pharmaceuticals Ltd. where they aim at identifying novel drugs interfering with biological mechanisms associated with cardiovascular and fibrotic diseases. Dr. Bolli has published close to 100 scientific papers and patent applications.*

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Historical Background

In 1997 Actelion Pharmaceuticals Ltd. was founded by Jean-Paul and Martine Clozel, Walter Fischli, and Thomas Widmann, four researchers who shared their passion for discovering and developing novel molecules interacting with the endothelin (ET) system. For about a decade, the four researchers and their teams had been working at the forefront of endothelin science at Roche. Their early studies described the ET system in physiology and pathology in great detail[1–5] and suggested that molecules interacting with the ET receptors could potentially hold great promise for the treatment of a large number of cardiovascular diseases.[6–10] Medicinal chemistry efforts at Roche soon led to the discovery of such molecules[11] and studies with a first orally active small molecule inhibitor of the two ET receptors underscored the pathological role of endothelin-1.[6] In 1995 a Phase II clinical trial had already been performed in chronic heart failure patients with bosentan (**2**), a potent orally active inhibitor of both the ET_A and the ET_B receptor.[12–14] While clinical development of bosentan was on-going in chronic heart failure (CHF) , the four researchers decided to create their own company named Actelion with the aim to extend bosentan's clinical development to other indications. When Roche discontinued clinical development of bosentan in CHF,[15] in 1998, Actelion was able to in-license bosentan and to follow its own clinical development plans. At the same time, it was the founders' clear vision to establish a research organisation within the newly formed company that embarked on the search for innovative drugs. Hence, shortly after its foundation Actelion hired a number of biologists, pharmacologists, and chemists with the mandate to fuel Actelion's research and development pipeline. This was the time when I joined Actelion – a very young company that just moved into empty lab and office space. One of our first tasks was to identify a novel endothelin receptor antagonist (ERA) with improved properties when compared to bosentan.

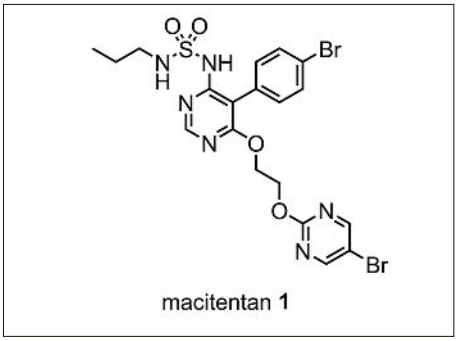


Fig. 1. Molecular structure of macitentan (**1**).

The Endothelin System

In 1988, Yanagisawa *et al.*[16] characterized a 21-amino acid peptide, endothelin-1 (ET-1) that they had isolated from the supernatant of porcine aortic endothelial cells as one of the most potent vasoconstrictors. At the same time, the Roche team had already demonstrated that this novel

vasoconstrictor is also produced by human endothelial cells and binds to a specific binding site on human vasculature smooth muscle cells.[1,3] Later it was found that this peptide is in fact a member of a family of three closely related peptides, ET-1, ET-2, and ET-3, that convey their biological activity through binding to two G-protein coupled receptors named ET_A and ET_B. While ET-1 and ET-2 bind and activate the ET_A receptor with high affinity, all three peptide homologs are potent agonists of the ET_B receptor. In addition to their vasoactivity, endothelins are upregulated in pathology and promote cell proliferation, inflammation, tissue remodelling and fibrosis.[17,18] Based on their mechanism of action, ERAs were proposed to be useful in a number of cardiovascular (*e.g.* hypertension, pulmonary hypertension, atherosclerosis, congestive heart failure, myocardial infarction), renal (*e.g.* chronic kidney failure), fibrotic (*e.g.* pulmonary fibrosis) and proliferative diseases (*e.g.* prostate cancer, ovarian cancer, melanoma).[9,17,19–22]

Under physiological conditions, blood vessels react in a bi-phasic fashion to the stimulation by exogenous ET-1 (Fig. 2). A transient vasodilation mediated by the activation of ET_B receptors on endothelial cells characterizes a first response. In a second phase, ET_A receptors that are predominantly expressed on vascular smooth muscle cells provoke a strong and sustained vasoconstriction. In animal models of cardiovascular diseases, however, not only ET-1 levels but also ET_B receptors are up-regulated in vascular tissue and no longer mediate vasodilation but rather enhanced vasoconstriction.[7,10,23–25] Dual ET_A and ET_B receptor antagonism therefore appeared superior to us in terms of efficacy as compared to selective ET_A-receptor blockade.[26–28] In addition, we believed dual ERAs provided a better safety profile as increased chronic ET_B receptor activation in the presence of ET_A selective blockade was associated with undesired cell proliferation and fluid retention.[29,30] The benefits of dual ET receptor blockade were further substantiated by pre-clinical as well as clinical studies.[25,26,28,31–34]

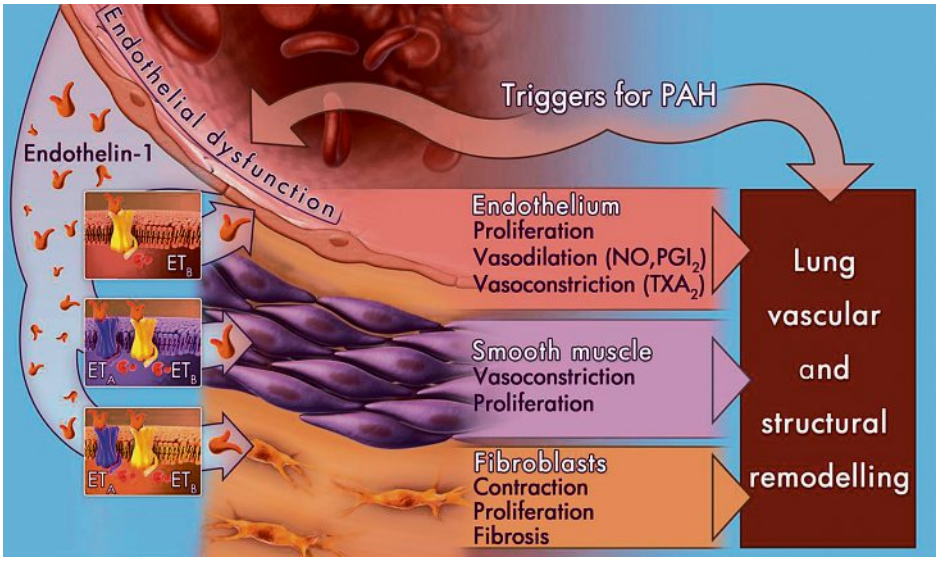


Fig. 2. Illustration of the effects of the endothelin system on the lung in pulmonary arterial hypertension. Endothelial cells produce endothelin-1 that can act in an autocrine and paracrine fashion. By activating ET_B receptors on endothelial cells, ET-1 induces cell proliferation, and vasodilation or vasoconstriction. On smooth muscle cells, stimulation of ET_A and ET_B by ET-1 leads to vasoconstriction and proliferation. Fibroblasts also express both ET receptors and contract and proliferate upon stimulation with ET-1. Adapted from Dupuis *et al.*[23]

Bosentan's Properties

Bosentan was characterised as a potent dual ERA showing efficacy in animal models of several cardiovascular diseases (reviewed in ref. [35]) such as chronic heart failure[26,36,37] and pulmonary hypertension.[38,39] In *in vitro* binding assays using membranes of recombinant CHO cells overexpressing the corresponding ET receptor, bosentan showed K_i values of 6.5 and 343 nM on the ET_A and ET_B receptor, respectively. In functional tests with isolated rat aorta and trachea rings, bosentan acted as a potent and competitive antagonist of ET-1 (aorta, ET_A) and sarafotoxin S6c (trachea, ET_B). Bosentan was able to reduce blood pressure in several rat models of hypertension.[35] In pithed rats, bosentan reduced the blood pressure increase induced by i.v. injection of big-ET-1 in a dose-dependent fashion.[12] Long-term treatment with bosentan of rats with chronic heart failure significantly decreased post-charge as measured by mean arterial pressure (MAP) and increased survival.[37] Furthermore, bosentan attenuated the monocrotaline induced increase in mean pulmonary arterial pressure (MPAP) in this rat model of pulmonary hypertension.[40] By 1998, phase II clinical trials with bosentan in chronic heart failure (CHF)[13,14,41] and essential hypertension[42] had been completed while phase III trials in pulmonary arterial hypertension (PAH) patients[43–45] were in preparation. In these trials increases in aminotransferase (ALT and AST) levels of more than three times the upper limit of normal (ULN) were observed in 10-15% of patients receiving bosentan and a dose of 125 mg b.i.d was identified as optimal balance of efficacy and safety.[46–48]

Project Goal & Screening Cascade

With this background we set out to identify a novel ERA optimized for chronic use, ideally being more efficacious, longer lasting and devoid of liver liability. In theory and reflecting in hindsight, we considered that this might be achieved by at least two alternative approaches. On one hand we could have aimed at improving the potency of the compound such that the exposures needed to achieve efficacy *in vivo* would be below the threshold that triggers liver toxicity. In such an approach, the profiling cascade for selecting compounds would focus on assays that characterise the compound's *in vitro* potency and *in vivo* efficacy. On the other hand, one could try to remove the liver liability *per se*. For this approach to be successful a relevant *in vitro* or *in vivo* assay that allows characterisation of the compounds with respect to their potential liver toxicity was required. Fattinger *et al.* developed an assay based on the observation that the clinical increases in liver transaminase are likely the consequence of inhibition of the canalicular bile salt export pump (BSEP) and the subsequent intrahepatic accumulation of bile salts by bosentan.[49] *In vitro*, bosentan inhibited taurocholate transport in vesicles overexpressing the rat or human export pump. In the rat, increased serum bile salt levels observed after i.v. injection of bosentan mimicked the cholestatic effect observed in patients with liver injury. We therefore decided to include plasma bile salt measurements after i.v. injection of our compounds to rats as a key characterisation step in our profiling cascade. By combining the two approaches mentioned above, our profiling cascade was based on the following cornerstones (Fig. 3): potency on the ET_A and ET_B receptor was first assessed by a ^{125}I-ET-1 competitive binding assay using membranes of CHO cells overexpressing either of the two receptors. In a second step, the functional potency of our compounds was assessed by measuring

their ability to inhibit the ET-1 and sarafotoxin S6c induced constriction of isolated rat aorta and trachea rings, respectively (Fig. 4). Efficacy of compounds that were at least as potent as bosentan in these prior assays was then assessed by measuring their ability to reduce blood pressure in hypertensive Dahl salt-sensitive rats equipped with a telemetry system (Fig. 5). This model has been validated with bosentan and the set-up of the experiment allowed testing of 4 to 5 compounds per week. At the selected screening dose of 3 mg/kg only about 12 mg of compound was required. Based on our experiences working in this area, we were able to rapidly test a relatively large number of compounds enabling us to study the *in vivo* structure activity relationship. In a second rat experiment, plasma ET-1 level increase after oral compound administration was measured as a means to assess efficient ET_B receptor blockade *in vivo*.[50,51] Finally, as mentioned above, absence of interference with hepatic bile salt transport was assessed by measuring plasma bile salt changes after i.v. administration of 25 mg/kg of the compound to rats. While our profiling cascade outlined in Fig. 3 was complemented with additional *in vitro* data *e.g.* on cytochrome P450 inhibition, hERG channel blockade, *etc.*, compound advancement heavily relied on *in vivo* rather than *in vitro* data. Indeed, our approach is in strong contrast to what is considered a standard medicinal chemistry approach in which many more properties would be assessed using *in vitro* and *in silico* methods (Fig. 6) to prioritize the compounds.

In the above assays, bosentan showed IC_{50} values of 45 and 202 nM on the ET_A and ET_B receptors, and pA_2 values of 6.78 and 6.47 in contraction experiments with isolated rat aorta and trachea, respectively. In hypertensive Dahl salt-sensitive rats, bosentan decreased blood pressure with an area between vehicle and treatment curve (ABC) of –88 mmHg × h at an oral dose of 3 mg/kg (Fig. 5B).[52] Ten minutes after intravenous injection of 25 mg/kg to Wistar rats, bosentan induced an 18 µM increase in plasma bile salts.[53] In order for our novel ERA to be superior to bosentan, we set the following thresholds for compound advancement in our profiling cascade: IC_{50} values of <20 and <400 nM on the ET_A and ET_B receptor, respectively; pA_2 values of >7 and >6 in the isolated rat aorta and trachea constriction experiment, respectively; an ABC of <–500 mmHg × h as calculated from the blood pressure recordings in Dahl rats; and no effect on plasma bile salt levels upon i.v. administration of 25 mg/kg of the compound to rats. These thresholds were set based on our own internal knowledge and creativity in attempting to discover a novel ERA.

Starting Points

It is interesting to note what Werner Neidhart, one of the inventors of bosentan, wrote about the challenge of finding small molecule ERAs in 1988: "… the general opinion then was that it would constitute a major challenge to substitute a large peptide hormone by a small molecule binder to compete for a G-protein coupled receptor…"[56] When we embarked on our program ten years later, our analysis of the competitive landscape of the ERA field revealed more than 150 patent applications disclosing small molecule ERAs and about a dozen compounds, mostly selective for the ET_A receptor, had already entered clinical trials for several indications (Fig. 7).[20,57–61] In view of this large number of structures known to interact with the endothelin receptors, we decided to select our starting points for a medicinal chemistry program from already known ERAs rather than to perform a high-throughput screen-

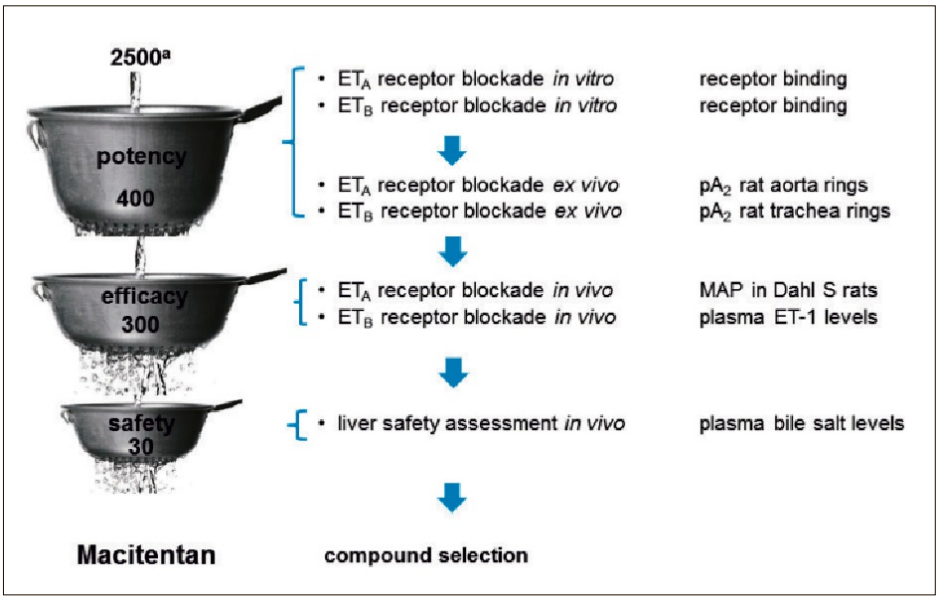


Fig. 3. The compound profiling cascade; [a]figures indicate number of compounds prepared and tested in the corresponding assays; MAP = mean arterial pressure.

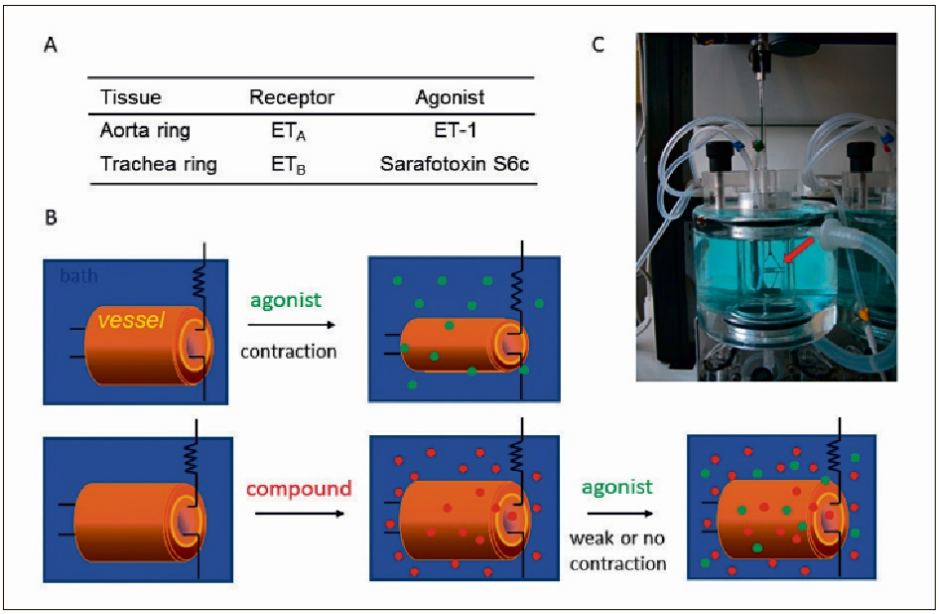


Fig. 4. *Ex vivo* functional potency assessment. A) ET receptor predominantly expressed in rat aorta and trachea rings and the corresponding agonist used. B) Rat aorta or trachea rings constrict upon stimulation with the endothelin receptor agonists ET-1 and sarafotoxin S6c, respectively. Pre-incubation of the tissue with an ERA attenuates/abolishes the constriction induced by the agonist. pA_2 values were calculated by Schild plot analyses of the dose-response curves; for example, at a concentration of 1 nM (10^{-9} M) a compound with a pA_2 value of 9 is able to shift the EC_{50}-value of the agonist by a factor 2; C) Organ bath with mounted tissue preparation (red arrow). For details see ref. [54].

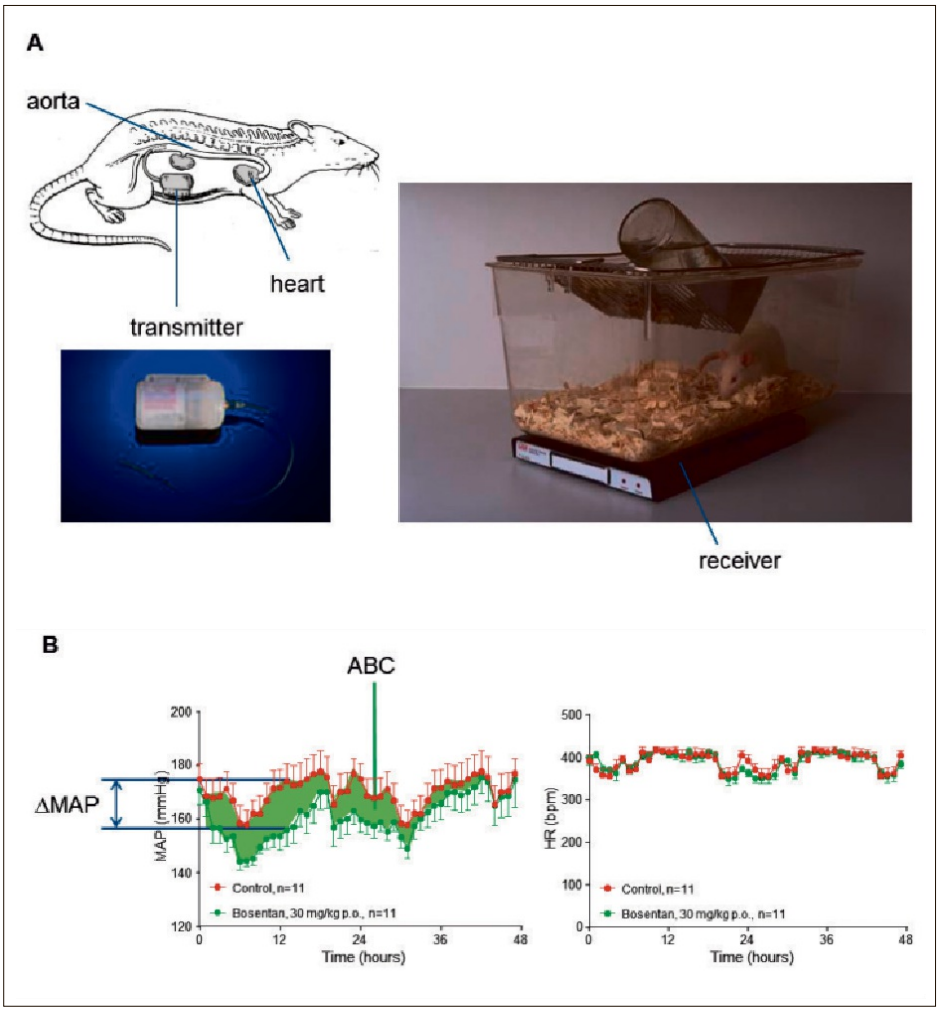


Fig. 5. A) Schematic view and picture of a rat equipped with a telemetry system measuring mean arterial blood pressure (MAP) and heart rate (HR). Adapted from Data Sciences International, St. Paul, MN, USA and refs [40, 55]. B) Example of MAP (left) and HR (right) recordings in telemetrized hypertensive Dahl salt-sensitive rats showing maximal ΔMAP (–14 ± 7 mmHg) and ABC (–343 mmHg × h) as calculated between the MAP curves before (control) and after administration of 30 mg/kg of bosentan. At a dose of 3 mg/kg the ABC was –88 mmHg × h (graph not shown). The HR is measured as a control to confirm that there is no HR increase as a response to the MAP reduction.

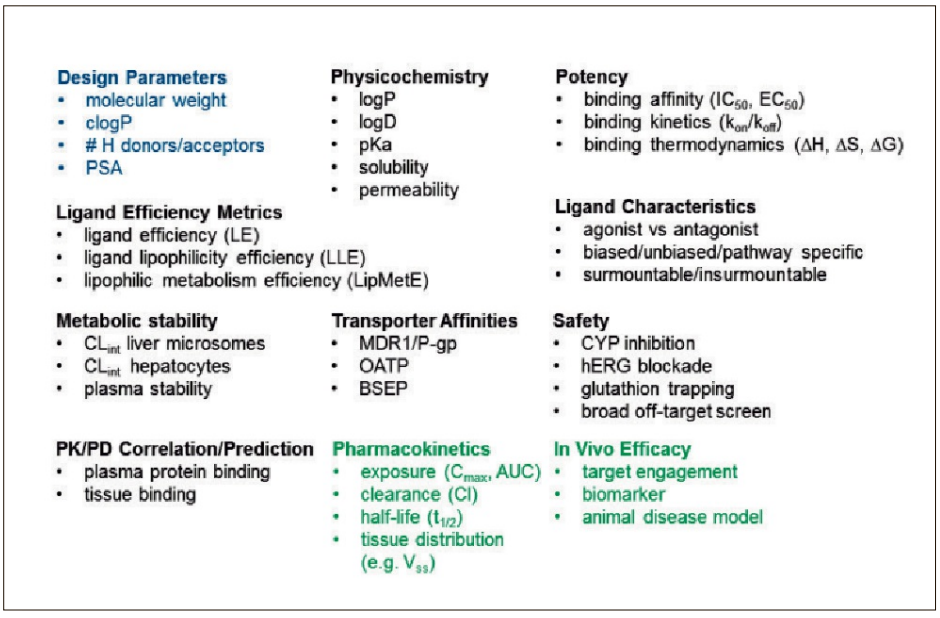


Fig. 6. Parameters that often form part of a screening cascade in a standard medicinal chemistry program; blue = calculated properties, black = properties measured *in vitro*, green = properties assessed *in vivo*.

ing. We based our selection of a small set of starting points on the compounds' structural diversity as well as their ability to block both the ET_A and the ET_B receptor. This ultimately led us to consider the five compounds shown in Fig. 8.

Project Progress

Preparing and testing analogues of the five starting points separated the wheat from the chaff (Fig. 9).[62] The synthesis of IRL-3630 (**14**)[63] analogues turned out to be more challenging than expected and as most of the prepared analogues were inactive this series was dropped early on. On the other hand, relatively easy synthetic access allowed to prepare a considerable number of analogues of pyrazole **16**.[64] However, none of these variations led to a promising affinity for the ET receptors. Similarly, analogues of Banyu/Merck's L-754142 (**15**)[65] did not live up to the initial expectations and the search for not yet patented structures retaining activity on the ET receptors was abandoned after a series of inactive molecules.

Hence, while we abandoned the three series that started from IRL-3630 (**14**), L-754142 (**15**) and the Roussel compound **16** relatively early, investigations in the series inspired by ambrisentan[66] and bosentan continued head to head. Already first novel derivatives of ambrisentan and bosentan showed interesting biological activities. On the one hand, rigidifying the structure of ambrisentan by introducing a ring between the methoxy group and one of the phenyl rings led to novel molecules that could be optimized readily for their affinity for the two ET receptors. In particular benzo[1,4]diazepin-2-one such as compound **17** (Fig. 10A) represented highly potent dual ERAs.

The series of pyrimidine derivatives that evolved from bosentan turned out to be the workhorse of our drug discovery program. As we explored the SAR of this series, we came across several surprises. Pyrimidine-based ERAs previously disclosed by Roche, Tanabe, Shionogi, Yamanouchi, and Kowa invariably contained a rather large substituent at the sulfonamide moiety suggesting that a bulky substituent is needed for high affinity.[60,67] However, as shown by the compounds in Table 1, even much smaller substituents (*e.g.* compounds **20**, **21**) in this part of the molecule led to potent ERAs.[52] In addition, replacing the alkyl chain in the sulfonamide by an alkylamino group to yield a sulfamide improved the compound's affinity for the two ET receptors, in particular ET_B (compare **22** with **20**, and compounds in Table 2). In fact, the affinity for the ET_B receptor increased with increasing chain

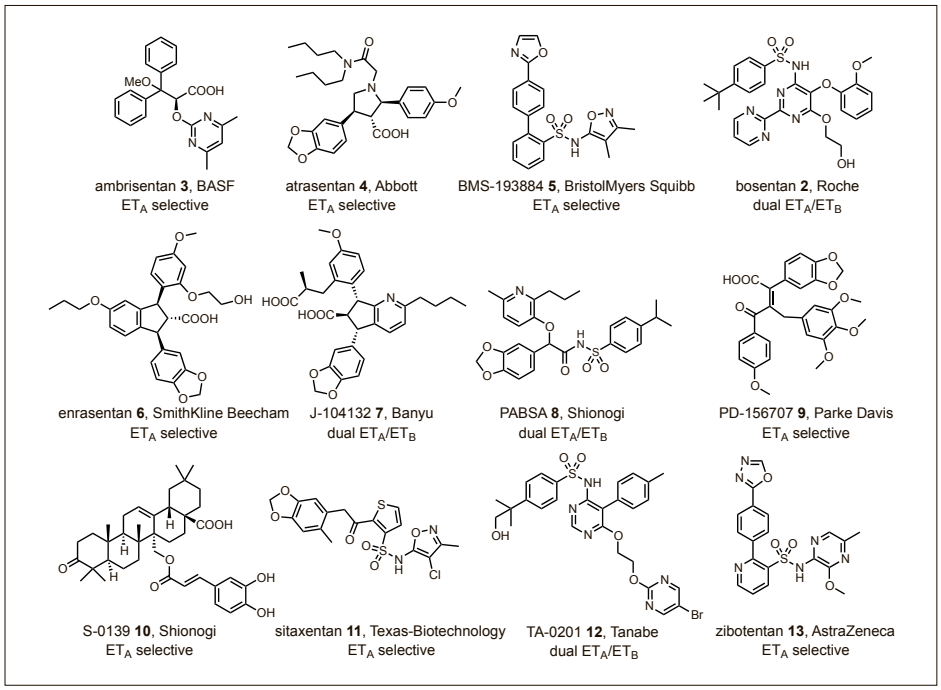


Fig. 7. Structures of some ERAs that entered clinical development up to 2001.

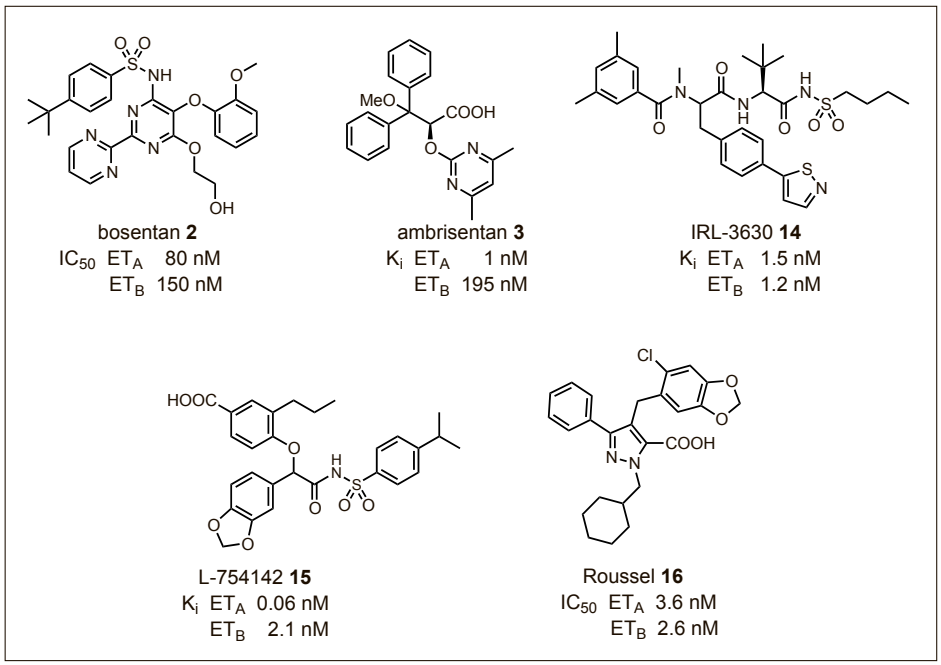


Fig. 8. The structures of the five starting points selected for our medicinal chemistry program.

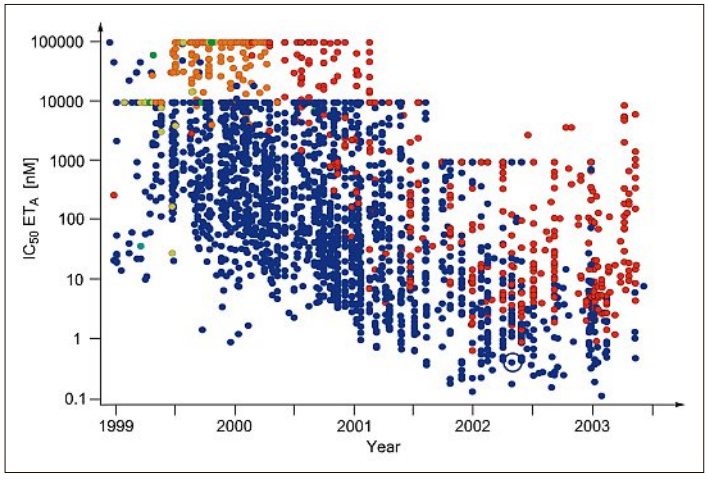


Fig. 9. Evolution of the ET_A affinity of the five compound series starting from: bosentan (**2**, blue), ambrisentan (**3**, red), IRL-3630 (**14**, yellow), L-754142 (**15**, green), and the Roussel compound (**16**, orange); macitentan (**1**, circled in dark blue).

length of the alkyl sulfamide moiety (**1**, **27** to **29**).These findings were not obvious or even remotely suggested in the literature. As most if not all previously described ERA contained an acidic functional group such as a carboxylic acid, an acyl or heteroaryl sulfonamide[60,67] we speculated that this acid plays an important role in the interaction with the receptor. One could therefore contemplate that the less acidic sulfamide (pK_a ~6) may lose affinity for the ET receptor when compared to the more acidic sulfonamide (pK_a ~5).[54] However, as illustrated by the examples in Table 1, this is not the case. More recent binding studies with receptor mutants confirmed that in contrast to the sulfonamides (*e.g.* bosentan) and carboxylic acids (ambrisentan), the sulfamides' affinity for the ET_A receptor does not depend on strong charge–charge interactions.[68]

As we obtained a growing number of novel pyrimidine and benzodiazepinone derivatives with high affinity for the ET receptors, we started to explore the functional potency of compounds that showed IC_{50} values below 20 nM and 400 nM on ET_A and ET_B in the binding assay, respectively. For both the pyrimidine as well as the benzodiazepinone series, the pA_2 values obtained in the isolated rat aorta and trachea constriction experiment correlated with the IC_{50} values of the ET_A and ET_B receptor binding, respectively, – albeit with significant scattering (Fig. 11; for IC_{50} ET_B vs pA_2 trachea see ref. [62]). These observations confirmed that the compound's ability to block agonist-induced vessel constriction not only depends on its affinity for the ET receptor but is also influenced by other factors. We speculated that compound lipophilicity, ability to penetrate tissue, unspecific tissue binding and receptor binding kinetics may be among these contributing factors but we were not able to unambiguously confirm these hypotheses. Nevertheless, the pA_2 values were a useful tool to further shortlist the most interesting compounds and we decided that compounds with a functional potency greater than the one of bosentan (pA_2 aorta >6.8) shall be characterized in the *in vivo* hypertension model.

The efficacy to reduce the mean arterial blood pressure of hypertensive Dahl salt-sensitive rats was assessed for about 70 benzodiazepinones and almost 300 pyrimidines. *In vivo* efficacy was assessed by calculating the area between the mean arterial pressure (MAP) recordings before and after oral compound administration. First experiments revealed that for the benzodiazepinones to show activity *in vivo* a dose of 30 mg/kg was needed, while for the pyrimidines 3 mg/kg was sufficient to obtain a significant pharmacological response. This 10-fold difference in required dose persisted throughout our discovery pro-

gram and clearly separated the pyrimidines from the benzodiazepinones (Fig. 12). Nevertheless, several representatives of the benzo[1,4]diazepin-2-one-derived ERAs showed efficacy in the hypertensive Dahl salt-sensitive rats that was superior to that of ambrisentan and bosentan at equal dose (*e.g.* Fig. 10).[52,69] Plotting the *in vivo* efficacy data (MAP ABC) of the pyrimidine and benzodiazepinone derivatives against the aorta pA_2 values revealed a clear trend for compounds with higher pA_2 to also show oral activity. Compounds reaching an ABC of >1000 mmHg × h typically had a pA_2 value of >7.5 on rat aorta rings, suggesting that a high pA_2 is necessary but not sufficient for a compound to show *in vivo* efficacy. This observation is not surprising as *in vivo* efficacy is of course not only driven by the compound's potency but also by its pharmacokinetic behaviour. However, rather than analysing the pharmacokinetic behaviour of our compounds in more detail, we tried to identify the structure–activity relationship driving *in vivo* efficacy. In the following, a few key observations are summarized. For instance, the nature of the substituent attached to the 5-position of the core pyrimidine significantly impacted the efficacy *in vivo*. The efficacy of the corresponding compounds often followed the order 2-methoxyphenoxy < 3-methoxyphenoxy < 3-methoxy-6-chlorophenoxy, 4-chlorophenyl, 4-bromophenyl (Table 3). In several series, the 4-bromophenyl moiety clearly delivered the most efficacious example (*e.g.* **30** to **34**, Table 4).[52] Another important observation is illustrated with the examples compiled in Table 2. In the sulfonamide (**23** to **26**) as well as in the sulfamide (**1**, **27** to **29**) series, the *in vivo* efficacy appeared to decrease with increasing chain length of the alkyl sulfonamide and sulfamide moiety. Together with the observation that the compounds' affinity for the ET_B receptor increased with increasing chain length (see **1**, **27** to **29**), the propyl chain represented the best compromise between high potency on ET_B and high efficacy *in vivo*. A last *in vivo* SAR element is illustrated with the nearly equipotent compounds listed in Table 5 incorporating different substituents in the 5-position of the lower pyrimidine. While the cyclopropyl (**37**) or a methoxy (**38**) derivative showed low *in vivo* efficacy at the tested dose, a methylthio (**39**) group or a bromine (**34**) atom yielded highly efficacious ERAs. For a handful of compounds, their ability to block the ET_B receptor *in vivo* was assessed by measuring plasma ET-1 levels after oral administration of the compound to Wistar rats (data not shown). These internal data provided a further selection criterion. Overall, our efforts to have a relatively easy access to reliable, relevant and translatable *in vivo* efficacy data facilitated the above SAR analyses that drove the discovery process.

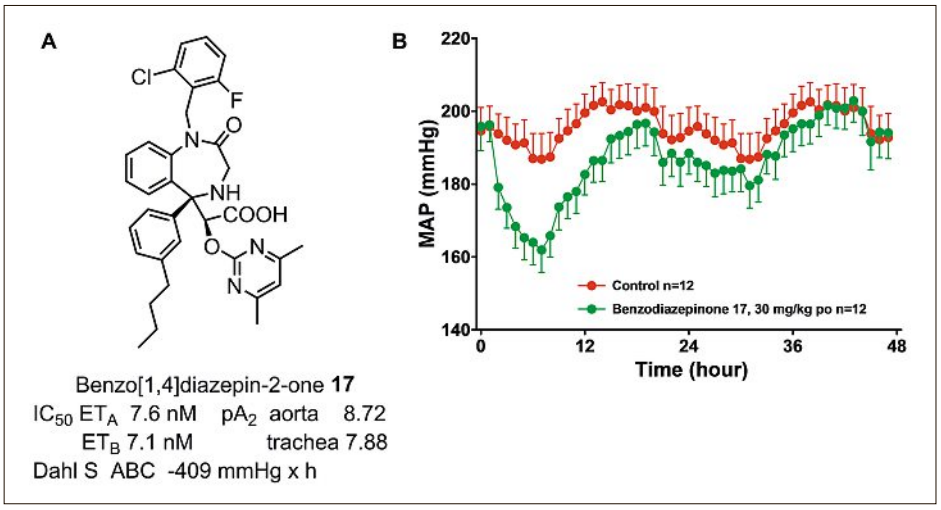
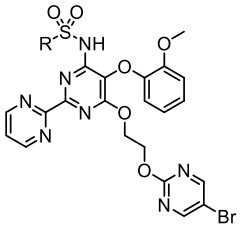


Fig. 10. A) Structure, *in vitro* and *in vivo* data of a highly potent and efficacious dual ERA derived from ambrisentan. B) Mean arterial blood pressure recordings 48 h before (control) and after administration of 30 mg/kg of benzo[1,4]diazepin-2-one (**17**) to hypertensive Dahl salt-sensitive rats. At the same dose, bosentan reached an ABC of –343 mmHg × h.

Table 1. Potent ERAs containing small substituents at the sulfonamide moiety.[52]

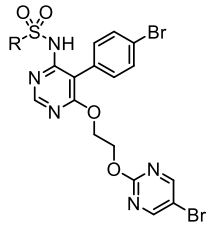


Compound	R	IC_{50} [nM]	
		ET_A	ET_B
18	4-*tert*-butyl phenyl	1.5	19.3
19	phenyl	10.4	341
20	ethyl	14	460
21	butyl	4.3	552
22	methyl-NH	7.9	194

Applying the criteria we set forth for compound advancement, about 30 compounds were found worthwhile to be tested in the plasma bile salt experiment. The results of these studies are shown in Fig. 13. None of the tested benzodiazepinones was inactive in this assay. However, while a few pyrimidine derivatives led to a plasma bile acid concentration increase similar to the one observed with bosentan, several representatives of this class caused no change in plasma bile salt levels. Unfortunately, the data set is too limited to derive a clear SAR. Mechanistic studies that were performed much later on the level of the hepatic transporters revealed that macitentan in fact inhibits BSEP with an IC_{50} value of 18 μM and is thus at least as potent as bosentan for which IC_{50} values of 25–77 μM were reported. However, while bosentan is significantly transported by OATP, accumulates in hepatocytes and thus becomes hepatotoxic, macitentan distributes mainly by passive diffusion, does not accumulate in the liver and therefore does not lead to cholestasis.[53]

Taking the data generated so far together, macitentan (**1**) stood out as the best molecule of the 2500 molecules prepared combining optimized *in vitro* and *ex vivo* potency on the two ET receptors, with high efficacy in the Dahl hypertension model and the absence of interference with hepatic bile salt transport *in vivo* (Fig. 14). Based on these data and a series of experiments that supported that the favourable efficacy and safety profile of macitentan in the rat would translate to humans, macitentan was selected for further development.

Table 2. *In vitro* potency and *in vivo* efficacy of sulfonamide and sulfamide derivatives.[52,67,82]



Compound	R	IC_{50} [nM]		ABC[a] [mmHg × h]
		ET_A	ET_B	
23	methyl	1.9	1310	–1639
24	ethyl	2.2	>1500	–1911
25	propyl	2.2	3060	–1116
26	butyl	1.1	2420	–734
27	methyl-NH-	1.7	812	–1045
28	ethyl-NH-	0.8	830	–598
1	propyl-NH-	0.5	390	–922
29	butyl-NH-	0.3	160	–443

[a]at 3 mg/kg p.o.

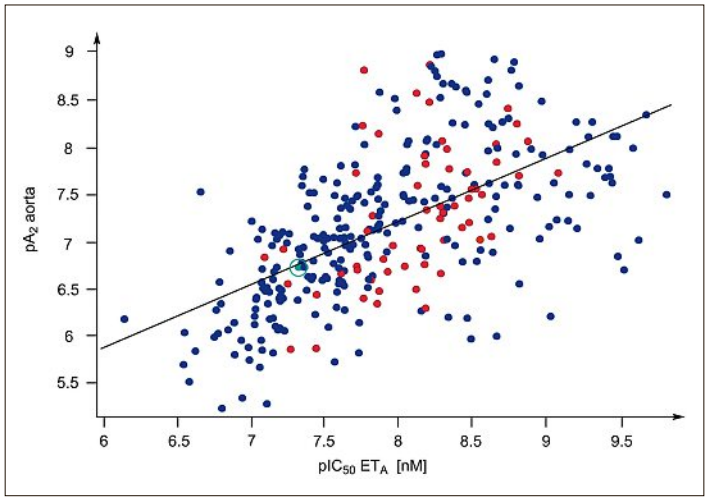


Fig. 11. pIC_{50} values determined in the ^{125}I-ET-1 competitive ET_A binding assay versus pA_2 values calculated from the isolated rat aorta constriction experiment; blue = pyrimidine series, light blue = bosentan; red = benzodiazepinone series.

Key Properties of Macitentan

Several additional attractive properties clearly differentiated macitentan from bosentan. Macitentan showed a reduced acidity (pK_a 6.2 vs 5.1) and increased lipophilicity ($logD_{7.4}$ 2.9 vs 1.3) when compared to bosentan. Detailed receptor binding studies revealed that macitentan has a significantly prolonged receptor occupancy half-life (17 min) when compared to bosentan (1 min). This increased receptor residence time was proposed to be the consequence of macitentan's increased lipophilicity and reduced acidity that made its interaction with the ET_A receptor less dependent on charge–charge interactions.[68,70] These altered physicochemical properties were also suggested to be responsible for macitentan's increased distribution into lung and right ventricle tissue in rats with bleomycin and monocrotalin induced pulmonary hypertension.[54,71] To investigate whether these differences in receptor binding kinetics and tissue distribution were pharmacologically meaningful, macitentan was administered on top of a maximally efficacious dose of bosentan to hypertensive Dahl salt-sensitive rats. In these animals the mean arterial blood pressure (MAP) was further reduced by the additional administration of macitentan. Similarly, macitentan was also able to further reduce the mean pulmonary artery pressure (MPAP) when given on top of a fully efficacious dose of bosentan to rats with bleomycin-induced pulmonary hypertension. This greater maximal efficacy was considered to be the consequence of macitentan's more potent and sustained ET receptor inhibition.[71,72]

Finally, in a pivotal long-term phase III clinical trial involving 742 PAH patients (SERAPHIN trial), macitentan at a dose of 10 mg q.d. significantly reduced the risk for the composite endpoint of morbidity/mortality by 45%. There was no sign for liver enzyme increases (ALT and AST) in patients receiving macitentan.[73] These results formed the basis for macitentan's approval for the longterm treatment of PAH by health authorities around the globe.

In vivo-based Compound Profiling Cascade – A Useful Concept?

Realizing that integrating a rapid *in vivo* screening step into the compound profiling cascade expedited the advancement of our medicinal chemistry program, we envisaged applying this approach to other medicinal chemistry projects. Indeed, our sphingosine-1-phosphate receptor 1 ($S1P_1$) agonist program offered such an opportunity. $S1P_1$ receptor agonists have been shown to sequester circulating lymphocytes to lymphoid organs and thus prevent autoreactive lymphocytes from doing harm to the tissue.[74–76] The blood lymphocyte count (LC) can be measured rapidly and reliably and therefore serves as a valuable marker for *in vivo* activity of an $S1P_1$ agonist. On this basis, we decided to include LC measurements as a key characterisation step in the compound profiling cascade. Similar to the endothelin project, the rapid availability and high reliability and relevance of LC data enabled us to build up the SAR linking structure, *in vitro* potency and *in vivo* activity that led to the discovery of ponesimod and cenerimod, two $S1P_1$ receptor agonists in clinical development. Fig. 15 illustrates the different behaviour of two subclasses of potent $S1P_1$ receptor agonists in the LC experiment. While in one subclass a particular moiety was bound to a thiophene *via* an oxadiazole, in the second class the two fragments were connected *via* an ethyl ketone linker. At 3 h post dosing, both subclasses show a similar behaviour. Compounds with EC_{50} of <20 nM usually show maximal LC reduction (LC<-60%) confirming a rapid onset of action. At 24 h, however, the two subclasses can clearly be differentiated. While the thiophene ketones lose activity (LC>-60%), the oxadiazole derivatives still show sustained LC reduction (LC <-70%). Some of the oxadiazoles even gained activity at 24 h.[77,78] A similar analysis revealed clear differences between potent $S1P_1$ agonists incorporating either a 2- or a 4-pyridine substituted

oxadiazole. For both series compounds with an EC_{50} value <20 nM efficiently reduce the LC at 3 h post dosing. At 24 h, the 2-pyridines clearly lose activity while most of the 4-pyridines retain full efficacy.[79–81]

Conclusions

We embarked on a medicinal chemistry program to identify a novel potent dual and orally active ERA starting from the structures of known ERAs and taking into account our knowledge acquired in prior years of research on the chemistry and pathophysiology of the ET system. To improve on our benchmark bosentan, we decided to set up a compound profiling cascade that emphasized on the use of reliable, relevant and translatable *ex vivo* and *in vivo* experimental models. Hence, the functional potency of our compounds was measured in isolated rat tissue while efficacy and liver safety were assessed in a rat model of hypertension and a model of hepatic bile salt transport interference, respectively. The corresponding efficacy and safety experiments certainly deliver composite readouts combining a multitude of parameters such as compound dissolution, absorption, potency on the target, binding kinetics, plasma protein binding, tissue distribution, metabolism, excretion, *etc.* While all these parameters can be studied individually, their importance and, in particular, their interplay is often difficult to assess. In this regard *in vivo* studies may offer a clear advantage as all these factors are incorporated in the study result. In our case, the ability to rapidly generate a large body of reliable and relevant *in vivo* data enabled us to establish SARs on *in vivo* efficacy and to monitor safety and only a limited number of experiments were needed to ensure that the data acquired in animals will positively translate to humans. Interestingly, a holistic approach emphasizing on *in vivo* data and an approach relying more on dissecting mechanistic studies may not necessarily lead to the same conclusions and decisions. This is illustrated with the result of the *in vivo* bile salt experiment. As outlined above, macitentan did not affect plasma bile salt levels upon i.v. injection in Wistar rats – a result that significantly contributed to its selection for further development. At this point the question whether we would have selected macitentan if we had based our selection process on the *in vitro* BSEP inhibition data collected later remains an open one.

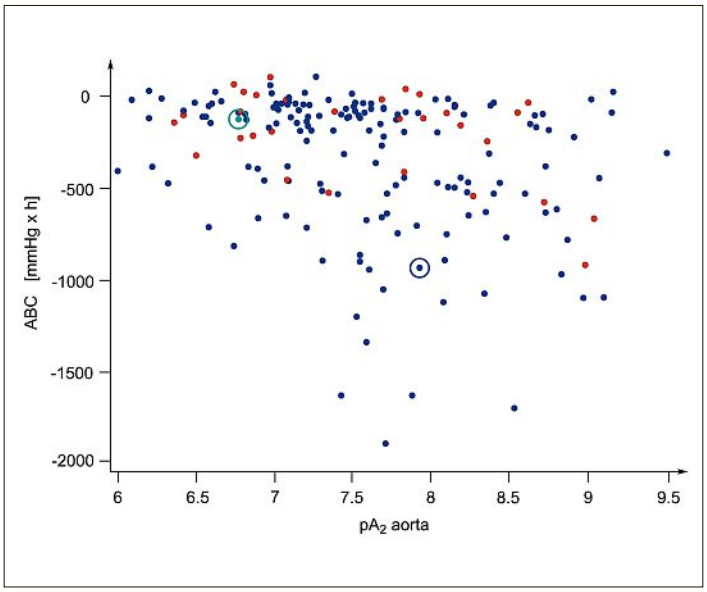


Fig. 12. ABC as calculated between the mean arterial blood pressure curves recorded before (control) and after administration of 3 mg/kg of pyrimidine (blue) or 30 mg/kg of benzodiazepinone (red) derivatives to hypertensive Dahl salt-sensitive rats *versus* pA_2 values obtained from the isolated rat aorta constriction experiment; bosentan (3 mg/kg, light blue circle, ABC = –88 mmHg × h), macitentan (blue circle, ABC = –922 mmHg × h).

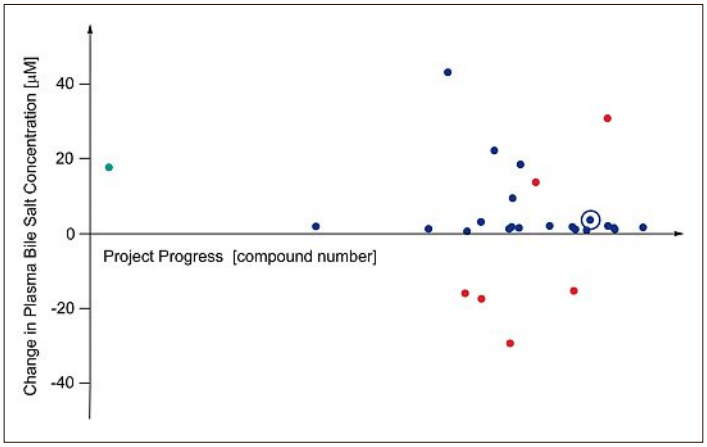
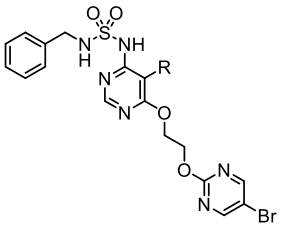


Fig. 13. Changes in plasma bile salt levels as measured 10 min after i.v. administration of 25 mg/kg of the compound to Wistar rats; pyrimidine series (blue), macitentan (in blue circle), bosentan (light blue), benzodiazepinones (red).

Table 3. *In vivo* SAR of the 5-substituent attached to the core pyrimidine.[52]



Compound	R	IC_{50} [nM]		pA_2		ABC[a] [mmHg × h]
		ET_A	ET_B	aorta	trachea	
30	2-methoxyphenoxy	6.9	530	n.d.	n.d.	–100
31	3-methoxyphenoxy	1.0	460	n.d.	n.d.	–245
32	3-methoxy-6-chloro-phenoxy	1.4	710	7.69	5.84	–639
33	4-chlorophenyl	1.4	510	8.80	5.63	–593
34	4-bromophenyl	1.6	460	8.44	n.d.	–445

[a]at 3 mg/kg p.o.

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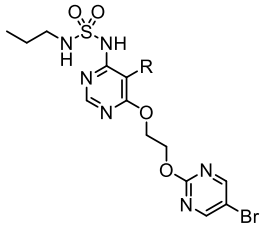
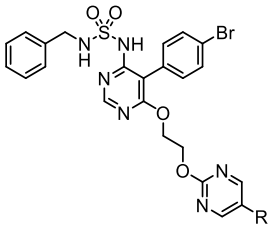
Table 4. *In vivo* SAR of the 5-substituent attached to the core pyrimidine.[52]



Compound	R	IC_{50} [nM]		pA_2		ABC[a] [mmHg × h]
		ET_A	ET_B	aorta	trachea	
35	3-methoxyphenoxy	1.9	468	n.d.	n.d.	–391
36	6-chloro-3-methoxyphenoxy	0.4	208	8.23	6.42	–500
1	4-bromophenyl	0.5	391	7.93	6.82	–922

[a]at 3 mg/kg p.o.

Table 5. *In vivo* SAR of the 5-substituent attached to the lower pyrimidine.[62]



Compound	R	IC_{50} [nM]		pA_2		ABC[a] [mmHg × h]
		ET_A	ET_B	aorta	trachea	
37	cyclopropyl	0.9	280	8.03	n.d.	–23
38	OCH_3	1.7	260	n.d.	n.d.	–85
39	SCH_3	0.4	60	8.40	6.76	–506
34	Br	1.6	460	8.44	n.d.	–445

[a]at 3 mg/kg p.o.

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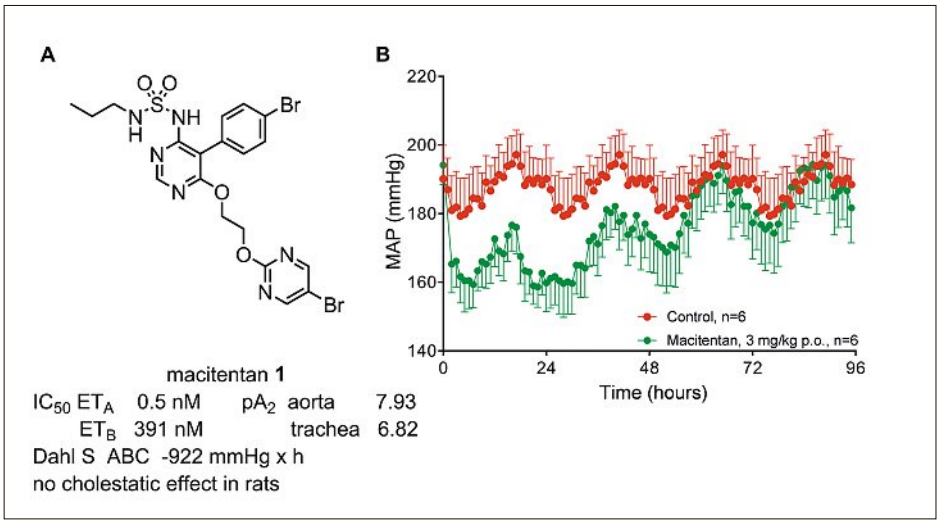

Fig. 14. A) Structure, *in vitro* and *in vivo* data of macitentan (**1**). B) MAP recordings 96 h before (control) and after administration of 3 mg/kg of macitentan to hypertensive Dahl salt-sensitive rats. ABC = area between the curve before and after compound administration.

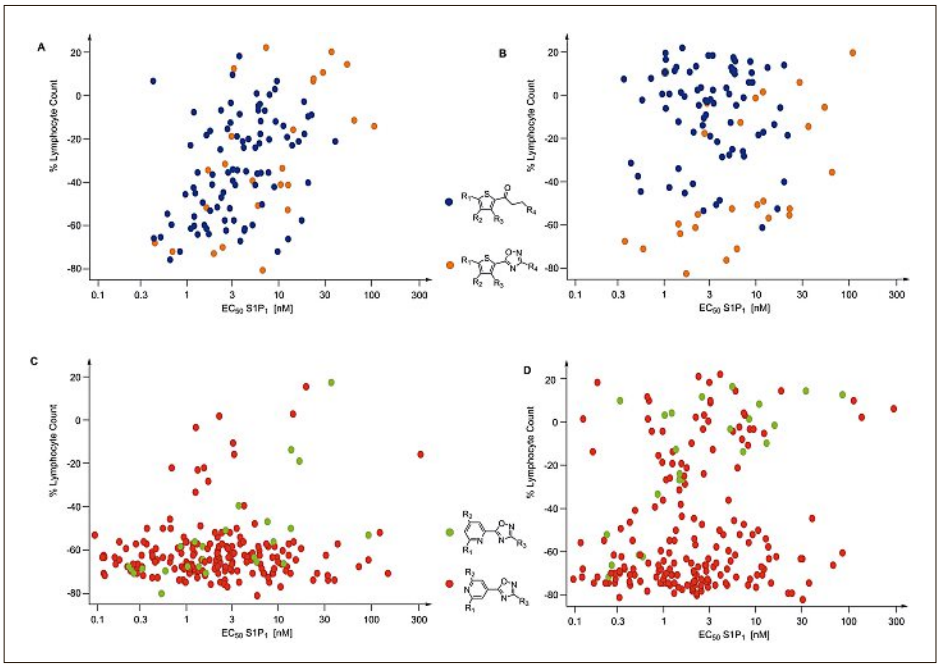

Fig. 15. Plotting $S1P_1$ GTPγS EC_{50} [nM] values against blood LC reduction [% of baseline] in Wistar rats 3 h (A, C) and 24 h (B, D) after receiving 10 mg/kg p.o.; dark blue = thiophene ketones, orange = thiophene oxadiazoles, light green = 2-pyridyl-oxadiazoles, red = 4-pyridyl-oxadiazoles.

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