Paper

Hypertension study in anaesthetized rabbits: protocol proposal for AT_1 antagonists screening

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Abstract

Introduction: The aim of this study was to establish an optimized fast and safe protocol for the pharmacological screening of AT_1 antagonists.

Materials and methods: The pharmaceutical prototype AT_1 antagonist losartan, its active metabolite EXP3174 and the synthetic compound MM1 were analysed in order to validate the protocol. Ang II was continuously infused while the animals received the drugs in two procedures.

Results: In the *post*-treatment procedure drugs were administered either in a single bolus dose or in a sequential manner. When losartan was administered in a single bolus dose, efficacy was evident until the 7th min (p=0.012) whilst EXP3174 infusion extended the efficiency up to the end of the study (p=0.006). In addition, the sequential injections of losartan prolonged the inhibitory time interval until the end of the study (p=0.045). In the *pre*-treatment procedure, results suggested a dose-dependent inhibitory effect for both antagonists. The pressor response to Ang II was unchanged after MM1 administration either in the post- or in the pre-treatment mode.

Conclusions: The proposed protocol appears to be safe, simple and fast for the pharmacological screening of AT_1 antagonists and enables the evaluation of new antagonists using lower doses than any other reported in the literature.

Introduction

The renin–angiotensin–aldosterone system (RAAS) plays an important role in the regulation of blood pressure. Recognized actions of this hormonal system contributing to blood pressure increase include peripheral vasoconstriction, sodium retention, and stimulation of fluid intake.[1,2] The peptide angiotensin II (Ang II), formed within the RAAS, is a vasoconstrictive hormone which leads to blood pressure increase even if it is administered exogenously.[3]

Previous studies have shown that blood pressure elevation after exogenous Ang II administration is dose dependent after either a single bolus or a continuous infusion.[4,5] However, termination of the Ang II infusion results in a return of mean arterial pressure to the normal levels within 10–20 seconds, an effect that is consistent with its half-life.[6]

The design of non-peptide AT_1 antagonists has been pursued for the past 20 years. The first available AT_1 antagonist approved in 1995 was losartan, an orally active drug with a selective and competitive profile.[7-9] The effectiveness of losartan has been studied in various animal models using different protocols.[10,11] Losartan inhibits the release of aldosterone[12] and abolishes the vasoconstriction caused by Ang II; thus, it results in blood pressure decrease.[13-15] When absorbed, losartan is metabolized in the liver to the active metabolite EXP3174. The metabolism of losartan has been studied both in humans and animals.[16,17]

A number of other AT_1 antagonists, such as irbesartan, telmisartan, candesartan, valsartan, and olmesartan have been approved for the treatment of arterial hypertension during the last 7 years. After the development of a novel receptor antagonist, identification of the in vitro affinity of the antagonist to its receptor is mandatory. Unfortunately, the in vitro results are not always correlated with the in vivo pharmacologic potency of a tested receptor antagonist. Although a high affinity of a drug to its receptor is very important, it is not the only requirement needed for a production of a potent antagonist in vivo. The discrepancies between in vitro and in vivo studies can be attributed to both pharmacokinetic and pharmacodynamic differences.

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Animal models have been widely used in order to study the pathogenesis of hypertension and in the discovery of new therapeutic procedures.[18,19] An ideal animal model should share similar cardiovascular anatomy and physiology with humans, resulting in the development of hypertension. Several protocols in different species such as rats, rabbits, and dogs have been used extensively in the past for the determination of blood pressure lowering activity.[20-23] Although Ang II has been infused both in conscious and anaesthetized animals in numerous studies,[24,25] anaesthetized rabbits were used only in a few hypertensive studies while the antihypertensive effect of drugs that precede or follow the Ang II infusion has received relatively little documentation.[26,27]

It is well known that a fast and safe protocol for the initial screening of the developed antihypertensive compounds is of importance. The aim of this research is to establish a quick optimized protocol for the pharmacological screening of AT_1 antagonists. We tested a quick, low stress, and painless protocol in an acute experimental setting, using low doses of the compounds under investigation as availability can be limited in early drug development. In our study, continuous infusion of Ang II in anaesthetized rabbits resulted in hypertension. In order to demonstrate the appropriateness and safety of the proposed protocol, we initially validated our protocol regarding both the effects of continuous Ang II infusion and the efficacy of the tested molecules compared with control. Finally, we tested two modes of compound administration, one post- and one pretreatment with Ang II, in order to achieve optimal and safe screening of AT_1 antagonists.

Methods

Seven groups (0, I, II_A, II_B, III_A, III_B and IV) of normotensive adult male, New Zealand white rabbits were used weighing from 2.5 to 3.3 kg each. Groups 0 and I served as the vehicle groups, while Groups II and III served as the main experimental groups. Four to five rabbits were used for each experiment. All procedures were approved by the Institutional Committee Governing Animal Care. Each rabbit was anaesthetized by sodium thiopentone injection (30 mg/kg, Pentothal, Abbott). The anaesthetic was slowly injected into an ear vein. A tracheotomy was performed and mechanical ventilation was applied using a positive pressure respirator for small animals, at a rate of 35 respirations/min, in order to keep blood gases within the normal range. Two polyethylene catheters were inserted, one in the carotid artery for continuous blood pressure monitoring via a transducer attached to a multichannel recorder (Nihon-Kohden, Model 6000, Japan) and the other in the jugular vein for the intravenous drug administration. After the surgical preparation, no action was performed for 3-4 min in order to stabilize the animals' blood pressure. During the subsequent experimental procedure no more anaesthetic was given to the animals.

Group 0: blind

In the blind experiment, four animals were infused continuously with dextrose 5% through a syringe pump. The rate of the infusion was 0.2 ml/min while each experiment lasted 20 min.

Group I: control

Ang II was dissolved in dextrose 5% at a final concentration of 5 μg/ml. An infusion rate of 0.2 ml/min (1 μg/min) was used in order to induce substantial vasoconstriction and blood pressure increase. Ang II was infused continuously for a 20-min period via a syringe pump (Harvard Apparatus Pump 22, Harvard Apparatus, Natick, MA, USA). Blood pressure measurements were recorded both during the infusion period and the first 3 minutes of the recovery period when Ang II infusion was stopped.

Group II (A&B): effects of losartan and EXP3174 administration during the Ang II infusion

Animals in this group were subjected to continuous Ang II infusion as already described for group I. In group $\mathbf{II_A}$, a single bolus dose of 0.2 mg/kg of either losartan $\mathbf{(II_{AL})}$ or EXP3174 $\mathbf{(II_{AE})}$ was injected in the 3rd minute of the study, while in group $\mathbf{II_B}$, losartan ($\mathbf{II_{BL}}$) or EXP3174 ($\mathbf{II_{BE}}$) were given in a sequential manner of 0.2, 0.4, and 0.4 mg/kg in the 3rd, 7th, and 12th min of the study, respectively. Losartan was dissolved in 0.9% NaCl solution and EXP3174 was dissolved in a 5% $NaHCO_3$/dextrose (50:50) solution. EXP3174 was provided by Merck Pharmaceutical Company.

Group III (A&B): study of losartan and EXP3174 pre-treatment effects

In Group III_A, animals were pre-treated with a single intravenous bolus dose of 0.2 mg/kg losartan $\mathbf{(III_{AL})}$ or EXP3174 $\mathbf{(III_{AE})}$. Ang II infusion started after a 4 min pre-treatment interval and lasted for a time period of 20 min. In order to study the pre-treatment dose dependence, the animals of Group III_B were given a single intravenous bolus dose of 0.4mg/kg losartan $\mathbf{(III_{BL})}$ or EXP3174 $\mathbf{(III_{BE})}$ while the rest of the experimental procedure was kept identical.

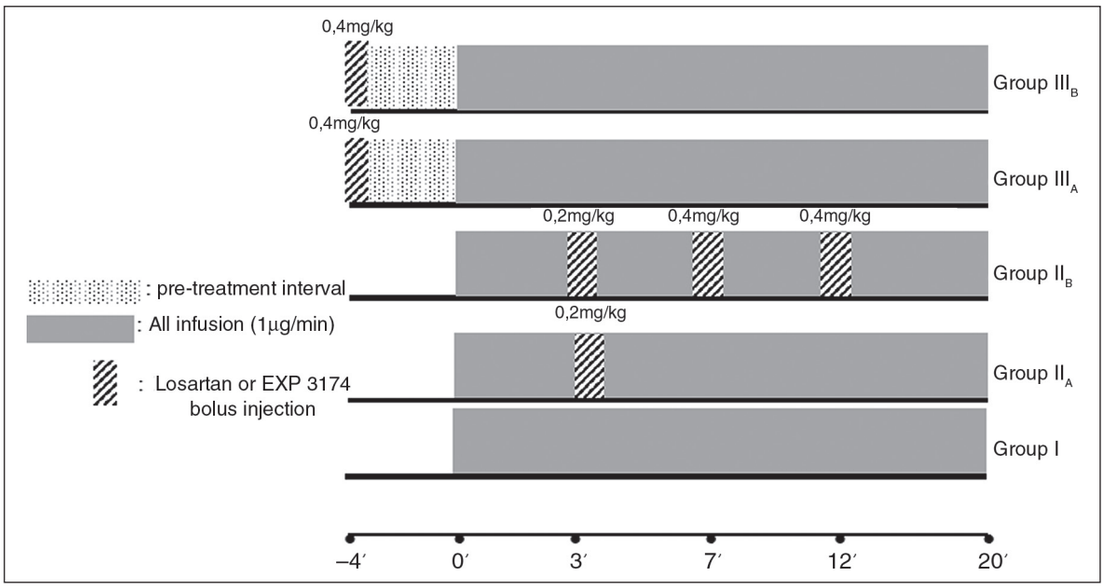

Figure 1
Schematic representation of the experimental groups showing infusion sequences and time duration of the experiment.

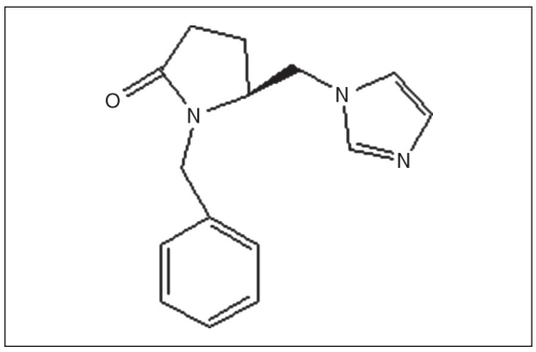

Figure 2
Chemical structure of the compound MMI.

The procedure that was followed in each experiment is presented in Figure 1.

Group IV: study of synthetic compound effects

In this group a synthetic compound, *(5S)*-5-(1*H*-imidazol-1-ylmethyl)-1-(phenylmethyl)-2-pyrrolidinone (MM1, see Figure 2), was used in order to validate the proposed protocol.[28] All of the procedures followed in the losartan and EXP3174 including post- and pre-treatment were also applied to the synthetic compound.

Statistical analysis

Blood pressure measurements were presented as mean arterial pressure, MAP (mmHg). All variables were expressed as mean ± standard deviation (SD). Student's paired *t*-test was used to compare MAP values and differences between different time intervals of the study. One-factor analysis of variance (ANOVA) was performed to determine the statistical significance of the variation of the MAP values between the control group and groups II, III, and IV (study groups) at time intervals t = 7, 12, and 20 min. A p-value less than 0.05, which rejects the null hypothesis, was considered as the level of statistical significance, indicating the efficacy of the administered drug. Statistical analysis was performed using SPSS 13 version (SPSS Inc., Chicago, IL, USA).

Results

The recorded MAP ± SD levels for all the experiments are presented in Table 1 and the associated graphs are depicted in Figures 3 and 4.

Group 0

The aim of this sub-experiment was to study any possible effect of the anaesthetic drug on arterial blood pressure. Pentothal was used as the anaesthetic for surgical preparation and no further administration was performed during the recording period in order to not interfere with the results. No significant difference was found regarding MAP levels after pentothal administration compared with baseline levels (p=0.42)

Group I

Group I actually served as a control group for all of the other study groups. Continuous infusion with 1 μg/min Ang II induced significant vasoconstriction during the study intervals. The Ang II infusion

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Table 1
Mean arterial pressure levels recordings in all groups both in post-treatment and pre-treatment experimental procedures.

	MAP ± SD (mmHg)							
Time / Group	***t* = –4**	***t* = –2**	***t* = 0**	***t* = 3**	***t* = 7**	***t* = 12**	***t* =20**	**Ang II infusion termination**
				Vehicle Groups				
Blind			123±7	122±7	120±9	115±12	118±14	
Control			100±9	172±6	165±6	154±7	145±13	100±25
				Post-treatment Groups				
Group II_{AL}			112±8	174±21	127±20	128±20	128±15	98±9
Group II_{AE}			109±15	182±31	97±17	99±13	104±4	87±2
Group II_{BL}			88±32	175±12	145±8	130±6	114±19	88±25
Group II_{BE}			105±22	171±15	81±18	74±13	81±13	81±12
Group IV_{A}			135±19	187±18	175±18	160±5	157±4	116±13
Group IV_{B}			103±29	162±19	149±13	141±16	134±13	103±18
				Pre-treatment Groups				
Group III_{AL}	117±8	114±8	108±9	173±9	150±5	140±16	128±11	98±10
Group III_{AE}	124±32	119±30	117±30	151±9	142±12	138±13	137±12	109±20
Group III_{BL}	120±9	117±11	107±12	132±18	135±10	137±10	135±9	102±15
Group III_{BE}	101±34	100±35	94±28	114±25	116±11	106±26	112±18	92±18
Group IV_{A}	116±9	118±9	113±9	183±10	167±11	152±9	144±8	103±13
Group IV_{B}	129±12	132±7	125±5	193±5	167±10	148±12	144±16	102±5

MAP, mean arterial pressure; SD, standard deviation.

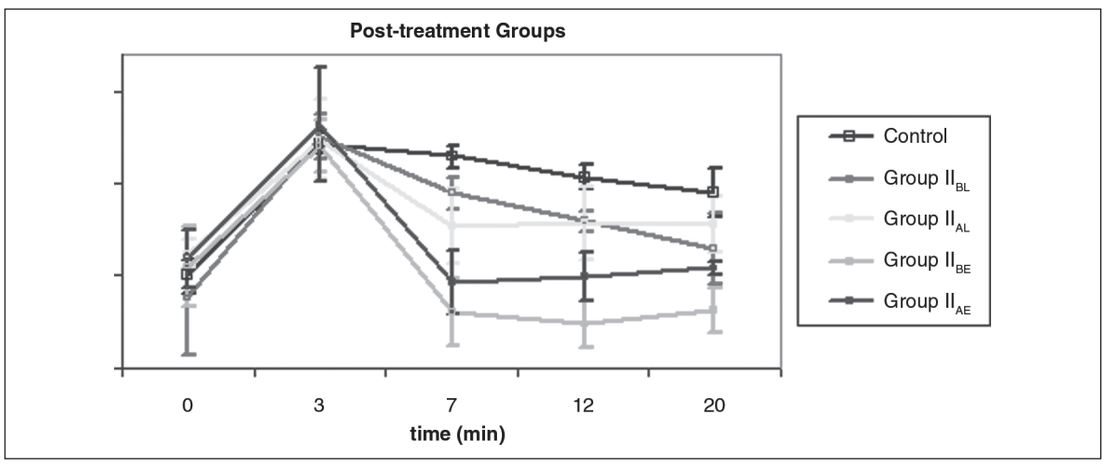

Figure 3
A time course of mean arterial pressure measurements (MAP)±SD due to continuous intravenous infusion of Ang II in control and post-treatment groups.

produced initially a progressive increase of blood pressure which reached its highest value at a time period of 3 min and was sustained until the 12th minute of the study. Loss of responsiveness to Ang II was observed after the 12th minute of the study since a significant decrease in MAP levels was noted between time periods t = 3 and 20 min. The decrease in MAP could possibly be attributed to the desensitization to Ang II infusion which became pronounced after the 12th minute of continuing infusion of Ang II.

Group II

The antihypertensive efficacy of losartan and EXP3174 in both groups, II_A and II_B, was assessed by measuring MAP at specific time intervals over 20 min.

Effects of post-treatment with losartan

The single bolus dose of 0.2 mg/kg losartan administered at the 3rd minute of the study markedly attenuated the pressor response to Ang II. The maximum inhibitory effect was evident 4–5 min

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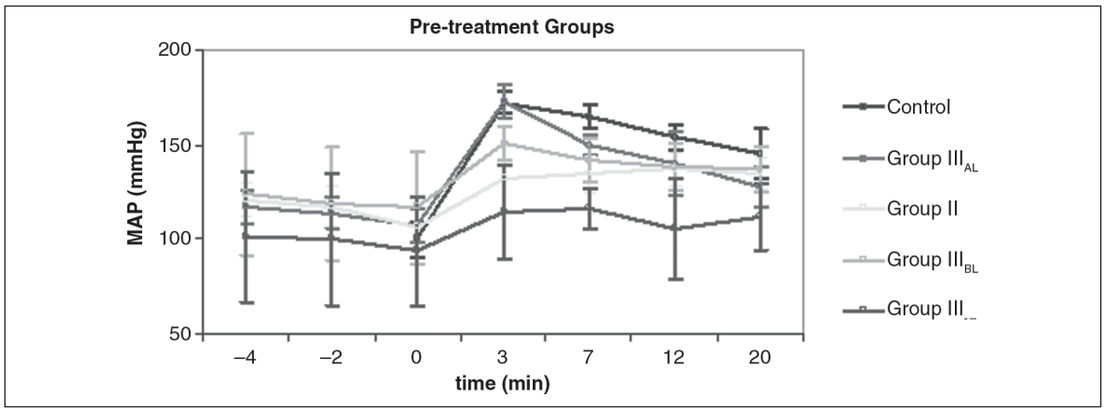

Figure 4
A time course of mean arterial pressure measurements (MAP)±SD due to continuous intravenous infusion of Ang II in control and pre-treatment groups.

after the injection (p=0.01, t = 7 min, Group II$_{AL}$, Figure 3). The efficiency of the drug was evident until the 7th minute of the study, as the statistical analysis shows (p=0.012). From the 7th to the 20th minute, no further decrease in MAP levels was seen. Seeking to prolong the inhibitory time interval of the drug, two more doses of losartan were injected at the 7th and 12th minute. The dose for the second and third bolus was 0.4 mg/kg each, as described previously.[28,29] However, as expected, sequential intravenous injections of losartan showed significant decrease in MAP levels during the whole study period compared with baseline levels and single-dose losartan administration (p=0.045, Group II$_{BL}$, Figure 3).

Effects of post-treatment with EXP3174

Compared with the control group, single 0.2 mg/kg EXP3174 administration inhibited the pressor response to Ang II. Specifically, normal MAP levels were evident 4–5 min after the injection and the inhibitory effect remained significant until the end of the experiment (p=0.06, Group II$_{AE,}$ Figure 3). When intravenous injections of EXP3174 were given at a sequence of 0.2, 0.4, and 0.4 mg/kg, the MAP lowered to normal levels from the initial dose of 0.2 mg/kg and remained within normal values until the end of the study, as it was expected (p=0.02, Group II$_{BE,}$ Figure 3). Although EXP3174, like losartan, presented a maximal inhibitory effect 4–5 min after the injection, it showed a significant decrease in MAP which was sustained until the end of the study (t=20), regardless of the drug protocol (single versus three doses).

Group III

Pre-treatment with losartan and EXP3174 (0.2 mg/kg for Group III$_A$ or 0.4 mg/kg for Group III$_B$) had no influence on baseline MAP levels during the pre-treatment period [t = –4 to 0 min, losartan 0.2 mg/kg (p=0.18), losartan 0.4 mg/kg (p=0.10), EXP3174 0.2mg/kg (p=0.75) and EXP3174 0.4 mg/kg (p=0.33)].

Effects of pre-treatment with losartan

Pre-treatment with losartan (0.2 mg/kg) does not prevent MAP from reaching its maximal value following infusion of Ang II (t=0) compared with the control (p=0.016, t=3, Group III$_{AL}$, Figure 4). The inhibitory efficiency of the drug is evident during the 7th minute after Ang II infusion. The profile of losartan efficiency parallels that of the post-treatment procedure. However, duplication of the pre-treatment dose of losartan results in a different profile. The vasoconstrictor response to Ang II is significantly reduced as shown in Figure 4. Use of a higher dose of losartan causes higher inhibitory effect, and even a longer duration of action (until the 12th minute of the study) as it is depicted by its more pronounced MAP lowering. This indicates that losartan causes a dose-dependent inhibitory effect. From the 12th until the 20th minute of the study, the efficacy of the drug did not increase.

Effects of pre-treatment with EXP3174

Pre-treatment with EXP3174 highlights the greater potency of the active metabolite of losartan. The administration of 0.2 mg/kg induced a more effective blockade of the receptor, showing a greater attenuation of maximal pressor response to Ang II compared with the same dose of losartan (Figure 4). In contrast, the duration of action of 0.2 mg/kg EXP3174 was the same as that of losartan, since significant MAP variations between Group III$_{AE}$ and control group were observed until the 7th minute (p=0.01). When the pre-treatment dose was doubled, EXP3174 showed inhibition of the

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pressor response of Ang II in a dose-dependent manner as mentioned with losartan. Although pre-treatment with 0.4 mg/kg EXP3174 showed similar blockade as 0.4 mg/kg losartan, the duration of action was increased for EXP3174 since it was sustained during the whole study period.

Validation study

The pressor response of Ang II on MAP was unchanged after MM1 administration either in the post- or in the pre-treatment mode of Ang II infusion as the statistical analysis has shown. Our results confirm the success of the protocol in screening novel synthetic molecules for being potential drugs since it is known that MM1 is a compound with a very low affinity at AT_1 receptors in vitro.[29]

Discussion

The main findings of the present study underline for the first time that continuous infusion of Ang II at a dose of 1 μg/min for 20 minutes in anaesthetized rabbits represents a rapid and safe pharmacological screening test for AT_1 antagonists.

All of the animals were anaesthetized in order to avoid any interference of stress on the results. No additional anaesthetic was given during MAP recordings and therefore a potentially undesirable drug interaction was avoided. For general anaesthesia a 20-minutes interval is safe and devoid of any stress reactions.

In the first step of the study, we tested the effects of the continuous Ang II infusion at a dose of 1 μg/min. Previous studies have reported that there is a hypertensive response to Ang II infusion at a higher concentration than 0.5 nmol/kg whilst the elevation of MAP with lower doses of Ang II is easily separated from the depressor response that usually follows.[30] Gavras and colleagues suggest that Ang II infusion in rabbits at a dose of 1.4 μg/kg/min, leads to Ang II plasma levels that are slightly higher than those observed in humans with malignant hypertension.[31] Based on previous studies,[32] we chose the continuous infusion of Ang II at a dose of 1 μg/min in order to induce substantial elevation in MAP and then to evaluate the effectiveness of the drugs under examination on the blood pressure response. The infusion rate in our study increased the MAP from 100±9 mmHg to 172±6 mmHg. This was the maximal response that was obtained 3 minutes after the start of infusion. A stable interval lasting until the 12th minute was followed and then loss of response to Ang II was recorded. The rapid desensitization to Ang II infusion is a known effect but the exact time interval depends on the concentration of infused Ang II and the animal model. Nielsen and colleagues have reported that the continuous infusion of Ang II in conscious rabbits at an identical concentration to that we used, caused a blood pressure elevation of 70 mmHg.[33] This effect was transient and the blood pressure returned to baseline values 1–2 hours after the initiation of Ang II infusion. Those findings confirm that a 20-minute study should be ideal in order to obtain secure results regarding the effectiveness of studied compounds.

Next in our study, we compared the efficacy of the drugs under investigation regarding blood pressure decrease using two methods of intervention, one post-treatment and one pre-treatment. This comparison provides additional information regarding the treatment regime that may be beneficial in terms of duration of action and efficacy of the drug as well as the appropriate concentration. Post-treatment experiments were carried out using either a single or three sequential injections of the drug molecules. The first dose was 0.2 mg/kg, and it was chosen based on previous in vivo studies in which it was well tolerated.[34] A comparison between single and sequential dosage revealed that there was a drug-dependent correlation. Using the single-dose protocol with losartan, a significant lowering of the blood pressure was maintained up to the 7th minute. MAP levels did not significantly differ after the 12th min compared with the control, probably due to the acute desensitization to Ang II. Although a further decrease in mean blood pressure was expected in the sequential protocol, this was not observed and the reason is not clear. However, it is possible that the study period was not suitable for the metabolism of the entire drug amount. The above results suggest that the sequential manner of administration is not an appropriate methodology for a dose-dependent evaluation of the study compound.

EXP3174 decreased the MAP to the normal levels even from the first chosen dose of 0.2 mg/kg. In the single-dose protocol, the duration of EXP3174 action was sustained from the 7th minute of the infusion up to the end of the study. The same pattern was observed when three sequential doses were used and normal MAP was observed from the first dose with no additional change after the second and third injection. These findings suggest that the use of a single-dose protocol is sufficient to provide a safe intervention with the molecule under investigation in the

post-treatment arm of the study. The maximal inhibitory effect of both molecules appeared after the 5th minute of the study, a time point where there is no evidence of desensitization to Ang II. In many previous reports the maximal inhibitory effect of losartan or EXP3174 was evident at 5 min post-injection regardless the dose, the experimental procedure or the animal model which was used.[13,17] This may suggest that the 20-minute study period of our protocol is a safe interval for the evaluation of a novel AT_1 antagonist.

The final series of experiments includes the study of a pre-treatment methodology. The infusion of either losartan or EXP3174 at each of the administered doses of 0.2 or 0.4 mg/kg had no effect, indicating that the activated RAAS system is significant for the expression of the activity. This was not unexpected as a large volume of evidence from both in vivo experimental studies and clinical studies in humans resulted in the conclusion that an AT_1 antagonist requires an activated RAAS in order to demonstrate its blood pressure lowering effect in normotensive subjects.[13,19] This may be attributed to AT_1 receptor activation through Ang II binding which can induce conformational change of the receptor.

In the pre-treatment methodology, both losartan and its metabolite EXP3174 inhibited the response to Ang II infusion. However, they demonstrated a different profile of AT_1 receptor blockade even if they were administered in the same dose. When a low dose (0.2 mg/kg) was used, the duration of the action for both drugs was similar but the maximum MAP response was different. EXP3174 induced a remarkable lowering of the maximum blood pressure, after Ang II administration, while losartan treatment did not cause a similar effect. By equalizing the dose of losartan and EXP3174 to 0.4 mg/kg, the duration of action was longer, an observation which is consistent with previous reports.[35] As was expected 0.4 mg/kg of losartan compared with 0.2 mg/kg resulted in further reduction of the maximum blood pressure response to Ang II infusion.

Pre-treatment with an insurmountable antagonist may actually prevent Ang II binding to the active site resulting in lowering of the maximum blood pressure response. However, both losartan and EXP3174 reduced the maximum blood pressure response. Nevertheless our results may indicate that the competitive or non-competitive nature of an AT_1 antagonist is possibly related to the concentration of the drug at least in the pre-treatment procedure.

Both post- and pre-treatment procedures can be used as fast screening methods for AT_1 antagonists. Our protocol enables the evaluation of AT_1 antagonists using lower doses (0.2mg/kg) than those reported by other protocols. The sequential methodology seems unable to give evidence about the dose-dependence correlation and separate experiments with different concentrations should be carried out in order to establish a dose-dependent correlation as emerges from pre-treatment experiments.

The use of anaesthetized models effectively minimizes the impact of the produced stress that influences the recording of true blood pressure levels. Moreover the fact that the animals we used for the study did not recover not only relieves the animals from pain but also avoids acute BP increases, as seen in restrained animals. Furthermore, the use of a direct method for blood pressure recording allows valuable continuous quantitative blood pressure estimation. Another advantage of our protocol is that it is not expensive, permitting almost any laboratory to use it. More specifically, since we used a short-term protocol in healthy animals, there was no reason to keep them for a long time period. In addition, only a small amount of the studied molecule is needed for safe and convincing results. This is worth mentioning, as in early drug development the amounts of the compounds synthesized are limited.

The fast protocol was validated using the synthetic, almost inactive MM1 compound which was designed to mimic the His^6-Pro^7-Phe^8 part of Ang II and is based on the (S)-pyroglutamic acid. The results confirmed previous findings in vitro, indicating that the applied protocol can differentiate active from inactive compounds in vivo, in a very short time period.

In conclusion, the protocol which we have proposed appears to be safe and effective for the pharmacological screening of novel AT_1 antagonists. Continuous infusion with Ang II in anaesthetized rabbits enables the evaluation of AT_1 antagonists using very low doses of the tested drug. Moreover, this protocol is characterized by its simplicity and rapid accomplishment.

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