Pharmacological Reports
2013, 65, 69–79
ISSN 1734-1140

Vasorelaxant and antihypertensive effects of ZCM298, a dihydropyridine derivative, are through inhibiting extracellular calcium influx

Li-Xin Zhang[1], Xin-lin Chen[2], Peng-bo Yang[2], San-Qi Zhang[3], Yong-Xiao Cao[1]

[1]Department of Pharmacology, [2]Institute of Neurobiology, [3]Department of Pharmacy, Xi'an Jiaotong University College of Medicine, Xi'an, Shaanxi, 710061, P.R. China

Correspondence: Yong-Xiao Cao, e-mail: yxy@xjtu.edu.cn

Abstract:
Background: ZCM298 is a novel 1,4-dihydropyridine derivative. The aim of the study was to investigate its vasodilation and hypotension, and the related mechanisms.
Methods: The isometric tension of artery ring segments was recorded using an *in vitro* myography system. The blood pressure of spontaneously hypertensive rats (SHRs) was measured *in vivo* using a non-invasive tail cuff blood pressure system. Changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) in the mesenteric artery were surveyed using real-time confocal microscopy. Regional cerebral blood flow (rCBF) in the pia mater was monitored by laser-Doppler flowmetry (LDP).
Results: ZCM298 (10^{-9}–10^{-4} M) relaxed rat mesenteric artery obviously and concentration-dependently, which was not affected by the removal of the endothelium. ZCM298 shifted the concentration-contractile curves of mesenteric arteries in response to phenylephrine, U46619, KCl and $CaCl_2$ towards the right in a non-parallel manner. The potency of ZCM298 on relaxing basilar artery was much higher than on mesenteric artery. ZCM298 did not depress the phenylephrine-induced vasoconstriction; however, it inhibited the contraction caused by the addition of $CaCl_2$ in Ca^{2+}-free solution. ZCM298 (10^{-6} M) inhibited the increase of $[Ca^{2+}]_i$ induced by KCl in the artery. ZCM298 improved the rCBF in the pia mater of rats at 0.03 and 0.06 mg/kg. ZCM298 depressed the systolic and diastolic blood pressure of SHRs in a dose-dependent manner.
Conclusions: ZCM298 relaxes arteries probably through inhibiting extracellular calcium influx and decreases the blood pressure of SHRs. ZCM298 is more potent in the basilar artery than in the mesenteric artery and improves rCBF in the pia mater of rats.

Key words:
1,4-dihydropyridine, vasorelaxation, hypotension, cerebral blood flow

Abbreviations: ACh – acetylcholine chloride, $[Ca^{2+}]_i$ – intracellular calcium concentration, HEPES – hydroxy-ethyl-piperazine ethanesulfonic acid, LDF – laser-Doppler flowmetry, PE – phenylephrine, PU – perfusion unit, rCBF – regional cerebral blood flow, ROCC – receptors' gated calcium channel, SHRs – spontaneously hypertensive rats, U46619 – (*Z*)-7-[(1*S*,4*R*,5*R*,6*S*)-5-[(*E*,3*S*)-3-hydroxyoct-1-enyl]-3-oxabicyclo-[2.2.1]heptan-6-yl]hept-5-enoic acid, VDCC – voltage-dependent calcium channel, ZCM298 – (E)-diethyl 2,6-dimethyl-4-{2-[3-oxo-3-(*tert*-pentyloxy)prop-1-enyl]phenyl}-1,4-dihydropyridine-3,5-dicarboxylate

Introduction

Although the pathogenesis of hypertension is associated with a variety of factors, elevated blood pressure is an essential feature of hypertension that is also a major risk factor for cardiovascular damage. In the clinic, the combination of calcium channel blockers and other types of antihypertensive drugs is considered to be an effective program for the treatment of

high risk patients [19]. 1,4-Dihydropyridines were well known as calcium channel blockers or activators such as nifedipine, nimodipine [22], nitrendipine [4], lacidipine [17], isradipine [3]. The privileged structures of 1,4-dihydropyridines have been studied in the search for new drugs through making minor changes of the existing compounds [23].

Lacidipine, a representative third generation 1,4-dihydropyridine calcium antagonist [24], has a unique molecular structure, in which the aromatic ring is a large orthotopic *tert*-butyl group with a high lipophilicity and it has a high membrane distribution coefficient, which allows it to be stored in the membrane lipid layer for slow release. Lacidipine has a short plasma half-life, a long clinical half-life, and a gentle, long-lasting antihypertensive effect [18, 25]. Moreover, lacidipine has been shown to promote anti-atherosclerosis [2], changes in metabolism, cardioprotective action [26], antimicrobial potential, and anti-oxidant effects [6, 15].

In animal studies, lacidipine was evaluated as a once-daily antihypertensive agent that had a markedly slower onset of action and a more pronounced vascular selectivity than nitrendipine [13]. The clinical safety assessment on lacidipine [8] revealed no unexpected adverse reactions. The advantages of lacidipine, including its long duration of action and once-daily dosage, indicate that it is a suitable agent for the first-line treatment of hypertension across a wide range of patients.

As a characteristic pharmacophore, 1,4-dihydropyridine represents a favorable starting point in the search for ligands [23]. Lacidipine contains a *tert*-butyl ester at the 2-position of 4-phenyl. To synthesise a new lacidipine's analogue, (E)-diethyl 2,6-dimethyl-4-{2-[3-oxo-3-(*tert*-pentyloxy)prop-1-enyl]phenyl}-1,4-dihydropyridine-3,5-dicarboxylate (ZCM298, Fig. 1), we changed the *tert*-butyl group into a *tert*-amyl group. The aim of the present study was to evaluate the vasodilation and hypotension of ZCM298 in rats.

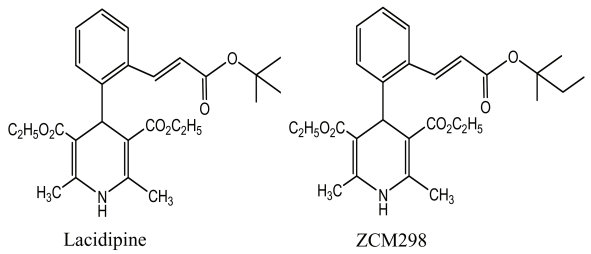

Fig. 1. The chemical structures of lacidipine and ZCM298

Materials and Methods

Animals and reagents

Sprague-Dawley rats were obtained from the Animal Center of Xi'an Jiaotong University (Xi'an, China). Male SHRs (20 weeks of age) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed and maintained under conventional laboratory conditions and allowed free access to food (standard pellet diet) and water. The protocol was approved by Ethics Committee of Xi'an Jiaotong University College of Medicine.

ZCM298 was synthesized in Department of Pharmacy, Xi'an Jiaotong University College of Medicine. Phenylephrine, acetylcholine chloride (ACh), caffeine, lacidipine, (*Z*)-7-[(1*S*,4*R*,5*R*,6*S*)-5-[(*E*,3*S*)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid (U46619), hydroxyethylpiperazine ethanesulfonic acid (HEPES) and Triton X-100 were purchased from Sigma Aldrich (St. Louis, USA). Fluo-3/AM was obtained from Biotium (Hayward, USA). All other reagents were of analytical reagent grade. In *in vitro* experiments, ZCM298, and Fluo-3/AM were dissolved in DMSO, and U46619 was dissolved in ethanol. All other substances were dissolved in double distilled water. Further dilutions were made in double distilled water and added just before the experiment. The concentrations were expressed as the final molar concentration in the solution. ZCM298 and lacidipine were dissolved in vehicle constituted by 10% Tween-80, 10% DMSO, and 80% double distilled water for the regional cerebral blood flow experiments, and by 30% Tween-80, 30% ethanol, and 40% double distilled water for blood pressure measurement experiments.

Krebs solution contained (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5. HEPES-Krebs solution contained (mM): NaCl 135, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 10 and HEPES 8.4. The pH of both solutions was 7.4.

Record of vessel isometric tension

Sprague-Dawley rats were sacrificed by CO_2 asphyxiation. The mesenteric, basilar, left anterior descending coronary and renal arteries were dissected from the adhering tissues under a stereomicroscope. Each vessel was cut into 4 to 8 1–2 mm-long ring seg-

ments and mounted in a multi myograph baths (DMT 610M, Denmark), which was defined in the 5 ml buffer solution at 37°C with 95% O_2 and 5% CO_2, continuously. The isometric tension of each vessel was recorded using a force displacement transducer. After the segments were stabilized with a 1 mN (in coronary and basilar artery) or 3 mN (in mesenteric artery and renal artery) resting tension for 1.5 h and the solutions in the baths were replaced by the same solution every 20 min, the contractile capacity of vessel segments was evaluated by exposure to K^+-rich (60 mM) Krebs solution in which NaCl was exchanged for an equimolar concentration of KCl.

In endothelium-denuded experiments, 0.1% Triton X-100 was poured into the vessels for 10 s to denude the endothelium, followed by incubation with Krebs solution for another 10 s [1]. The denuded segments were mounted in the baths and removal of the endothelium was confirmed by the loss of relaxant response to 10^{-5} M ACh.

Determination of tissue $[Ca^{2+}]_i$ in the artery

Sprague-Dawley rats weighing 100–110 g were sacrificed by CO_2 asphyxiation. The superior mesenteric arteries were quickly removed and immersed into cold HEPES-Krebs solution. The artery was dissected free of adhering tissues under a stereomicroscope. Each artery was cut into approximately 3 mm-long ring segments and mounted in a U shaped stainless steel wire [20]. The artery ring segments with the stainless steel wire were placed in the bottom of a confocal dish which had been added a mixture of 10^{-6} M Fluo-3/AM and DMSO, ZCM298 (10^{-7} M) or lacidipine (10^{-7} M). The operation was gentle and absolutely keeping segments steadily. A real-time confocal microscope (Olympus, FV1000) was used in the investigation. The image formation was continuously acquired every 1.108 s and stored in the hard disk. $[Ca^{2+}]_i$ of the arterial segments was observed immediately after dye loading. The fluorescence intensity was calculated from each individual image using FV10 ASW software (version 1.7, Olympus, Japan). The changes of fluorescence intensity *versus* time were plotted. The change rate of fluorescence intensity by KCl was calculated based on the equation as [(fluorescence intensity after exposure to KCl – fluorescence intensity before exposure to KCl) / fluorescence intensity before exposure to KCl] × 100%.

Measurement of blood pressure

The blood pressure of SHRs was measured daily over five days using a non-invasive tail cuff blood pressure system (Chengdu TME Technology Co., Ltd., China) to adapt the rats to the operation. The systolic and diastolic blood pressures were measured before and after drug administration. Blood pressure was measured 3 times at each time point and the mean value was used as the blood pressure measurement. Decreased rate of the blood pressure was calculated based on the equation as [(blood pressure before the administration – blood pressure after the administration) / blood pressure before the administration] × 100%.

Measurement of regional cerebral blood flow

Sprague-Dawley rats weighing 250–300 g were divided into three groups (ZCM298, lacidipine and vehicle) randomly. After anesthetization with 10% chloral hydrate, the head of the rat was placed on a stereotaxic apparatus (Narishige, SN-2 N, Japan) and the body temperature was maintained at 37°C with a heating blanket. A 0.5 × 0.4 cm^2 bony window was made in the parietal bone (anteroposterior –0.2 cm; lateral 0.1 cm) with the dental drill. The dura mater of rat was uncovered gently. Then, laser-Doppler probe (probe 407, tip diameter 1.0 mm, fiber separation 0.25 mm) was fixed on a micromanipulator and touched on the pia mater (anteroposterior –0.6 cm; lateral 0.2 cm), avoiding large pia vessels. rCBF in the pia mater was monitored by laser-Doppler flowmetry (Perimed, Stockholm, Sweden) continuously, which has a spatial resolution of 1 mm^3 and a temporal resolution of 0.1 s. The system was stabilized for 10–20 min to obtain the basal level of rCBF. Throughout the process, room temperature was maintainted at 25°C and environments were kept quiet.

All recordings were stored in the computer using the software of PeriSoft for Windows (PSW) v.2.50 and connected with LDF. Relative changes of rCBF were dealed as relative changes of perfusion unit (PU). Relative changes of PU after treatment were calculated as percentage of the baseline using the software PSW v.2.50.

Statistical analysis

Data are shown as the mean ± SEM. The differences between the means were evaluated using SPSS for Windows version 13.0. Statistical analysis was per-

formed with two-way analysis of variance (ANOVA) followed by *post-hoc* test and unpaired Student's *t*-test. The p value of less than 0.05 was considered to be significant.

Concentration response data were obtained by cumulative concentration's agonist or ZCM298 to the solution. The tensions of arterial ring segments were expressed as percentage of contraction from pre-contracted by 60 mM K^+. Relaxation responses were expressed as percentage of pre-contraction induced by agonists. E_{max} and R_{max} values referred to the maximum contraction and relaxant effect, respectively. The pEC_{50} (negative logarithm of the molar concentration that produced half maximum relaxation) was calculated from the straight line equation between the concentration above and below the midpoint of the concentration response curve. The pA_2', the dissociation constant of antagonist, was calculated using the equation:

$$pA_2' = -\log([B] / (r-1))$$

in which [B] denotes the molar concentration of the antagonist and r denotes the ratio of the EC_{50} values with the antagonist and the vehicle.

Results

Vascular reactivity

Relaxation of ZCM298 on mesenteric artery pre-contracted by K^+

In order to verify vasodilation of ZCM298, after the superior mesenteric artery ring segments of rats with intact endothelium or denuded endothelium were pre-contracted by 60 mM K^+, ZCM298 (10^{-9} – 10^{-4} M) was added to the baths cumulatively. The relaxation curve of ZCM298 was obtained and showed that ZCM298 relaxed the mesenteric artery in a concentration-dependent manner (Fig. 2). In intact endothelium artery ring, the R_{max} was 80 ± 5% and the pEC_{50} value was 5.40 ± 0.17. In denuded endothelium artery ring, the R_{max} was 82 ± 5% and the pEC_{50} value was 5.31 ± 0.13. There was no significant difference of the R_{max} and the pEC_{50} values between intact endothelium and denuded endothelium artery rings. These results indicate that removal of endothelium does not affect the relaxation effect of ZCM298.

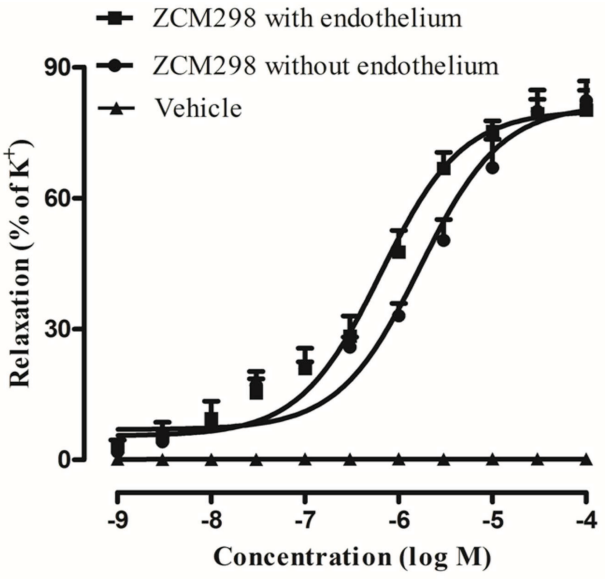

Fig. 2. The relaxation effects of ZCM298 on the superior mesenteric artery of rats induced by 60 mM K^+. Data are given as the mean ± SEM, n = 8

The comparison of vasodilation between ZCM298 and lacidipine was studied (Fig. 3). The results showed that the relaxative effects of ZCM298 and lacidipine at 10^{-7} M were 20.2 ± 4.0% and 23.1 ± 5.2%, respectively ($p > 0.05$). The relaxative effects of ZCM298 and lacidipine at 10^{-6} M were 54.9 ± 5.0% and 50.2 ± 2.9%, respectively ($p > 0.05$). There was no significant difference of the relaxant effects between ZCM298 and lacidipine.

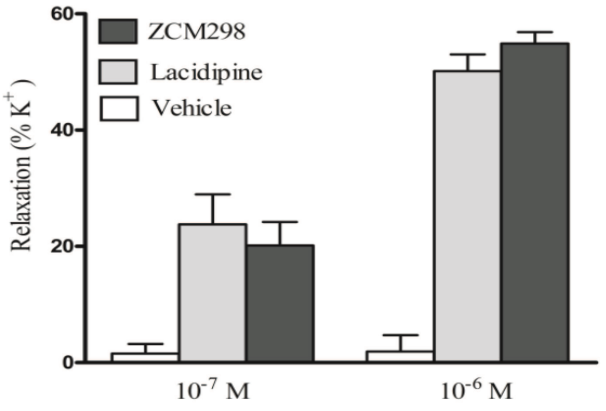

Fig. 3. The comparison of vasorelaxation between ZCM298 and lacidipine on the superior mesenteric artery. Data are expressed as the mean ± SEM, n = 8

Effects of ZCM298 on KCl- and $CaCl_2$-induced artery contraction curves

After the mesenteric arterial rings were incubated in the presence of ZCM298 (3×10^{-8} M, 3×10^{-7} M) or vehicle in the baths for 60 min, the normal Krebs solution of the baths was replaced by K^+-Krebs solution containing KCl (10, 20, 40 and 80 mM) successively. The concentration-contraction curve induced by KCl was obtained. The results showed that ZCM298 shifted the curve induced by KCl towards the right in a non-parallel manner. ZCM298 at 3×10^{-8} M and 3×10^{-7} M depressed the E_{max} of KCl from 110 ± 4% in vehicle to 49 ± 5% and 34 ± 4%, respectively. The pA_2' value of ZCM298 to KCl was 7.88 (Fig. 4A).

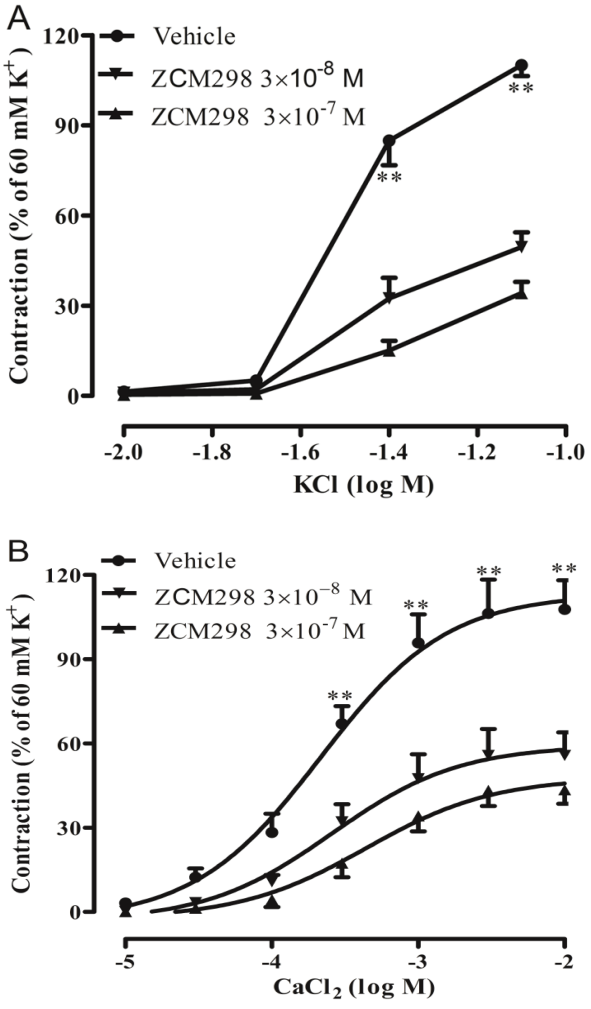

Fig. 4. Effects of ZCM298 on the concentration-response curves in the mesenteric artery induced by KCl (**A**) and $CaCl_2$ (**B**). Values are denoted as the mean ± SEM, n = 8, ** $p < 0.01$ compared with vehicle

After the mesenteric arterial segments were incubated in Ca^{2+}-free K^+-rich (60 mM) solution containing ethylenediamine tetraacetic acid (10^{-4} M) in the presence of ZCM298 (3×10^{-8} M, 3×10^{-7} M) or vehicle for 60 min, $CaCl_2$ (10^{-5} – 10^{-2} M) was added to the baths cumulatively. Figure 4B showed that ZCM298 (3×10^{-8} M, 3×10^{-7} M) shifted the concentration-contraction curve induced by $CaCl_2$ towards the right in a non-parallel manner with the decreased E_{max} from 107 ± 10% in vehicle to 56 ± 8% and 44 ± 5%, respectively. The pA_2' value of ZCM298 to $CaCl_2$ was 7.31.

Effects of ZCM298 on phenylephrine- and U46619-induced vasoconstriction

After the mesenteric arterial segments were incubated in the presence of ZCM298 (3×10^{-8} M, 3×10^{-7} M) or vehicle for 60 min, phenylephrine (10^{-9} – 3×10^{-5} M) was added to the baths cumulatively and the concentration-contraction curve was obtained. ZCM298 shifted the concentration-contractile curve induced by phenylephrine towards the right in a non parallel manner. ZCM298 3×10^{-8} M and 3×10^{-7} M decreased the E_{max} from 103 ± 9% in vehicle to 49 ± 6% and 40 ± 5%, respectively. The pA_2' value of ZCM298 to phenylephrine was 7.69 (Fig. 5A).

Figure 5B showed that ZCM298 (10^{-8} M, 3×10^{-7} M) shifted the concentration-contractile curve induced by U46619 (10^{-10} – 3×10^{-6} M) towards the right in a similar manner. ZCM298 10^{-8} M and 3×10^{-7} M decreased the E_{max} from 139 ± 9% in vehicle to 115 ± 5% and 62 ± 8%. The pA_2' value of ZCM298 to U46619 was 6.70.

Reactivities of ZCM298 on phenylephrine- and $CaCl_2$-induced contraction in the Ca^{2+}-free Krebs solution

After incubation of the mesenteric arterial ring segments in the Ca^{2+}-free Krebs solution containing EDTA (10^{-4} M) in the presence of ZCM298 (3×10^{-8} M, 3×10^{-7} M and 3×10^{-6} M) or vehicle for 60 min, phenylephrine (10^{-5} M) was added to the baths. Phenylephrine induced a rapid, transient, and weak contraction of the segments in vehicle group and ZCM298 treatment groups. There was no significant difference of the contraction tension between vehicle and ZCM298 treatment groups. On the basis of the phenylephrine-induced contraction, $CaCl_2$ (2×10^{-3} M) was added to the baths to contract the segments again. The $CaCl_2$ in-

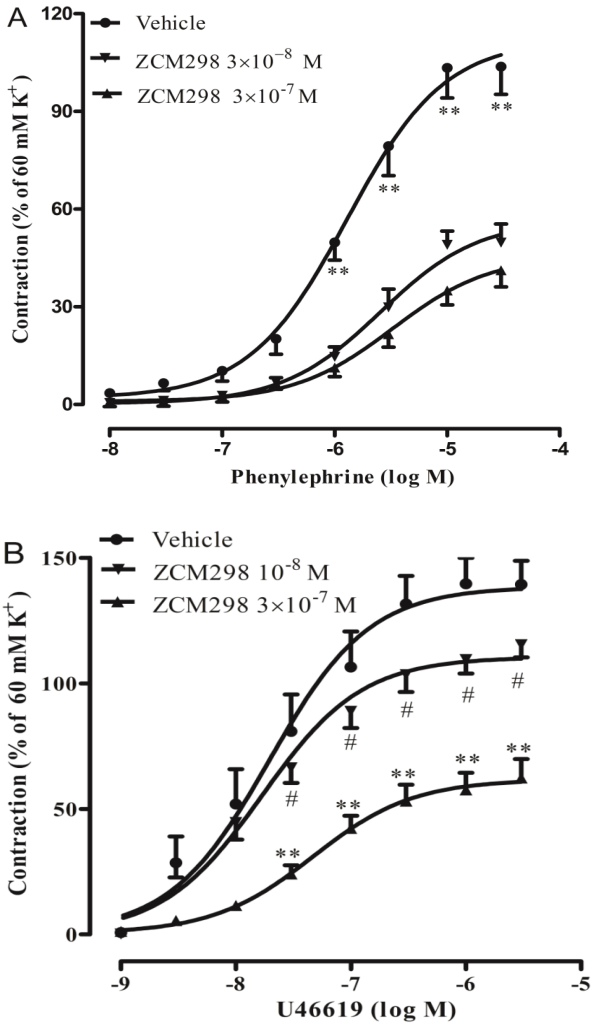

Fig. 5. The effects of ZCM298 on concentration-contractile curves in the superior mesenteric artery induced by phenylephrine (**A**) and U46619 (**B**). Values are expressed as the mean ± SEM, n = 8. ** $p < 0.01$ compared with vehicle, # $p < 0.05$ compared with 3×10^{-7} M

duced 88 ± 10% contraction of segments in vehicle. In the presence of ZCM298 (3×10^{-8} M, 3×10^{-7} M and 3×10^{-6} M), $CaCl_2$-induced contraction was 81 ± 13%, 63 ± 7% and 44 ± 5%, respectively (Fig. 6). There was a significant difference of $CaCl_2$-induced contraction between vehicle and ZCM298 (3×10^{-6} M) ($p < 0.05$, n = 6), suggesting that ZCM298 depressed the contraction induced by $CaCl_2$.

Effects of ZCM298 on caffeine-induced vasoconstriction

Following the arterial segments were incubated in the presence of ZCM298 (3×10^{-6} M) in Ca^{2+}-free Krebs

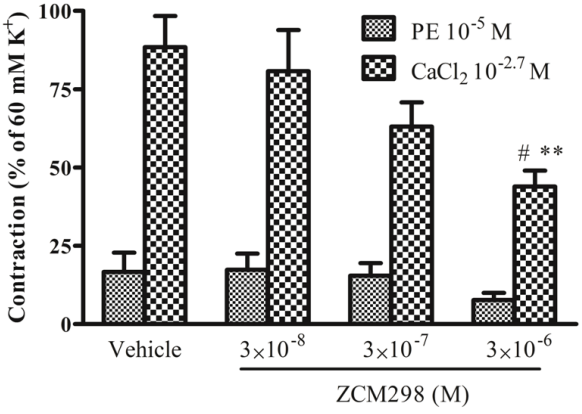

Fig. 6. The effects of ZCM298 on the rat mesenteric artery induced by phenylephrine and $CaCl_2$ in Ca^{2+}-free Krebs solution. Values are given as the mean ± SEM, n = 6. ** $p < 0.01$ compared with vehicle, # $p < 0.05$ compared with 3×10^{-8} M

solution for 60 min, caffeine (3×10^{-2} M) was added to baths. The results showed that caffeine induced a rapid and short-term contraction of 13 ± 2% in vehicle and 11 ± 1% in ZCM298 groups, respectively ($p > 0.05$, n = 6), suggesting that ZCM298 does not affect the caffeine-induced vasoconstriction.

The reactivities of ZCM298 on different arteries

In order to study the effects of ZCM298 on different arterial reactivity, the superior mesenteric artery, renal artery, basilar artery and coronary artery of the same rat were mounted in the baths synchronously. After U46619 (3×10^{-6} M) was added to the baths and pre-contraction was obtained, ZCM298 was added to the baths cumulatively ($10^{-10} - 3 \times 10^{-5}$ M). The concentration-relaxation curves of different arteries were obtained (Fig. 7). The results showed that there was no significant difference of the R_{max} among the arteries. The pEC_{50} values of ZCM298 in the basilar artery, coronary, renal artery and mesenteric artery were 7.09 ± 0.28, 6.81 ± 0.21, 6.11 ± 0.16 and 5.78 ± 0.26, respectively.

There was a significant difference of the pEC_{50} values between the basilar artery and renal artery or mesenteric artery ($p < 0.05$, n = 9), suggesting that ZCM298 is more potent in basilar artery than in renal artery and mesenteric artery.

The reactivity of ZCM298 on different arteries induced by 60 mM K^+ was similar to that by U46619 (data not shown).

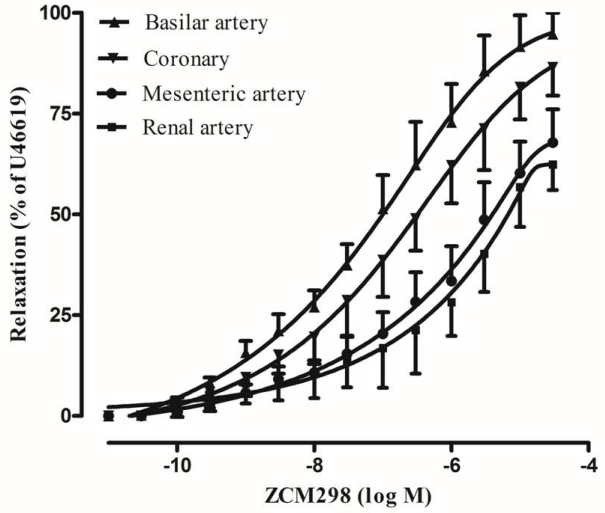

Fig. 7. The different sensitivity of ZCM298 on the coronary artery, basilar artery, renal artery and mesenteric artery ring segments of rats induced by 3×10^{-6} M U46619. Values are expressed as the mean ± SEM, n = 9

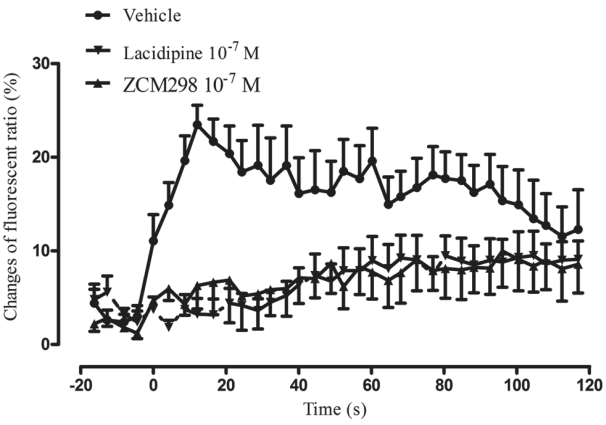

Fig. 8. The effect of ZCM298 on Ca^{2+} fluorescence intensity in the mesenteric artery of rats. The change rate of fluorescence intensity reflects the change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Values are expressed as the mean ± SEM, n = 6

Effects of ZCM298 on Ca^{2+} fluorescence intensity in mesenteric artery

Figure 8 displayed the change rate of fluorescence intensity induced by 50 mM KCl in the mesenteric artery of rats over time. The prepared segments were placed under the confocal laser microscope for 40 s and the fluorescent image of the vessel in the resting state was acquired in HEPES-Krebs solution. After the acquisition of resting response, 50 mM KCl was added to the HEPES-Krebs solution of confocal dish and the images were acquired continuously [12]. The dynamic image for 120 s showed a rapid, transient change of fluorescence intensity and a relative lasting contraction in vehicle group, but not in ZCM298 and lacidipine treatment groups. The results hinted that ZCM298 inhibits the $[Ca^{2+}]_i$ concentration increase induced by KCl in the mesenteric arteries as same as lacidipine.

Effects of ZCM298 on regional cerebral blood flow

After the basal value of rCBF was obtained, the rats were cumulatively administrated ZCM298 or lacidipine at 0.01, 0.03, 0.06, 0.15 and 0.3 mg/kg *via* intraperitoneal injection every 20 min. The changes of perfusion unit (PU) were recorded continuously. Figure 9 showed that the PU of vehicle group had a decrease trend over the time, but there was no significant difference between before and after vehicle. ZCM298 at 0.01, 0.03 and 0.06 mg/kg increased the PU by 4%, 14% and 10%, respectively. The PU in lacidipine group did not change obviously. There was a significant difference between ZCM298 and vehicle or lacidipine in the same time point ($p < 0.05$, n = 8), suggesting that ZCM298 increases the regional cerebral blood flow of rats.

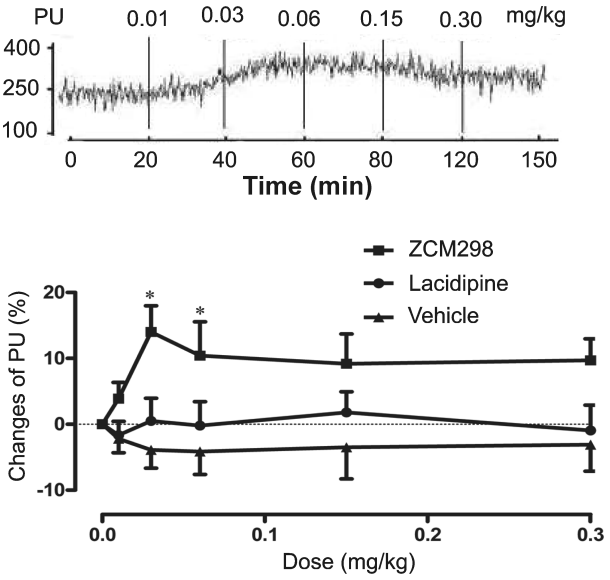

Fig. 9. Effect of ZCM298 on regional cerebral blood flow. Values are expressed as the mean ± SEM, n = 8. * $p < 0.05$ compared with vehicle or lacidipine

Tab. 1. Effects of ZCM298 by gavage on blood pressure of SHRs (n = 8)

Groups	Dose (mg/kg)	Systolic pressure (mmHg)			Diastolic pressure (mmHg)		
		Before drugs	Decreased values	Decreased percent (%)	Before drugs	Decreased values	Decreased percent (%)
Vehicle	–	191 ± 2	–4 ± 2.5	–2 ± 3.3	169 ± 7	–6 ± 4.7	–4 ± 3.4
Lacidipine	0.05	203 ± 1	16 ± 2	8± 3.0*	171 ± 2	11 ± 5.7	6 ± 3.3*
Lacidipine	0.2	198 ± 2	44 ± 2.4	22 ± 2.8** #	166 ± 6	31 ± 6.0	19 ± 3.2** #
ZCM298	0.1	208 ± 2	14 ± 2.5	7± 1.5**	180 ± 4	14 ± 2.9	8 ± 1.6**
ZCM298	0.2	208 ± 3	34 ± 2.8	16 ± 3.6** #	178 ± 1.8	31 ± 8.2	17 ± 4.3** #
ZCM298	0.4	201 ± 3	49 ± 3.9	24 ± 2.1** #	170 ± 2.6	42 ± 2.3	25 ± 1.4** #

The blood pressure was measured *via* a non-invasive tail-cuff blood pressure system. Values are expressed as the mean ± SEM. Values of systolic blood pressure and diastolic blood pressure are measured 1 h after administration; * $p < 0.05$, ** $p < 0.01$ compared with vehicle; # $p < 0.05$ compared with low dose

Effects of ZCM298 on blood pressure of SHRs

SHRs were divided into 6 groups: ZCM298 (0.1, 0.2 and 0.4 mg/kg), lacidipine (0.05 and 0.2 mg/kg) and vehicle groups were administrated by gavage in 5 ml/kg. After administration agents for 1 h, the blood pressure was measured. There was no significant change of the blood pressure in vehicle group before and after gavage (Tab. 1). The decreased rates of systolic pressure and diastolic pressure of ZCM298 (0.1, 0.2 and 0.4 mg/kg) groups and lacidipine (0.05 and 0.1 mg/kg) groups were higher than that of vehicle group obviously ($p < 0.01$, $n = 8$). The decreased rates of blood pressure of ZCM298 0.4 mg/kg group were higher than that of ZCM298 0.1 mg/kg group ($p < 0.05$, $n = 8$), suggesting that the hypotensive effect of ZCM298 was acting in a dose dependent manner.

Discussion

Dihydropyridines are known as calcium channel blockers, and they have obvious vasorelaxant effects. ZCM298 is a newly synthesized 1,4-dihydropyridine derivative that was created by changing the *tert*-butyl ester group of lacidipine into *tert*-butyl amide group. We predicted that ZCM298 would have the same vasorelaxation effects as lacidipine. To verify this hypothesis, we investigated vasorelaxation in response to ZCM298 *in vitro*. On the basis of the vasorelaxation of ZCM298, we compared ZCM298 and lacidipine by assessing the change of $[Ca^{2+}]_i$ in the artery, the antihypertensive effect and the impact on rCBF.

The vasorelaxation experiment showed that ZCM298 relaxed the mesenteric artery in a concentration-dependent manner following K^+-induced contraction, and the R_{max} reached 80%. There was no significant difference in vasodilation between intact and denuded endothelium arteries in response to ZCM298. This effect suggested that the vasodilation of ZCM298 mainly acted on the vascular smooth muscle. So, the further arterial ring segments experiments were conducted in intact endothelium artery and the change of $[Ca^{2+}]_i$ in the artery also were investigated on the vascular smooth muscle. The comparison of vasodilation showed that there was no significant difference of the relaxant effects between ZCM-298 and lacidipine in the same concentration.

Intracellular calcium is the most important factor for life activities [5]. When the concentration of extracellular K^+ rises, the cell membrane depolarizes and

voltage-dependent calcium channels (VDCC) open, causing extracellular calcium influx and inducing the contraction of vascular smooth muscle [21]. In this study, ZCM298 relaxed mesenteric artery pre-contracted by high K^+ concentration-dependently and shifted the concentration-contractile curves induced by KCl towards the right with the decreased $E_{max,}$ suggesting that role in the VDCC may be the one way in the vasodilation of ZCM298.

Phenylephrine induces vasoconstriction by binding α_1-adrenoceptors on the cell membrane, which activates phosphorus lipase C to generate inositol triphosphate. This leads to calcium release from the intracellular inositol triphosphate sensitive calcium pool, and increases the intracellular calcium concentration [7, 16]. Phenylephrine also activates cell membrane receptors' gated calcium channel (ROCC) and leads to phosphorylation of voltage dependent calcium channels, which both enhance calcium influx [9, 10]. In this study, ZCM298 shifted the concentration-response curves of phenylephrine towards the right in a non-parallel manner, suggesting that effecting ROCC may be another mechanism by which ZCM298 induces vasodilation. However, in Ca^{2+}-free Krebs solution, ZCM298 did not inhibit calcium release induced by phenylephrine and inhibited calcium influx by $CaCl_2$. Although we can not conclude that the relaxative effect of ZCM298 is due to the inhibition of calcium release, we show that ZCM298 relaxes vascular smooth muscle by inhibiting extracellular calcium influx.

Real-time laser confocal microscopy and fluorescent probe are widely employed to monitor the dynamic changes of $[Ca^{2+}]_i$ in various cells [11]. Dynamic changes of $[Ca^{2+}]_i$ in the isolated artery ring were monitored using real-time laser confocal microscopy to monitor the intensity of reflected fluorescence $[Ca^{2+}]_i$. In the study, we surveyed this change in the mesenteric artery of rats. The results showed that Ca^{2+} fluorescence intensity was immediately enhanced upon the addition of high K^+ and contraction of artery ring wall was clearly visible. ZCM298 and lacidipine decreased the Ca^{2+} fluorescence intensity induced by K^+, suggesting that ZCM298 inhibited the increase of $[Ca^{2+}]_i$, induced by KCl in rat mesenteric arteries to the same extent as lacidipine. These data further demonstrate that the effects of ZCM298 on vasodilatation involves decreasing $[Ca^{2+}]_i$. In these experiments, the mesenteric arteries of rats weighing 100–110 g were fit for a U-shaped stainless steel wire so that the ring segments in the confocal dish could maintain a fixed state. In the process of operation, it was the most basic requirement that the segments were to maintain a fix state [12].

The response of arteries in the diverse parts of the body is different [14]. In our study, the mesenteric, renal, basilar and coronary arteries of the same rat were mounted in baths to synchronously compare the sensitivity of different arteries to ZCM298. These results showed that ZCM298 relaxed various arteries that were pre-contracted by U46619 and high K^+. The rank order of potency of ZCM298 was as follows: basilar artery > coronary artery > mesenteric artery > renal artery. These data suggest that ZCM298 may have a relative high affinity and selectivity in the brain.

To further explore the effect of ZCM298 on cerebral circulation, we studied the rCBF. As the dose of ZCM298 was altered, fluctuations of rCBF appeared. ZCM298 at 0.03 and 0.06 mg/kg increased the rCBF, which was consistent with the results of the reactivities of different arteries *in vitro*. We believe that this increase in the rCBF is due to the vasorelaxation induced by ZCM298. It was interesting that lacidipine did not increase rCBF at the same dose as ZCM298.

The vasodilation of resistance arteries decreases the system blood pressure. We also investigated the effect of ZCM298 on blood pressure in SHRs. We found that ZCM298 caused hypotensive effects in a dose dependent manner. Lacidipine 0.2 mg/kg depressed the systolic pressure and diastolic pressure by 22 ± 2.8% and 19 ± 3.2%, respectively; while ZCM298 (0.2 mg/kg) depressed the systolic pressure and diastolic pressure by 16 ± 3.6% and 17 ± 4.3%, respectively. Although there was no significant difference in the hypotension caused by ZCM298 and lacidipine at 0.2 mg/kg, the effect of lacidipine showed a higher trend. The hypotensive effect of lacidipine at 0.05 mg/kg (decreased systolic and diastolic pressure by 8% and 6%) was almost equal to that of ZCM298 at 0.1 mg/kg (decreased systolic and diastolic pressure by 7% and 8%), suggesting that the antihypertensive effect of lacidipine is stronger than that of ZCM298.

However, in the experiments assessing the effect of ZCM298 on rCBF, when the dose of ZCM298 was higher than 0.06 mg/kg, the PU value declined gradually, suggesting that this phenomenon was the result of hypotension of ZCM298. In a state of hypotension, the rCBF decrease offsets the increase caused by vasodilation after ZCM298. Thus, we can infer that

the dose of ZCM298 that increases CBF is lower than that which causes antihypertension.

In summary, the new derivative, ZCM298, causes vasodilation probably *via* the inhibition of extracellular calcium influx. The selectivity on the cerebral artery is higher than peripheral resistance vessels, and its anti-hypertensive effect is slightly weaker than that of lacidipine. These results suggest that ZCM298 could be a potential candidate to treat ischemic cerebrovascular disease with hypertension.

Acknowledgment:
This work was supported by the Specialized Research Fund for the Doctoral Program of Higher Education of China (SRFDP) under Grant No. 20100201110054.

References:

1. Adner M, Cantera L, Ehlert F, Nilsson L, Edvinsson L: Plasticity of contractile endothelin-b receptors in human arteries after organ culture. Br J Pharmacol, 1996, 119, 1159–1166.
2. Anacak GY, Ustunes L, Dilsiz OY, Ergul A: Lacidipine has antiatherosclerotic effects independent of its actions on lipid metabolism and blood pressure. Vascul Pharmacol, 2010, 53, 193–199.
3. Barhwal K, Hota SK, Baitharu I, Prasad D, Singh SB, Ilavazhagan G: Isradipine antagonizes hypobaric hypoxia induced CA1 damage and memory impairment: Complementary roles of L-type calcium channel and NMDA receptors. Neurobiol Dis, 2009, 34, 230–244.
4. Bellemann P, Ferry D, Lubbecke F, Glossman H: [^{3}H]-nitrendipine, a potent calcium antagonist, binds with high affinity to cardiac membranes. Arzneimittelforschung, 1981, 31, 2064–2067.
5. Dai S, Hall DD, Hell JW: Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol Rev, 2009, 89, 411–452.
6. Dasgupta A, Jeyaseeli L, Dutta NK, Mazumdar K, Karak P, Dastidar SG, Motohashi N, Shirataki Y: Studies on the antimicrobial potential of the cardiovascular drug lacidipine. In Vivo, 2007, 21, 847–850.
7. Eckert RE, Karsten AJ, Utz J, Ziegler M: Regulation of renal artery smooth muscle tone by α_1-adrenoceptors: Role of voltage-gated calcium channels and intracellular calcium stores. Urol Res, 2000, 28, 122–127.
8. Endersby CA, Brown EG, Perelman MS: Safety profile of lacidipine: A review of clinical data. J Cardiovasc Pharmacol, 1991, 17, Suppl 4, S45–47.
9. Ford WR, Broadley KJ: Effects of adenosine receptor agonists on induction of contractions to phenylephrine of guinea-pig aorta mediated via intra- or extracellular calcium. Gen Pharmacol, 1999, 33, 143–150.
10. Garaliene V, Barsys V, Jakuška P, Benetis R: Action of calcium antagonists and agonists on isolated human thoracic arteries used for coronary artery bypass grafting. Pharmacol Rep, 2012, 64, 733–738.
11. Hashimoto T, Ohata H, Nobe K, Honda K: Comparative study of calcium ion dynamics and contractile response in rat middle cerebral and basilar arteries. J Pharmacol Sci, 2009, 109, 546–555.
12. Hashimoto T, Ohata H, Nobe K, Sakai Y, Honda K: A novel approach for the determination of contractile and calcium responses of the basilar artery employing real-time confocal laser microscopy. J Pharmacol Toxicol Methods, 2007, 56, 79–86.
13. Heber ME, Broadhurst PA, Brigden GS, Raftery EB: Effectiveness of the once-daily calcium antagonist, lacidipine, in controlling 24-hour ambulatory blood pressure. Am J Cardiol, 1990, 66, 1228–1232.
14. Kawada T, Sun HT, Nakazawa M, Imai S: In vitro effects of the new calcium antagonist lacidipine. Jpn J Pharmacol, 1993, 62, 289–296.
15. Keles MS, Bayir Y, Suleyman H, Halici Z: Investigation of effects of lacidipine, ramipril and valsartan on DNA damage and oxidative stress occurred in acute and chronic periods following isoproterenol-induced myocardial infarct in rats. Mol Cell Biochem, 2009, 328, 109–117.
16. Kobayashi S, Gong MC, Somlyo AV, Somlyo AP: Ca^{2+} channel blockers distinguish between G protein-coupled pharmacomechanical Ca^{2+} release and Ca^{2+} sensitization. Am J Physiol, 1991, 260, C364–370.
17. Micheli D, Collodel A, Semeraro C, Gaviraghi G, Carpi C: Lacidipine: A calcium antagonist with potent and long-lasting antihypertensive effects in animal studies. J Cardiovasc Pharmacol, 1990, 15, 666–675.
18. Motomura S, Wu ZJ, Hashimoto K: Lacidipine, a new long-acting dihydropyridine calcium antagonist, has high vascular selectivity against all intracardiac variables. Heart Vessels, 1993, 8, 16–22.
19. Oparil S, Weber M: Angiotensin receptor blocker and dihydropyridine calcium channel blocker combinations: An emerging strategy in hypertension therapy. Postgrad Med, 2009, 121, 25–39.
20. Sun T, Liu R, Cao YX: Vasorelaxant and antihypertensive effects of formononetin through endothelium-dependent and -independent mechanisms. Acta Pharmacol Sin, 2011, 32, 1009–1018.
21. Takeuchi M, Watanabe J, Horiguchi S, Karibe A, Katoh H, Baba S, Shinozaki T, Miura M et al.: Interaction between L-type Ca^{2+} channels and sarcoplasmic reticulum in the regulation of vascular tone in isolated rat small arteries. J Cardiovasc Pharmacol, 2000, 36, 548–554.
22. Towart R, Kazda S: The cellular mechanism of action of nimodipine (BAY e 9736), a new calcium antagonist [proceedings]. Br J Pharmacol, 1979, 67, 409P–410P.
23. Triggle DJ: 1,4-Dihydropyridines as calcium channel ligands and privileged structures. Cell Mol Neurobiol, 2003, 23, 293–303.
24. Triggle DJ: Calcium channel antagonists: Clinical uses – past, present and future. Biochem Pharmacol, 2007, 74, 1–9.

25. Vertkin AL, Topolianskii AV: Lacidipine: a new third generation calcium channel blocker (Russian). Kardiologiia, 2002, 42, 100–103.
26. Zanchetti A, Hennig M, Baurecht H, Tang R, Cuspidi C, Carugo S, Mancia G: Prevalence and incidence of the metabolic syndrome in the european lacidipine study on atherosclerosis (ELSA) and its relation with carotid intima-media thickness. J Hypertens, 2007, 25, 2463–2470.

Received: March 11, 2012; **in the revised form:** August 22, 2012; **accepted:** September 4, 2012.