The T- and L-Type Calcium Channel Blocker (CCB) Mibefradil Attenuates Leg Edema Induced by the L-Type CCB Nifedipine in the Spontaneously Hypertensive Rat: A Novel Differentiating Assay

Terry C. Major, Shantanu Dhamija, Nicole Black, Serguei Liachenko, Brandy Morenko, Gregg Sobocinski, Carlin Okerberg, Paula Tinholt, Steven Madore and Mark C. Kowala

Cardiovascular and Atherosclerosis Biology (TCM, MCK)
Center of Emphasis-Bioimaging (SD, NB, SL)
Worldwide Comparative Medicine (BM)
World Wide Safety Sciences (GS, CA)
Discovery Biomarkers (PT, SM)
Pfizer Global Research & Development
Pfizer, Inc.
Eastern Point Road
Groton, CT 06340
Running Title: Mibefradil Attenuates Peripheral Edema as Measured via MRI

Corresponding Author: Serguei Liachenko
Bioimaging Center of Emphasis
Pfizer Global Research & Development
Pfizer, Inc.
Eastern Point Rd 118W-339
Groton, CT 06340
Telephone: (860) 686-9526
Fax: (860) 715-0338
E-mail: serguei.liachenko@pfizer.com

Total text pages: 35
No. of tables: 1
No. of Figures: 7
No. of References: 29
Abstract word count: 244
Introduction word count: 741
Discussion word count: 1498

Non-standard abbreviations: CCB – calcium channel blocker; LSE – least square error; MBP – mean arterial blood pressure; MRI – magnetic resonance imaging; Mibefradil, (1S,2S)-2-[2[[3-(2-
Benzimidazolyl)propyl]methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate dihydrochloride hydrate; Nifedipine, 1,4-Dihydro-2,6-dimethyl-4-(2-
nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester
RAAS - renin-angiotensin-aldosterone system; RF – radiofrequency; ROI – region of interest; SD – Sprague-Dawley; SHR – spontaneously hypertensive rat.

Section assignment: Cardiovascular
ABSTRACT

Among the L-type calcium channel blockers (CCBs), particularly dihydropyridines like nifedipine, a common adverse effect is vasodilatory edema. Newer CCBs such as the T- and L-type CCB, mibefradil, demonstrate antihypertensive efficacy similar to that of their predecessors but appear to have a reduced propensity to cause edema. Using magnetic resonance imaging (MRI) T2 mapping technique, we investigated the ability of mibefradil to reduce extracellular water accumulation caused by the L-type CCB, nifedipine, in the hindleg skeletal muscle of the spontaneously hypertensive rat (SHR). Mibefradil (10 mg/kg IV) and nifedipine (1 mg/kg IV) lowered mean arterial blood pressure (MBP) by 97±5 and 77±4 mmHg, respectively. MRI edema index (expressed as % increase of integral T2 over predrug control) was significantly higher with nifedipine (2606±86%; p<0.05) than with mibefradil (981±171%) measured 30-60 minutes after the start of drug infusion. The hindleg edema caused by nifedipine was dose-dependently decreased by co-administration of mibefradil (0, 0.3 or 3 mg/kg). The hindleg edema formation was not due to albumin leakage into the interstitial space based on immunostaining. However, a 4.2 fold increase in the arterial L- to T-type CC mRNA expression ratio was observed compared to the venous L:T ratio as shown by quantitative RT-PCR. These results demonstrate the novel utility of MRI to measure extravascular water after acute exposure to CCBs and indicate that T-type CCB activity may reduce L-type CCB-induced vasodilatory edema in the skeletal muscle vasculature, possibly by a differential effect on arteriole and venule dilatation.
Introduction

Calcium channel blockers comprise a class of powerful, well-tolerated, and safe antihypertensive agents that are widely used either alone or as a key component of combination therapy for hypertension. Unfortunately a common adverse effect of calcium channel blockers (CCB) is vasodilatory edema which results in peripheral leg edema. Vasodilatory edema is related to several mechanisms, including arteriolar dilation (Hayashi et al., 2005), stimulation of the renin-angiotensin-aldosterone system (RAAS) (He et al., 2005; Schiffrin, 2003) and fluid volume retention (Messerli, 2002). The most widely held theoretical mechanism for this edema is a disproportionate decrease in arteriolar versus venular resistance which increases hydrostatic pressure in the capillary circulation and drives fluid shifts into the interstitial compartment. Vasodilatory edema is common and dose-dependent with first generation CCBs such as verapamil and nifedipine (Messerli, 2002; Safak and Simsek, 2006). Once edema is present it can be slow to resolve without intervention. A number of strategies exist to treat CCB-related edema, including switching CCB classes, reducing the dosage, adding known venodilators such as nitrates, or adding RAAS inhibitors such as angiotensin-converting enzyme inhibitors, or angiotensin-receptor blockers to the treatment regimen (Messerli, 2001; Weir et al., 2001). Diuretics may remediate the edema state somewhat, but at the expense of further reducing plasma volume. Traditional measures such as limiting the amount of time that a patient is upright and/or considering use of graduated compression stockings are useful adjunctive therapies.

Not all CCBs appear to have the same propensity for causing edema. For example in the COHORT study, two lipophilic CCBs, lercanidipine and lacidipine, in elderly hypertensives
caused edema in 9% and 4% of patients, respectively, compared to 19% for amlodipine (Leonetti et al., 2002). The mechanism for the reduced incidence of edema with more lipophilic CCBs like lercanidipine, lacidipine and mibefradil still remains unclear.

Mibefradil (Brogden and Markham, 1997), which is structurally and pharmacologically different than traditional calcium antagonists, recently has been shown in clinical studies to reduce the incidence of leg edema to 5% compared to 26% and 17% for the dihydropyridines, amlodipine and nifedipine, respectively (Kobrin, 1997). Mibefradil represents a new generation of CCBs that blocks both the T- and L-type Ca$^{2+}$ channels, whereas the dihydropyridines selectively block L-type Ca$^{2+}$ channels. Previous work has shown that the L-type Ca$^{2+}$ channel binding affinities for nifedipine and mibefradil, measured at the alpha1C subunit, had a Ki value of 8.2 and 156 nM, respectively (Huber et al., 2000). The T-type Ca$^{2+}$ channel (i.e., the alpha 1H subunit) binding affinities determined electrophysiologically for nifedipine and mibefradil had IC$_{50}$ values of > 10 µM and 140 nM, respectively, with mibefradil having a 10-15 fold preference for the T-type versus the L-type Ca$^{2+}$ channel (Martin et al., 2000). These reported Ca$^{2+}$ channel affinity data illustrate the difference in binding capabilities between nifedipine and mibefradil and establish the proposed mechanism in the edema formation between nifedipine and mibefradil.

In order to explain the functional differences of T- and L-type Ca$^{2+}$ channels in vascular tissue, previous studies have demonstrated the differential dilatory effects of CCBs on arteries and veins (Magnon et al., 1995; Harris et al., 1980; Ozawa et al., 2001). Recently, Feng et al. have shown that CCBs with combined T- and L-type activity such as pimozide and mibefradil equally dilate efferent and afferent arterioles in the renal circulation and provide protection.
against hypertensive glomerular injury unlike L-type CCBs (Feng et al., 2004). These studies are consistent with the notion that CCBs with T-type activity have an attenuated ability to cause peripheral edema due to their equal vasodilatory effects on pre- and postcapillary vessels. The molecular mechanism involved may be dependent on the differential T- and L-type Ca\textsuperscript{2+} channels in arterial and venous tissue.

In this study we examined whether MRI could be used to quantitatively detect CCB-induced peripheral edema in a well-characterized model of hypertension, the SHR, as compared to the normotensive rat. After establishing that peripheral edema can be measured by this technique, we examined whether mibefradil could attenuate edema caused by nifedipine. The functional MRI results indicate that, at equally antihypertensive doses, mibefradil caused less vasodilatory edema than nifedipine, which is consistent with a mechanism that may equalize hydrostatic pressure across the capillary bed by decreasing venous resistance. This equalized capillary hydrostatic pressure may be due to either directly having equal T-type Ca\textsuperscript{2+} channel expression on the arterial and venous sides of the capillary or indirectly through a simple reduction in L-type Ca\textsuperscript{2+} channel expression on the arterial side.
METHODS

Materials

Mibefradil and nifedipine were obtained from Sigma Chemical Co. (St.Louis, MO). Intravenous solutions of both drugs were prepared using a cardiovascular-inert vehicle consisting of 5% N-methylpyrrolidone/45% polyethylene glycol 400/50% 50 mM lactic acid.

Animal Preparation

The animal handling and imaging procedures were approved by the Pfizer Institutional Animal Care and Use Committee according to NIH Guidelines for the Care and Use of Laboratory Animals. Thirty-eight spontaneously hypertensive rats (SHRs, 360±65 g) and 6 Sprague-Dawley normotensive rats (SD, 450±50 g) were anesthetized with isoflurane in O₂ (3% v/v induction in a chamber followed by 1-1.5% v/v maintenance via a nose mask). Catheters (PE50) were surgically placed into the left carotid artery (for blood pressure measurement) and the right jugular vein (for drug delivery). The animal was then placed supine on a heated bed and the hind paws were secured to the bed. The rat was allowed to breathe spontaneously. Respiration and mean arterial blood pressure (MBP) were monitored throughout the experiment with a chest pneumatic transducer and a blood pressure transducer connected to an animal monitoring system. Temperature was measured with the rectal fluoroptic thermometer and maintained at 36±1°C.
**Magnetic Resonance Imaging Protocol**

A T$_2$ mapping MRI technique was adapted for the present studies (Patten et al., 2003). Briefly, MRI was performed using a 7 Tesla Bruker MRI system. A 72 mm volume coil (Bruker Biospin, Billerica, MA) was used for RF excitation and reception. Preliminary experiments were performed on phantoms in order to optimize the sequence for T$_2$ measurement. A vial with water titrated with gadopenteate dimeglumine (Magnevist®, Berlex Pharmaceuticals, Wayne, NJ) to a known T$_2$ (served as a quality control imaging phantom) was placed between the hind paws of the rat.

A multi-slice multi-echo spin echo sequence was used to obtain axial images of the upper hindleg region for the calculation of the T$_2$ maps. The imaging parameters were as follows: matrix size 128×128, field of view (FOV) 5.5×5.0 cm, 16 slices, slice thickness 2 mm, echo time 7 ms, 12 echoes, 2500 ms repetition time, 2 averages. The edge of the slice pack was carefully positioned at the center of the knee joint, which served as a fiducial mark for reproducible slice planning. Each T$_2$ map acquisition took approximately 11 minutes.

**Experiment Protocol**

In order to test for the edema-generating mechanism of the L-type Ca$^{2+}$ channel, nifedipine, both hypertensive SHRs as well as the normotensive SD rats were studied for MRI-measured hindleg edema, femoral artery/vein mRNA and extracellular skeletal muscle albumin...
staining. In another set of experiments, the pharmacological effect of the mixed L- and T-type CCB, mibefradil, was assessed both alone and in combination with nifedipine to assess the influence of the T-type Ca\textsuperscript{2+} channel expression on peripheral edema formation in the SHR.

MBP was allowed to stabilize for 30 minutes. The experiment was aborted if the predrug control mean MBP was greater than 130 mm Hg for the SD rat or less than 130 mmHg for the SHR.

Two T\textsubscript{2} map scans, 9 minutes apart, were acquired to establish the baseline condition. If the baseline was not achieved (i.e. there were statistical differences between average T\textsubscript{2} values in these two scans) the animal was excluded from experiment in the postprocessing stage. The rat was then dosed intravenously (IV) with nifedipine (1, 0.1 or 0.01 mg/kg; n=3 rats/dose), mibefradil (10, 1 or 0.1 mg/kg; n=3 rats /dose), or vehicle (1 ml/kg bolus + 1 ml/kg/60 min; n=3 rats). The compound was delivered as an IV loading dose over 2 minutes, immediately followed by a maintenance infusion for over 60 minutes using a syringe infusion pump. Two T\textsubscript{2} map scans were acquired, one at 30 and 50 minutes after the start of the infusion respectively. These scans from the drug infusion were averaged together for comparison to the pre-drug control scans. The blood pressure lowering was determined as the difference between baseline and a point with a minimum blood pressure, which invariably occurred right at the end of loading dose delivery.

The protocol for the combination of mibefradil with nifedipine involved the same MRI scanning paradigm as described above. The dose selection criteria for the combination of nifedipine and mibefradil was determined in separate experiments (not shown) that demonstrated low, medium and maximum blood pressure lowering.

MRI Data Analysis
Data were zero-padded to matrix size of 256×256 and analyzed using the AFNI software package (NIH, Bethesda, MD). T2 maps were calculated by monoexponential fitting of multi-echo images using the least square error (LSE) fitting. Percent increase in T2 during drug treatment was calculated pixel-by-pixel. To limit the number of false positives, all increases in T2 of less than 10% were set to zero. Regions of interest (ROI) were drawn on the muscle tissue on seven contiguous slices on anatomical images 8 mm distal from the knee joint (tibial plateau), which served as a fiducial mark. The stack of these ROIs was treated as a single volume ROI. Regions with bone structures (tibia and fibula) were excluded from ROI. The number of non-zero voxels (N) and the average change in T2 (ΔT2) over these voxels was calculated in a volume ROI. The integral increase in T2 (I = N × ΔT2) was used as quantitative biomarker for the severity of edema (MRI edema index). This could also be described as the sum of ΔT2 of all voxels within volume ROI, which changed for more than 10%. The quality control phantom had an average T2 value of 44 ms. The data set was considered good for further analysis if there were no voxels in the phantom showing change in T2 more than 10% during a single experiment.

The AFNI software package was utilized for volume measurements of the hindleg skeletal muscle in selected data sets. For volume calculations two-dimensional ROIs were manually drawn on the anatomical images to cover the same regions (i.e., the same number of slices) that were analyzed for T2 maps. The best judgment of human operator was used to discriminate between muscle and non-muscle tissue. Volume was calculated as the sum of the ROI areas. The percent increase in muscle volume was calculated from the pre-drug and post-drug scans.
**Immunohistochemistry**

In a separate set of animals for immunohistochemistry, formalin-fixed, paraffin-embedded sections were used for each SHR proximal hindleg skeletal muscle specimen to assess if extracellular protein accumulation induced by the L-type CCB, nifedipine, caused hindleg edema. Briefly, 1 mg/kg nifedipine or vehicle were delivered as an IV loading dose over 2 minutes, immediately followed by a maintenance infusion for 30 minutes using a syringe infusion pump to three SHRs. Animal euthanasia was immediately performed following the IV infusion of nifedipine or vehicle. For comparison, the same muscle masses in three normotensive Sprague Dawley rats were used as normal skeletal muscle specimens. The tissue blocks were sectioned at 3 µm thick, placed on charged slides and immunohistochemically stained on a Ventana Discovery autostainer. Unless specified, all reagents are from Ventana Medical Systems (Tucson, AZ). Slides were deparaffinized, blocked for 30 minutes in Dako serum-free blocking serum (X0909, Dako) incubated in rabbit anti-human albumin antibody (A0001, Dako 1:4000) for one hour at 37º C, blocked with avidin and biotin blocking reagents for 18 minutes each, then incubated in a biotinylated, anti-rabbit link (H+L) (BA-1000, Vector Labs 1:1,000) for 30 minutes at 37º C. Slides were developed with a DAB kit and counterstained with hematoxylin and bluing reagent for four minutes each, removed from the autostainer, dehydrated, and coverslipped with Permount (Fisher Scientific).

**Real-time Polymerase Chain Reactions**
TaqMan real-time RT-PCR was performed to evaluate the mRNA expression levels in the major vessels that supply the hindleg skeletal muscle, namely the femoral artery and vein. These large vessels were used so that enough mRNA for expression work could be obtained for vascular tissue that perfuses a majority of skeletal muscle. The validation that the femoral vessels can serve as tissue surrogates for the pre- and post capillary vessels on a molecular level has not directly been demonstrated but pharmacologically the conduit femoral arteries have been shown to function similarly to the small femoral arteries (200-300 µm) (Li and Schiffrin, 1996). One microgram of each total RNA sample was used to generate cDNA using the High Capacity cDNA Archive Kit following the manufacturer's protocol (Applied Biosystems, Foster City, CA). PCR assays were performed to measure the expression levels of two differentially expressed genes using rat sequence-based TaqMan Gene Expression assays on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The forward and reverse primers for the L-type Ca\(^{2+}\) channel subtype alpha 1C (CACNA1C; amplicon = 236 bp) were 5’-CAAGAGTTGGTGAGGAGAAGCC and 5’-TGAAGCTCAGAGAGTGGTCG, respectively. The forward and reverse probes for the T-type Ca\(^{2+}\) channel subtype alpha 1H (CACNA1H; amplicon = 177 bp) were 5’-TCGAGGAGGACTTCCACAAG and 5’-TGCATCCAGGAATGGTGAG, respectively. The fluorescence TaqMan probe contained a 6-carboxyfluorescein phosphoramidite (FAM™ dye) label at the 5’ end of the gene and a minor groove binder and nonfluorescent quencher at the 3’ end designed to hybridize across exon junctions. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA expression levels were used for normalization. For each target gene, cDNA corresponding to 20 ng RNA was used per 20 µl reaction. Each reaction was performed in duplicate on a 384-well plate using the standard conditions determined by the
manufacturer. The level of expression was calculated based upon the PCR cycle number (Ct) at which the exponential growth in fluorescence from the probe passes a certain threshold value. Relative expression was determined by the difference in the Ct values for the target genes after normalization to RNA input level, using GAPDH ribosomal RNA Ct values ($\Delta$Ct=$Ct$ of the target gene minus $Ct$ of the internal control GAPDH ribosomal RNA). Relative quantification between different samples (e.g., arterial vs. venous) was determined according to the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct=\Delta Ct$ SHR sample$-\Delta Ct$ normotensive sample) (Livak and Schmittgen, 2001).

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Comparison between vehicle-treated and drug-treated groups were analyzed by the Student’s t-test method. Values of $p<0.05$ were considered statistically significant. For both the mRNA expression and mibefradil/nifedipine combination studies, differences were evaluated using an one-way ANOVA with the Hsu’s mean comparison with the best (Hsu J.C., 1996).
RESULTS

Effects of Nifedipine and Mibebradil on Edema as measured by MRI

To ascertain if MR imaging is capable and sensitive to measure static, interstitial water as a marker for peripheral edema, the L-type CCB, nifedipine (total dose after 60 minutes equals 1 mg/kg), which is known to elicit peripheral edema, was assessed by T2 mapping in the rat hindleg skeletal muscle. As shown in Figure 1, representative T2 maps after 60 minutes of nifedipine constant IV infusion show that nifedipine increases the T2 values measured in a 7 Tesla MRI system compared to the vehicle. The T2 change became measurable after only 30 minutes into the nifedipine infusion and continued to increase slightly during the following 20 min, while MBP dropped immediately after bolus dose delivery and gradually recovered during infusion of the rest of the compound (Figure 2).

To confirm that the observed increase in the integral T2 reflects edema, we measured the volume of muscle tissue on the same MRI scans we used for T2 calculations in both the vehicle and nifedipine treated groups, which represent two extremes – baseline and positive control. The muscle volume did not change in the vehicle treated group (1.5 ± 1.6%, P=0.389 compared to zero) and increased in nifedipine-treated group (7.4 ± 1.3%, P=0.031 compared to zero, P=0.041 compared to the vehicle group). The correlation coefficient between integral T2 and the volume changes were statistically significant (R = 0.741, P = 0.033, Figure 3). Based on this strong correlation, we used the integral T2 increase values as a surrogate for peripheral edema.
Edema Assessment in SHR Skeletal Muscle at Equal Blood Pressure Lowering Doses for Nifedipine or Mibefradil

The average pre-drug mean MBP across all the SHRs was 141 ± 3 mmHg. Nifedipine and mibefradil each decreased mean MBP in a dose-dependent manner with nifedipine being approximately 10 times more potent than mibefradil (Figure 4A). The mean MBP decrease caused by 1 mg/kg nifedipine (-77 ± 4 mmHg; p<0.05) was not statistically different compared with 10 mg/kg mibefradil (-97 ± 5 mmHg).

Compared with the vehicle group, the edema formation during the 30-60 minute IV infusion of nifedipine and mibefradil showed a dose-dependent increase, but the edema caused by mibefradil was significantly (p<0.05) less than the edema caused by equal or less effective antihypertensive doses of nifedipine (Figure 3B). At similar mean BP lowering, nifedipine at 1 mg/kg increased MRI edema index in the SHR hindleg muscle to 2606 ± 86% while the high dose of mibefradil (10 mg/kg) increased the MRI edema index to 981 ± 171% (p<0.05 between treatments; Figure 4B). When compared to the vehicle, the 0.1 mg/kg dose of nifedipine and 1 mg/kg mibefradil significantly increased MRI edema index (1301 ± 419% and 554 ± 129%; p<0.05, respectively) while the low doses of either drug did not increase MRI edema index in the SHR hindleg muscle.

In addition, the capacity of nifedipine to induce peripheral edema was compared between the SHR and a normotensive rat model where antihypertensives have been shown to exert attenuated activity. As shown in Table 1, the BP lowering effect of 1 mg/kg nifedipine was significantly less in the normotensive SD rat when compared to the same dose in the SHR. The MRI edema index was significantly less in the normotensive rat compared to the SHR.
Albumin Expression in SHR Hindleg Muscle Specimens after Nifedipine Infusion

Peripheral edema can be caused by either an increase in capillary hydrostatic pressure forcing water into the extracellular space or by increased extracellular osmotic pressure due to increased protein leakage through the capillary wall. To rule out that protein extravasation into the extracellular space of the SHR hindleg muscle was possibly the mechanism for the rat hindleg edema, immunostaining for albumin in the skeletal muscle formalin-fixed sections was used. As shown in Figure 5C and 5D, no albumin was present in the extracellular space of the muscle after a 30 minute nifedipine (1 mg/kg) or vehicle IV infusion in the SHR. The 30 minute infusion was used since MRI studies shown there was edema developed already at this time point.

Normotensive Sprague Dawley (SD) rats were also investigated as a control for the hypertension in the SHR and no albumin immunostaining was observed in the SD rat after 1 mg/kg nifedipine compared to vehicle (Figure 5B and 5A, respectively). Immunostaining for albumin within vessels in the muscle sections acted as an internal positive control for the albumin antibody in both the SHR and SD rat tissue sections.

Alpha 1H (a T-type Ca\(^{2+}\) channel subunit) and Alpha 1C (a L-type Ca\(^{2+}\) channel subunit) mRNA Expression in the Femoral Artery and Femoral Vein

The mRNA expression levels of the T-type Ca\(^{2+}\) channel subunit, alpha 1H, and the L-type Ca\(^{2+}\) channel subunit, alpha 1C, from arterial and venous tissues were analyzed by the
TaqMan quantitative RT-PCR technique. In the SHR femoral artery, the fold change (from GAPDH housekeeping gene) in alpha 1C (Figure 6A) was $5.3 \pm 2.2$ while the femoral vein alpha 1C (Figure 6B) fold change was $1.4 \pm 0.4$. The fold change in the T-type Ca$^{2+}$ channel mRNA expression was $1.1 \pm 0.1$ in the SHR femoral artery versus $1.2 \pm 0.1$ in the SHR femoral vein. The arterial L- to T-type expression ratio is 4.2 fold compared to the venous expression ratio.

As a comparison, mRNA from femoral arteries and veins in the normotensive SD rat was evaluated to assess L- and T-type Ca$^{2+}$ channel expression levels. As shown in Figure 6A, the alpha 1C expression level was significantly reduced in the femoral artery compared to the SHR artery levels. The venous alpha 1C expression levels were, however, not different between the SHR and normotensive SD rat. As for the T-type Ca$^{2+}$ channel expression levels (i.e., alpha 1H mRNA), there were no significant differences between the arterial or venous tissue levels as well as no difference between the SHR and SD rat.

**Effects of Mibefradil in Combination with Nifedipine-induced Edema in SHR Skeletal Muscle**

Since L-type Ca$^{2+}$ channel expression is greater in the arterial tissue (consistent with a hydrostatic edema mechanism) and T-type expression more prevalent in the veins we thought that adding a T-type CCB might attenuate L-type-induced edema. To determine if the mixed T- and L-type CCB, mibefradil, could attenuate the edema induced by the L-type CCB nifedipine, a combination IV infusion of nifedipine at 1 mg/kg plus varying doses of mibefradil (0, 0.3 or 3 mg/kg) was assessed for edema formation in the SHR hindleg muscle. Representative MRI $\Delta T_2$ maps for the nifedipine and mibefradil combinations are shown in Figure 7. Figure 7A is the 1
mg/kg nifedipine dose alone and shows the greatest increase in integral T2 (i.e., edema) compared to either the nifedipine plus 0.3 mg/kg mibefradil (Figure 7B) or nifedipine plus 3 mg/kg mibefradil combinations (Figure 7C). Upon quantification of the increase in integral T2, a dose-dependent attenuation of the level of edema measured by MRI in the SHR hindleg muscle was observed as shown in Figure 7D. Nifedipine at 1 mg/kg plus vehicle elicited an edema response of 2606 ± 86% while nifedipine plus 0.3 mg/kg mibefradil and nifedipine plus 3 mg/kg mibefradil significantly reduced edema index (1725 ± 306% and 1139 ± 279%, respectively).

The maximum BP lowering of nifedipine alone, nifedipine plus 0.3 mg/kg mibefradil and nifedipine plus 3 mg/kg mibefradil was -77 ± 4, -75 ± 3 and -100 ± 7, respectively. Only the nifedipine plus 3 mg/kg mibefradil combination produced MBP lowering that was significantly different then the other treatment groups.
DISCUSSION

L-type CCBs, although highly effective in lowering BP, also cause lower extremity edema. The incidence is dose-dependent as the result of vasodilation (Messerli, 2003). The edema likely develops by the mechanism of distal arteriolar dilation with capillary leakage common with many CCBs including late generation agents amlodipine and isradipine. To meet the increasingly challenging BP guidelines, the physician is faced with either uptitrating the dose of the CCB or resorting to combination therapy with the addition of an angiotensin converting enzyme inhibitor (ACEI), angiotensin receptor blocker (ARB) and/or diuretic. The development of the non-dihydropyridine CCBs, such as the mixed T- and L-type CCB mibefradil, has improved tolerability by producing less vasodilatory edema (Karch et al., 1997; Kobrin et al., 1997). However, predicting the level of edema caused by novel agents preclinically has plagued new compound development. We report the use of MRI to demonstrate significantly less edema formation with the mixed T- and L-type CCB, mibefradil, compared to the L-type CCB, nifedipine, at equal antihypertensive doses in the SHR model of hypertension. Edema, measured by the MRI parameter, T2, confirms the difference observed clinically in the incidence of edema caused by nifedipine and mibefradil.

Peripheral edema is clinically characterized by a diffuse swelling in the lower extremities and is the most frequent adverse effect reported by patients receiving CCBs. The reported incidence of swollen ankles has been the major measurement for edema due to CCB treatment.
However, determining edema comes late in the drug discovery process and renders a large risk to development of safer CCBs. Objective methods for measuring edema involve the evaluation of foot-ankle volume by water displacement. The changes in foot volume are greater with the less lipophilic CCBs nifedipine and amlodipine than with more lipophilic CCBs manidipine or lercanidipine (Van Hamersvelt et al., 1996; Lund-Johansen et al., 2003). However, only large changes in foot-ankle volume can be detected. A more sensitive measurement of edema earlier in the preclinical and clinical evaluation would enable the discovery and development of safer antihypertensive agents.

The ability to assess edema by MRI currently exists in the hospital setting since most MRI practices are able to perform the T2 mapping for detecting many pathological conditions including muscle edema (May et al., 2000; Patten et al., 2003). Ababneh et al. have shown that the T2 proton relaxation can be fit with two exponential decay components, one short and another long. These components presumably correspond to intracellular (short decay component) and extracellular water (long component) (Ababneh et al., 2005). It was found that edema had a marked dependence on an increase in the T2 long decay component while exercise moderately changed T2 short decay component (Ploutz-Snyder et al., 1997). We used a single-exponential model for T2 relaxation fit for simplicity. During method development (data not shown) we found that the number of refocusing (180°) radio frequency (RF) pulses should be limited to <16 due to RF power deposition and subsequent heating of the subject, which may affect T2 measurement significantly. We have observed a consistent change in T2 values measured with a monoexponential fit after the induction of edema (from 31.2 ± 0.7 sec to 44.8 ± 1.0 sec after 60 min infusion of 1 mg/kg of nifedipine). To increase the dynamic range and sensitivity of the
measurement, we combined both the intensity of the change in T2 ($\Delta T_2$) and the affected area (N, number of pixels with $\Delta T_2 > 10\%$ over baseline) to derive integral T2 parameter, or MRI edema index. Although T2 changes were not quite reaching the plateau 50 min after drug infusion (see Figure 2) we chose to use predetermined time points to quantify the MRI edema index rather than wait for T2 stabilization. To increase the throughput and decrease the effect of anesthesia on edema we decided to limit scanning to 60 min after drug infusions. To validate, at least partially, the use of T2 mapping for detecting edema, we have measured the changes of muscle volume in response to the CCB treatment. These changes were very subtle and detectable only for two extreme cases: the vehicle-treated and nifedipine-treated groups. There was a significant correlation between changes in muscle volume and integral T2 values, which confirms the specificity of T2 mapping method to measure edema. However, the sensitivity of the integral T2 is greater by several orders of magnitude. It should be noted that the change in volume of the muscle is the potential source of error in estimation of integral T2 change as it is based on pixel-by-pixel comparisons. The pixels in the edematous images are slightly shifted comparing to the baseline. However, the extent of the linear shift is very small, as the volume of the muscle changed for only 7.4\% in the extreme case. It should have not changed pixel-by-pixel comparisons significantly.

The next question was whether integral T2 increase is sensitive enough to differentiate between the edema-causing dihydropyridine CCBs (nifedipine) and the mixed T- and L-type CCB, mibefradil. This study describes the novel utility of MRI in differentiating between compounds by measuring their ability to produce edema. A mechanistic limitation inherent in this study stems from the lack of a selective T-type Ca$^{2+}$ CCB. Even though mibefradil has T-
type Ca\textsuperscript{2+} channel blocking activity it also has L-type CCB activity which can confound data interpretations. However, our results implicate that the lessening of the L-type CCB activity and/or increasing the T-type CCB activity can reduce peripheral edema.

Edema formation is increased by nifedipine, in the SHR hindleg muscle in a short period of time (i.e., < 1 hour). A possible mechanism for the increase in extracellular water in the skeletal muscle involves an increased capillary hydrostatic pressure rather than extravasation of serum protein since immunostaining for albumin in muscle was negative. The ability of mibefradil to attenuate edema induced by the nifedipine may indicate a differentiating mechanism in the vasodilatory effects of the two CCBs on precapillary arterioles and the postcapillary venules. This study suggests that acute water accumulation in skeletal muscle is a hemodynamic mechanism versus any vessel wall restructuring, i.e., albumin leakage. The mRNA results for alpha 1H and 1C differential expression demonstrating a 4.2 fold increase in the arterial L- to T-type mRNA expression ratio versus veins are consistent with the hypothesis that peripheral edema caused by CCBs results from selective arterial dilation and increased capillary hydrostatic pressure. The differential inhibitory effects between arteries and veins of various CCBs have been demonstrated where the L-type CCBs dilated arterioles more than venules (Magnon et al., 1995; Harris et al., 1980; Ozawa et al., 2001). Even though the differential T- and L-type Ca\textsuperscript{2+} channel expression between the femoral artery and vein in this study is on major vessels, the results confirm the published differences of particularly the L-type Ca\textsuperscript{2+} channel involvement in dilating the precapillary side to a greater extent than the postcapillary vessels (Li and Schiffrin, 1996). To support the hypothesis that the greater the L-type Ca\textsuperscript{2+} channel present on the arterial side of the capillary the more edema that could result was tested normotensive SD
rats. These studies confirmed that less edema developed in the normotensive rat compared to the SHR at the same dose of nifedipine. This data plus the differential L-type Ca\(^{2+}\) channel mRNA expression provide strong support of the degree of differential L-type Ca\(^{2+}\) channel between the arterial and venous sides which can drive the level of edema. This study has shown that extracellular protein accumulation is not driving the mechanism of CCB-induced peripheral edema and that L-type differential expression between the arterial and venous sides may actually play a large role in driving the peripheral edema formation due to L-type CCBs.

General anesthesia may have confounding effects on animals’ BP and response to hypertensive therapy. However, it was shown that isoflurane had the least effect on cardiovascular parameters especially if the minimal levels of anesthesia were used (Dardai and Heavner, 1987). These levels are what the authors used in this paper. Since cardiovascular parameters drive the edema response the minimal effects should be seen on edema formation using isoflurane.

In summary, this study reveals for the first time that the MRI T\(_2\) mapping provides highly sensitive biomarker of edema, which could be used for drug research. Due to relative simplicity this method has the potential for clinical translatability. At equal BP lowering doses, compounds with both T- and L-type Ca\(^{2+}\) channel blocking activity have significantly less increase in integral T\(_2\) or edema formation than compounds with selective L-type Ca\(^{2+}\) channel blocking activity. In addition, mibefradil can dose-dependently attenuate the peripheral edema induced by nifedipine. This study may indicate that mixed T- and L-type CCBs may equalize the hydrostatic pressure across the capillary bed by equally dilating arterioles and venules, reducing vasodilatory
edema. These findings suggest that the design of novel CCBs should incorporate a mix of T- and L-type Ca^{2+} channel activity to reduce the risk of edema. Thus, the development of a mixed T- and L-type CCB could replace the edema-laden L-type CCBs as antihypertensives.
ACKNOWLEDGEMENTS

The authors wish to thank Drs. David G. Taylor and Robert Leadley for their scientific discussions and editorial assistance in preparing this manuscript.
References


Tolerability of long-term treatment with lercanidipine versus amlodipine and lacidipine in

Li JS and Schiffrin EL (1996) Effect of calcium channel blockade or angiotensin-converting
enzyme inhibition on structure of coronary, renal, and other small arteries in spontaneously

Livak KJ and Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-

Lund-Johansen P, Stranden E, Helberg S, Wessel-Aas T, Risberg K, Ronnevik PK, Istad H, and
Madsbu S (2003) Quantification of leg oedema in postmenopausal hypertensive patients treated
with lercanidipine or amlodipine. *J Hypertens* **21**:1003-1010.

Magnon M, Gallix P, and Cavero I (1995) Intervessel (arteries and veins) and heart/vessel
selectivities of therapeutically used calcium entry blockers: variable, vessel-dependent indexes. *J
Pharmacol.Exp.Ther.* **275**:1157-1166.

Martin RL, Lee JH, Cribbs LL, Perez-Reyes E, and Hanck DA (2000) Mibefradil block of


Footnotes

The authors declare no other sources of funding beyond that of Pfizer, Inc.

The authors declare no conflicts of interest.
Legends for Figures

Figure 1. Representative MRI $\Delta T_2$ maps for vehicle and the L-type CCB, nifedipine, in SHR hindleg muscles. (A) $T_2$-weighted images before and 50-60 min after the IV administration of 1 mg/kg nifedipine. The $\Delta T_2$ is calculated as the percent change in $T_2$ relaxation time measured before and during compound infusion based on the image signal intensity decay over 12 echoes with an MRI echo spacing equal to 7 ms. The third panel from left is a $\Delta T_2$ map image showing the extracellular water accumulation levels with the green-blue areas the highest water content (100%) and the orange-red the lowest level of water accumulation (0%). (B) Similar $T_2$-weighted images and $\Delta T_2$ maps as in (A) but with the vehicle control. Notice that no $T_2$ changes occurred with the vehicle. A phantom was used in MR imaging to ensure stability of $T_2$ measurements during each single experiment.

Figure 2. Time course of MBP (solid line, left Y-axis) and ROI averaged $T_2$ (dashed line, right Y-axis) in SHRs after infusion of nifedipine (1 mg/kg, black circles) and mibefradil (10 mg/kg, open circles). The beginning of loading dose delivery is marked as zero time point. Data are means ± SEM.

Figure 3. Correlation between integral $T_2$ change and muscle volume change after infusion of vehicle (open circles) and nifedipine (1 mg/kg, black circles). Correlation coefficient ($R = 0.741$, $P = 0.033$) was computed with Pearson Product Moment statistic.
Figure 4. Effects of nifedipine and mibefradil on SHR blood pressure and increase in % integral $T_2$ ($N \times \text{average } \Delta T_2$) as an MRI index of peripheral edema. (A) The MBP lowering effects of nifedipine (0.01, 0.1 and 1 mg/kg) and mibefradil (0.1, 1 and 10 mg/kg) in the anesthetized SHR. Nifedipine is 10 fold greater potency in BP lowering than mibefradil. (B) Dose-response to nifedipine (0.01, 0.1 and 1 mg/kg) and mibefradil (0.1, 1 and 10 mg/kg) on SHR hindleg edema formation. Nifedipine has a 2.5 fold greater propensity to form edema than mibefradil. The data in both (A) and (B) are means ± SEM. * = significant difference from vehicle group, P<0.05.

Figure 5. Immunostaining for albumin shows no protein in the rat hindleg muscle interstitial space. Albumin expression was determined by immunohistochemistry. Representative images of immunostaining for both formalin-fixed, paraffin-embedded normotensive Sprague Dawley (A and B) and SHR (C and D) hindleg skeletal muscle. Arrows indicate specific albumin staining (dark brown areas) inside blood vessels as positive staining control for antibody. Vehicle control tissues for both animals (A and C) as well as the edema-inducing L-type CCB, nifedipine tissues (B and D) show no albumin staining in the muscle. The albumin-specific antibody was visualized by diaminobenzidine (DAB) chromogen; magnification, x20.

Figure 6. Alpha 1H (a T-type Ca$^{2+}$ channel subunit) and Alpha 1C (a L-type Ca$^{2+}$ channel subunit) mRNA expression in the femoral artery and femoral vein of the rat. For
quantitative RT-PCR data, the relative levels (fold change) of the alpha 1C (A) and alpha 1H (B) mRNA expressions were normalized to the housekeeping gene coding for GAPDH ribosomal RNA. The data are means ± SEM with significance determined at the p<0.05 level using an one-way ANOVA with mean comparison by Hsu’s MCB. * = significance for alpha 1C gene expression compared to normotensive SD rat femoral artery, SHR femoral vein and normotensive SD rat femoral vein.

Figure 7.  Mibefradil attenuates edema formation induced by nifedipine. ∆T2 maps were determined as increase of T2 from the baseline to 30-60 minutes into the IV co-infusion of 1 mg/kg nifedipine plus vehicle (A), 1 mg/kg nifedipine plus 0.3 mg/kg mibefradil (B) and 1 mg/kg nifedipine plus 3 mg/kg mibefradil (C). The highest level of T2 change is depicted in the orange-red colors (100%) while the lowest T2 change is shown in green-blue colors (0%). The highest integral T2 increase was observed with the nifedipine/vehicle group and the lowest integral T2 change with the nifedipine plus mibefradil (3 mg/kg). (D) Dose-response to mibefradil (0, 0.3 and 3 mg/kg) in attenuating SHR hindleg edema formation induced by 1 mg/kg nifedipine. Mibefradil at 3 mg/kg was able to reduce the edema formed by nifedipine by approximately 2.5 times compared to the nifedipine/vehicle combination. The data are mean ± SEM; 1N + 0M = 1 mg/kg nifedipine plus vehicle, 1N + 0.3M = 1 mg/kg nifedipine plus 0.3 mg/kg mibefradil, 1N + 3M = 1 mg/kg nifedipine plus 3 mg/kg mibefradil. Asterisk denotes significance of 1N + 0.3M and 1N + 3M vs 1N + 0M, p<0.05.
Tables.

TABLE 1  Comparison of Hindleg Edema and Blood Pressure Lowering of 1 mg/kg Nifedipine between the SHR and SD rat.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Hindleg Edema (% increase in integral T2)</th>
<th>Max MBP Lowering (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>2606 ± 86 (n= 6)</td>
<td>-77 ± 4 (n= 6)</td>
</tr>
<tr>
<td>SD rat</td>
<td>1439 ± 129* (n= 3)</td>
<td>-19 ± 3* (n= 3)</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
* p<0.05 Student’s t-test; SHR vs. SD rat
Figure 2

The graph shows the changes in MBP (mmHg) and ROI averaged T₂ (ms) over time before and after the loading dose infusion. The x-axis represents the time before and after the loading dose infusion in minutes, ranging from -40 to 40. The y-axis on the left represents MBP, ranging from 20 to 160 mmHg, and the y-axis on the right represents ROI averaged T₂, ranging from 20 to 60 ms.

Different lines and markers represent different conditions and treatments:
- Solid black dots with error bars: MBP, Nifedipine
- Dashed white dots with error bars: MBP, Mibefradil
- Solid black line: ROI averaged T₂, Nifedipine
- Dashed white line: ROI averaged T₂, Mibefradil

The graph illustrates the impact of different medications on blood pressure and ROI averaged T₂ over time.
Figure 3

![Graph showing the relationship between integral T2 change and muscle volume change with different markers for Vehicle and Nifedipine, 1 mg/kg](image)

R = 0.741
(P = 0.033)
Figure 5

Sprague Dawley

A

B

SHR

C

Vehicle Control

D

1 mg/kg Nifedipine
Figure 7

(A) 1N+0M
(B) 1N+0.3M
(C) 1N+3M

(D) Peripheral Edema

% Increase in T2

1N + 0M  1N + 0.3M  1N + 3M