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Metabolism of the dihydropyridine calcium channel blockers mebudipine and dibudipine by isolated rat hepatocytes

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Abstract

The prototype 1,4-dihydropyridine (1,4-DHP) nifedipine, indicated for the management of hypertension and angina pectoris, has drawbacks of rapid onset of vasodilating action and a short half-life. Several newer analogues have been designed to offset these problems and these include mebudipine and dibudipine. These analogues contain *t*-butyl substituents that have been selected to alter the fast metabolism without altering pharmacological activity. In this study, the metabolism of mebudipine and dibudipine by isolated rat hepatocytes has been investigated. These compounds were extensively metabolized in 2 h by oxidative pathways, analogous to those known for nifedipine, and by *O*-glucuronidation after hydroxylation of the *t*-butyl substituents. The in-vitro half-lives of mebudipine (22 ± 7.1 min) and dibudipine (40 ± 9.8 min) were significantly longer than that of nifedipine (5.5 ± 1.1 min), which was investigated in parallel in this study. These newer 1,4-DHPs address the problem of the short half-life of nifedipine and have potential for further development in view of their comparable potency to nifedipine.

Introduction

The 1,4-dihydropyridines (1,4-DHPs) inhibit the trans-membrane influx of calcium ions into cardiac and vascular muscle cells and are indicated in the management of hypertension and angina pectoris (Roden 2001). The prototype, nifedipine (see Figure 1), is clinically effective but has a number of undesirable pharmacokinetic and pharmcodynamic properties, which include a rapid onset of vasodilating action, a short half-life and side effects such as reflex tachycardia, flushing, headache and dizziness (van Zwieten et al 1993; Borchard 1994). Nifedipine undergoes significant first-pass metabolism to highly soluble inactive metabolites. Several newer 1,4-DHP analogues, which seek to overcome these undesirable properties, have been developed and include amlodipine, felodipine, lacidipine, nicardipine, nitrendipine and barnidipine (Roden 2001).

Recently, we reported the synthesis of two new 1,4-DHP calcium channel blockers, mebudipine (see Figure 3) and dibudipine (see Figure 2) (Mahmoudian et al 1997). These compounds contain *t*-butyl substituents selected to decrease the fast metabolism of the 1,4-DHPs without altering their pharmacological activity. The incorporation of *t*-butyl substituents, as in terfenadine, has been used previously in drug development to manipulate the pharmacological half-life with some success. The half-life of the di (*t*-butyl) substituted dibudipine (2.5 h) determined in-vivo in the rat was longer than those of other DHPs such as nicardipine (0.1 h), benidipine (0.5 h), nisoldipine (0.4 h), nitrendipine (1.3 h), felodipine (1.5 h) and nilvadipine (1.3 h) (Teramura et al 1997) and comparable with that of the long-acting amlodipine (Stopher et al 1988). Also in a previous study, the pharmacological potencies of mebudipine and dibudipine were evaluated by studying their effects on guinea-pig isolated ileum and the pIC50 values for the inhibition of the contractile response to electrical stimulation were found to be similar to that of nifedipine (Mahmoudian et al 1997). The compounds also antagonize the contractile responses of K^+ depolarized guinea-pig ileum to cumulative concentrations of calcium with the inhibitory effects in the order mebudipine > nifedipine = dibudipine. The vasorelaxant actions of mebudipine and dibudipine have been reproduced in a human vascular preparation (Mahmoudian et al 1999).

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In this study, we have investigated the metabolism of these novel 1,4-DHPs by freshly isolated rat hepatocytes. The effect of the *t*-butyl substituent in attenuating the fast metabolism of the parent 1,4-DHP molecule has also been examined.

Materials and Methods

Materials

Mebudipine ((±)-*t*-butyl, methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl-3,5-pyridine dicarboxylate) and dibudipine (bis-*t*-butyl-1, 4-dihydro-2,6-dimethyl-4-(3-nitrophenyl-3,5-pyridine dicarboxylate) were synthesized by the method of Mahmoudian et al (1997) and the purity determined by LC-MS (≥ 95%). HPLC-grade acetonitrile, methanol and ethylacetate were obtained from BDH Laboratory Supplies (Poole, UK). All other chemicals were of analytical reagent grade unless otherwise stated.

Hepatocyte incubations

Freshly isolated hepatocytes were obtained from male Sprague Dawley rats (190–210 g). Anaesthesia was induced in rats with 60 mg kg^{-1} intraperitoneal phenobarbital sodium (Sagatal 60 mg mL^{-1}; Aventis, UK) and hepatocytes were isolated from whole livers by a two-step collagenase perfusion as described previously (Moldeus et al 1978). The viability of the cells as determined by Trypan blue exclusion was typically ≥ 85%. Portions (10 μL) of dibudipine, mebudipine and nifedipine solutions (100 mM in dimethyl sulfoxide (DMSO)) were added to 10 mL of hepatocyte suspension (2.4×10^6 cells/mL) in Krebs-Henseleit buffer pH 7.6 containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (12.5 mM) in rotating 50 mL round-bottom flasks to produce a final concentration of 100 μM. The flasks were maintained at 37 °C in a water bath in an atmosphere of O_2/CO_2 (95:5% v/v) and samples taken at specific intervals over a 2-h incubation period. The samples were immediately frozen at −20°C before analysis. Either the compounds or hepatocytes were omitted from control incubations. All experiments using animals were carried out under Home Office project licence PPL 60/2055.

Sample preparation

The hepatocyte incubations were sampled at specific times (0, 5, 15, 30, 60 and 120 min after the addition of the 1,4-DHP analogue) and immediately stored at −20°C before sample preparation and analysis. Samples were thawed and deproteinated with perchloric acid (60% v/v, 20 μL/1 mL of cell suspension) followed by centrifugation at 1500 *g* for 5 min. The supernatant was loaded onto an Isolute SPE cartridge (500 mg sorbent, 3 mL capacity, C4 for dibudipine and C8 for mebudipine and nifedipine), which had been activated and conditioned with methanol (1 mL) and water (1 mL), respectively. Interferents were washed with ammonium formate buffer (2 mM, pH 3.5, 3 × 1 mL) and the retained analytes eluted with ethyl acetate (3 mL). The ethyl acetate extract was reduced to dryness at 45°C under a stream of nitrogen and the residue reconstituted in methanol (200 μL) before analysis by HPLC. The amount of the parent 1,4-DHP analogue remaining after the specified times was quantified by HPLC using an external standard and the data were fitted to a first-order exponential decay model using GraphPad Prism software Version 2.01 (GraphPad Software Inc., CA) to determine the half-lives and elimination constants.

To confirm glucuronidation, the dried ethyl acetate extract was reconstituted in sodium acetate buffer (0.1 M, 1 mL, pH 5.0), hydrolysed in the presence of β-glucuronidase Type II (from *Helix pomatia*) at 37°C for 24 h and subsequently sampled for HPLC.

Liquid chromatography–mass spectrometry (LC-MS)

Full scanning and selected ion monitoring (SIM) data were obtained on a TSQ7000 triple quadrupole spectrometer fitted with an in-line electrospray source. Metabolites and drug standards were eluted from an ODS column (5 μm, 150 × 4.6 mm i.d.; Thermo Hypersil-Keystone, UK) at ambient temperature. The following gradient programmes with a mobile phase composed of a mixture of acetonitrile and either 0.5% formic acid in water (for nifedipine) or ammonium formate buffer (2 mM, pH 3.5; for dibudipine and mebudipine) were used in analyses: nifedipine–acetonitrile (10–45% in 50 min); mebudipine–acetonitrile (5–85% in 45 min); dibudipine–acetonitrile (10–65% in 20 min, then 65% for 25 min). The eluent was delivered at a flow-rate of 0.7 mL min^{-1} using a Spectra system P4000 quaternary pump equipped with an AS3000 autosampler (20 μL fixed loop) and analytes were monitored in-line before MS analysis using a Spectra System UV6000LP photodiode array detector. Nitrogen was used as the nebulizing and drying gas in MS analysis at a capillary temperature of 300°C. Full scanning mass spectra were acquired between m/z 100–900 at 1 scan/s. Daughter spectra were acquired under the following conditions: ESI spray voltage 4.5 kV; collision energy 30 eV, collision cell pressure 1.0 mT, scan range m/z 15–900 at 1 scan/s using argon as the collision gas.

Statistical analyses

Statistical analysis of variance was performed using Minitab statistical software (Vs 13.1, Minitab Inc.) followed by the Newman–Keuls range test (at a 5% significance level) to determine the differences between the half-lives of nifedipine, mebudipine and dibudipine.

Results and Discussion

Nifedipine, the prototype of the 1,4-DHP calcium channel blockers, is metabolized rapidly in-vivo and has a half-life of approximately 1.2 h in the rat (Mohri et al 2000). In 1,4-DHP-type calcium channel blockers, such as nifedipine (Scherling et al 1992), felodipine (Baarnhielm et al 1986), nitrendipine (Scherling et al 1991a) and amlodipine (Beresford et al 1988),

oxidation of the 1,4-DHP ring to its corresponding pyridine is the primary route of metabolism. However, this biotransformation leads to the loss of pharmacological activity (Ramsch et al 1986). Therefore, synthetic analogues and controlled-release pharmaceutical formulations have been used to address the loss of activity due to the rapid hepatic biotransformation of the parent 1,4-DHP ring.

The new 1,4-DHPs dibudipine and mebudipine were shown in previous studies to possess pharmacological potencies comparable with that of nifedipine. In this study, the metabolic profiles of dibudipine and mebudipine, which were designed by incorporating bulky *t*-butyl substituents to modulate the undesirable pharmacokinetics of nifedipine, have been evaluated. The in-vivo metabolic pathways of nifedipine are well documented (Scherling et al 1992) and have therefore been used to validate the in-vitro hepatocyte system used in this study. Using LC-MS/MS analyses, the major pathways of metabolism of nifedipine reported for the rat in-vivo were reproduced with the present in-vitro model (Figure 1). Nifedipine underwent the characteristic 1,4-DHP oxidation to the pyridine metabolite (N-1), methyl ester hydrolysis (N-8), oxidation of the C-2 methyl substituent (N-3) with subsequent oxidation to the carboxylic acid (N-4) and *O*-glucuronidation (N-5). Nifedipine formed the characteristic lactone metabolite (N-2), which proceeds to hydroxy carboxylic acid metabolites N-6 and N-7. Based on UV detector responses, metabolites N-7 and N-8 were identified as the two major metabolites of nifedipine, an observation consistent with the in-vivo metabolism in the rat (Scherling et al 1992).

Under identical metabolism conditions, dibudipine (100 μM) and mebudipine (100 μM) were metabolized extensively by rat hepatocytes and were not detectable in the total cell incubation system after 2 h. In dibudipine, both methyl esters in nifedipine have been replaced with the relatively bulkier *t*-butyl ester moiety whereas mebudipine has only one substituted methyl ester. LC analyses of incubations of dibudipine with hepatocytes demonstrated the presence of at least seven secondary peaks (D1–D7) for dibudipine and nine secondary peaks (M1–M9) with mebudipine (Table 1), which were absent in controls.

LC-MS analyses identified the presence of the oxidized dihydropyridines D-1 and M-1. The shift in UV absorbance of these metabolites (λmax 265 nm) relative to the parent

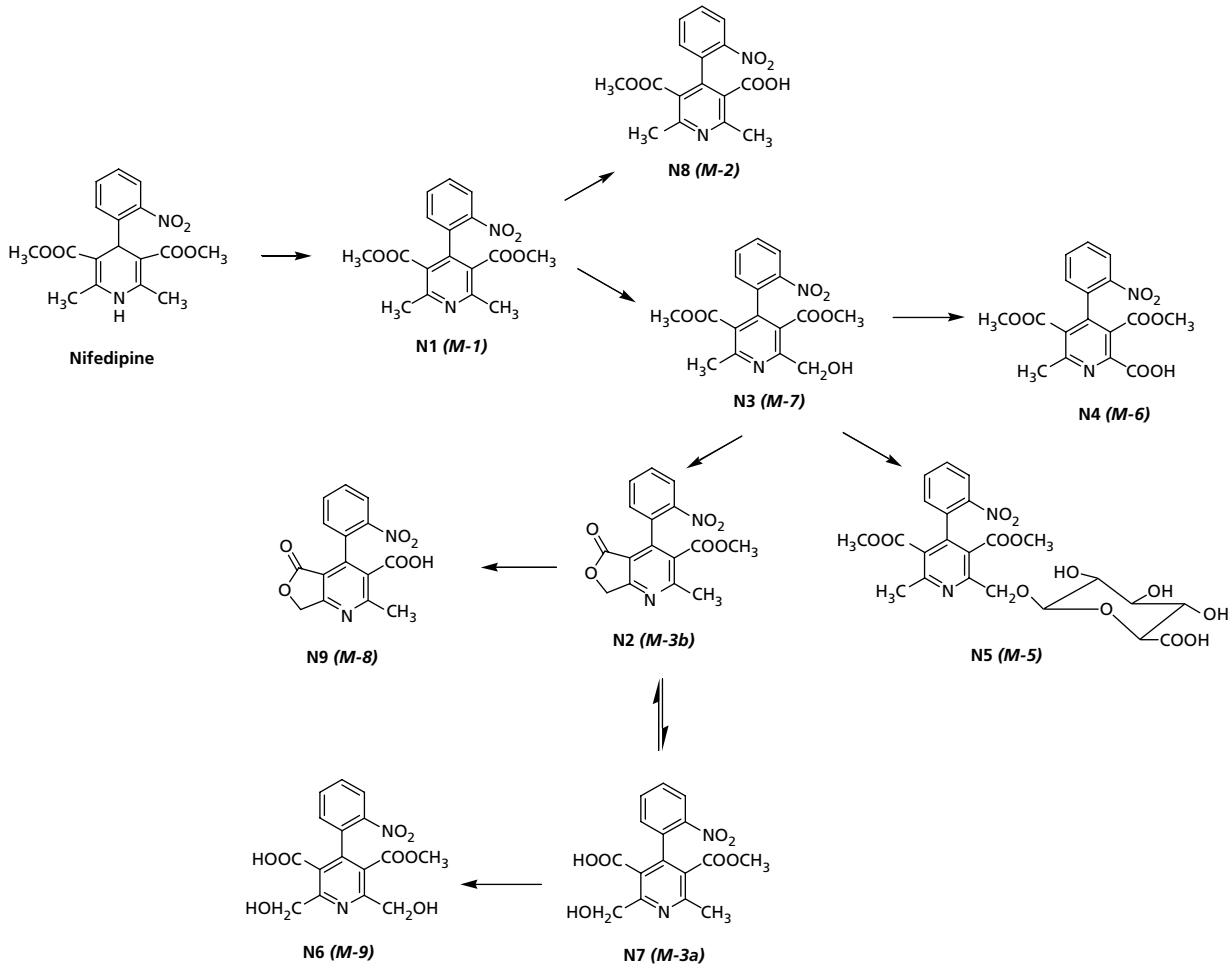

Figure 1 Pathway for the metabolism of nifedipine by rat isolated hepatocytes. Designations in parenthesis represent the corresponding metabolite reported by Scherling et al (1992).

Table 1 Mass spectra (MS/MS fragmentation) and chromatographic retention times for the metabolites of dibudipine (D-1 to D-7) and mebudipine (M-1 to M-9)

Dibudipine	Mebudipine
D-1 (m/z 429, t_R 32.7 min) m/z 373 $[M - (H_2C{=}C(CH_3)_2]^+$, 317* $[M - 2(CH_2{=}C(CH_3)_2]^+$, 271 $[M - 2(H_2C{=}C(CH_3)_2) - NO_2]^+$	M-1 (m/z 387, t_R 40.2 min) m/z 387, 331* $[M - (H_2C{=}C(CH_3)_2]^+$, 285 $[M - (H_2C{=}C(CH_3)_2) - NO_2]^+$ M-2 (m/z 403, t_R 28.7 min) m/z 347* $[M - H_2C{=}C(CH_3)_2]^+$, 329 $[M - H_2C{=}C(CH_3)_2 - H_2O]^+$, 297 $[M - H_2C{=}C(CH_3)_2 - H_2O - CH_3OH]^+$
D-2 (m/z 445, t_R 27.8 min) m/z 389 $[M - (H_2C{=}C(CH_3)_2]^+$, 333* $[M - 2(H_2C{=}C(CH_3)_2]^+$, 315 $[M - 2(H_2C{=}C(CH_3)_2) - H_2O]^+$, 297 $[M - 2(H_2C{=}C(CH_3)_2) - 2(H_2O)]^+$	
D-3 (m/z 445, t_R 24.6 min) m/z 389 $[M - (H_2C{=}C(CH_3)_2]^+$, 373 $[M - H_2C{=}C(CH_3)CH_2OH]^+$), 317* ($[M - H_2C{=}C(CH_3)_2 - H_2C{=}C(CH_3)CH_2OH]^+$	M-3 (m/z 357, t_R 28.3 min) m/z 301* $[M - H_2C{=}C(CH_3)_2]^+$, 269$[M - H_2C{=}C(CH_3)_2 - CH_3OH]^+$, 257 $[M - H_2C{=}C(CH_3)_2 - CO_2]^+$, 225 $[M - H_2C{=}C(CH_3)_2 - CO_2 - CH_3OH]^+$
D-4 (m/z 621, t_R 14.4 min) m/z 565 $[M - (CH_2{=}C(CH_3)_2]^+$, 509* ($[M - 2(CH_2{=}C(CH_3)_2]^+$, 445 $[M - DHGA]^+$, 389 $[M- CH_2{=}C(CH_3)_2 - DHGA]^+$, 333 $[M - 2(H_2C{=}C(CH_3)_2) - DHGA]^+$, 297 $[M - 2(H_2C{=}C(CH_3)_2) - DHGA - 2(H_2O)]^+$	M-4 (m/z 403, t_R 24.6 min) m/z 331 $[M - H_2C{=}C(CH_3)CH_2OH]^+$
D-5 (m/z 621, t_R 13.5 min) m/z 565 $[M - CH_2{=}C(CH_3)_2]^+$, 445 $[M - DHGA]^+$, 389 $[M - H_2C{=}C(CH_3)_2 - DHGA]^+$, 373 $[M - (H_2C{=}C(CH_3)_2 - (DHGA + O)]^+$, and 317* $[M - (DHGA + O) - H_2C{=}C(CH_3)CH_2OH]^+$, 299 $[M - (DHGA + O) - H_2C{=}C(CH_3)CH_2OH - H_2O]^+$, 271 $[M - (DHGA + O) - H_2C{=}C(CH_3)CH_2OH - 2(H_2O)]^+$	M-5 (m/z 579, t_R 18.9 min) m/z 523 $[M - H_2C{=}C(CH_3)_2]^+$, 347 $[M - H_2C{=}C(CH_3)_2 - DHGA]^+$
D-6 (m/z 373, t_R 11 min) m/z 317* $[M - (H_2C{=}C(CH_3)_2]^+$, 271 $[M - H_2C{=}C(CH_3)_2 - NO_2]^+$	M-6 (m/z 579, t_R 17.3 min) m/z 579, 403 $[M - DHGA]^+$, 331* $[M - DHGA - H_2C{=}C(CH_3)CH_2OH]^+$
D-7 (m/z 389, t_R 10 min) m/z 333* $[M - H_2C{=}C(CH_3)_2]^+$, 315 $[M - H_2C{=}C(CH_3)_2 - H_2O]^+$, 297* $[M - H_2C{=}C(CH_3)_2 - 2(H_2O)]^+$, 251 $[M - H_2C{=}C(CH_3)_2 - 2(H_2O) - NO_2]^+$	M-7 (m/z 373, t_R 17.1 min) m/z 317* $[M - H_2C{=}C(CH_3)_2]^+$, 299 $[M - H_2C{=}C(CH_3)_2 - H_2O]^+$, 271 $[M - H_2C{=}C(CH_3)_2 - NO_2]^+$
	M-8 (m/z 389, t_R 16.0. min) m/z 333 $[M - H_2C{=}C(CH_3)_2]^+$, 315 $[M - H_2C{=}C(CH_3)_2 - H_2O]^+$, 297* $[M - H_2C{=}C(CH_3)_2 - 2(H_2O)]^+$
	M-9 (m/z 331, t_R 10.6 min) m/z 285 $[M - NO_2]^+$, 251 $[M - NO_2 - OCH_3]^+$, 240 $[M - NO_2 - COOH]^+$, 210 $[M - NO_2 - COOH - OCH_3]^+$

Base peak, dehydroglucuronic acid (DHGA)

compounds (λmax 235 nm) is consistent with aromatization of the DHP ring (Scherling et al 1991a). Two conjugated metabolites (D-4 and D-5) identified by LC-MS as *O*-glucuronides of dibudipine, underwent the loss of *t*-butyl susbtituents and the diagnostic elimination of dehydroglucuronic acid (Table 1). In the production of fragment ions in MS/MS analyses, the loss of the *t*-butyl and hydroxylated *t*-butyl moieties was characterized by the neutral loss of isobutene (56 Da) and 3-hydroxy-2-methylprop-1-ene (72 Da), respectively, from the protonated molecular ions. Following the loss of dehydroglucuronic acid and 3-hydroxy-2-methylprop-1-ene, D-5 yielded ions at m/z 299 and 271. This represents the sequential loss of two water molecules due to a MacLafferty rearrangement and internal esterification, an observation that is consistent with hydroxylation of either the 2- or 6-methyl substituents (Scherling et al 1992). Enzymatic hydrolysis of the conjugates using β-glucuronidase resulted in an increase in the chromatographic peaks due to the putative aglycone metabolites D-2 and D-3, the daughter spectra (Table 1) of which are consistent with aromatization of the dihydropyridine ring and methyl hydroxylation at the C-2 position or the C-3 of the *t*-butyl moiety. A product of *t*-butyl ester hydrolysis (D-6) and of methyl hydroxylation and t-butyl ester hydrolysis (D-7) were also identified by LC-MS. A summary of the proposed pathway for the metabolism of dibudipine by rat isolated hepatocytes is shown in Figure 2.

Mebudipine was biotransformed to conjugated metabolites M-5 and M-6. These were identified by LC-MS as

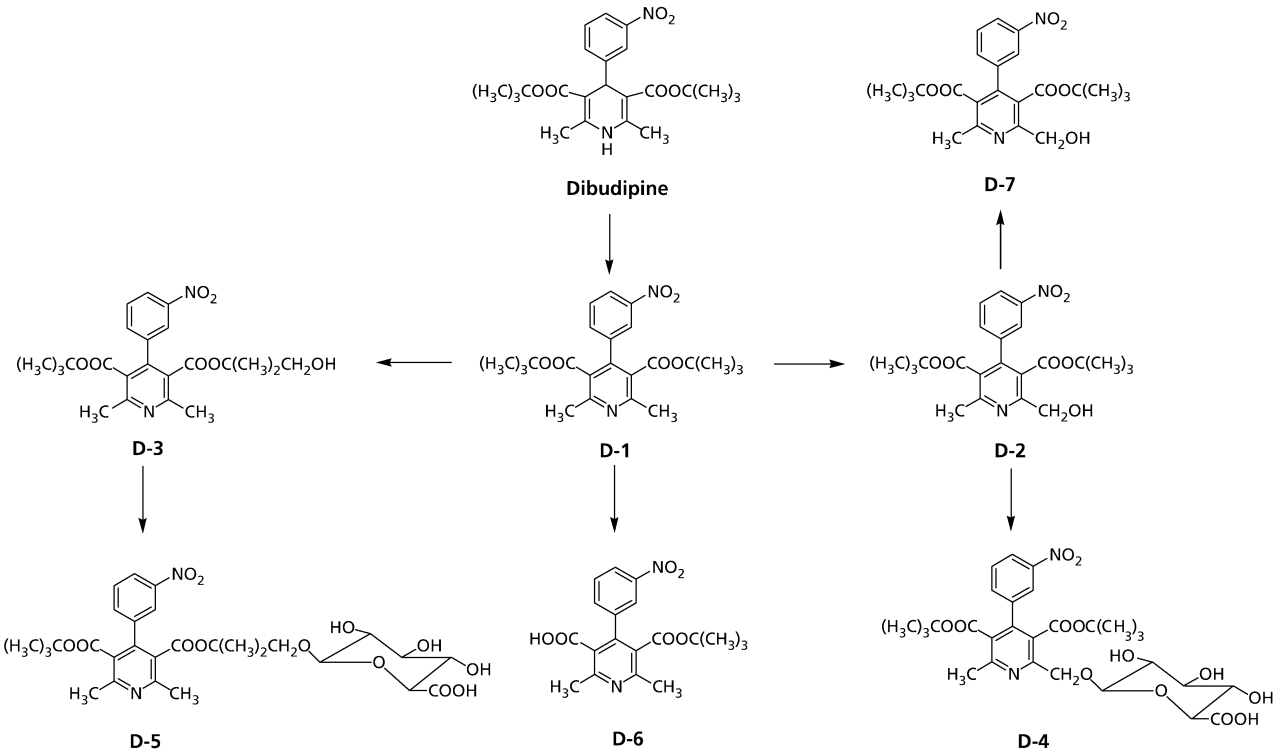

Figure 2 Proposed pathway for the metabolism of dibudipine (100 μM) by rat isolated hepatocytes.

glucuronides and underwent the diagnostic elimination of dehydroglucuronic acid (176 amu). Enzymatic hydrolysis of the conjugates using β-glucuronidase resulted in an increase in the peaks due to the putative aglycone metabolites M-2 and M-4, the spectra of which are consistent with aromatization of the dihydropyridine ring and the hydroxylation pathways described for dibudipine.

In mebudipine, the aromatic nitro moiety was reduced to an amine (M-3). Although this pathway was not detected with either nifedipine or dibudipine, a similar biotransformation has been reported for nitrendipine (Scherling et al 1991a), benidipine (Kobayashi & Kobayashi 1998) and nimodipine (Scherling et al 1991b). The methyl ester at C-4 in mebudipine was hydrolysed to form metabolite M-7 analogous to the methyl ester hydrolysis in nifedipine. Hydrolysis of the *t*-butyl ester resulted in the formation of M-9. Metabolite M-8, a product of methyl hydroxylation of M-1, yielded daughter spectra containing ions at 333 ($[M - C(CH_3)_3]^+$), 315($[M - C(CH_3)_3\text{-}H_2O]^+$), 297 ($[M - C(CH_3)_3\text{-}2H_2O]^+$) and 251 ($[M - C(CH_3)_3\text{-}2H_2O\text{-}NO_2]^+$). The proposed pathway for the metabolism of mebudipine by rat isolated hepatocytes is shown in Figure 3.

Dibudipine and mebudipine undergo extensive oxidative metabolism similar to that reported for nifedipine (Scherling et al 1992). The extensive first-pass metabolism of nifedipine to the pyridine and other highly soluble metabolites is a critical step in the loss of therapeutic effect. Potential clinical substitutes for nifedipine will require metabolic stability, similar pharmacological/therapeutic profiles and a longer half-life in the biological system. In this study, the rate of metabolism of the parent 1,4-DHP was determined for nifedipine, dibudipine and mebudipine by HPLC quantification of the amount(s) of parent compound remaining at specific time points (0, 5, 15, 30, 60, 90, 120 min) after the start of the incubation. The data were fitted to a first-order exponential decay model using Prism software to determine the half-lives and elimination constants. The $t_{1/2}^{1}$ values for the three compounds were found to be significantly different and were observed to increase with the number of *t*-butyl substituents (e.g. dibudipine (40.1 ± 9.8 min) > mebudipine (22.0 ± 7.1 min) > nifedipine (5.5 ± 1.1 min, mean $\pm$ s.d., n = 3 separate incubations)). The mean elimination constants ((K_{el}) were determined as 0.02 ± 0.003, 0.03 ± 0.01 and $0.13 \pm 0.03\ min^{-1}$ for dibudipine, mebudipine and nifedipine, respectively.

Conclusion

The metabolism of the novel 1,4-DHP calcium channel blockers mebudipine and dibudipine by rat isolated hepatocytes has been evaluated. These compounds are metabolized extensively through oxidative pathways and by *O*-glucuronidation. In contrast to nifedipine, these compounds did not show evidence of lactone formation, which is produced by hydroxylation followed by internal esterification. However, the nature of the major metabolites identified in this study is consistent with those reported for the closely related analogues. In a previous study, mebudipine and dibudipine were demonstrated to have identical pharmacological effects to the prototype 1,4-DHP nifedipine. In this study, it has been demonstrated that the substitution of the methyl-ester moiety

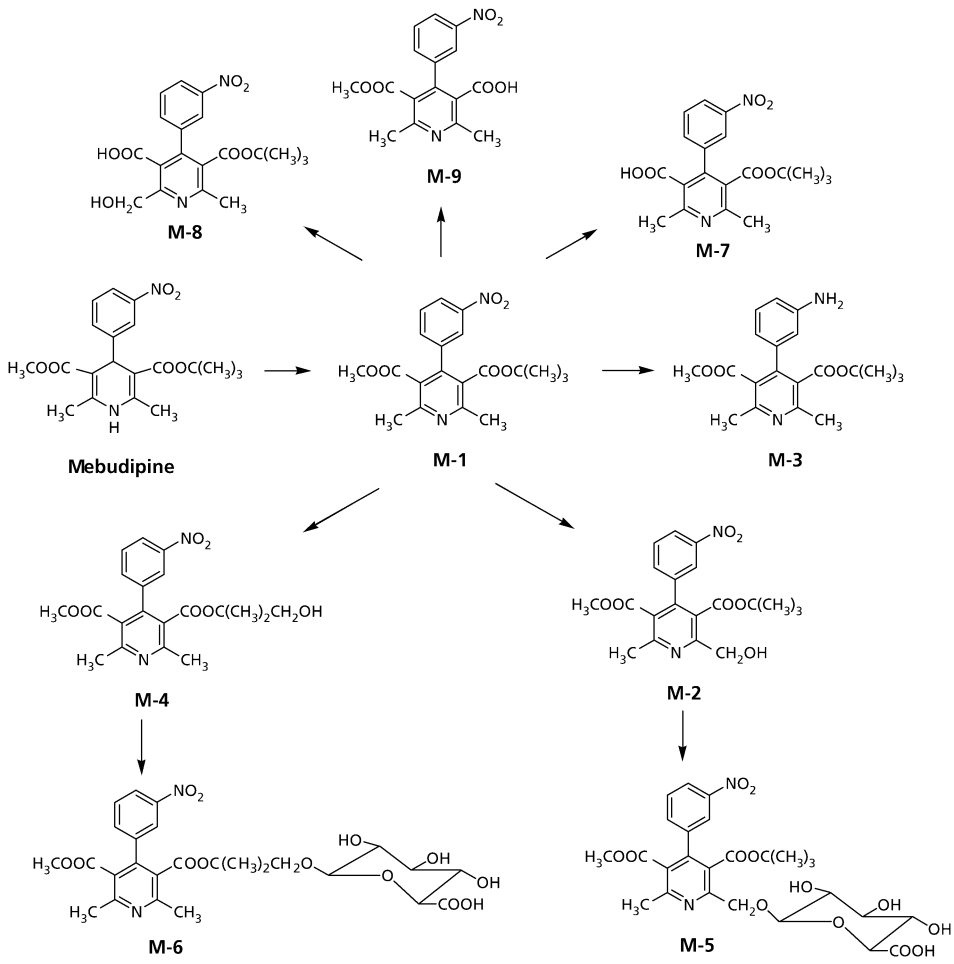

Figure 3 Proposed pathway for the metabolism of mebudipine (100 μM) by rat isolated hepatocytes.

with *t*-butyl esters, as in mebudipine and dibudipine, leads to a reduction in the rate of conversion of the parent 1,4-DHP to the inactive metabolites. These newer 1,4-DHPs address the problem of the short half-life of nifedipine, are metabolically more stable and possess pharmacological activity comparable with that of nifedipine and may therefore be suitable for further development as potential therapeutic alternatives to the existing 1,4-DHP calcium channel blockers.

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