Eur J Med Chem (1996) 31, 71–75

Short communication

Synthesis, binding affinity and antioxidant activity of new 1,4-dihydropyridines

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(Received 18 July 1995; accepted 20 September 1995)

Summary — The synthesis and *in vitro* pharmacology of a series of triaryl-1,4-dihydropyridines were investigated. Molecules containing a nitro group on the phenyl ring had a higher affinity for specific [^{3}H](+)-PN 200-110 binding sites. All the compounds possessed a radical scavenging effect and an antiperoxidant activity. These properties were more marked for molecules with 2-pyridinyl and 2-thienyl substituents. The synthesis of new triaryl-1,4-dihydropyridines with both a higher binding affinity and antioxidant activity might be effective in cerebral ischemic disease treatment.

triaryl-1,4-dihydropyridine / binding affinity / radical scavenging effect / antioxidant

Introduction

It is now recognized that disturbances in the homeostasis of intracellular Ca^{2+} may cause a variety of toxicological and pathological processes. Calcium ions mediate or propagate ischemic cell damage, for instance by increasing the damage caused by oxygen free radicals to the mitochondrial electron transport chain [1]. Furthermore, studies showed that lipid peroxidation may be implicated in cerebral injury by ischemia and reoxygenation [2, 3]. These data suggest that molecules that combine both radical scavenging and calcium antagonist properties may be of particular interest in protecting against ischemia reperfusion damage. An increase in the number of 1,4-dihydropyridine (DHP) binding sites (approximately 60%) suggesting an increase of L-type calcium channels was observed *in vitro* and *in vivo* in rat brain after short-term experimental ischemia [4, 5] and DHP L-type calcium channel blockers were proposed in cerebral ischemic disease treatment [6, 7]. Their main recognized mechanism is the inhibition of the entry of calcium ions across the cell membrane but recent studies showed an antiperoxidant activity for several DHP [8]. The introduction of aromatic rings in the structure of the DHPs may be increase the capacity of free radical scavenging without important decrease of the affinity with DHP receptors. Taking into account the compatibility between the dual activities of new potential active compounds, we decided to synthesize triaryl-1,4-dihydropyridines. We wish to report the affinity for DHP receptors labeled with [^{3}H](+)-PN 200-110 and the antioxidant activity of these triaryl-dihydropyridines.

Chemistry

Recently, we have studied the possibilities for the preparation of a series of imine-enamines **1** [9]. These compounds were generally unstable and quickly hydrolysed to ketones. Yet, under certain conditions and with favorable structures, the imines **1** were isolated. The condensation of these imines with unsaturated compounds like the Knoevenagel product **2** thus gave triaryl-1,4-dihydropyridines **3** according to the Hantzsch condensation (scheme 1, table I).

Pharmacology

The pharmacological activity of the compounds was evaluated by determining their affinity for the DHP receptors and by measuring their antioxidant activity.

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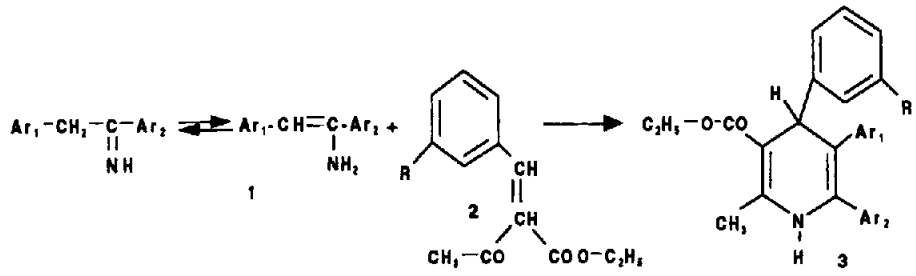

Scheme 1.

Table I. Synthesized triaryl-1,4-dihydropyridines.

Compound	*R*	*Ar_1*	*Ar_2*	*Melting point (°C)*	*Yield (%)*
3a	H	2-Pyrazinyl	2-Pyridinyl	48	75[a]
3b	H	2-Pyrazinyl	2-Furyl	138	80[a]
3c	H	2-Pyrazinyl	2-Thienyl	177	72[a]
3d	NO_2	2-Pyrazinyl	2-Pyridinyl	173	32[a]
3e	NO_2	2-Pyrazinyl	2-Furyl	190	82[a]
3f	NO_2	2-Pyrazinyl	2-Thienyl	171	89[a]
3g	NO_2	2-Pyridinyl	2-Thienyl	179–180	45[b]

[a]Based on isolated imine; [b]based on the Knoevenagel compound (imines was not isolated).

The brain hippocampus membrane preparation for the DHP binding assay and liver microsomal fractions to determine inhibition of lipid peroxidation were obtained from male Wistar rats (15 weeks old) from Iffa–Credo (L'Arbresle, France). [^{3}H](+)-PN 200-110 (85 Ci/mM) was purchased from Amersham (Les Ulis, France). Nicardipine was donated by Sandoz (Rueil-Malmaison, France); butylated hydroxytoluene (BHT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma. DHPs were dissolved in ethanol and dilutions were performed with distilled water.

DHP receptor binding assay

Male Wistar rats were sacrificed by decapitation and their brains were rapidly removed. Membranes from the hippocampus were prepared and DHP receptor binding assayed as previously described [10] with minor modifications. Tissues were homogenized with Ultra-Turrax (IKA, Labortechnick, T 25) in 10 vol of ice-cold 0.32 M sucrose. The nuclear materials were removed by centrifugation (1000 *g*, 10 min, 4°C) and the supernatants were stored at 4°C. The pellets were resuspended in 0.32 M sucrose and recentrifuged (1000 *g*, 10 min, 4°C). The two supernatants were combined and centrifuged at 50 000 *g* for 10 min at 4°C (Heraeus 20 RS centrifuge). The resulting pellets were rehomogenized in ice-cold Tris-HCl 50 mM (pH 7.6) buffer and recentrifuged at 50 000 *g* for 10 min at 4°C. The final pellets were resuspended in the same buffer.

Samples of membrane preparation (0.7 mg protein/assay) were incubated at 25°C for 90 min with 0.3 nM [^{3}H](+)-PN 200-110 and different concentrations of the test compound or nicardipine. The reaction was

stopped by rapid filtration under vacuum through GF/C filters (Whatman). Filters were immediately washed twice with 5 ml ice-cold buffer and suspended in 7.5 ml premixed liquid scintillation fluid (Optiphase Hisafe II LKB). Radioactivity was measured 12 h later in a Packard Tri-Carb 2050 CA counter at 40% efficiency.

Specific binding was calculated as the difference between the total binding of [^{3}H](+)-PN 200-110 and the non-specific binding determined in the presence of 1 μM nicardipine, and was about 75–85% of the total binding. Binding in the absence or presence of competing ligands was determined in triplicate samples from four animals. The inhibition of binding was expressed as a percentage and a graph of dose against inhibition was plotted. From this, the IC_{50} (the concentration inhibiting binding by 50%) was determined.

Radical scavenging effect

Free radical scavenging capacity of the DHPs was determined using DPPH [11]. An ethanol DPPH solution (0.1 mM) was mixed with different concentrations of compound and the optical density change at 515 nm was measured 10 min later with a spectrophotometer (Uvikon 940, Kontron). Colorization in the absence or presence of DHP was measured at least three times. The inhibition of colorization was expressed as a percentage and the IC_{50} was obtained from the inhibition curve. The results were compared with those observed with BHT.

Inhibitory effect on lipid peroxidation

Lipid peroxidation was assayed in microsomal fractions by measuring thiobarbituric acid reactive substances [12]. Rat liver microsomes were prepared by tissue homogenization with 5 volumes of ice-cold 0.15 M KCl. Microsomal fractions were isolated in Tris-HCl 0.05 M / KCl 0.15 M, pH 7.4, by removal of the nuclear fraction at 800 *g* for 15 min, removal of the mitochondrial fraction at 15 000 *g* for 15 min and sedimentation at 105 000 *g* for 60 min. Fractions were washed twice in buffer by centrifugation, with subsequent sedimentation at 125 000 *g* for 15 min. Microsome pellets were stored at –80°C for a maximum of one week.

For the test, microsomal fractions were diluted with 50 mM Tris-HCl, pH 7.4, containing KCl (150 mM). The final protein concentration in the incubation mixture amounted to 0.75 mg/ml. Lipid peroxidation was initiated either with $FeCl_2$ (10 μM) alone or with $FeCl_2$ (10 μM) and NADPH-regenerating system (0.5 mM NADP, 5 mM G6P and 1.5 mIU G6PD). In two cases, samples were incubated with different concentrations of compound or BHT in a shaking water-bath at 37°C for 30 min. Thiobarbituric acid reactive material was determined and the absorbance was measured at 532 nm. Measurement in the absence or presence of DHP was repeated at least three times. The inhibition of lipid peroxidation was expressed as a percentage and the IC_{50} value was obtained from the inhibition curve.

Results and discussion

DHP receptor binding assay

The affinity of DHPs for [^{3}H](+)-PN 200-110 specific binding sites in hippocampus membranes are shown in table II. Three compounds (**3e, 3f, 3g**) inhibited [^{3}H](+)-PN 200–110 binding with an IC_{50} value between 0.17 and 0.70 μM and three compounds (**3b, 3c** and **3d**) with an IC_{50} of 2–3 μM. The DHPs of the second group were thus approximately 10-fold less potent than others. For **3a**, at 150 μM, the highest concentration tested the [^{3}H](+)-PN 200-110 specific binding was not fully displaced. Inhibition of [^{3}H](+)-PN 200-110 binding by nicardipine yielded an IC_{50} of 3 nM in agreement with published data [13].

These results showed that DHP receptor binding affinity was increased when a 3-nitro substituent was present on the aromatic ring, as is usual for 4-phenyl-1,4-DHP. Furthermore, replacement of an ester group by an aromatic structure Ar_1 in the 5-position decreased binding affinity as compared to DHP reference (Nicardipine). Molecule with a 2-pyridinyl substituent (Ar_1) seemed to be more efficient than those with 2-pyrazinyl in the same position (**3g** *vs* **3f**).

Table II. Inhibition of the specific binding of [^{3}H](+)-PN 200-110 by DHP in hippocampus membranes of rat.

Compound	*IC_{50} (μM)*[a]
3a	11.31 ± 0.42
3b	2.74 ± 0.16
3c	2.93 ± 0.12
3d	2.55 ± 0.15
3e	0.70 ± 0.02
3f	0.52 ± 0.02
3g	0.17 ± 0.01
Nicardipine	0.0030 ± 0.0001

[a]Membranes (0.7 mg protein/assay) were incubated with 0.3 nM [^{3}H](+)-PN 200-110 and different concentrations of compounds. Each result is the mean of four experiments performed in triplicate ± SEM.

The addition in the 6-position (Ar_2) of a 2-thienyl group induced a higher binding affinity than 2-furyl, which itself was more active than 2-pyridinyl.

Radical scavenging effect

Data for the decolorization of a DPPH solution by the DHPs are shown in table III which express the free radical scavenging capacity. Four compounds (**3b, 3e, 3f** and **3g**) inhibited the colorization with an IC_{50} value of 3 or 7 mM. The IC_{50} values for **3a**, **3c** and **3d** were approximately 5–33 times higher than for four of the other DHPs. Nicardipine was devoid of free radical scavenging capacity. BHT inhibited the colorization with an IC_{50} approximately 3000-fold lower than for DHP, the most active. These data indicate that molecules with two aromatic cycles showed an enhanced free radical scavenger activity, which was not observed with Nicardipine.

Inhibitory effect on lipid peroxidation

The effect of DHPs on lipid peroxidation induced by $FeCl_2$ alone, or $FeCl_2$ and NADPH-regenerating system, are shown in table IV. In these two assays, the DHPs may be classified into three groups based on inhibitory aspect and the order of inhibition was: **3b** ≈ **3e** ≈ **3g** > **3c** ≈ **3f** > **3a** ≈ **3d**. The IC_{50} values for the second and third group was approximately twice and 5–10 times higher than that for first group, respectively.

Table III. Inhibition of the colorization of DPPH free radical by DHP (**3a–g**), nicardipine and BHT.

Compound	*IC_{50} (μM)*[a]
3a	97.1 ± 2.0
3b	3.0 ± 0.1
3c	40.3 ± 1.8
3d	51.4 ± 1.7
3e	3.2 ± 0.2
3f	7.3 ± 0.3
3g	3.1 ± 0.1
Nicardipine	510 ± 16
BHT	0.0010 ± 0.0001

[a]DPPH solution was incubated for 10 min with different concentrations of compounds. Each value is the mean of three experiments performed in duplicate ± SEM.

Nicardipine inhibited lipid peroxidation with an IC_{50} intermediate between that of second and third group. The IC_{50} value for BHT was approximately 14 and 23 times lower than for the most active DHP (**3g**) on peroxidation induced by $FeCl_2$ alone and by $FeCl_2$ and NADPH-regenerating system, respectively.

The 3-nitro group did not seem to influence antioxidant activity. Except for molecule **3f**, DHP antiradical activity parallels the antioxidant effect.

Table IV. Inhibition of the lipid peroxidation initiated with $FeCl_2$ alone, or $FeCl_2$ + NADPH-regenerating system, by DHP and BHT in liver rat microsomes.

Compound	*$FeCl_2$ IC_{50} (μM)*[a]	*$FeCl_2$ + NADPH-regenerating system IC_{50} (μM)*[a]
3a	52.1 ± 2.0	250 ± 13
3b	8.2 ± 0.4	25.2 ± 1.1
3c	17.0 ± 0.5	45.5 ± 1.9
3d	48.3 ± 1.4	210 ± 10
3e	8.4 ± 0.3	34.1 ± 1.7
3f	14.2 ± 0.4	56.0 ± 4.2
3g	7.1 ± 0.3	23.2 ± 1.0
Nicardipine	24.8 ± 0.9	115 ± 6
BHT	< 0.5	< 1

[a]Microsomal suspension (0.75 mg protein/assay) was incubated with oxidant agent and different concentrations of compounds. Lipid peroxidation was assayed by measuring thiobarbituric acid reactive substances. Values are the means of three experiments performed in duplicate ± SEM.

Conclusion

The results of the present experiments show the weaker affinity of the compounds compared with that observed with Nicardipine for DHP binding sites in the rat brain membranes and the higher antioxidant activity of certain compounds compared with that obtained with Nicardipine for both radical scavenging and antiperoxidant effect on rat liver microsomes.

Further studies will attempt to improve the compromise observed with **3g**, *ie* to increase the DHP binding affinity without loss of the antioxidant effect.

Experimental protocols

Melting points were measured using a Köfler apparatus and are uncorrected. Infrared spectral data were acquired with a ATI-UNICAM Genesis apparatus. ^{1}H-NMR spectra were recorded on Varian EM 360 and Bruker 200 AC spectrometers. ^{13}C-NMR spectra were recorded on a Bruker 200 AC spectrometer. Elemental analyses were performed on a Perkin-Elmer 240 apparatus and were within the acceptable limits of ±0.4% of the theoretical values.

*General procedure for the synthesis of 1,4-dihydropyridines **3a–g**. Method A*

Imines-enamines were prepared according to reference [9]. A mixture of imine-enamine **1** (5 mmol), the Knoevenagel compound **2** (5 mmol) and anhydrous ethanol (60 ml) was refluxed 8–9 h under a nitrogen atmosphere. After cooling, the solid was filtered and separated by chromatography on neutral alumina.

*Ethyl-2-methyl-4-phenyl-5-(2-pyrazinyl)-6-(2-pyridyl)-1,4-dihydropyridine-3-carboxylate **3a***
IR (KBr) (cm^{-1}) 3350 (NH), 2958 (CH_3), 1692 (C=O), 1610 (C=C); ^{1}H-NMR ($CDCl_3$): δ 1.3 (t, 3H, CH_3), 2.45 (s, 3H, CH_3), 4.1 (q, 2H, CH_2), 5.1 (s, 1H, H4), 7–7.5 (m, 9H, 8H Ar + NH), 8.05 (m, 1H), 8.2 (m, 1H), 8.45 (m, 1H), 8.55 (m, 1H).

*Ethyl-2-methyl-4-phenyl-5-(2-pyrazinyl)-6-(2-furyl)-1,4-dihydropyridine-3-carboxylate **3b***
IR (KBr) (cm^{-1}): 3300 (NH), 1680 (C=O), 1615 (C=C); ^{1}H-NMR ($CDCl_3$): δ 1.2 (t, 3H, CH_3), 2.4 (s, 3H, CH_3), 4.0 (q, 2H, CH_2), 5.0 (s, 1H, CH), 6.0 (s, 1H, NH), 6.3 (m, 2H), 7.2 (m, 6H), 8.1 (m, 1H), 8.25 (m, 1H), 8.4 (m, 1H).

*Ethyl-2-methyl-4-phenyl-5-(2-pyrazinyl)-6-(2-thienyl)-1,4-dihydropyridine-3-carboxylate **3c***
IR (KBr) (cm^{-1}): 3300 (NH), 1670 (C=O), 1630 (C=C); ^{1}H-NMR ($CDCl_3$): δ 1.2 (t, 3H, CH_3), 2.35 (s, 3H, CH_3), 4.05 (q, 2H, CH_2), 5.2 (s, 1H, CH), 6.5 (s, 1H, NH), 6.9–7.4 (m, 8H Ar), 7.85 (m, 1H), 8.1 (m, 1H), 8.5 (m, 1H); ^{13}C-NMR ($CDCl_3$): δ: 14.2 (q), 19.6 (q), 42.5 (d), 59.6 (t), 111.9 (s), 126.3 (d), 127.6 (d), 127.7 (d), 128.05 (d), 128.4 (d), 131.5 (s), 136.1 (s), 137.9 (d), 143.9 (d), 145.1 (s), 146.3 (s), 154.7 (s), 167.4 (s).

*Ethyl-2-methyl-4-(3-nitrophenyl)-5-(2-pyrazinyl)-6-(2-pyridyl)-1,4-dihydropyridine-3-carboxylate **3d***
IR (KBr) (cm^{-1}): 3400 (NH), 1690 (C=O), 1625 (C=C); ^{1}H-NMR ($CDCl_3$): δ 1.25 (t, 3H, CH_3), 2.5 (s, 3H, CH_3), 4.1 (q, 2H, CH_2), 5.3 (s, 1H, CH), 6.8-8.7 (m, 12H, 11H Ar + NH).

*Ethyl-2-methyl-4-(3-nitrophenyl)-5-(2-pyrazinyl)-6-(2-furyl)-1,4-dihydropyridine-3-carboxylate **3e***
IR (KBr) (cm^{-1}): 3400 (NH), 1660 (C=O), 1620 (C=C); ^{1}H-NMR ($CDCl_3$): δ 1.2 (t, 3H, CH_3), 2.4 (s, 3H, CH_3), 4.05 (q, 2H, CH_2), 5.1 (s, 1H, CH), 6.1 (s, 1H, NH), 6.35 (m, 2H), 7.15–7.9 (m, 5H), 8.05 (m, 1H), 8.15 (m, 1H), 8.4 (m, 1H).

*Ethyl-2-methyl-4-(3-nitrophenyl)-5-(2-pyrazinyl)-6-(2-thienyl)-1,4-dihydropyridine-3-carboxylate **3f***
IR (KBr) (cm^{-1}): 3400 (NH), 1660 (C=O), 1620 (C=C); ^{1}H-NMR ($CDCl_3$): δ 1.2 (t, 3H, CH_3), 2.35 (s, 3H, CH_3), 4.1 (q, 2H, CH_2), 5.25 (s, 1H, CH), 6.1 (s, 1H, NH), 6.9–8.4 (m, 10H).

*Synthesis of ethyl-2-methyl-4-(3-nitrophenyl)-5-(2-pyridyl)-6-(2-thienyl)-1,4-dihydropyridine-3-carboxylate **3h**. Method B*

Imine-enamine **1** was not purified. The mixture of **1** was evaporated, the residue was dissolved in anhydrous ethanol and condensed with the Knoevenagel compound **2** (5 mmol). The yield was calculated from compound **2**. IR (KBr) (cm^{-1}): 3430 (NH), 1650 (C=O), 1630 (C=C); ^{1}H-NMR ($CDCl_3$): δ 1.2 (t, 3H, CH_3), 2.45 (s, 3H, CH_3), 4.1 (q, 2H, CH_2), 5.35 (s, 1H, CH), 5.85 (s, 1H, NH), 6.7 (d, 1H), 6.95 (m, 3H), 7.3 (m, 4H), 7.7 (d, 1H), 8.0 (m, 1H), 8.02 (m, 1H), 8.55 (m, 1H); ^{13}C-NMR ($CDCl_3$): δ: 14.25 (q), 20.0 (q), 43.5 (d), 59.6 (t), 115.3 (s), 121.1 (d), 122.8 (d), 125.1 (d), 127.05 (d), 127.3 (d), 127.9 (d), 128.7 (d), 129.25 (s), 134.2 (d), 135.1(d), 136.8 (s), 146.5 (s), 148.2 (s), 149.1(s), 157.15 (s), 167.3 (s).

Acknowledgments

The authors thank A Guettier, R Pontcharraud and S Mairesse-Lebrun for excellent technical assistance.

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