Studies on Agents with Mixed NO-Dependent and Calcium Channel Antagonistic Vasodilating Activities

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Purpose. To obtain new cardiovascular agents with mixed Ca^{2+}-channel antagonistic and NO-donor properties, a series of "hybrid" 1,4-dihydropyridines (1,4-DHPs), bearing NO-donating furoxan moieties on the 3-positioned lateral ester chain were synthesized and pharmacologically characterized. Furazan analogues were also prepared and investigated for control purposes, because they are unable to release NO.[4]

Methods. Synthesis of the models was achieved by a modified Hantzsch approach. All of the final furoxan 1,4-DHPs were assessed for their ability to produce nitrite in the presence of a large excess of cysteine by the Griess reaction. Vasodilating activity was evaluated on rat aorta and expressed as EC_{50} and EC_{50}^{MB} values, obtained in the absence and in the presence of methylene blue (MB) respectively, a well-known guanylate cyclase inhibitor. Affinities to 1,4-DHP receptor on Ca^{2+}-channels, expressed as IC_{50} values, were determined through displacement experiments of [^{3}H]-nitrendipine on rat cortex homogenates.

Results. Some hybrid compounds (derivatives **15a, 15b, 16a,** and **16b**) displayed vasodilating activity depending predominantly on their Ca^{2+}-channel blocker properties. By contrast, some others (derivatives **17a, 17b,** and **21**) behaved as well-balanced hybrids with mixed Ca^{2+}-channel blocking and NO-dependent vasodilating activities.

Conclusion. This work demonstrates the possibility of obtaining well-balanced hybrids endowed with mixed NO-donor and Ca^{2+}-channel blocker properties using appropriate 1,4-DHP and furoxan moieties. A procedure for the individual evaluation of the NO-dependent vasodilator component and that due to Ca^{2+}-channel blocking is proposed.

KEY WORDS: NO donors; 1,4-dihydropyridines; Ca^{2+}-channel blockers; hybrid drugs; furoxans; vasodilation.

INTRODUCTION

Medicinal chemical hybridization is a well-known approach to drug design. It involves the combination of two complementary biological activities by joining appropriate pharmacophoric groups directly or via spacers (1,2). We have designed a number of drugs by combining suitable pharmacophoric groups with different NO-donor moieties (3–6). Among these, we also realized new 1,4-dihydropyridines (DHPs) with mixed calcium channel antagonist and nitric oxide-like vasodilator activities. These compounds, which are structurally related to Nifedipine **1** and to its *meta*-analogue **2** (Fig. 1), are characterized by having furoxan substructures endowed with different NO-donor properties at the *ortho* and *meta* position of the 4-phenyl ring (Fig. 1, derivatives **3a–8a** and **3b–8b**). A number of these compounds behaved as well-balanced hybrids, because they were able to display NO-dependent and Ca^{2+}-antagonist dependent vasodilating properties in the same range of concentration. In particular, 1,4-DHPs bearing appropriately substituted furoxans at *ortho* position were as potent as the references **1** and **2** (5).

In this paper, we report the preparation and the pharmacological characterization of a series of 4-(3-nitrophenyl)-1,4-dihydropyridines analogues of **1**, having furoxan moieties on the 3-positioned lateral ester chain (Fig. 2, derivatives **15a, 15b; 16a, 16b; 17a, 17b;** and **21**). Furoxan substituents were appropriately chosen, in order to modulate NO-release of the final products. These compounds should display differences over other known NO-donor 1,4-DHPs bearing nitrate groups in their ester side chain (7,8). In fact the presence of suitable substituted furoxan moieties gives rise to a series of compounds widely modulated in their NO-release properties. In addition, these products could lack significant tolerance development (9,10). In this paper, related furazan derivatives (Fig. 2, derivatives **15, 16, 17,** and **22**) were also considered for control purposes, because they are unable to release NO. Nifedipine **1** and its *meta*-nitro analogue **2** were used as references.

MATERIALS AND METHODS

Chemistry

Synthesis

Melting points were measured on a Büchi 530 capillary apparatus and are uncorrected. Melting points with decomposition were determined after introducing the sample into the bath at a temperature 10°C lower than the melting point. A heating rate of 3°C min^{-1} was used. The compounds were routinely checked by infrared spectroscopy (Shimadzu FT-IR 8101M, Shimadzu Italia, Milan, Italy) and mass spectroscopy (Finnigan-Mat TSQ-700 spectrometer, Finnigan, Milan, Italy), and ^{1}H and ^{13}C nuclear magnetic resonance at 200 and 50 MHz, respectively (Bruker AC-200 spectrometer, Bruker, Milan, Italy). All of the spectra were in keeping with the proposed structures. Column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM, Merck, Milan, Italy) with the indicated solvent system. Anhydrous magnesium sulfate was used as the drying agent of the organic extracts. Petroleum ether (PE) 40–60°C was used. Solvent removal was achieved under reduced pressure at room temperature. Elemental analyses of the new compounds were performed by REDOX (Cologno Monzese, Italy) and the results are within ±0.4% of theoretical values. Intermediates **9a** (11), **9b** (5), and **18** (12) were synthesized according to procedures described in the literature. Derivatives **10a** and **10b** were a gift from Dr. K. Schönafinger, Aventis Pharma (Frankfurt am Main, Germany). Compounds **11, 13,** and **14** are commercial reagents (Aldrich Chemical Co., Milwaukee, WI, USA).

3-(3-(Benzenesulfonyl)furoxan-4-yloxy)propanol, ***19.*** A 50% w/w NaOH aqueous solution (2.19 g, 27.3 mmol),

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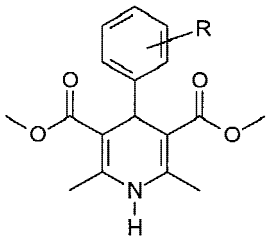
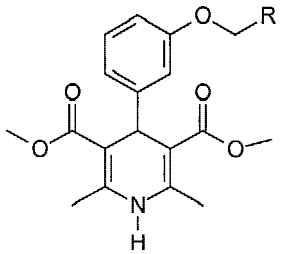
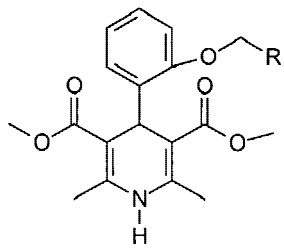
[4] In this paper, the term nitric oxide (NO) is used as a family name. When necessary in the discussion, we specify the particular redox form to which we refer.



1, R = *ortho*-NO_2
2, R = *meta*-NO_2





3, R = A **3a**, R = B **3b**, R = C
4, R = D **4a**, R = E **4b**, R = F
5, R = G **5a**, R = H **5b**, R = I

6, R = A **6a**, R = B **6b**, R = C
7, R = D **7a**, R = E **7b**, R = F
8, R = G **8a**, R = H **8b**, R = I

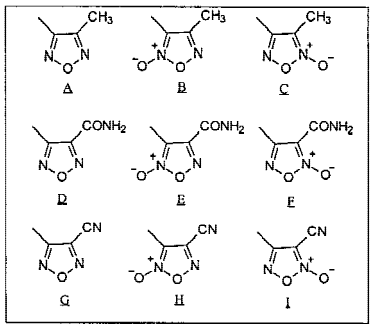


Fig. 1. Nitrosubstituted 1,4-DHPs and related furazan and furoxan derivatives.

was slowly added at room temperature to a stirred solution of **18** (10.9 g, 27.3 mmol) and 1,3-propandiol (20.5 ml, 273 mmol) in tetrahydrofuran (THF) (80 ml). The reaction mixture was stirred for 30 min and then an additional amount (1.09 g) of 50% NaOH was added (13.7 mmol). The solution was concentrated under vacuum at room temperature. The residue was treated with EtOAc and the resulting mixture was washed twice with water. Aqueous phases were extracted with EtOAc. The combined organic layers were dried, and evaporated under vacuum at room temperature to give a residue that was purified by flash chromatography (PE 6/EtOAc 4, yield 69%, mp 114°C (isopropyl ether).

^{1}H-NMR ($CDCl_3$): 1.82 (t, 1H, CH_2OH), 2.15 (qi, 2H, CH_2CH_2OH), 3.89 (q, 2H, CH_2CH_2OH), 4.59 (t, 2H, OCH_2CH_2), 6.1–7.6 (m, 5H, C_6H_5). ^{13}C-NMR ($CDCl_3$): 31.20 (CH_2CH_2OH), 59.29 (CH_2CH_2OH), 69.19 (OCH_2CH_2), 110.35 (C3-Furox.), 158.79 (C4-Furox.) 128.43, 129.57, 135.56, 137.84 (C_6H_5). Anal. ($C_{11}H_{12}N_2O_6S$) C, H, N.

General Procedure for the Synthesis of Acetoacetate ***11a, 11b, 12a, 12b,*** *and* ***20.*** A solution of the appropriate furoxanmethanol derivatives (**9a, 9b, 10a, 10b,** and **19**) and 2,2,6-trimethyl-4H-1,3-dioxin-4-one (**11**) in toluene (20 ml) was refluxed. Solvent removal gave a residue that was purified by flash chromatography. Reagent ratio, reaction times, chromatography eluents, yields, melting points, and crystallization solvents were as follows.

11a: Reagent ratio 1:2, reaction time 10 min, eluent PE 7/EtOAc 3, yield 42%, oil. ^{1}H-NMR (DMSO-d_6): 2.18 (s, 3H, $COCH_3$); 2.40 (s, 3H, CH_3); 3.72 (s, 2H, $COCH_2CO$); 5.08 (s, 2H, CH_2O). ^{13}C-NMR (DMSO-d_6): 7.37 (CH_3); 30.0 ($COCH_3$); 49.1 ($COCH_2CO$); 53.8 (CH_2O); 113.3 (C3-Furox.); 155.7 (C4-Furox.); 170.5 (COO); 201.3 ($COCH_3$). Anal. ($C_8H_{10}N_2O_5$) C, H, N.

11b: Reagent ratio 1:2, reaction time 2 h, eluent PE 7/EtOAc 3, yield 60%, oil. ^{1}H-NMR (DMSO-d_6): 2.16 (s, 3H, $COCH_3$); 2.20 (s, 3H, CH_3); 3.76 (s, 2H, $COCH_2CO$); 5.29 (s,

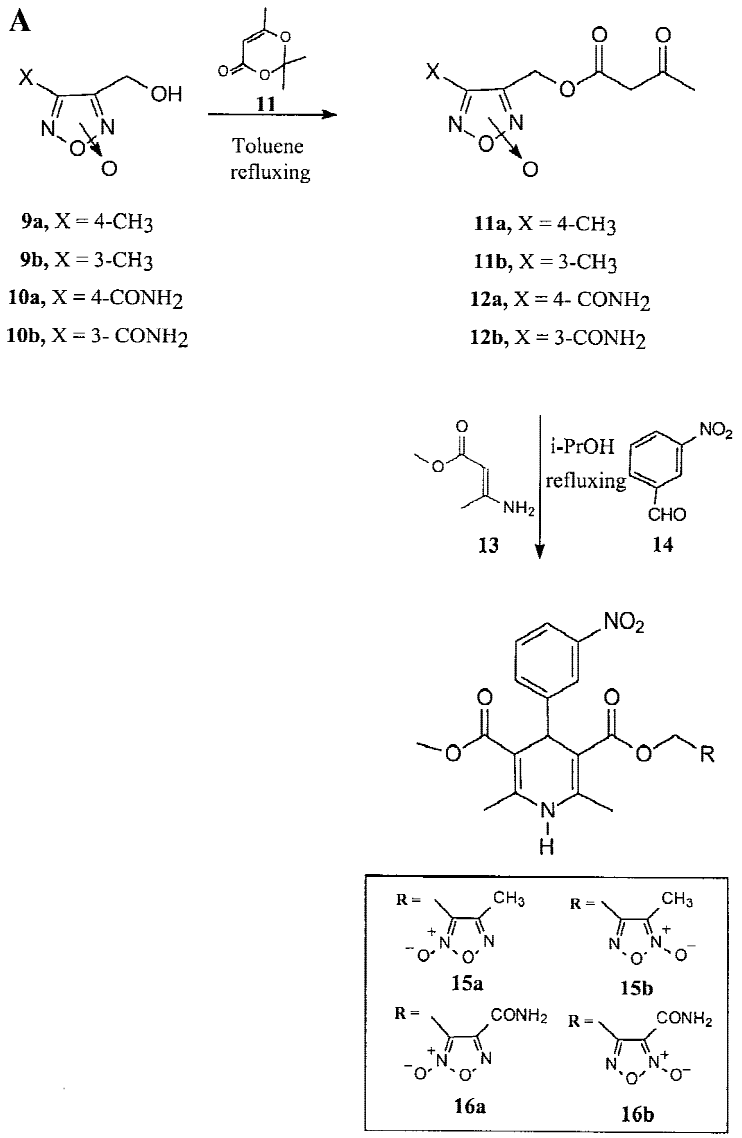


Fig. 2. Synthesis of furazan and furoxan 1,4-DHPs.

2H, CH_2O). ^{13}C-NMR (DMSO-d_6): 7.47 (CH_3); 30.1 ($CO\mathit{CH_3}$); 49.2 ($CO\mathit{CH_2}CO$); 59.9 (CH_2O); 113.4 (C3-Furox.); 154.8 (C4-Furox.); 167.0 (COO); 201.4 ($\mathit{C}OCH_3$). Anal. ($C_8H_{10}N_2O_5$) C, H, N.

12a: Reagent ratio 1:1, reaction time 4 h, yield 60%, mp 73–74°C (EtOAc/PE). ^{1}H-NMR (DMSO-d_6): 2.18 (s, 3H, CH_3); 3.67 (s, 2H, $COCH_2CO$); 5.23 (s, 2H, CH_2O); 8.26, 8.60 (2s, 2H, $CONH_2$). ^{13}C-NMR (DMSO-d_6): 30.0 ($CO\mathit{CH_3}$); 49.1 ($CO\mathit{CH_2}CO$); 54.0 (CH_2O); 111.7 (C3-Furox.); 151.6 (C4-Furox.); 158.1 ($CONH_2$); 166.8 (COO); 201.1 ($\mathit{C}OCH_3$). Anal. ($C_8H_9N_3O_6$) C, H, N.

12b: Reagent ratio 1:1, reaction time 4 h, yield 68%, mp 96–97°C (EtOAc/PE). ^{1}H-NMR (DMSO-d_6): 2.22 (s, 3H, CH_3); 3.73 (s, 2H, $COCH_2CO$); 5.42 (s, 2H, CH_2O); 7.81, 8.51 (2s, 2H, $CONH_2$). ^{13}C-NMR (DMSO-d_6): 30.1 ($COCH_3$); 49.2 ($CO\mathit{CH_2}CO$); 57.3 (CH_2O); 110.3 (C3-Furox.); 154.5 (C4-Furox.); 155.7 ($CONH_2$); 166.6 (COO); 201.0 ($\mathit{C}OCH_3$). Anal. ($C_8H_9N_3O_6$) C, H, N.

20: Reagent ratio 1:1, reaction time 2 h, yield 60%, mp 87–88,5°C (EtOAc /PE). ^{1}H-NMR (DMSO-d_6): 2.11 (m, 2H, $CH_2\mathit{CH_2}CH_2$); 2.19 (s, 3H, CH_3); 3.65 (s, 2H, $COCH_2CO$); 4.21 (t, 2H, $COOCH_2$); 4.48 (t, 2H, CH_2O); 7.72–8.05 (m, 5H, C_6H_5). ^{13}C-NMR (DMSO-d_6): 27.4 ($CH_2\mathit{CH_2}CH_2$); 30.2 ($CO\mathit{CH_3}$); 49.6; ($CO\mathit{CH_2}CO$); 60.8 ($COOCH_2$); 68.2 (CH_2O); 110.3 (C3-Furox.); 158.9 (C4-Furox.); 128.4, 130.1, 136.3, 137.2 (C_6H_5); 167.4 (COO); 201.8 ($\mathit{C}OCH_3$). Anal. ($C_{15}H_{16}N_2O_8S$) C, H, N.

General Procedure for the Synthesis of Furoxan 1,4-DHPs ***15a, 15b, 16a, 16b,*** *and* ***21.*** A solution of the appropriate acetoacetate (**11a, 11b, 12a, 12b,** and **20**) (1 mmol), 3-nitrobenzaldehyde (**14**) (0.15 g, 1 mmol) and methyl 3-aminocrotonate (**13**) (0.12 g, 1 mmol) in 2-propanol (20 ml) was refluxed for 8 h. Solvent removal gave a residue, which was purified by flash chromatography (eluent PE 7/EtOAc 3). Yields, melting points, crystallization solvents, ^{1}H-NMR, and chemical shifts of the products were as follows (^{13}C-NMR data are reported in Table I).

15a: 66%, mp 153–154°C softens 150°C ($CHCl_3$/PE). ^{1}H-NMR (DMSO-d_6): 2.20 (s, 3H, $-CH_3$); 2.29/2.30 (2s, 6H, C_2–, C_6–CH_3); 3.56 (s, 3H, $-OCH_3$); 4.86–5.09 (q, 2H, $-OCH_2$);

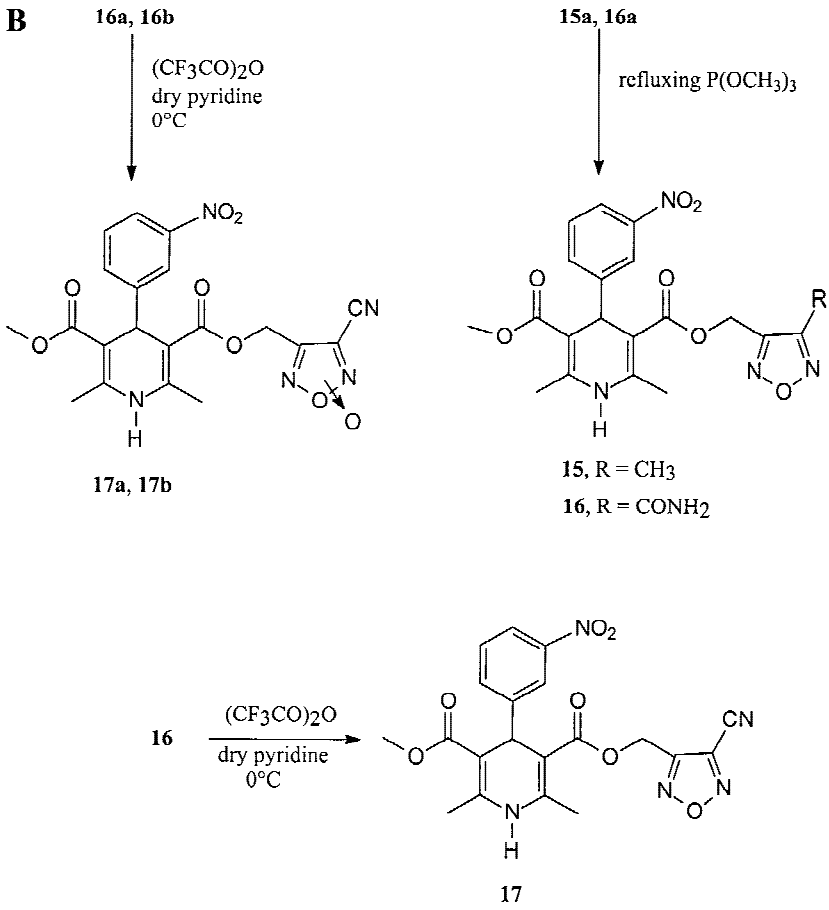


Fig. 2. Continued.

4.94 (s, 1H, C_4–H); 7.48–8.03 (m, 4H, Ph); 9.22 (s, 1H, NH). Anal. ($C_{20}H_{20}N_4O_8$) C, H, N.

15b: 61%, mp 163–164°C ($CHCl_3$/PE). ^{1}H-NMR (DMSO-d_6): 1.92 (s, 3H, –CH_3); 2.30/2.34 (2s, 6H, C_2–, C_6–CH_3); 3.58 (s, 3H, –OCH_3); 4.99 (s, 1H, C_4–H); 5.06–5.31 (q, 2H, –OCH_2); 7.51–8.03 (m, 4H, Ph); 9.24 (s, 1H, NH). Anal. ($C_{20}H_{20}N_4O_8$) C, H, N.

16a: 55%, mp 191–192°C ($CHCl_3$/PE). ^{1}H-NMR (DMSO-d_6): 2.28/2.29 (2s, 6H, C_2–, C_6–CH_3); 3.57 (s, 3H, –OCH_3); 4.89 (s, 1H, C_4–H); 5.11–5.33 (q, 2H, –OCH_2); 7.49–8.01 (m, 4H, Ph); 8.22/8.57 (2s, 2H, $CONH_2$); 9.19 (s, 1H, NH). Anal. ($C_{20}H_{19}N_5O_9 \cdot 0.5\ H_2O$) C, H, N.

16b: 74%, mp 223–224°C, softens 220°C ($CHCl_3$/PE). ^{1}H-NMR (DMSO-d_6): 2.32/2.33 (2s, 6H, C_2–, C_6–CH_3); 3.56 (s, 3H, –OCH_3); 5.01 (s, 1H, C_4–H); 5.24-5.41 (q, 2H, –OCH_2); 7.52–7.98 (m, 4H, Ph); 7.77/8.46 (2s, 2H, $CONH_2$); 9.21 (s, 1H, NH). Anal. ($C_{20}H_{19}N_5O_9$) C, H, N.

21: 50%, mp 166–167°C (EtOH). ^{1}H-NMR (DMSO-d_6): 2.01–2.1 (m, 2H, $CH_2CH_2CH_2$); 2.29/2.33 (2s, 6H, C_2–, C_6–CH_3); 3.56 (s, 3H, –OCH_3); 4.03–4.19 (m, 2H, $COOCH_2$); 4.28 (t, 2H, CH_2O); 4.98 (s, 1H, C_4–H); 7.46–8.02 (m, 9H, C_6H_5/C_6H_4–DHP); 9.11 (s, 1H, NH). Anal. ($C_{27}H_{26}N_4O_{11}S$) C, H, N.

General Procedure for the Synthesis of Furazan 1,4-DHPs ***15, 16, 22.*** A solution of the appropriate furoxan 1,4-DHPs (**15a, 16a,** and **21**) (2 mmol) in trimethyl phosphite (15 ml) was refluxed for 9 h. Then the reaction mixture, cooled at room temperature, was poured into 2 N HCl (150 ml). The resulting precipitate was filtered off, washed several times with water and dried. Yields, melting points, crystallization solvents and ^{1}H -NMR chemical shifts of the products were as follows (^{13}C-NMR data are reported in Table 1):

15: 70%, mp 145–146°C, softens 143°C ($CHCl_3$/PE). ^{1}H-NMR (DMSO-d_6): 2.18 (s, 3H, –CH_3); 2.30/2.34 (2s, 6H, C_2–, C_6–CH_3); 3.56 (s, 3H, –OCH_3); 4.98 (s, 1H, C_4–H); 5.17–5.36 (q, 2H, –OCH_2); 7.47–8.05 (m, 4H, Ph); 9.23 (s, 1H, NH). Anal. ($C_{20}H_{20}N_4O_7$) C, H, N.

16: 90%, mp 182–184°C ($CHCl_3$/PE). ^{1}H-NMR (DMSO-d_6): 2.32 (s, 6H, C_2–, C_6–CH_3); 3.56 (s, 3H, –OCH_3); 5.01 (s, 1H, C_4-H); 5.42–5.43 (q, 2H, –OCH_2); 7.52–8.02 (m, 4H, Ph); 8.20, 8.58 (2s, 2H, $CONH_2$); 9.22 (s, 1H, NH). Anal. ($C_{20}H_{19}N_5O_8$) C, H, N.

22: 50% , mp 144–145°C, softens at 140°C (EtOH). ^{1}H-NMR (DMSO-d_6): 2.0–2.06 (m, 2H, $CH_2CH_2CH_2$); 2.29/2.31 (2s, 6H, C_2–, C_6–CH_3); 3.57 (s, 3H, –OCH_3); 3.98–4.08 (m, 2H, $COOCH_2$); 4.25 (t, 2H, CH_2O); 4.96 (s, 1H, C_4-H); 7.47-8.08 (m, 9H, C_6H_5/C_6H_4–DHP); 9.11 (s, 1H, NH). Anal. ($C_{27}H_{26}N_4O_{10}S \cdot 0.25\ H_2O$) C, H, N.

General Procedure for the Synthesis of Furazan and Furoxan 1,4-DHPs ***17a, 17b, 17.*** Trifluoroacetic anhydride (3 mmol) was added dropwise to a stirred and ice-salt cooled solution of the appropriate DHPs **16a, 16b**, and **16** (1 mmol) in dry pyridine (5 ml). The cooling bath was removed, and strirring was continued for 30 min at room temperature. The reaction mixture was poured into water and the solution, acidified with 2 N HCl, was extracted with EtOAc. The com-

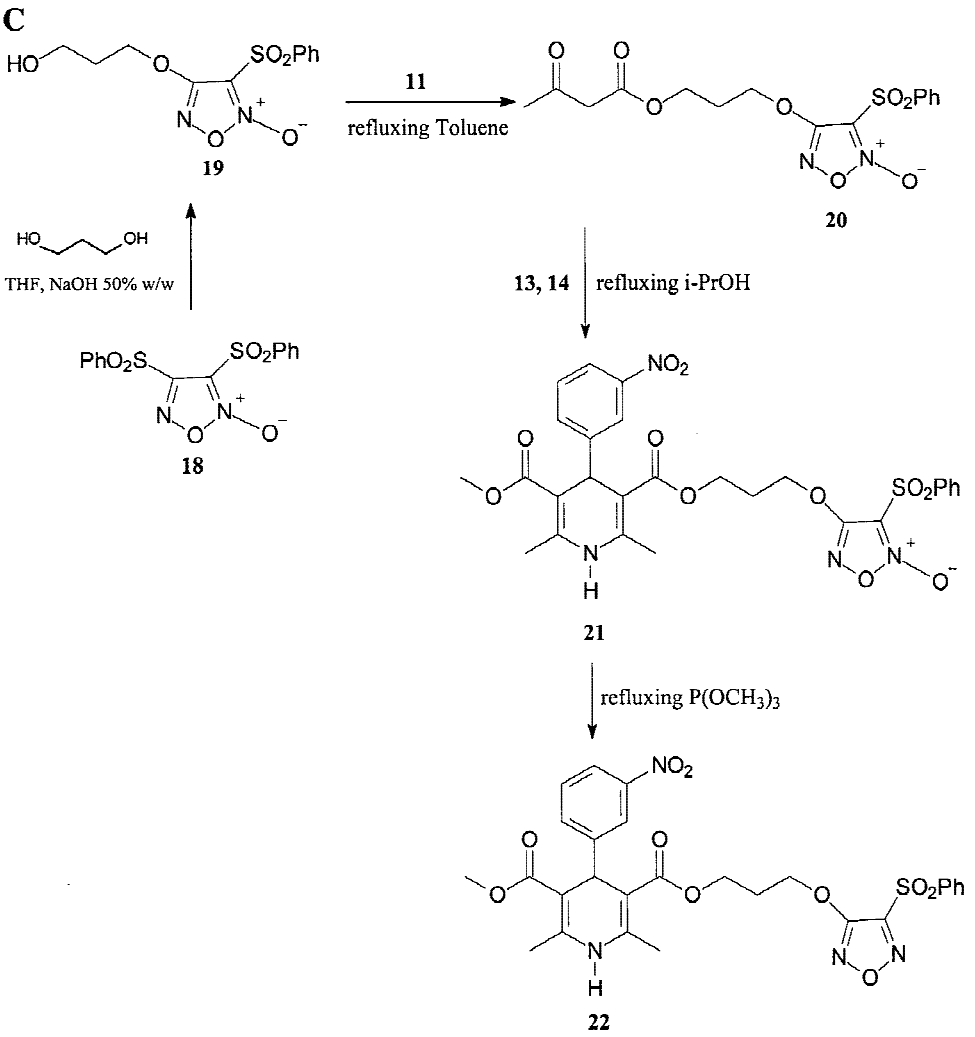


Fig. 2. Continued.

bined organic layers were washed with 2 N HCl, dried and evaporated to afford a residue that was purified by flash chromatography (PE 7/EtOAc 3). Yields, melting points, crystallization solvents, and ^{1}H -NMR chemical shifts of the products were as follows (^{13}C-NMR data are reported in Table I).

17: 60%, mp 145–146°C ($CHCl_3$/PE). ^{1}H NMR (DMSO-d_6): 2.31/2.35 (2s, 6H, C_2–, C_6–CH_3); 3.56 (s, 3H, –OCH_3); 5.02 (s, 1H, C_4–H); 5.37–5.53 (q, 2H, –OCH_2); 7.48–8.03 (m, 4H, Ph); 9.29 (s, 1H, NH). Anal. ($C_{20}H_{17}N_5O_7$) C, H, N.

17a: 57%, mp 161–162°C ($CHCl_3$/PE). ^{1}H-NMR (DMSO-d_6): 2.30/2.32 (2s, 6H, C_2–, C_6–CH_3); 3.56 (s, 3H, –OCH_3); 4.98 (s, 1H, C_4–H); 5.05–5.16 (q, 2H, –OCH_2); 7.57–8.05 (m, 4H, Ph); 9.27 (s, 1H, NH). Anal. ($C_{20}H_{17}N_5O_8$) C, H, N.

17b: 67%, mp 107–108°C ($CHCl_3$/PE). ^{1}H-NMR (DMSO-d_6): 2.37/2.39 (2s, 6H, C_2–, C_6–CH_3); 3.60 (s, 3H, –OCH_3); 5.17 (s, 1H, C_4–H); 5.39 (s, 2H, –OCH_2); 7.5–8.0 (m, 4H, Ph); 8.36 (s, 1H, NH). Anal. ($C_{20}H_{17}N_5O_8$) C, H, N.

Detection of Nitrite

Nitrite production by furoxan DHPs was assessed in the presence of a large excess of cysteine (1:50) in 50 mM phosphate buffer (pH 7.4) at 37°C according to the procedure previously described (13).

Pharmacology

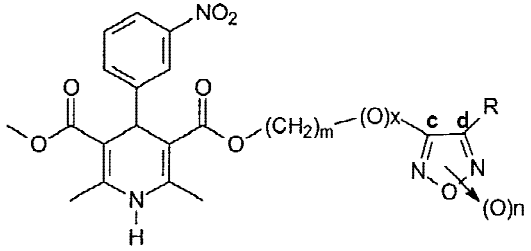
All experiments were done in accordance with the *Principles of Laboratory Animal Care* (National Institutes of Health Publication No. 85-23, revised 1985).

Vasoactivity Determination

Thoracic aortae were isolated from male Wistar rats weighing 180–200 g. The vessels were helically cut, the endothelium removed, and three strips were obtained from each aorta. The tissues were mounted under 0.7 g tension in organ baths containing 30 ml of Krebs-bicarbonate buffer of the following composition (mM): NaCl 111.2, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.0, $NaHCO_3$ 12.0, glucose 11.1, maintained at 37°C and gassed with 95% O_2–5% CO_2 (pH 7.4).

The aortic strips were allowed to equilibrate for 1 h and were then depolarized by addition of a solution of KCl to a final K^+ concentration 50 mM. The preparations were then extensively washed with Krebs-bicarbonate buffer and a second contraction was evoked by K^+-depolarization (50 mM).

Table I. ^{13}C-NMR Chemical Shifts in δ (ppm) Units (*Interchangeable) of the 1,4-Dihydropyridines Analogues (DMSO-d_6)



	R	n	x	m	2–,6–CH_3	OCH_3	C_4 (DHP)	C_3C_5 (DHP)	C_2C_6 (DHP)	3–,5– COO	DHP–C_6H_4–NO_2						$(CH_2)_m$	c	d	R
15	4-CH_3	0	0	1	18.4 18.6	51.0	39.2	101.6 99.7	146.3 147.6	165.8 167.0	121.4	121.7	129.8	134.1	148.5*	149.7	55.5	148.7*	151.8	7.6
15a	4-CH_3	1	0	1	18.3 18.5	51.0	38.8	101.6 99.6	146.2 147.6	165.9 167.0	121.3	121.6	129.8	134.0	148.6	149.8	52.6	113.3	155.4	10.5
15b	3-CH_3	1	0	1	18.3 18.6	51.0	38.7	101.7 99.6	146.7 147.6	165.7 167.0	121.3	121.7	129.8	134.2	148.7	149.9	55.5	155.3	112.8	7.2
16	4-$CONH_2$	0	0	1	18.3 18.7	51.0	39.1	101.4 100.0	146.4 147.7	167.0 165.8	121.3	121.8	129.7	134.1	148.3*	149.9	55.2	148.7*	152.3	158.1
16a	4-$CONH_2$	1	0	1	18.3 18.5	50.9	38.8	101.6 99.8	146.4 147.6	167.0 165.8	121.3	121.7	129.6	134.1	148.3	149.8	53.1	112.0	151.5	158.1
16b	3-$CONH_2$	1	0	1	18.3 18.6	51.0	38.8	101.5 99.9	146.4 147.6	167.0 165.7	121.3	121.8	129.7	134.2	148.4	149.8	56.5	155.1*	110.3	155.6*
17	4-CN	0	0	1	18.3 18.8	51.0	39.5	101.8 99.3	146.2 147.7	167.0 165.7	121.7	121.4	129.8	134.1	149.1*	149.7	53.7	148.5*	155.3	107.4
17a	4-CN	1	0	1	18.3 18.6	51.0	38.8	101.8 99.2	146.2 147.7	167.0 166.0	121.3	121.7	129.8	134.1	149.1	149.6	52.6	112.5	135.8	107.9
17b	3-CN	1	0	1	18.1 18.6	50.5	39.7	100.3 103.1	145.7 148.4	167.1 165.8	121.3	122.5	129.4	134.5	149.1	150.3	55.9	154.5	97.9	105.7
22	4-Ph–SO_2	0	1	3	18.4	50.9	38.9	100.6 101.2	146.4 147.2*	167.1 166.4	121.6	121.8	129.7	134.2	147.6*	150.0	59.5, 27.5, 70.7	148.7	160.7	128.7, 130.2, 136.2, 136.6
21	3-Ph–SO_2	1	1	3	18.4 18.6	50.9	38.3	100.6 101.2	146.4 147.2*	167.1 166.5	121.2	121.8	129.7	134.2	147.6*	150.0	59.6, 27.3, 68.1	158.8	110.5	128.4, 130.0, 136.2, 137.2

When the contraction plateaued, cumulative concentrations of the vasodilator agents were added every 45 min, according to Christiaans (2). Development of antagonism occurred so slowly that increasing doses had to be given at established times, without waiting for a complete equilibrium to be reached.

The effect of methylene blue (MB) 10 μM on relaxation was evaluated in a separate series of experiments by adding this compound 5 min before K^+-depolarization. All experiments were performed avoiding exposure to light, because of the photolability of the 1,4-DHPs. The experimental room was darkened and the organ bath system was shielded from light.

Receptor Binding Assay

Cerebral cortices, isolated from male Wistar rats (180–200 g), were homogenized in 20 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.4 at 4°C) in an Ultra Turrax Homogenizer. The homogenate was centrifuged at 43,000 *g* for 10 min and the pellet was resuspended in the buffer. This process was repeated an additional two times. The final pellet was stored at −80°C until required. As in the vasoactivity study, the binding experiments were performed in a darkened room and both incubation tubes and filtration device were shielded from light.

All binding assays were carried out according to Christiaans (2), by adding 200 μl of Tris-buffer 50 mM (pH 7.4), 100 μl of rat brain membrane suspension (170 μg protein ml^{-1}), 100 μl of [^{3}H]-nitrendipine solution (1 nM), and 100 μl of the drug concentration to each incubation tube for a final volume of 0.5 ml. Triplicate tubes were used for each condition. Specific binding was defined as the difference between total binding (measured in the absence of any added ligand) and nonspecific binding (determined in the presence of 1 μM nifedipine). Reaction tubes were incubated for 60 min at 37°C, then diluted with 4 ml of ice-cold Tris-buffer (50 mM, pH 7.4) and filtered under reduced pressure through Whatman GF/C glass fiber filters, treated with a 0.1% polyethylenimine solution. Tubes and filters were washed two additional times with ice-cold buffer. The amount of radioactivity retained on the filters was quantified by liquid scintillation counting, using a Beckman liquid scintillation spectrophotometer.

Saturation experiments were performed by incubating increasing concentrations of [^{3}H]-nitrendipine from 0.03 nM up to 2 nM with 50 μl of rat cortical membranes (170 mg protein ml^{-1}) and with Tris-HCl buffer 50 mM (pH 7.4) at 37°C for a total volume of 0.250 ml. Nonspecific binding was determined in the presence of 1 μM nifedipine. Equilibrium dissociation constant (K_D) of 0.83 ± 0.16 nM and the maximal binding (B_{max}) of 245 ± 70 fmol mg^{-1} protein of [^{3}H]-nitrendipine, and IC_{50} values (the concentration of each compound able to display 50% of [^{3}H]-nitrendipine binding) of all the compounds tested were calculated with the nonlinear fitting program INPLOT 4.0.

RESULTS AND DISCUSSION

Synthesis of furoxan 1,4-DHPs containing methyl (Fig. 2A, derivatives **15a** and **15b**), carbamoyl (Fig. 2A, derivatives **16a** and **16b**), and phenylsulfonyl (Fig.2C, derivative **21**) moieties required preliminary preparation of a series of acetoacetic esters. Intermediates **11a, 11b, 12a,** and **12b** were synthesized by treating the appropriate furoxanmethanol derivatives **9a, 9b, 10a,** and **10b** with **11**, a synthon of acetylketene, in refluxing toluene. Acetoacetate **20** (Fig. 2C) was prepared by the same method, starting from intermediate **19**. During the reactions, no furoxan thermal isomerisation was observed. Reaction of acetoacetates with *ortho*-nitrobenzaldehyde (**14**) and β-aminocrotonate (**13**) in boiling isopropanol afforded the expected 1,4-DHPs. Again, no furoxan isomerization was observed. Furazan DHPs **15, 16,** and **22** were easily obtained by reduction of the furoxan analogues **15a, 16a** (Fig. 2B), and **21** (Fig. 2C) in boiling trimethylphosphite. Finally, the cyano-substituted 1,4-DHPs **17, 17a,** and **17b** were prepared by dehydration of the amide analogues dissolved in dry pyridine in the presence of trifluoroacetic anhydride (Fig. 2B). All of the final furoxan DHPs were assessed for their ability to produce nitrite (NO_2^-), in the presence of a large excess of cysteine (Table II). Nitric oxide center dot ($NO^{\cdot}$), or nitroxyl anion (NO^-), or both, may be involved in thiol-induced NO-donation by furoxans (14,15). These two redox species are both able to activate soluble guanylate cyclase (sGC) (16).

Table II. Vasodilating Potencies (EC_{50}, EC_{50}^{MB}), Radioligand Binding Affinities (IC_{50}), Interpolated Values from Regression Eq. (2) (EC_{50}^{calcd}), and NO Releasing Properties ($\%NO_2^-$) of 1,4-DPH Derivatives

Compound	EC_{50} ± SE (nM)	EC_{50}^{MB} ± SE (nM)	IC_{50} ± SE (nM)	EC_{50}^{calcd}(CL 95%) (nM)	$\%NO_2^-$ ± SE mol/mol
1	3.2 ± 0.4		2.7 ± 0.4		
2	3.4 ± 0.6		2.6 ± 0.7		
15	3.5 ± 0.6		2.7 ± 0.8		
15a	6.6 ± 0.8	5.7 ± 0.8	6.4 ± 1.1	5.8 (4.6–7.2)	2.0 ± 0.1
15b	6.7 ± 0.6	7.0 ± 0.6	8.3 ± 1.3	7.4 (5.9–9.3)	<0.5
16	50 ± 8		49 ± 15		
16a	82 ± 12	83 ± 6	81 ± 1	68 (55–83)	17.4 ± 0.1
16b	64 ± 4	64 ± 2	71 ± 19	59 (48–72)	12.0 ± 0.2
17	1.4 ± 0.3		2.2 ± 0.5		
17a	6.7 ± 0.8	21 ± 3	28 ± 10	24 (19–29)	40.6 ± 0.5
17b	4.8 ± 0.5	22 ± 3	30 ± 9	26 (21–32)	35.8 ± 0.5
22	0.48 ± 0.07		0.65 ± 0.27		
21	8.6 ± 1.0	16 ± 2	17 ± 6	15 (12–18)	26.8 ± 1.3

Under physiological conditions, the principal oxidation product of NO· is NO_2^-. The reaction evolves through oxidating and nitrosating NO_x intermediates not fully characterized. By contrast, the fate of nitroxyl anion NO^- is more complex. It can be converted to N_2O, but its rapid reaction with O_2 competitively generates peroxy nitrite ($^-OON=O$). In turn, this last product partially decomposes to nitrite. Also oxidation of NO^- to NO· has been claimed. Another source of nitrite is the slower reaction of NO^- with NO·. In our experimental conditions the whole picture is complicated by the presence of thiols, which in turn can interact with the two NO-redox species and their transient oxidation products. This complex picture is discussed in reference 14. In summary, nitrite detection serves for inferring the previous presence of NO only in first approximation.

Vasodilator activity of all 1,4-DHPs was evaluated on K^+-depolarized rat thoracic aorta strips. EC_{50} values (Table 2) were calculated from concentration-response curves. The experiments for the NO-donor furoxan DHPs were repeated in the presence of MB, a well-known inhibitor of soluble guanylate cyclase (sGC). For the compounds **17a, 17b,** and **21,** which are good producers of nitrite, a rightward shift of the concentration response curves was observed. In Fig. 3, the concentration response curves for compounds **17a** and **21** are reported. The potencies obtained in this new set of experiments, expressed as EC_{50}^{MB}, are shown in Table 2. All DHP derivatives were subjected to a competition study of inhibition of ^{3}[H]-nitrendipine binding to cerebral cortices (see Materials and Methods). IC_{50} values (Table 2) were determined from the competition curves. Analysis of the data reported in the table shows that the introduction of the furazan moieties into the 3-ester lateral chain of reference **2** gives rise to potent Ca^{2+}-channel antagonist vasodilators. In fact, all of these compounds are as active as or more active than **2** and Nifedipine **1**. Only the carbamoyl analogue **16** is less potent and this could be principally dependent on the low lipophilicity of the $CONH_2$ moiety. Furoxan analogues are less active vasodilators than the corresponding furazans, but their potencies are still comparable to the reference compounds. A problem that arises with these compounds is individual evaluation of the NO-dependent vasodilator component and that due to Ca^{2+}-channel block. To solve this problem we used the same approach discussed in a previous paper (5). In that work, by plotting log $1/EC_{50}$ values versus log $1/IC_{50}$ values of the DHP compounds not containing NO-donor moieties (derivatives **1, 2**, and **3–8**), we obtained Eq. (1):

$$\text{Log } 1/EC_{50} = 0.947\ (\pm 0.042)\ \log 1/IC_{50} + 0.443\ (\pm 0.299) \qquad (1)$$

where $N = 8$, $r^2 = 0.988$, and $s = 0.14$ (figures in brackets are SE).

Using a set of 12 compounds, namely the eight structures described in the previous work and the four furazan DHPs described in this paper (derivatives **15, 16, 17, 22**), Eq. (1) is changed to Eq. (2):

$$\text{Log } 1/EC_{50} = 0.971\ (\pm 0.032)\log 1/IC_{50} + 0.286\ (\pm 0.246) \qquad (2)$$

where $N = 12$, $r^2 = 0.989$, and $s = 0.14$.

No outlier is present and all of the compounds show residuals (observed values minus estimated values) lower than 1.5 standard deviation(s). Slopes and intercepts of these two linear correlations are the same, if the SEs are considered. Equation (2) indicates a very good relationship between DHP receptor occupancy and functional effect. The value of the slope is ~1 and the intercept is ~0. From Eq. (2) and the tabulated IC_{50} values, it is possible to calculate EC_{50}^{calcd} values (Table 2) for all of the furoxan DHPs of the present work. Analysis of the data shows a very good correlation between EC_{50}^{MB} and EC_{50}^{calcd} values. Consequently, EC_{50}^{MB} values are a measure of the vasodilator potencies of these compounds, largely dependent on their Ca^{2+}-blocking properties. Data in the table show that EC_{50}^{MB}/EC_{50} ratio is ~1 for the compounds **15a, 15b, 16a,** and **16b**. Therefore, they are not well-balanced hybrids, because their vasodilating action, in the tested concentration range, is due to their Ca^{2+}-channel antagonist activities. By contrast, DHPs **17a, 17b,** and **21**, which are more potent NO-donors, show EC_{50}^{MB}/EC_{50} ratio between 2 and 5 and behave as well-balanced hybrids. This means that, for a large part of the tested concentration range, they display vasodilating activity dependent both on their NO-donor and on their Ca^{2+}-blocker properties (Fig. 3). Fi-

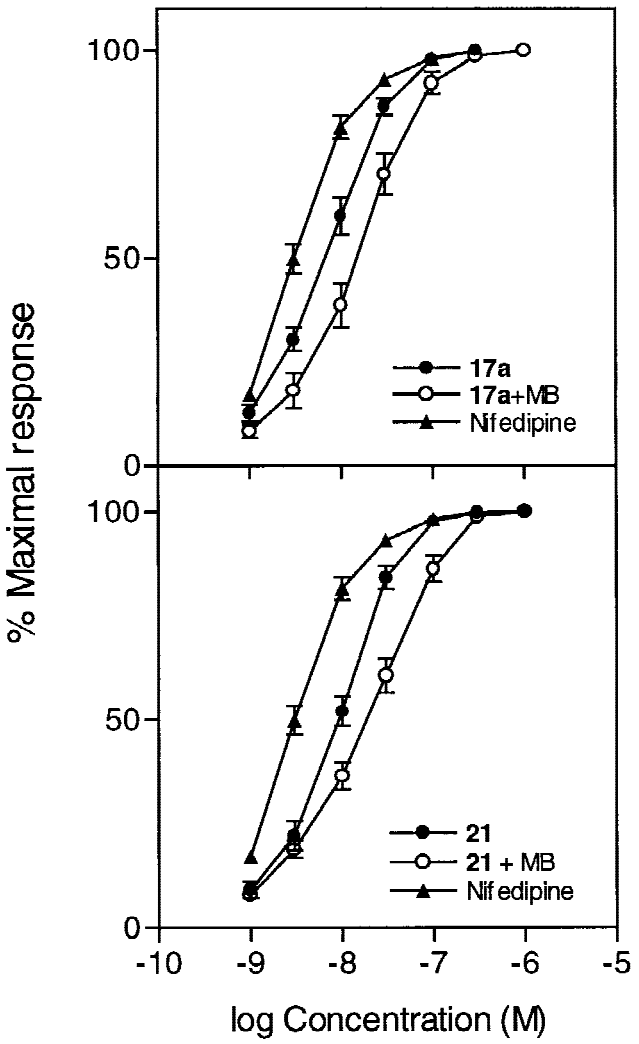


Fig. 3. Concentration-response curves for vasodilating activity of compounds **17a** and **21** in the presence and absence of MB. All points are mean values ± SE from independent experiments.

nally, analysis of EC_{50}^{MB} values for furoxan DHPs indicates that substitution of furazan moieties with the corresponding furoxans produces a decreased affinity for DHP receptors.

In conclusion, the manipulation of the 3-ester lateral chain in **2** gave rise to well-balanced hybrids as potent as those obtained by manipulation of the *ortho*-position of the 4-phenyl ring.

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