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Synthesis and Pharmacological Study of Rho-Kinase Inhibitors: Pharmacomodulations on the Lead Compound Fasudil

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With a view to specifying structure–activity relationships we have synthesised a new series of analogues of the Rho-kinase inhibitor 1-(5-isoquinolinesulfonyl)-homopiperazine (Fasudil). The structural modifications concerned the isoquinolinyl heterocycle and the sulfonyl group which are the two main features of this lead compound. These analogues were evaluated on the actin cytoskeleton and on the enzymatic activity of Rho-kinase. Most of the chemical modifications result in a loss of activity showing that interactions of Fasudil with the catalytic domain of Rho-kinase seem to be particularly definite and sensitive to structural variations. The presence of an isoquinolinyl nitrogen and a basic amino group separated by a spacer bearing a sulfonamide function are of utmost importance. Only the tetrahydroisoquinoline analogue 3 shows the same activity as Fasudil. Moreover, this compound is unable to inhibit PKC biological activity contrary to Fasudil. The loss of the aromatic property could increase the selectivity level in favour of compound 3.

Keywords: Rho-kinase, Protein-kinase C, Inhibitors, Fasudil, Structure–activity relationships

INTRODUCTION

Protein kinases are implicated in various physiological processes. They are known to play an important role for cellular signal transduction and regulation of a variety of cellular events.[1] Targeting of these enzymes is a relatively recent activity for medicinal chemists. Consequently, selective inhibitors of particular protein kinases may have therapeutic value in a wide range of diseases, such as cancer, diabetes, arthritis and hypertension.[2] Among these inhibitors, Fasudil (Figure 1) has been shown to inhibit Rho-kinase activity in a manner competitive with ATP.[3,4] However, the ATP binding site of protein kinases is relatively conserved, therefore inhibitor selectivity is an important concern.

In the present study, chemical modifications of the structure of Fasudil (Figure 1) have been carried out in order to specify structure-activity relationships and eventually to improve the pharmacological profile of this compound. These modifications affected the isoquinolinyl heterocycle and the sulfonyl group which could be involved in ionic and/or hydrogen bonds with the ATP binding site of Rho-kinase. With the aim of evaluating the selectivity level of these compounds that showed a Rho-kinase inhibitory effect, they were also tested on another protein-kinase: protein-kinase C (PKC).

Rho-Kinase

A novel serine/threonine protein kinase, Rho-kinase/ROKα/ROCK II, was identified as an effector of the small guanosine triphosphatase (GTPase) RhoA.[5,6] ROKβ/ROCK I is an isoform of Rho-kinase.[5,7] Rho-kinase has a kinase domain in its N-terminal domain, a coiled-coil domain in its

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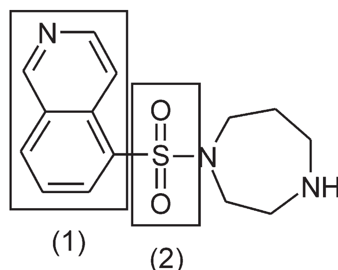


FIGURE 1 Structure of Fasudil with the two components which have been modified.

middle portion, and a putative pleckstrin-homology (PH) domain in its C-terminal domain that is split by the insertion of a cystein-rich region. Rho-kinase binds to and is activated by the GTP-bound active form of Rho.[7–9] Rho-kinase appears to regulate various cellular responses downstream of Rho:[10] stress fiber and focal adhesion formations,[5,7,9] smooth-muscle contraction.[11–13] Rho-kinase regulates the phosphorylation of myosin light chain (MLC), resulting in actomyosin contractility, by the direct phosphorylation of MLC[11] and by the inactivation of myosine phosphatase through the phosphorylation of myosin binding subunit.[12]

Hypercontraction or abnormal smooth-muscle contraction may be a major cause of disease states such as hypertension and asthma, and a smooth-muscle relaxant that modulates this process would be therapeutically useful. To evaluate the inhibitory potential of the synthesised compounds, two tests were used. The first test analysed their effects on the actin cytoskeleton, and so on stress fiber formation. The second test was a direct measure of the enzymatic activity of Rho-kinase. This test was only conducted if the first test results showed an inhibitory activity.

Protein Kinase C

Protein kinase C, a serine/threonine kinase, was discovered by Nishizuka.[14] PKC exists in several isoforms divided into three subclasses: classical, novel and atypic. Classical PKC (α, βI, βII, γ) can be stimulated by Ca^{2+}, various lipids like diacylglycerol (DAG), phosphatidylserine (PS) or analogs like phorbol esters. Novel PKC (δ, ϵ, η, θ) can be activated by DAG and PS but not by Ca^{2+}. Stimulation of the atypical PKC (ζ, λ) is also discussed, most of studies have shown this activation is independent of Ca^{2+} or DAG and its analogue,[15] whereas some results conclude that phorbol esters can stimulate these enzymes.[16] In each case, as a result of the stimulation, PKC is translocated from the cytoplasm to the plasmic membrane to attain its enzymatic capacity.

In this study, we took into consideration the implication of PKC on oxidative stress in human neutrophils. This stress can be responsible for the production of reactive oxygen species which involve damage to cellular structures. To evaluate the inhibitory activity of our compounds on PKC, three tests were used. The first one checked their activity on oxidative stress by the measure of superoxide anion ($O_2^{\cdot -}$) production. The activator was phorbol-12-myristate-13-acetate (PMA), a compound capable of activating neutrophils by action on PKC.[17] The second test analysed the binding variation between PKC and a radioactive phorbol ester, a specific ligand of this enzyme. The final test was the visualisation, by Western blot, of the translocation or not of PKC during its activation.

MATERIALS AND METHODS

Instrumentation

Melting points were determined on a BUCHI 535 apparatus and are not corrected. IR spectra were obtained in KBr pellets with a Vector 22 Bruker spectrometer. ^1H-NMR spectra were obtained using an AC 300 Bruker spectrometer in d_6-DMSO or $CDCl_3$ as solvent. Chemical shifts are expressed as δ values (ppm) relative to Me_4Si as internal standard. Elemental analyses for new substances were performed by CNRS Laboratories (Vernaison, France) and the results obtained were within ±0.4% the theoretical values. All reactions were monitored by thin-layer chromatography (TLC) using 0.2 mm-thick silica gel plates 60F 254 (5735 Merck).

Synthesis

1-(1-Naphthylsulfonyl)-1,4-diazepane, Hydrochloride 2a

To an ice-cold CH_2Cl_2 solution (100 ml) of homopiperazine (0.90 g, 8.8 mmol) was added dropwise a solution of 1-naphthalenesulfonyl chloride **1a** (1.00 g, 4.4 mmol) in CH_2Cl_2 (100 mL). The reaction mixture was stirred for 2 h at room temperature and then evaporated under reduced pressure. The residue was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm) and eluted with $MeOH/CH_2Cl_2$ gradients to give a colorless oil. The residue was transformed into its hydrochloride salt and recrystallized from EtOH to give a white crystalline solid (0.50 g, 1.5 mmol) (35% yield) **2a**, m.p. = 203–205°C. ^1H-NMR (d_6-DMSO) δ 2.05 (m, 2H, CH_2), 3.30–3.00 (m, 4H, $2CH_2$), 3.80–3.40 (m, 4H, $2CH_2$), 8.35–7.60 (m, 6H, H_{ar}), 8.55 (d, 1H, J = 8.48 Hz, H_{ar}), 9.60 (signal, 2H, NH_2^+). Found: C, 55.24; H, 5.88; N, 8.65.

$C_{15}H_{18}N_2O_2S{\cdot}HCl$ requires: C, 55.07; N, 5.81; N, 8.56%.

5-(1,4-Diazepane-1-ylsulfonyl)-1,2,3,4-tetrahydro-isoquinoline, Dihydrochloride 3

To a stirred solution of Fasudil **2b** (0.70 g, 2.4 mmol) in AcOH (60 mL) was added PtO_2 (0.10 g). The mixture was hydrogenated for 48 h at room temperature under atmospheric pressure or until a reasonable conversion was reached (TLC control). The catalyst was filtered off and the filtrate was concentrated under reduced pressure to give a colorless oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with $MeOH/CH_2Cl_2$ gradients gave a colorless oil. The residue was transformed into its hydrochloride salt and recrystallized from isopropanol/H_2O (9/1) to give a white crystalline solid (0.55 g, 1.5 mmol) (62% yield) **3**, m.p. > 260°C. ^{1}H-NMR (d_6-DMSO) δ 2.00 (m, 2H, CH_2), 3.80–2.80 (m, 12H, $6CH_2$), 4.35 (s, 2H, CH_2), 7.60–7.40 (m, 2H, H_{ar}), 7.75 (d, 1H, $J = 7.49$ Hz, H_{ar}), 10.00–9.30 (signals, 4H, $2NH_2^+$). Found: C, 45.69; H, 6.38; N, 11.22. $C_{14}H_{21}N_3O_2S{\cdot}2HCl$ requires: C, 45.65; N, 6.29; N, 11.41%.

tert-Butyl 4-(5-isoquinolylsulfonyl)-1,4-diazepane-1-carboxylate 4

To a mixture of *t*BuOH /H_2O (100/20 ml), were added Fasudil (3.40 g, 11.7 mmol), a solution of 1N NaOH (25 ml) and di-*tert*-butyl dicarbonate (3.05 g, 14.0 mmol). The reaction was stirred for 5 h at room temperature and evaporated under reduced pressure. The organic layer was extracted with EtOAc, dried over $MgSO_4$, filtered and concentrated under reduced pressure to give an oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with acetone/toluene/cyclohexane gradients gave a colorless oil (2.00 g, 5.1 mmol) (44% yield) **4**. ^{1}H-NMR (d_6-DMSO) δ 1.35 (s, 9H, $3CH_3$), 1.75 (m, 2H, CH_2), 3.60–3.25 (m, 8H, $4CH_2$), 7.85 (m, 1H, H_{ar}), 8.55–8.25 (m, 3H, H_{ar}), 8.70 (d, 1H, $J = 6.16$ Hz, H_{ar}), 9.50 (s, 1H, H_{ar}).

tert-Butyl 4-(1,2,3,4-tetrahydro-5-isoquino linylsulfonyl)-1,4-diazepane-1-carboxylate 5

The procedure was the same described above for the preparation of **3** using compound **4** (1.80 g, 4.6 mmol) except that MeOH (60 mL) was used instead of AcOH. The catalyst was filtered off and the filtrate was concentrated under reduced pressure to give a colorless oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with $MeOH/CH_2Cl_2$ gradients gave a colorless oil (1.30 g, 3.3 mmol) (72% yield) **5**. ^{1}H-NMR ($CDCl_3$) δ 1.45 (s, 9H, $3CH_3$), 2.00 (m, 2H, CH_2), 2.60 (signal, 1H, NH), 3.70–3.00 (m, 12H, $6CH_2$), 4.10 (s, 2H, CH_2), 7.40–7.10 (m, 2H, H_{ar}), 7.70 (m, 1H, H_{ar}).

5-(1,4-Diazepan-1-ylsulfonyl)-2-methyl-1,2,3,4-tetrahydroisoquinoline, Dihydrochloride 6

To a solution of **5** (1.20 g, 3.0 mmol) in acetone (60 mL) were added K_2CO_3 (1.25 g, 9.1 mmol) and methyl iodide (0.20 mL, 3.3 mmol). The reaction was stirred under refluxed for 72 h until TLC showed a reasonable conversion. After cooling, the reaction was filtered and concentrated under reduced pressure to give an oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with $MeOH/CH_2Cl_2$ gradients gave a colorless oily residue which was treated with diethyl ether saturated with gaseous hydrochloric acid in a minimum volume of methanol. The reaction mixture was kept overnight at room temperature. The crude white solid was filtered, washed with Et_2O and recrystallized from EtOH to give a white crystalline solid (0.38 g, 1.0 mmol) (33% yield) **6**, m.p. > 260°C. ^{1}H-NMR (d_6-DMSO) δ 2.00 (m, 2H, CH_2), 3.85–3.10 (m, 15H, $6CH_2$ and CH_3), 4.70 (s, 2H, CH_2), 7.65–7.45 (m, 2H, H_{ar}), 7.90 (m, 1H, H_{ar}), 9.80–9.50 (signals, 3H, NH_2^+ and NH^+). Found: C, 47.17; H, 6.20; N, 10.60. $C_{15}H_{23}N_3O_2S{\cdot}2HCl$ requires: C, 47.12; N, 6.59; N, 10.99%.

General Procedure for the Preparation of Compounds 8a–b

To an ice-cold aqueous solution of 12N HCl (40 mL) were added the appropriate amine **7a–b** (42.3–15.1 mmol) and dropwise an ice-cold solution of $NaNO_2$ (46.5–16.61 mmol) in H_2O (10 mL). The reaction was stirred for 1 h at O°C. The resulting mixture was added dropwise to an ice-cold AcOH solution (20–40 mL) of a copper(II) chloride (12.7–4.5 mmol in H_2O)/sulfur dioxide gas (46.3–130 mmol) mixture. The reaction mixture was allowed to stir for 16 h at room temperature and filtered. The precipitate was recrystallized from cyclohexane. Experimental data for the compounds are reported below.

3-cyano-1-benzenesulfonyl chloride **8a**

It was prepared using compound **7a** (5.00 g, 42.3 mmol): orange crystalline solid 4.43 g (22.0 mmol) (52% yield), m.p. = 53–55°C. ^{1}H-NMR ($CDCl_3$) δ 7.85 (m, 1H, H_{ar}), 8.05 (m, 1H, H_{ar}), 8.45–8.25 (m, 2H, H_{ar}).

2-(CYANOMETHYL)-1-BENZENESULFONYL CHLORIDE **8b**

It was prepared using compound **7b** (2.00 g, 15.1 mmol): white crystalline solid 2.18 g (10.1 mmol) (67% yield), m.p. = 109–111°C. ^{1}H-NMR ($CDCl_3$) δ 4.40 (s, 2H, CH_2), 7.65 (m, 1H, H_{ar}), 7.95–7.80 (m, 2H, H_{ar}), 8.19 (dd, 1H, $J = 7.75$ Hz and $J' = 1.06$ Hz, H_{ar}).

3-(1,4-Diazepan-1-ylsulfonyl)benzonitrile 9a

The procedure was the same described above for the preparation of **2a** using compound **8a** (4.00 g, 19.8 mmol). The reaction mixture was allowed to stir for 16 h at room temperature and then evaporated under reduced pressure. The residue was recrystallized from cyclohexane to give a white crystalline solid (4.45 g, 16.8 mmol) (85% yield) **9a**, m.p. = 86–87°C. ^{1}H-NMR ($CDCl_3$) δ 1.85 (m, 2H, CH_2), 3.05–2.85 (m, 4H, $2CH_2$), 3.50–3.30 (m, 4H, $2CH_2$), 7.65 (m, 1H, H_{ar}), 7.85 (d, 1H, $J = 7.95$ Hz, H_{ar}), 8.05 (d, 1H, $J = 7.95$Hz, H_{ar}), 8.10 (s, 1H, H_{ar}).

2-[2-(1,4-Diazepan-1-ylsulfonyl)phenyl]acetonitrile 9b

The procedure was the same described above for the preparation of **2a** using compound **8b** (2.00 g, 9.3 mmol). The reaction mixture was allowed to stir for 16 h at room temperature and then evaporated under reduced pressure. The residue was recrystallized from cyclohexane to give a white crystalline solid (2.07 g, 7.4 mmol) (80% yield) **9b**, m.p. = 76–78°C. ^{1}H-NMR (d_6-DMSO) δ 1.65 (m, 2H, CH_2), 2.90–2.70 (m, 4H, $2CH_2$), 3.30 (t, 2H, $J = 5.16$ Hz, CH_2), 3.40 (t, 2H, $J = 5.16$ Hz, CH_2), 4.30 (s, 2H, CH_2), 7.60 (m, 1H, H_{ar}), 7.80–7.68 (m, 2H, H_{ar}), 7.90 (d, 1H, $J = 7.68$ Hz, H_{ar}).

General Procedure for the Preparation of Compounds 10a–b

To a solution of the appropriate sulfonamide (**9a–b**) (2.8–2.1 mmol) in EtOH (80 mL) saturated with gaseous ammonia was added Raney nickel (0.075–0.060 g). The reaction mixture was stirred under a 50 atm hydrogen pressure, at 60°C, for 5 h. The catalyst was filtered off and the filtrate was concentrated under reduced pressure to give a colorless oily residue which could be transformed into its hydrochloride or oxalate salts to give a white solid. The obtained compounds were further recrystallized from EtOH. Experimental data for the compounds are reported below.

3-(1,4-DIAZEPAN-1-YLSULFONYL)BENZYLAMINE, DIOXALATE **10a**

It was prepared using compound **9a** (0.75 g, 2.8 mmol): white crystalline solid 0.37 g (0.8 mmol) (30% yield), m.p. = 225–227°C. ^{1}H-NMR (d_6-DMSO) δ 1.95 (m, 2H, CH_2), 3.25–3.05 (m, 4H, $2CH_2$), 3.60–3.25 (m, 4H, $2CH_2$), 4.20 (s, 2H, CH_2), 7.68 (m, 1H, H_{ar}), 7.90–7.75 (m, 2H, H_{ar}), 7.97 (s, 1H, H_{ar}). Found: C, 43.02; H, 5.26; N, 9.25. $C_{12}H_{19}N_3O_2S{\cdot}2H_2C_2O_4$ requires: C, 42.76; N, 5.16; N, 9.35%.

2-[2-(1,4-DIAZEPAN-1-YLSULFONYL)PHENYL]-1-ETHANAMINE, DIHYDROCHLORIDE **10b**

It was prepared using compound **9b** (0.60 g, 2.1 mmol): white crystalline solid 0.38 g (0.1 mmol) (51% yield), m.p. = 137–139°C. ^{1}H-NMR (d_6-DMSO) δ 2.10 (m, 2H, CH_2), 3.55–3.00 (m, 10H, $5CH_2$), 3.70 (m, 2H, CH_2), 7.70–7.45 (m, 3H, H_{ar}), 7.80 (d, 1H, $J = 8.04$ Hz, H_{ar}), 8.30 (signal, 3H, NH_3^+), 9.60 (signal, 2H, NH_2^+). Found: C, 44.12; H, 6.39; N, 11.76. $C_{13}H_{21}N_3O_2S{\cdot}2HCl$ requires: C, 43.82; N, 6.51; N, 11.79%.

tert-Butyl 4-[(3-cyanophenyl)sulfonyl]-1,4-diazepan-1-carboxylate 11a

The procedure was the same described above for the preparation of **4** using compound **9a** (4.20 g, 15.8 mmol). The residue was recrystallized from cyclohexane to give a white crystalline solid (3.63 g, 10.0 mmol) (63% yield) **11a**, m.p. = 110–112°C. ^{1}H-NMR (d_6-DMSO) δ 1.38 (s, 9H, $3CH_3$), 1.70 (m, 2H, CH_2), 3.50–3.20 (m, 8H, $4CH_2$), 7.82 (m, 1H, H_{ar}), 8.20–8.05 (m, 2H, H_{ar}), 8.30 (s, 1H, H_{ar}).

tert-Butyl 4-{[2-(cyanomethyl)phenyl]sulfonyl}-1,4-diazepane-1-carboxylate 11b

The procedure was the same described above for the preparation of **4** using compound **9b** (1.70 g, 6.1 mmol). The residue was recrystallized from cyclohexane to give a white crystalline solid (1.82 g, 4.8 mmol) (79% yield) **11b**, m.p. = 115–117°C. ^{1}H-NMR (d_6-DMSO) δ 1.45 (s, 9H, $3CH_3$), 2.00 (m, 2H, CH_2), 3.45–3.30 (m, 4H, $2CH_2$), 3.65–3.50 (m, 4H, $2CH_2$), 4.20 (s, 2H, CH_2), 7.50 (m, 1H, H_{ar}), 7.75–7.60 (m, 2H, H_{ar}), 7.85 (m, 1H, H_{ar}).

tert-Butyl 4-[(3-formylphenyl)sulfonyl]-1,4-diazepane-1-carboxylate 12

To a solution of dry toluene (100 mL) were added nitrile **11a** (3.00 g, 8.2 mmol) and dropwise a 1 M solution of DIBAL-H in THF (19.70 mL, 0.0197 mmol) over 15 min. The reaction was stirred overnight at room temperature. The resulting mixture was hydrolysed with aqueous 1N HCl until pH = 4.0, then 50 mL of CH_2Cl_2 was added and the mixture agitated vigorously for 10 min. The layers were separated, and the aqueous solution was extracted with CH_2Cl_2. The combined organics were dried over $MgSO_4$, filtered and

concentrated under reduced pressure to give an oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with $MeOH/CH_2Cl_2$ gradients gave a colorless oil (2.17 g, 6.0 mmol) (73% yield) **12**. ^{1}H-NMR (d_6-DMSO) δ 1.40 (s, 9H, $3CH_3$), 1.95 (m, 2H, CH_2), 3.40–3.20 (m, 4H, $2CH_2$), 3.60–3.40 (m, 4H, $2CH_2$), 7.72 (dd, 1H, J = 7.95 and 7.62 Hz, H_{ar}), 8.05 (d, 1H, J = 7.62 Hz, H_{ar}), 8.10 (d, 1H, J = 7.95Hz, H_{ar}), 8.30 (s, 1H, H_{ar}), 9.80 (signal, 1H, CHO).

tert-Butyl 4-({3-[(isopropylamino)-methyl]phenyl}sulfonyl)-1,4-diazepane-1-carboxylate 13

To a solution of aldehyde **12** (1.20 g, 3.2 mmol) in MeOH (40 mL) were added $NaBH_3CN$ (0.22 g, 3.2 mmol) and isopropylamine (0.40 mL, 4.9 mmol). The reaction was stirred under refluxed for 16 h. After cooling, the reaction was concentrated under reduced pressure to give a colorless oily residue which was dissolved in CH_2Cl_2 (30 mL). The organic layer was washed with H_2O, dried over $MgSO_4$, filtered and concentrated under reduced pressure to give an oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with EtOAc gave a colorless oil (0.52 g, 1.3 mmol) (40% yield) **13**. ^{1}H-NMR (d_6-DMSO) δ 1.10 (d, 6H, J = 6.18 Hz, $2CH_3$), 1.40 (s, 9H, $3CH_3$), 1.55 (signal, 1H, NH), 1.95 (m, 2H, CH_2), 2.85 (m, 1H, CH), 3.35–3.15 (m, 4H, $2CH_2$), 3.60–3.40 (m, 4H, $2CH_2$), 3.85 (s, 2H, CH_2), 7.50 (t, 1H, J = 7.77 Hz, H_{ar}), 7.60 (d, 1H, J = 7.77 Hz, H_{ar}), 7.65 (d, 1H, J = 7.77 Hz, H_{ar}), 7.75 (s, 1H, H_{ar}).

N-[3-(1,4-Diazepan-1-ylsulfonyl)benzyl]-N-isopropylamine, Dihydrochloride 14

A solution of amine **13** (0.45 g, 1.1 mmol) in Et_2O (30 mL) saturated with gaseous hydrochloric acid was stirred for 24 h at room temperature. The reaction was concentrated under reduced pressure to give a crude white solid which was washed with Et_2O without any further purification to give a white solid (0.31 g, 0.8 mmol) (74% yield) **14**, m.p. = 229–231°C. ^{1}H-NMR (d_6-DMSO) δ 1.35 (d, 6H, J = 6.36 Hz, $2CH_3$), 2.00 (m, 2H, CH_2), 3.25–3.05 (m, 4H, $2CH_2$), 3.70–3.25 (m, 5H, $2CH_2$ and CH), 4.25 (m, 2H, CH_2), 7.70 (m, 1H, H_{ar}), 7.85 (d, 1H, J = 8.22 Hz, H_{ar}), 7.93 (d, 1H, J = 7.74 Hz, H_{ar}), 8.10 (s, 1H, H_{ar}), 9.70–9.30 (signals,4H, $2NH_2^+$). Found: C, 46.70; H, 7.17; N, 10.83. $C_{15}H_{25}N_3O_2S{\cdot}2HCl$ requires: C, 46.87; N, 7.08; N, 10.93%.

tert-Butyl 4-{[2-(2-aminoethyl)phenyl]sulfonyl}-1,4-diazepane-1-carboxylate 15

The procedure was the same described above for the preparation of **10a–b** using compound **11b** (1.70 g, 4.5 mmol). The catalyst was filtered off and the filtrate was concentrated under reduced pressure to give a colorless oily residue (1.55 g, 4.0 mmol) (90% yield) **15**. ^{1}H-NMR ($CDCl_3$) δ 1.45 (s, 9H, $3CH_3$), 1.60 (m, 2H, CH_2), 2.00 (m, 2H, CH_2), 3.20–2.90 (m, 4H, CH_2 and NH_2), 3.45–3.35 (m, 4H, $2CH_2$), 3.60–3.50 (m, 4H, $2CH_2$), 7.55–7.25 (m, 3H, H_{ar}), 7.70 (m, 1H, H_{ar}).

tert-Butyl 4-({2-[2-(dimethylamino)ethyl]-phenyl}sulfonyl)-1,4-diazepane-1-carboxylate 16

To an ice-cold solution of amine **15** (0.70 g, 1.8 mmol) in MeOH (15 mL) were added $NaBH_3CN$ (0.23 g, 3.6 mmol), AcOH glacial (0.30 mL, 4.7 mmol) and dropwise an aqueous solution of 30% formaldehyde (0.55 mL, 5.4 mmol) in MeOH (15 mL). The reaction mixture was stirred for 3 h at room temperature before adding saturated K_2CO_3 solution (5 mL). The MeOH was evaporated under reduced pressure and H_2O was added to the residue. The organic layer was extracted with EtOAc, dried over $MgSO_4$, filtered and concentrated under reduced pressure to give an oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with $MeOH/CH_2Cl_2$ gradients gave a colorless oil (0.63 g, 1.5 mmol) (85% yield) **16**. ^{1}H-NMR (d_6-DMSO) δ 1.45 (s, 9H, $3CH_3$), 2.00 (m, 2H, CH_2), 2.40 (s, 6H, $2CH_3$), 2.70 (t, 1H, J = 8.17 Hz, CH_2), 3.20 (t, 1H, J = 8.17 Hz, CH_2), 3.45-3.35 (m, 4H, $2CH_2$), 3.60–3.50 (m, 4H, $2CH_2$), 7.55–7.25 (m, 3H, H_{ar}), 7.70 (m, 1H, H_{ar}).

N,N*-Dimethyl-2-[2-(1,4-diazepan-1-ylsulfonyl)-phenyl]-1-ethanamine, Dioxalate 17*

The procedure was the same described above for the preparation of **14** using compound **16** (0.60 g, 1.5 mmol). The reaction was concentrated under reduced pressure before adding 10% K_2CO_3 aqueous solution (10 mL). The organic layer was extracted with Et_2O, dried over $MgSO_4$, filtered and concentrated under reduced pressure to give an oily residue which was transformed into its oxalate salt to give a white solid (0.37 g, 0.7 mmol) (50% yield) **17**, m.p. = 229–231°C. ^{1}H-NMR (d_6-DMSO) δ 2.05 (m, 2H, CH_2), 2.75 (s, 6H, $2CH_3$), 3.35–3.05 (m, 8H, $4CH_2$), 3.45 (t, 2H, J = 5.86 Hz, CH_2), 3.65 (m, 2H, CH_2), 7.73–7.46 (m, 3H, H_{ar}), 7.77 (d, 1H, J = 7.20 Hz, H_{ar}). Found: C, 46.80; H, 6.20; N, 8.84. $C_{15}H_{25}N_3O_2S{\cdot}2H_2C_2O_4$ requires: C, 46.43; N, 5.95; N, 8.55%.

5-Isoquinolinecarbonitrile 20

To a solution of the aryl bromide **19** (1.00 g, 4.8 mmol) in deoxygenated DMF (10 mL) were added zinc cyanide (0.35 g, 2.9 mmol) and tetrakis-(triphenylphosphine) palladium(0) (0.22 g, 0.2 mmol). The reaction was stirred for 6 h under nitrogen, at 80°C. After cooling, the resulting mixture was diluted with toluene (15 mL) and washed twice with 2N ammonium hydroxyde (40 mL). The toluene solution was washed with brine (20 mL) and concentrated under reduced pressure to give a crude white solid which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with petroleum ether/Et_2O gradients gave a white solid (0.55 g, 3.6 mmol) (75% yield) **20**, m.p. = 138–140°C. ^{1}H-NMR (d_6-DMSO) δ 7.85 (m, 1H, H_{ar}), 7.95 (d, 2H, $J = 5.90$ Hz, H_{ar}), 8.57–8.40 (m, 2H, H_{ar}), 8.75 (d, 1H, $J = 5.90$ Hz, H_{ar}), 9.52 (s, 1H, H_{ar}).

1,4-Diazepan-1-yl(5-isoquinolyl)methanone 22

To a stirred solution of the acid hydrochloride **21** (1.00 g, 4.7 mmol) in CH_2Cl_2 (50 mL) was added dropwise thionyl chloride (3.70 mL, 50 mmol). After being heated under reflux for 5 h, the resulting solution was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (40 mL) and added dropwise to an ice-cold CH_2Cl_2 solution (20 mL) of homopiperazine (0.95 g, 9.5 mmol). The reaction mixture was stirred for 16 h at room temperature, filtered and evaporated under reduced pressure. The residue was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm) and eluted with MeOH/CH_2Cl_2 gradients to give a yellow oil (0.50 g, 1.9 mmol) (40% yield) **22**. ^{1}H-NMR (d_6-DMSO) δ 1.45 (m, 1H, CH), 1.85 (m, 1H, CH), 3.05–2.55 (m, 4H, 4CH), 3.80–3.10 (m, 5H, 4CH and NH), 7.60 (m, 1H, H_{ar}), 8.30–7.65 (m, 3H, H_{ar}), 8.70 (d, 1H, $J = 5.81$ Hz, H_{ar}), 9.45 (s, 1H, H_{ar}). Found: C, 70.73; H, 6.33; N, 16.86. $C_{15}H_{17}N_3O$ requires: C, 70.57; N, 6.71; N, 16.46%.

Pharmacology

Chemicals and Drugs

Texas Red-Dnase I was obtained from Molecular Probe (Leiden, The Netherland). The chromatography column was purchased from Pharmacia Biotech AB (Uppsala, Sweden). Primary and secondary antibodies were purchased from Tebu (Le Perray en Yvelines, France). ECL Western blotting detection reagents and Hyperfilm ECL were purchased from Amersham International plc (Bucks, UK). All other reagents were purchased from Sigma (Saint Quentin Fallavier, France).

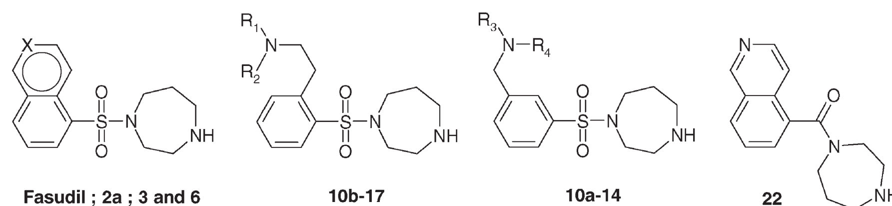
Smooth Muscle Cell Culture

Smooth muscle cells from young rat (45 g) aorta were isolated by enzymatic dissociation as previously described.[18] Cells were cultured in DMEM with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Secondary cultures were obtained by serial passages after the cells were harvested with 0.5 g/l trypsin and 0.2 g/l EDTA (Trypsin-EDTA) and reseeded in fresh DMEM containing 10% FCS and antibiotics.

Actin Staining

After dissociation, aortic myocytes were cultured in DMEM with 10% FCS on glass coverslips for 2 days. The cells were maintained in culture for another day (24 h) in the absence or presence of compounds to be tested. Cells were then fixed for 30 min in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and then rinsed in phosphate-buffered saline (PBS). For polymerized (F) actin staining, cells were incubated with FITC-conjugated phalloidin (5 μg/ml) for 45 min at room temperature then washed with PBS. Actin staining was also performed with a monoclonal anti-α-smooth muscle actin antibody followed by FITC-conjugated anti-mouse antibody which gave results similar to those obtained with FITC-conjugated phalloidin. When dual labeling was performed, cells were simultaneously stained with FITC-conjugated phalloidin and Texas red-labeled DNase I (10 μg/ml) to localize monomeric G-actin,[19] and then washed in PBS. Coverslips were mounted on a glass slide and examined with a fluorescence microscope (Eclipse E-600, Nikon, Champigny-sur-Marne, France). The background fluorescence signal was estimated by collecting planes from areas of the slide without cells and was electronically subtracted before analysis. Images were collected with a cool-SNAP camera (Princeton Instruments, Evry, France) and stored and analyzed using Metamorph software (Universal Imaging, West Chester, PA). For each area examined, images of FITC-phalloidin and Texas Red-DNase I fluorescence were collected. The time of measurements and image capturing and the image intensity gain at both wavelength were optimally adjusted and kept constant. The ratio of fluorescence of FITC-phalloidin and Texas Red-DNase I (F- to G-actin ratio), used to quantify actin cytoskeleton organization, and so stress fiber formation, was calculated for at least 20 cells in each experimental condition and expressed as a percentage of the ratio obtained under control condition. A decrease in the F- to G-actin ratio was assumed to represent depolymerization of actin filaments and so the percentage of inhibition of the compounds. This results for this test will be expressed at a 10^{-5} M concentration (Table I–Test A-1).

TABLE I Inhibition values for the stress fiber formation (Test A-1) and the enzymatic activity for Rho-kinase (Test A-2).



Compound	X	R_1	R_2	R_3	R_4	Test A-1 % of inhibition at 10^{-5}M	Test A-2 % of inhibition at 10^{-5}M
Fasudil	N					73.5 ± 6.1	64
3	HN					55.6 ± 2.85	58
2a	HC					Inactive	
6	H_3C—N					Inactive	
10b		H	H			Inactive	
17		CH_3	CH_3			Inactive	
10a				H	H	Inactive	
14				H	CH-$(CH_3)_2$	Inactive	
22						Inactive	

Rho-Kinase Assay

The kinase reaction was carried out in a 50 μl kinase buffer (20 mM Tris, 25 mM β-glycerol phosphate, 1 mM EGTA, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, pH 7.5) containing 90 μM [γ-^{32}P]ATP (2.2 mCi/mmol), 50 μM S6 kinase substrate peptide, 20 mU Rho-kinase (Upstate Biotechnology), with or without 10 μM of potential Rho-kinase inhibitor. After incubation for 30 min at 30°C with agitation, the reaction mixtures were spotted onto Whatman P81 paper. The paper was washed three times for 5 min with 0.75% phosphoric acid and once for 3 min with acetone. ^{32}P incorporation into the substrate was then determined by Cerenkov counting (Table I–Test A-2).

Polymorphonuclear Separation Technique

The 15 ml of freshly drawn human heparinized blood, obtained from a healthy donor, was diluted 1:2 with a 0.1 M phosphate buffer saline; pH 7.4; 10 ml of histopaque®-1077 was placed at the bottom of a conical tube. After centrifugation (400 × g for 30 min at 20°C), 10 ml of plasma was put aside, the supernatant was eliminated, the pellet was resuspended with the plasma, and the suspension was diluted 1:10 with an ammonium chloride solution (0.15 M ammonium chloride; 10 mM sodium bicarbonate; pH 7.4). After beginning the haemolysis, the tube was stored at 4°C and gently shaken all the time; the polymorphonuclears were recovered by centrifugation (400 × g at 4°C for 10 min), then the cells were washed twice with Hank's Hepes buffer.[20] Cell viability, determined by Trypan blue exclusion was over 95%. The cells were kept at 4°C until use. The cytotoxicity of the compound was estimated by determining lactate dehydrogenase activity in the cellular supernatant of a polymorphonuclear suspension.[21]

Anion Superoxide Assay

The polymorphonuclears ($5{\cdot}10^6$ GrN/ml) were incubated with the inhibitor and the cells were activated with 160 nM PMA at 37°C for 5 min. After incubation, ferricytochrome C at 0.2 mg/ml was added to the cellular suspension, and when this compound was reduced, it caused a change in color measured at 550 nm.[22] The concentration of anion superoxide was calculated using the extinction coefficient: $E_{550\,nm} = 2.1{\cdot}10^{-2}\ \mu M^{-1}\,cm^{-1}$ (Table II–Test B-1).

An acellular model could determine a scavenger effect. Anion superoxide was produced by a hypoxanthine-xanthine oxidase system.[23] Absorbance was measured at 550 nm.

TABLE II Inhibition results for superoxide anion production (Test B-1), for binding phorbol-12,13-dibutyrate (Test B-2) and for translocation of protein kinase C (Test B-3)

	Test B-1 IC_{50} (μM)	Test B-2 IC_{50} (μM)	Test B-3 Inhibition at 10^{-3} M
Fasudil	118 ± 21	1.317 ± 0.057	yes
3	682 ± 80	>10	No

Binding

The protein kinase C was purified by chromatography.[24] 20 μg of purified protein was used per tube and the compound was incubated with the enzyme for 15 min at 30°C in a specific buffer (20 mM TRIS; 100 mM potassium chloride; 0.5 mM calcium chloride; 50 μg/ml phosphatidylsérine; 0.17% DMSO; 0.2% ethanol; pH 7.5). Then 30 mM [^{3}H] phorbol-12,13-dibutyrate (activity 18.6 Ci/mmol) was placed in each tube to start the reaction, the final volume being 325 μl. The mixture was put at 30°C for 10 min, and the reaction was stopped by adding 1 ml of ice-cold 0.5% DMSO solution. The tube was emptied on a GF/C filter, before being plunged into a 0.3% polyethylenimine solution. The filter was washed five times with the DMSO solution and then dried by aspiration.[25] Residual radioactivity was measured with a scintillation counter. Non-specific binding was determined using cold phorbol-12, 13-dibutyrate (Table II–Test B-2).

Preparation of Cells for Western Blot

5×10^6 cells/ml were incubated for 20 min with 57 μM PMSF at 4°C and then centrifuged (400 × g for 10 min at 4°C) to eliminate the PMSF solution. They were then resuspended in a Hank's Hepes buffer pH 7.4, incubated with the inhibitor at 37°C and then activation started by 160 nM PMA at 37°C for 5 min. The final volume was 5 ml. Stimulation was stopped by addition of 20 ml of ice cold Hank's Hepes buffer and centrifuging (400 × g for 10 min at 4°C). The pellet was resuspended in 500 μl of buffer (20 mM TRIS; 5 mM EGTA; 2 mM EDTA, 50 mMβ-mercaptoethanol; 1 mM PMSF; 330 μM leupeptin; 350 μM antipain; A 350 μM pepsatin; 40 μM chymostatin; 3.1 μM aprotinin; 0.25 mM sucrose; pH 7.5) and sonicated 6 times for 5 s. The lysate protein was centrifuged (100 000 × g for 30 min at 4°C) and the supernatant was kept. The pellet was resuspended with 200 μl of the previous buffer, each sample was mixed with a SDS sample buffer (32 mM TRIS; 71 mM SDS; 1.35 mM glycerol; 0.76 mM bromophenol blue) and boiled for 10 min.[26] The protein concentration was determined by the Folin assay.

Western Blot

The equivalent of 130 μg of protein was analysed by polyacrylamide gel electrophoresis on 10% SDS-PAGE for 15 h at 40 mA. The proteins were electrophoretically transferred to the nitrocellulose membrane for 2 h at 36 v. The membrane was saturated with a solution of 10% fat-free dried milk in TRIS buffer saline 0.05% Tween 20 (TBS-Tween 20) for 30 min, followed by incubation with rabbit polyclonal antibodies [anti-PKCβI, anti-PKCβII or anti-PKCζ (1/1000 dilution)] for 1.5 h at room temperature. The membrane was washed twice for 10 min with TBS-Tween 20, followed by incubation with (1/10 000) dilution of a rabbit peroxidase conjugated secondary antibody for 45 min at room temperature. The membrane was abundantly washed with TBS-Tween 20, then incubated with an ECL mixture of two reagents for 20 min and the films were developed in a dark room (Table II–Test B-3).

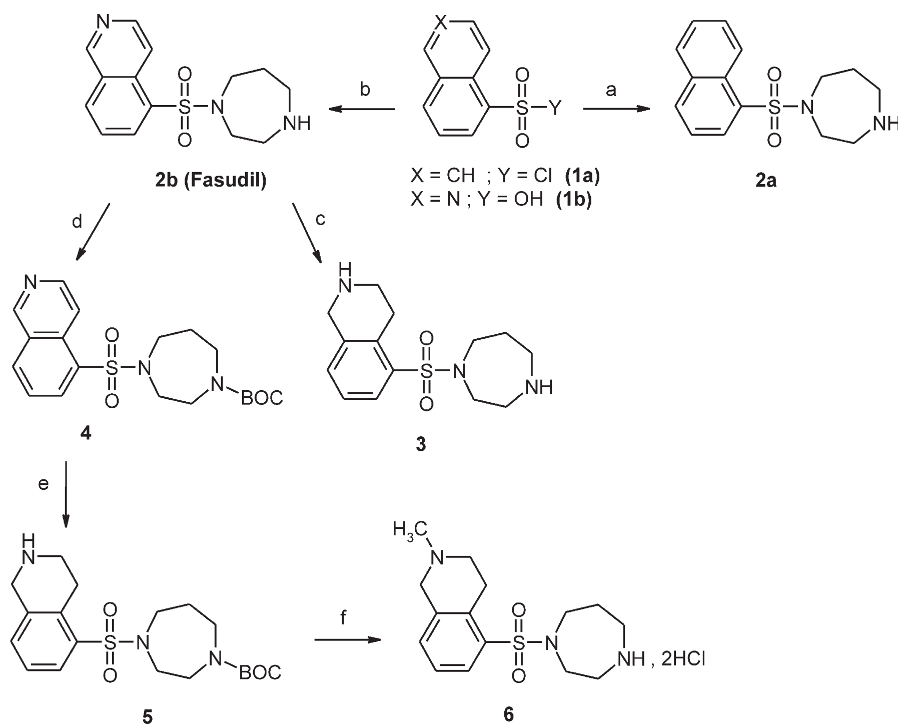
RESULTS AND DISCUSSION

Chemistry

Fasudil and compounds **19** and **21** were prepared according to existing literature procedure.[27–29,34] The sulfonyl chloride **1a** and the amines used as starting materials were commercially available.

The synthesis of sulfonamides **2a**, **3** and **6** is shown in Scheme 1. Treatment of the 1-naphthalenesulfonyl chloride **1a** with homopiperazine in methylene chloride afforded sulfonamide **2a**. Sulfonamide **3** was obtained from Fasudil **2b** by hydrogenation in the presence of platinum oxide in acetic acid under hydrogen at atmospheric pressure. Condensation of Fasudil with di-*tert*-butyl dicarbonate in a *tert*-butanol/water mixture under basic conditions gave the *N*-Boc-protected amine **4** which led to the secondary amine **5**, by hydrogenation in the presence of platinum oxide in methanol under hydrogen at atmospheric pressure. This compound was first treated with methyl iodide in acetone under basic conditions (the corresponding N-methyl sulfonamide was not isolated) and removal of the *N*-Boc-protecting group under acidic conditions provided sulfonamide **6** as a chlorhydrate salt.

Sulfonamides **10a–b**, **14** and **17** were obtained following the synthetic route described in Scheme 2. Treatment of the amines **7a–b** with hydrochloric acid and sodium nitrite gave the not isolated corresponding diazonium salts which led to the desired sulfonyl chlorides **8a–b** by the use of a copper(II) chloride/sulfur dioxide mixture in acetic acid.[30,31] Condensation with homopiperazine afforded the sulfonamides **9a–b** which gave the appropriate primary amines **10a–b** by hydrogenation in the presence of Raney nickel in ethanol saturated with gaseous ammonia under a 50 atm hydrogen pressure. Treatment of the sulfonamides **9a–b** with di-*tert*-butyl dicarbonate in a *tert*-butanol/water mixture under basic conditions gave the *N*-Boc-protected amines **11a–b**. The secondary amine **14** was synthesised from **11a**. Reduction of the nitrile function by diisobutyl-aluminum hydride in toluene and further acidic



Scheme 1 Synthesis of compounds **2a**, **3** and **6**. Reagents: (a) homopiperazine, CH_2Cl_2 for **1a**; (b) i) $SOCl_2$, CH_2Cl_2 ii) homopiperazine, CH_2Cl_2 for **1b**; (c) H_2, PtO_2, AcOH; (d) Boc_2O, *t*BuOH, H_2O, 1N NaOH; (e) H_2, PtO_2, MeOH; (f) i) CH_3I, K_2CO_3, acetone ii) HCl gas, Et_2O.

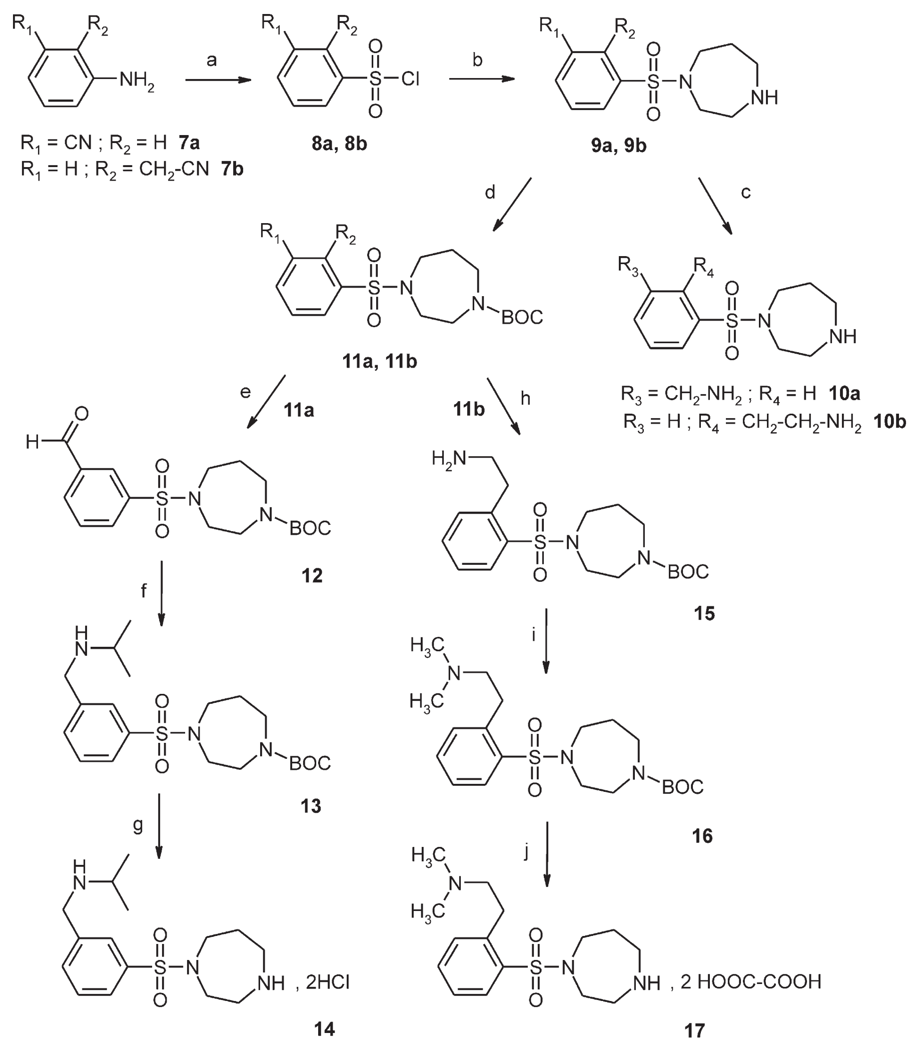
hydrolysis afforded the corresponding aldehyde **12**.[32] Reductive amination of this compound with isopropylamine and sodium cyanoborohydride in methanol led to the secondary amine **13** which gave the sulfonamide **14** by removal of the *N*-Boc-protecting group under acidic conditions. The tertiary amine **17** was obtained from **11b**. Hydrogenation of the nitrile function in the presence of Raney nickel in ethanol saturated with gaseous ammonia under a 50 atm hydrogen pressure afforded the primary amine **15**. Reductive amination of this compound with formaldehyde and sodium cyanoborohydride gave the tertiary amine **16**,[33] which led to sulfonamide **17** by first removal of the *N*-Boc-protecting group under acidic conditions and further transformation into its oxalate salt.

Amide **22** was obtained by the route illustrated in Scheme 3, from 5-aminoisoquinoline **18**, according to a previously described procedure.[34] Treatment with hydrobromic acid and sodium nitrite gave the not isolated corresponding diazonium salt which led to the bromo derivative **19**, by the use of copper(I) bromide in acidic conditions. Substitution of the bromo group with zinc cyanide in the presence of tetrakis(triphenylphosphine)palladium(0) in dimethylformamide afforded the cyano-derivative **20**.[35] Acidic hydrolysis of this compound gave the carboxylic acid **21** which led to amide **22** by first treatment with thionyl chloride and further with homopiperazine.

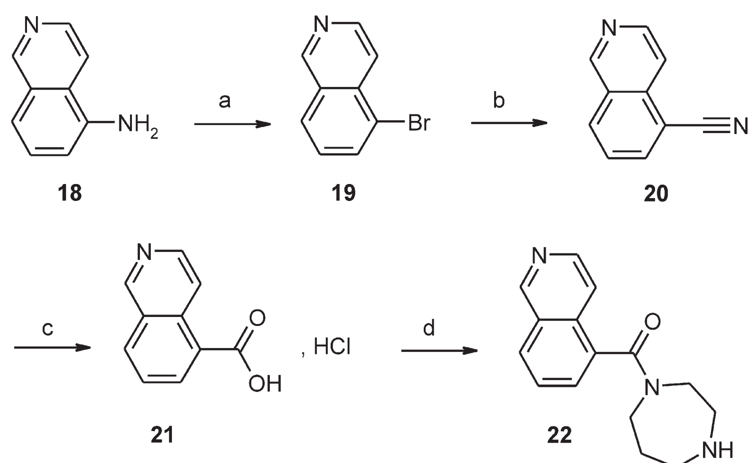
Pharmacology

The aim of this study was first to evaluate the role of the structural features of the compound Fasudil with regard to its Rho kinase inhibitory activity. We therefore rationally modified the 2 functional groups which could be implicated in the interaction with the enzyme active site i.e. the isoquinolinyl heterocycle and the sulfonyl group.

The isoquinolinyl heterocycle could be involved in an ionic or hydrogen bond with the enzyme active site. This hypothesis is corroborated by the various pharmacomodulates carried out on this feature. The first important point to note is that the pyridyl group in Fasudil can be substituted by the corresponding aliphatic heterocycle **3** without any loss of inhibitory activity in tests A-1 and A-2. However, replacing the NH piperidyl group of **3** by a N-methyl one led to the inactive compound **6**. This discrepancy could be explained by steric parameters at this level of interaction. The same result was obtained with the suppression of the nitrogen atom in **2a** or when the piperidyl ring of **3** was replaced by ring-opened derivatives leading to primary **10a–b**, secondary **14** or tertiary **17** amines. These modifications indicate that the isoquinolinyl nitrogen is concerned with hydrogen or ionic interaction with the drug target and a strict spatial arrangement imposed by the rigid characteristic of the heterocycle is required at this level.



Scheme 2 Synthesis of compounds **10a–b**, **14** and **17**. Reagents: (a) i) 12N HCl, $NaNO_2$ ii) SO_2 gas, $CuCl_2$, AcOH; (b) homopiperazine, CH_2Cl_2; (c) H_2, Raney-Ni, EtOH/NH_3 gas; (d) Boc_2O, *t*BuOH, H_2O, 1N NaOH; (e) i) DIBAL-H, toluene ii) 1N HCl for **11a**; (f) isopropylamine, $NaBH_3CN$, MeOH; (g) HCl gas, Et_2O; (h) H_2, Raney-Ni, EtOH/NH_3 gas for **11b**; (i) formaldehyde, $NaBH_3CN$, AcOH, MeOH; (j) i) HCl gas, Et_2O ii) HOOC-COOH, EtOAc.



Scheme 3 Synthesis of compound **22** Reagents: (a) i) 48% HBr, $NaNO_2$ ii) CuBr; (b) $Zn(CN)_2$, $Pd(PPh_3)_4$, DMF; (c)12N HCl; (d) i) $SOCl_2$ ii) homopiperazine, NEt_3, CH_2Cl_2.

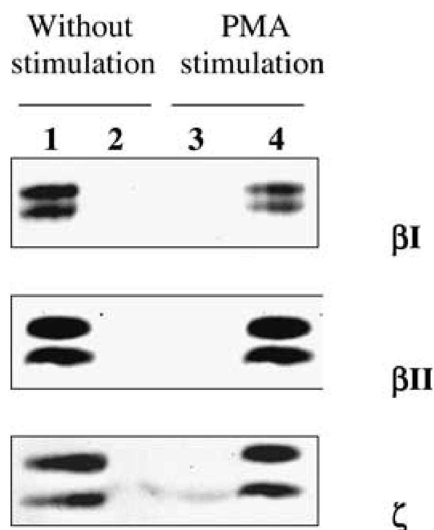


FIGURE 2 For the reference, cells were incubated for the same period for each activator, then cytoplasm (lane 1) and plasmic membrane (lane 2) were separated by centrifugation. For the activated cells, stimulation was made with 160 nM PMA for 5 min, after centrifugation, the cytoplasm (lane 3) was separated from the plasmic membrane (lane 4). Results are representative of three experiments.

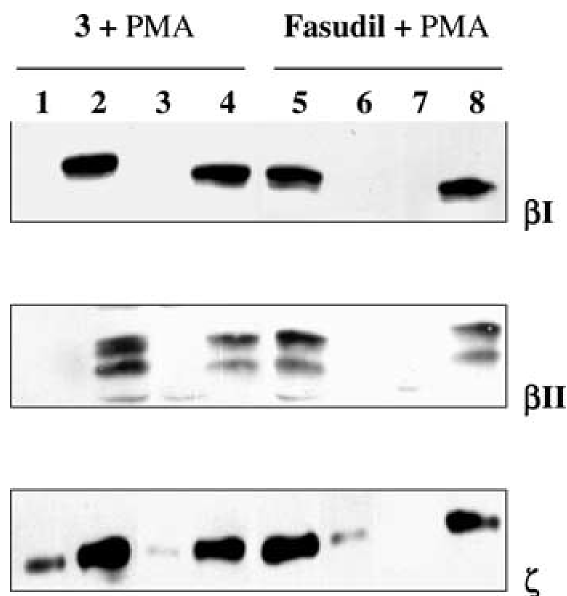


FIGURE 3 Action of Fasudil and **3** on the translocation of PKC. Lanes 1 and 5, cytoplasm of incubated cells with 1 mM of compound; lanes 2 and 6, membrane of incubated cells with 1 mM of compound; lanes 3 and 7, cytoplasm of incubated cells with 0.01 mM of compound; lanes 4 and 8, membrane of incubated cells with 0.01 mM of compound. Results are representative of three experiments.

Generally, a sulfonamide group has a structural role in holding other functionalities in a particular geometry. This is probably the case for the sulfonamide spacer of Fasudil since replacement by the isosteric amide **22** induces a loss of inhibitory activity.

The second part of this study was then to evaluate the selectivity level of compound **3**, which showed a Rho kinase inhibitory effect. Therefore, we have determined its activity on another protein-kinase: PKC.

We first investigated the involvement of compound **3** on superoxide anion production. This compound is 6-fold less active than Fasudil in Test B-1. On the other hand, to prove this inhibition was due to an action on PKC, we have directly evaluated its activity on this enzyme. Binding with a compound having high affinity for PKC (phorbol-12,13-dibutyrate) showed an IC_{50} more than 7-fold less important for compound **3** than Fasudil in test B-2. Moreover PKC has to be translocated from cytoplasm to plasmic membrane to acquire its enzymatic activity (Figure 2). This stimulation can be executed by PMA. Translocation was totally inhibited at 1 mM Fasudil but not at the same concentration for compound **3** in test B-3, with the PMA as activator (Figure 3).

CONCLUSION

Our main objective was to clarify the relationship between the structure of compound Fasudil and its Rho kinase inhibitory activity. All the pharmacomodulations achieved on the structural features of this lead compound result in a loss of activity, showing that its interactions with the enzyme active site are particularly definite and sensitive to structural variations. The pharmacophoric pattern constituted by an isoquinolinyl heterocycle and a secondary amine separated by a spacer bearing a sulfonamide function, is of utmost importance. Only the tetrahydroisoquinoline analogue **3** retains the same activity as Fasudil. Moreover this compound is unable to inhibit the biological activity of PKC contrary to Fasudil. The loss of the aromatic property could increase the selectivity level in favour of compound **3** so leading to new drug design research.

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