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Design, synthesis and biological evaluation of a sulfonylcyanoguanidine as thromboxane A_2 receptor antagonist and thromboxane synthase inhibitor

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Abstract

The synthesis and the structure of N-isopropyl-N′-[2-(3′-methylphenylamino)-5-nitrobenzenesulfonyl] urea (**14**) was drawn from two thromboxane A_2 receptor antagonists structurally related to torasemide. Compound **14** showed an IC50 value of 22 nM for the thromboxane A_2 (TXA_2) receptor of human washed platelets. Compound **14** prevented platelet aggregation induced by arachidonic acid (0.6 mM) and U-46619 (1 μM) with an IC50 value of 0.45 and 0.15 μM, respectively. Moreover, **14** relaxed the rat isolated aorta and guinea-pig trachea precontracted by U-46619, a TXA_2 agonist. Its efficacy (IC50) was 20.4 and 5.47 nM, respectively. Finally, **14** (1 μM) completely inhibited TXA_2 synthase of human platelets. The pK_a value and the crystallographic data of **14** were determined and used to propose an interaction model between the TXA_2 antagonists related to torasemide and their receptor.

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Introduction

Thromboxane A_2 (TXA_2, **1**) is an arachidonic acid metabolite playing a crucial role in vasoconstriction, bronchoconstriction and platelet-aggregation. It is involved in the aetiology of cardiovascular and pulmonary diseases such as myocardial infarction and asthma (Ogletree 1987; Devillier & Bessard 1997). With the aim to cure these pathologies, TXA_2 synthase inhibitors, TXA_2 receptor antagonists and combined TXA_2 synthase inhibitor/receptor antagonist compounds have been developed (Dogné et al 2000). The lack of efficacy of TXA_2 synthase inhibitors was attributed to the accumulation of prostaglandin H_2 (PGH_2) which activated the TXA_2 receptor (Baghwat et al 1985; Fiddler & Lumley 1990). The efficacy of dual-acting molecules as TXA_2 synthase inhibitors/receptor antagonists would prevent the biosynthesis of TXA_2 and the action of accumulated PGH_2. A wide variety of TXA_2 receptor antagonists have been synthesized. These antagonists (Figure 1) are distributed into the sulfonamide derivatives from which sulotroban (**2**) is the lead compound (Gresele et al 1984), into the potent ω-alkylcarboxylic compounds with SQ-29548 (**3**) (Ogletree et al 1985) and seratrodast (**4**) (Kurokawa et al 1994) as prototypes, and into a class of various tricyclic molecules (Hall et al 1987; Jessup et al 1988; Ford-Hutchinson et al 1989; Miki & Ishii 1992; Theis et al 1992; Romstedt et al 1993; Takeuchi et al 1998). In 1992, torasemide (**5**, Figure 2), a diuretic sulfonylurea acting by inhibiting the Na^+ K^+ $2Cl^-$ co-transporter (Friedel & Buckley 1991), was described as a poor TXA_2 receptor antagonist able to relax the canine coronary artery precontracted with TXA_2 (Uchida et al 1992). A

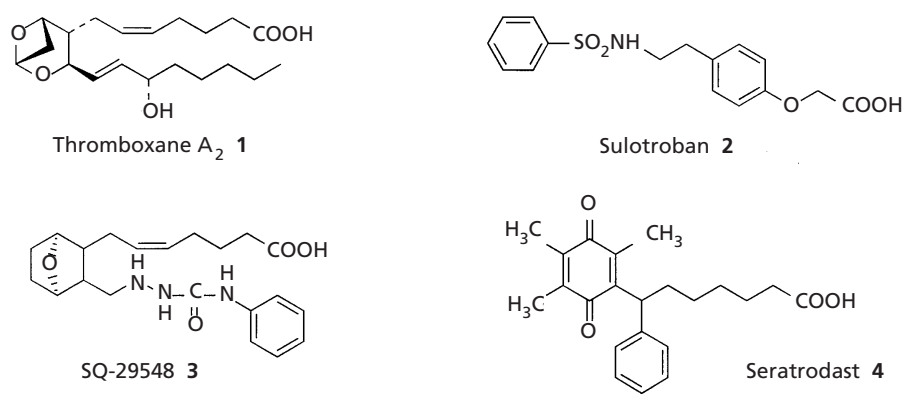


Figure 1 Structure of thromboxane A_2 and some antagonists.

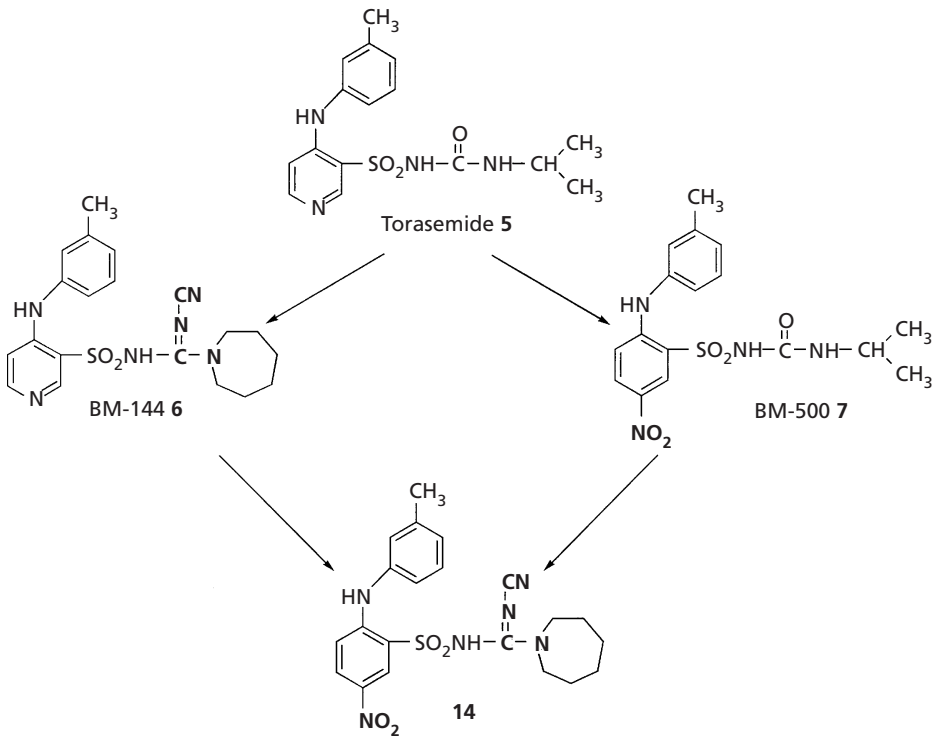


Figure 2 Design and origin of compound **14**.

screening of molecules chemically related to torasemide led to the discovery of two TXA_2 receptor antagonists, BM-144 (**6**) and BM-500 (**7**) (Figure 2) (Masereel et al 1999). Their potency was higher than that of the parent compound and similar to that of sulotroban. Moreover both torasemide derivatives lost the diuretic properties of torasemide. As compared with the structure of torasemide, the improvement of the TXA_2 antagonism observed with BM-144 was due to the replacement of the isopropylsulfonylurea group with an unusual sulfonylcyanoguanidine side chain. BM-500 differed from torasemide by the presence of a nitrobenzene instead of a pyridine ring. To reinforce the TXA_2 antagonism, we have prepared the molecule **14** bearing the side chain of BM-144 and the nitrobenzene ring of BM-500 (Figure 2). To study the effect of the acidic proton of the sulfonamide function, BM-500 was methylated (**15**).

Materials and Methods

Chemistry

Melting points were determined on a Büchi B-540 capillary apparatus. IR spectra were recorded as KBr pellets on a Perkin-Elmer 1750 FT spectrophotometer. The ^{1}H NMR spectra were taken on a Jeol JNM-EX 400 (400 MHz) instrument in DMSO-d_6 with hexamethyldisilane as an internal standard; chemical shifts were reported in δ values (ppm) relative to internal hexamethyldisilane. The abbreviations used are as follows; s = singlet, d = doublet, t = triplet, m = multiplet, and b = broad signal. Elemental analyses (C, H, N, S) were performed on a Carlo-Erba NA 1500 elemental analyser and were within ± 0.4 % of the theoretical values. All reactions were routinely checked by TLC on silica gel Merck $60F_{254}$.

*2-Chloro-5-nitrobenzenesulfonamide (**10**)*

An aqueous solution of sodium nitrite (0.1 mol, 10 mL) was added dropwise to a cooled solution (-5°C) of 2-chloro-5-nitroaniline (**8**; 10 g, 58 mmol) in acetic acid (100 mL) and 12 M HCl (40 mL). Copper chloride (4 g, 30 mmol) dissolved in water (10 mL) was poured into acetic acid (160 mL) previously saturated with SO_2. This last mixture was added to the diazonium salt and stirred for 2 min. After the addition of crush-ice (200 g), the precipitate **9** was collected by filtration and washed with cold water. The resulting sulfonyl chloride (**9**) was rapidly dissolved in 150 mL aqueous NH_4OH (28–32 %). The solution was concentrated to 50 mL under reduced pressure and acidified with 5 M HCl to precipitate the title compound **10** which was collected, washed with water and dried. Yield: 57 %, 7.82 g; mp 177–179°C; IR (KBr) 1538 and 1354 (NO_2), 1386 and 1166 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz). δ 8.70 (s, 1H, 6*H*-NO_2phenyl), 8.44 (d, 1H, 4*H*-NO_2phenyl), 7.96 (d, 1H, 3*H*-NO_2phenyl). Anal. ($C_6H_5N_2O_4SCl$). Expected (C, H, N, S in %) 30.45, 2.13, 11.84, 13.55; found 30.62, 2.23, 11.81, 13.92.

*2-(3′-Methylphenylamino)-5-nitrobenzene sulfonamide (**11**)*

2-Chloro-5-nitro-benzenesulfonamide (**10**; 10 g, 42 mmol) was refluxed for 3 h with 3-methylaniline (10 mL, 93 mmol) in 3-chlorotoluene (20 mL), the solvent was evaporated under reduced pressure and the residue dissolved in 2.5 M NaOH (100 mL). The solution was extracted three times with cyclohexane (100 mL) and adjusted to pH 1 with 5 M HCl. The precipitate was collected, washed with water and crystallized in methanol (60 mL) to give the title compound **11**. Yield: 73 %, 9.48 g; mp 153–155°C; IR (KBr) 1490 and 1338 (NO_2), 1331 and 1148 (SO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz). δ 8.59 (s, 1H, 6*H*-NO_2phenyl), 8.20 (d, 1H, 4*H*-NO_2phenyl), 7.38 (d, 1H, 3*H*-NO_2phenyl), 7.11–7.22 (m, 4H, phenyl), 2.36 (s, 3H, C*H*$_3$). Anal. ($C_{13}H_{13}N_3O_4S$). Expected (C, H, N, S in %) 50.81, 4.26, 13.67, 10.43; found 51.17, 4.13, 13.58, 13.81.

*1-(1′-Hexamethyleneimino)-1-methylthio-2-cyanoimine (**13**)*

Hexamethyleneimine (3.1 mL, 27.5 mmol) was refluxed with N-cyano-S,S′-dimethyldithioiminocarbonate (Hantzsch & Wolvekamp 1904) (**12**; 3.6 g, 24.6 mmol). After 4-h of reaction, the medium was evaporated under reduced pressure, the crude residue added to 50 mL 0.1 M HCl, and the bottom layer was extracted three times with $CHCl_3$. The organic phase was dried with anhydrous $MgSO_4$ and chloroform evaporated under reduced pressure to give the title compound **13**. Yield: 64 %, 3.1 g; mp 35–37°C; ^{1}H NMR (DMSO-d_6, 400 MHz). δ 3.33 (m, 4H, C*H*$_2$-N-C*H*$_2$), 2.72 (s, 3H, C*H*$_3$S), 1.50–1.70 (2m, 8H, -(C*H*$_2$)$_4$-). Anal. ($C_9H_{15}N_3S$). Expected (C, H, N, S in %) 54.79, 7.66, 21.30, 16.25; found 55.02, 7.48, 21.09, 16.17.

*N″-[2-(3′-methylphenylamino)-5-nitrobenzenesulfonyl]-N-cyano-N′N′-hexamethylene guanidine (**14**)*

The sodium salt of 2-(3′-methylphenylamino)-5-nitrobenzene sulfonamide (**11**; 1.0 g, 3.3 mmol) and 1-(1′-hexamethyleneimino)-1-methylthio-2-cyanoimine (**13**; 1.1 g, 5.6 mmol) were dissolved in a mixture of 1,4-dioxane (3 mL) and *N*,*N*-dimethylformamide (2 mL), and refluxed for 7 h. After evaporation of solvents under reduced pressure the residue was dissolved in water (50 mL) and 2.5 M NaOH (2 mL). The solution was extracted three times with diethyl ether (50 mL) and adjusted to pH 1 with dilute HCl. The precipitate was collected by filtration, washed with water, dried and crystallized from boiling methanol (40 mL) to afford the title compound **14**. Yield: 30 %, 0.44 g; mp 161–163°C; IR (KBr) 1502 and 1306 (NO_2), 1160 and 1329 (SO_2), 2182 C≡N cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz). δ 8.80 (b, 1H, N*H*-phenyl), 8.49 (s, 1H, 6*H*-NO_2phenyl), 8.12 (d, 1H, 4*H*-NO_2phenyl), 7.33 (d, 1H, 3*H*-NO_2phenyl), 7.02–7.20 (m, 4H, phenyl), 3.48 (m, 4H, C*H*$_2$-N-C*H*$_2$), 2.32 (s, 3H, C*H*$_3$-phenyl), 1.41–1.57 (2m, 8H, -(C*H*$_2$)$_4$-). Anal. ($C_{21}H_{24}N_6O_4S$). Expected (C, H, N, S in %) 55.25, 5.30, 18.41, 7.02; found 55.36, 5.41, 18.23, 7.14.

N-isopropyl-N′-[2-(3′-methylphenylamino)-5-nitrobenzenesulfonyl] urea (7)

The sodium salt of 2-(3′-methylphenylamino)-5-nitrobenzene sulfonamide (**11**; 1.0 g, 3.3 mmol) was dissolved in 20 mL methanol and tetrahydrofuran (50:50). Isopropyl isocyanate (0.65 mL, 6.8 mmol) was added and the mixture refluxed for 30 min. The solvents were evaporated under reduced pressure and the residue dissolved in water (50 mL) and 2.5 M NaOH (2 mL). The solution was extracted three times with diethyl ether (50 mL) and adjusted to pH 1 with dilute HCl. The precipitate was collected by filtration, washed with water, dried and crystallized from boiling methanol–water (30 mL) to afford the title compound **7**. Yield: 76%; IR (KBr) 1581 and 1307 (NO_2), 1332 and 1158 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz). δ 10.88 (br s, 1H, -CO-N*H*-C<) 8.95 (br s, 1H, N*H*-phenyl), 8.77 (s, 1H, 6*H*-NO_2phenyl), 8.36 (d, 1H, 4*H*-NO_2phenyl), 7.52–7.27 (m, 5H, 3*H*-NO_2phenyl and phenyl), 3.67 (m, 1H, -C*H*<), 2.50 (s, 3H, CH_3-phenyl), 1.19 (2s, 6H, C(CH_3)$_2$. Anal. ($C_{17}H_{20}N_4O_5S$). Expected (C, H, N, S in %) 52.03, 5.14, 14.28, 8.17; found 51.94, 5.23, 14.37, 8.28.

*N-isopropyl-N′-methyl-N′-[2-(3′-methylphenylamino)-5-nitrobenzenesulfonyl] urea (**15**)*

N-isopropyl-N′-[2-(3′-methylphenylamino)-5-nitrobenzenesulfonyl] urea (**14**; 1.0 g, 2.5 mmol) was dissolved in methanol (20 mL) containing KOH (2.5 mmol). After cooling (4°C), methyl iodide (0.19 mL, 3 mmol) was added and the mixture stirred for 1 h. Methanol was evaporated under reduced pressure, the crude residue was crystallized from water-methanol to afford the title compound. Yield: 68%; mp 143–145°C; IR (KBr) 1576 and 1308 (NO_2), 1335 and 1161 (SO_2) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz). δ 10.90 (br s, 1H, -CO-N*H*-C<) 8.93 (br s, 1H, N*H*-phenyl), 8.76 (s, 1H, 6*H*-NO_2phenyl), 8.38 (d, 1H, 4*H*-NO_2phenyl), 7.50–7.27 (m, 5H, 3*H*-NO_2phenyl and phenyl), 3.70 (m, 1H, -C*H*<), 3.01 (s, 3H, SO_2-N-CH_3) 2.51 (s, 3H, CH_3-phenyl), 1.21 (2s, 6H, C(CH_3)$_2$. Anal. ($C_{18}H_{22}N_4O_5S$). Expected (C, H, N, S in %) 53.19, 5.46, 13.78, 7.89; found 52.94, 5.62, 13.89, 8.11.

Receptor binding assay

Washed human platelets were prepared as described by Masereel et al (1999). Incubation (1 mL) containing 500 μL platelet suspension (2×10^8 cells mL^{-1}), 100 μL [^{3}H]SQ-29548 (5 nM final) and 400 μL drug at a fixed concentration was performed at 25°C for 60 min. The incubation medium was (in mM): NaCl 137, KCl 2.7, NaH_2PO_4 0.4, $NaHCO_3$ 12, D-glucose 5, HEPES pH 7.40. The reaction was terminated by addition of 4 mL ice-cold Tris-HCl buffer (10 mM, pH 7.40), followed by rapid filtration through Whatman GF/C glass filters. Non-specific binding was defined as the amount of radioactivity bound in the presence of a large molar excess (10 μM) of SQ-29548. It is 5–7% of the total binding determined by the radioactivity in absence of competing ligand. The IC50 was defined as the drug concentration required to displace 50% [^{3}H]SQ-29548 bound to the TXA_2 receptor. Values are the mean of three concentration–response curves performed in triplicate and calculated by non-linear regression (GraphPad Prism software).

Platelet aggregation

Blood was obtained from the antecubital vein of volunteers who had not taken any medication within the previous 10 days. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as described by Masereel et al (1999). The PRP was diluted to 3×10^8 platelets mL^{-1}, and the PPP was used to adjust the photometric measurement to the minimum optical density. The drug (or vehicle) was added to the PRP and stirred at 1100 rev min^{-1} for 3 min at 25°C (aggregometer Chronolog Corporation). The aggregation was induced by addition of a freshly prepared solution of the sodium salt of arachidonic acid (0.6 mM final) or U-46619 (1 μM). The aggregation curve was recorded for 5 min. The drug concentration (IC50) reducing platelet aggregation by 50% was calculated by non-linear regression analysis (GraphPad Prism software) from at least four dose–response curves.

Rat aorta and guinea-pig trachea contraction

Rat aortic (male Wistar rats) and guinea-pig tracheal rings were taken from anaesthetised animals (80 mg kg^{-1} nembutal). The rings were suspended under 1g tension, equilibrated for 1 h in an organ bath containing 20 mL Krebs solution (in mM: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2; $CaCl_2$ 2.5, $NaHCO_3$ 25, D-glucose 5, indomethacin 1) kept at 37°C and bubbled continuously with a mixture of O_2/CO_2 (95/5). The aortic and tracheal rings were then exposed to 20 and 10 nM U-46619, respectively. When the tension was stable, the TXA_2 receptor antagonists (50 μL) were added to the bath at cumulatively increasing concentrations until relaxation had reached maximum. The tension was measured with an isometric transducer and recorded by the Iox acquisition data software (Emka

Technologies, Paris, France). The IC50 value of each drug was assessed for six concentration–response curves and expressed as the concentration evoking 50% inhibition of the plateau induced by U-46619. The IC50 values and their standard error were calculated by non-linear regression analysis (GraphPad Prism software).

Thromboxane synthase inhibition

PRP (3×10^8 platelets mL^{-1}) and PPP were prepared as described by Masereel et al (1999). Six minutes after the addition of drug (or vehicle) to the PRP, the thromboxane synthesis was stimulated by addition of arachidonic acid (0.6 mM). The incubation (25°C) was stopped after 4 min by addition of indomethacin (1 mM), the PRP was centrifuged (16000 *g* for 10 s) and two samples (400 μL) of the supernatant assayed for TXB_2. Serum TXB_2, the stable metabolite of TXA_2, was measured by enzyme immunoassay (TXB_2 EIA kit, Cayman Chemical). Results were expressed as the percentage of TXB_2 production compared with that of PRP incubated with arachidonic acid in absence of any drug (100%). The basal production of TXB_2 by unstimulated platelets was 3.2%. Results are the mean $\pm$ s.d., n = 3.

pK_a determination

Compound **7** was dissolved at a concentration of 20 μM in a mixture containing 10, 20 or 30% CH_3OH and the appropriate buffer prepared with sodium citrate/HCl (pH 1–4.5) or Na_2HPO_4/KH_2PO_4 (pH 4.5–8.0). The pH of each solution was measured, and its optical density (230–500 nm) was recorded against blank with a double-beam UV-visible spectrophotometer (Perkin-Elmer Lambda 20). The wavelength of the maximum absorption was plotted against the pH value (Figure 4). For each percentage of CH_3OH, the inflection point of the sigmoid curve calculated by non-linear regression analysis (GraphPad Prism software) gave the pK_a value. Finally, the pK_a values obtained at 10, 20 and 30% (v/v) CH_3OH were plotted against the percentage of co-solvent used, and the linear regression led to extrapolate the pK_a value of **7** at 0% of CH_3OH (Figure 4): $pK_a = (0.0176 \times \%CH_3OH) + 3.905$; $r = 0.9999$; $P < 0.001$ (GraphPad Prism software).

X-ray crystallography

The crystal used to collect X-ray diffraction data of **14** was obtained by slow evaporation of a concentrated solution in a chloroform/toluene mixture. Cell parameters (triclinic, $a = 9.980(1)$, $b = 12.020(10)$, $c = 13.85(2)$, $\alpha = 94.30(1)$, $\beta = 97.56(1)$, $\gamma = 103.22(1)$, $V = 1593.8(3)$ Å^3) were refined from 25 well-centred reflections. A total of 5985 reflections were measured. Data were corrected for background, Lorentz-polarization, and absorption ($T_{min} = 0.42$, $T_{max} = 0.63$) effects. The structure was solved by direct methods and refined (full matrix least squares on intensities) with Shelxl97 (Sheldrick & Schneider 1997). Molecule **14** crystallized with one molecule of chloroform and one molecule of toluene. Final statistics: $C_{21}H_{24}N_6O_4S$-$CHCl_3$-C_7H_8, Mr = 668.0, P-1, $D_x = 1.392$, Z = 2, F(000) = 696, $\mu = 3.59$, R1 = 0.0745 for 4215 $I > 2\sigma(I)$ and wR = 0.198, S = 1.037, $\triangle\rho$ max = 1.05 (close to the methyl group of the toluene solvent molecule).

Molecular modelling

Docking simulations of **6**, **7**, and **14** in the human TXA_2 receptor were performed. A model of the transmembrane spanning helices of the receptor was built using the GPCR mode of SwissModel and bacteriorhodopsin (1bac.pdb) as the template (Guex & Peitsch 1997, 1999; Guex et al 1999). The alignment of the target and template sequences was taken from the work of Yamamoto et al (1993). The crystal geometry of **14** was used as input for the co-ordinates of the ligands. The geometry for **6** and **7** were obtained by replacing the nitrobenzene or the *N*-cyano-*N'N'*-hexamethyleneguanidine moiety of **14** by a pyridine or *N'*-isopropylurea group, respectively. Starting geometry for the complexes were obtained using a Monte-Carlo procedure available from the Affinity program of MSI (San Diego, CA) using default parameters. Parameters of the extensible and sytematic force field were used along the entire procedure. According to previous studies (Yamamoto et al 1993; Wouters et al 1999) a binding site was defined in the receptor that comprised residues: Met-112, Ile-113, Phe-115, Gly-116, Leu-120, Leu-161, Leu-163, Gly-164, Leu-165, Leu-166, Pro-167, Leu-168, Leu-169 Val-171, Ser-201, Met-202, Gly-204, Gly-205, Leu-206, Val-208, Leu-210, Phe-212, Leu-262, Arg-295. The best structures of the complex of the human TXA_2 receptor with **6**, **7**, and **14** were optimized further with the Discover program (MSI, San Diego, CA) using a first molecular dynamics run (300 K, 1000 fs) followed by energy minimization (steepest descent and conjugated gradient) to a final convergence of 1 kcal mol^{-1}. The conformation of the ligands and the lateral chains of all amino acid residues of the binding site were allowed to move. The solvent effect was approached by using a distance dependent dielectric constant (1*r).

Results and Discussion

Chemistry

The diazonium salt prepared from 2-chloro-5-nitroaniline (**8**) reacted with sulfur dioxide to afford the corresponding sulfonyl chloride **9** which reacted immediately with NH_4OH to give 2-chloro-5-nitrobenzenesulfonamide (**10**; Figure 3). The sulfonamide **10** refluxed with 3-methylaniline to give 2-(3′-methylphenylamino)-5-nitro-benzenesulfonamide (**11**). The N-cyano-S,S′-dimethyldithioiminocarbonate **12** (Hantzsch & Wolvekamp 1904) refluxed with an excess (1.1 equiv.) of hexamethyleneimine to afford 1-(1′-hexamethyleneimino)-1-methylthio-2-cyanoimine (**13**). Finally, N″-[2-(3′-methylphenylamino)-5-nitro-benzenesulfonyl]-N-cyano-N′N′-hexamethyleneguanidine (**14**) was prepared by reaction of **13** with the sodium salt of the sulfonamide **11**. The sulfonylurea **7** was prepared by the reaction of the sodium salt of **11** with isopropylisocyanate, then the potassium salt of **7** was methylated by CH_3I to give **15**.

Biological evaluation

The affinity for the TXA_2 receptor of human washed platelets was evaluated as the drug concentration (IC50) required to displace 50% [^{3}H]SQ-29548, a potent competitive and specific ligand of this site (Table 1) (Ogletree et al 1985). Compound **14** was found as active as SQ-29548 and more active than the parent compounds (**6**

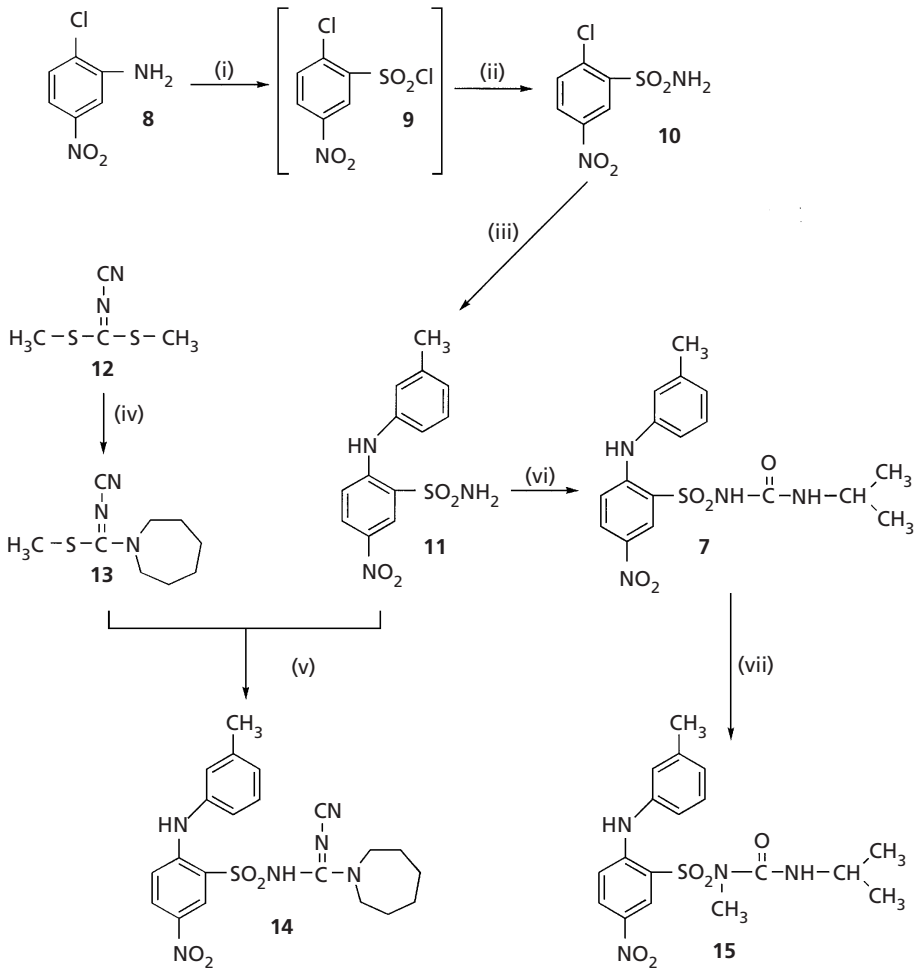


Figure 3 Synthesis of TXA_2 receptor antagonists related to torasemide. i, CH_3COOH, HCl, $NaNO_2$; CH_3COOH, SO_2, $CuCl_2$; ii, NH_4OH; iii, 3-CH_3-C_6H_4-NH_2; iv, hexamethyleneimine; v, 1 equiv. NaOH, dimethylformamide, dioxane; vi, NaOH, $(CH_3)_2CH$-N=C=O; vii, KOH, CH_3OH, CH_3I.

Table 1 Affinity for the TXA_2 receptor, anti-aggregating efficacy on human platelets, rat aorta and guinea-pig trachea relaxation induced by reference molecules, torasemide and its related compounds.

Drug	Affinity for the TXA_2 receptor (IC50 μM)[a]	Platelet aggregation		Aorta IC50 (nM)[c]	Trachea IC50 (nM)[c]
		Arachidonic acid (IC50 μM)[b]	U-46619 (IC50 μM)[b]		
Sulotroban (**2**)	0.93 ± 0.15	12.3 ± 2.1	10.1 ± 1.7	1620 ± 182	465 ± 39
SQ-29548 (**3**)	0.021 ± 0.001	0.034 ± 0.002	0.035 ± 0.002	21.1 ± 0.8	3.75 ± 0.56
Seratrodast (**4**)	nd	35.0 ± 4.3	5.1 ± 0.7	48.5 ± 4.5	5.68 ± 0.96
Torasemide (**5**)	2.69 ± 0.07	> 100	> 100	3608 ± 260	4020 ± 380
6	0.28 ± 0.02	9.4 ± 0.8	12.9 ± 1.0	120 ± 10.3	110 ± 13.4
7	0.080 ± 0.08	14.0 ± 0.3	9.5 ± 0.7	190 ± 16.6	112 ± 7.5
14	0.022 ± 0.002	0.45 ± 0.04	0.15 ± 0.02	20.4 ± 1.8	5.47 ± 0.95
15	> 100	nd	nd	nd	nd

[a]Concentration displacing 50% of [^{3}H]SQ-29548 specifically bound to the TXA_2 receptor of human washed platelets. [b]Concentration required to reduce by 50% platelet aggregation induced by arachidonic acid (0.6 mM) or U-46619 (1 μM). [c]Concentration required to reduce by 50% the rat aortic tonus and the guinea-pig tracheal tonus induced by 20 and 10 nM U-46619, respectively; nd, not determined. Values are mean ± s.d., n = 3–5.

and **7**). Its IC50 value was 122- and 42-times lower than that of torasemide and sulotroban, respectively. The Hill coefficient of **14** (1.035 ± 0.08) was similar to that of SQ-29548 (1.023 ± 0.03). This suggested a competitive binding to the TXA_2 receptor. The N-methylsulfonylurea **15** had no affinity.

TXA_2 antagonism was evaluated by the ability of the compounds to inhibit human platelet aggregation induced by arachidonic acid, the natural precursor of TXA_2, or by U-46619, a TXA_2 agonist (Coleman et al 1981; Liel et al 1987). When using arachidonic acid, the anti-aggregating potency of **14** was higher than that of **6**, **7**, sulotroban and seratrodast (Table 1). SQ-29548 remained the most powerful antagonist studied. Similar results were obtained when U-46619 was used as the aggregating agent (Table 1). Indeed, SQ-29548 was still four-times more active than **14**. The molecules related to torasemide showed a discrepancy between the IC50 values calculated from the binding experiment on washed platelets and from the aggregating tests. This could be assigned to the high affinity of sulfonamide compounds for plasma proteins present in the aggregating experiment (Cozzi et al 1994; Soyka et al 1994).

The TXA_2 antagonism of **14** was confirmed also by its ability to relax the rat aortic ring precontracted by U-46619 (20 nM). Its potency was similar to that of SQ-29548. Once more, **14** was six- and ten-times more potent than **6** and **7**, respectively. As seratrodast, the only TXA_2 receptor antagonist launched, is indicated in the treatment of asthma, we evaluated the ability of torasemide and its derivatives to relax the guinea-pig isolated trachea contracted by U-46619 (10 nM). Compound **14** was 20-times more active than its precursors (**6**, **7**). The potency of **14** was similar to that of seratrodast and SQ-29548 (Table 1).

Table 2 Inhibition of the TXA_2 synthase is expressed as the reduction of the production of TXB_2 induced by 0.6 mM arachidonic acid (control = 100%) on human platelets. The TXB_2 production of unstimulated platelets was 2.7 ± 0.4%.

Drug	Concn (μmol L^{-1})	% of TXB_2 production
Control		100 ± 5.9
Furegrelate	1	105 ± 6.8
Furegrelate	10	32.5 ± 4.7***
6	10	98.8 ± 8.6
7	10	99.3 ± 1.3
14	0.1	104 ± 4.1
14	1	4.4 ± 1.1***
14	10	3.7 ± 0.1***

Values are mean ± s.d., n = 3. ***$P < 0.001$ compared with the control value.

According to the interest of a combined TXA_2 receptor antagonist/synthase inhibitor therapy, their inhibitory potency on thromboxane synthase of human platelets was evaluated. The inhibition of thromboxane synthase was estimated by the reduction of the platelet production of TXB_2, the stable metabolite of TXA_2, induced by 0.6 mM arachidonic acid (Table 2). At 1 μM, **14** was found more active than furegrelate, a TXA_2

synthase inhibitor chosen as reference (Johnson et al 1986), while **6** and **7** were inactive at 10 μM.

Dissociation constants (pK_a)

Except for **6** and **7**, all the TXA_2 receptor antagonists described bear a carboxylic function, which is claimed to interact with the TXA_2 receptor (Yamamoto et al 1993; Masereel et al 1999). For this reason, the pK_a value of the sulfonylurea moiety of **7** was determined by UV-spectrophotometry. As observed for many pH indicators, **7** revealed a bathochromic effect associated to the decrease of pH (Figure 4). For **7**, the maximum optical densities (λ_{max}) of the molecular and anionic forms were 375 and 391 nm, respectively. Due to the poor solubility of **7** in aqueous buffers, methanol (10, 20 and 30%) was used as co-solvent. As shown, the pH value corresponding to the inflection point was the pK_a value of **7**, where the concentration of both forms was equal. Extrapolated to 0% of methanol, the pK_a value of **7** was 3.90 ± 0.01 (Figure 4). Compared with torasemide (**5**; $pK_a = 6.68$) (Masereel et al 1994), the replacement of the pyridine by a nitrobenzene ring strongly dropped the pK_a value of the sulfonylurea function. These results indicated clearly that **7** was under its anionic form at physiological pH (7.4) and was able to establish an ionic bond with the TXA_2 receptor.

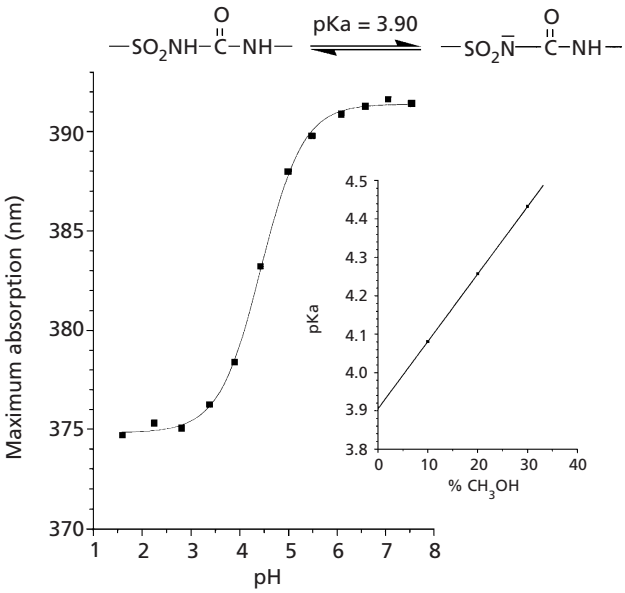


Figure 4 Variation of the maximum of the optical density of **7** related to the pH in presence of methanol (30%, v/v), and determination of its pK_a value by extrapolation to 0% of methanol.

Crystallography

Molecule **14** crystallized with one molecule of chloroform and one molecule of toluene. An Oak Ridge Thermal Ellipsoid Plot diagram of the crystal structure is given in Figure 5. In the crystal structure, **14** adopted a folded conformation (Figure 5) with the cyano group facing the nitrophenyl ring to favour van der Waals interactions. Stacking between the toluene solvent molecule and the toluyl ring of **14** also stabilized the molecules in the solid state. Crystal cohesion was further reinforced by intra (N8-H8 ... O20 : 2.786(5) Å, 138.6°) and inter (N21-H21 ... N25 (1-x, 1-y, 1-z): 2.880(5) Å, 177.6°) molecular hydrogen bonds.

Bond lengths and valence angles corresponded to the standard values measured for torasemide and its related compounds. Based on the values of the torsion angles $\phi 1$, $\phi 2$, $\phi 3$ and $\phi 4$ measured in the crystal, four theoretical conformations (α, β, γ, δ) were defined for these molecules (Table 3) (Masereel et al 1995). Nevertheless, the conformation of **14** differed from the δ one observed with the pyridylsulfonylcyanoguanidine (Dupont et al 1995) and from those adopted by torasemide and its related pyridylsulfonylureas (Dupont et al 1978, 1991). Indeed, the conformation deduced from the crystal structure of **14** did not corresponded to any one described among our series (Table 3). More structure determinations of other analogues are required to see if this is specific to the nitrobenzene series.

Molecular modelling and receptor–ligand interactions

Compounds **6**, **7**, and **14** have been docked into a model of the human TXA_2 receptor to compare their interactions with the protein (Figure 6). Starting geometry for the complexes were obtained using a Monte-Carlo approach (Affinity, MSI, San Diego, CA) and optimized further by a molecular dynamics run followed by energy minimization (Discover, MSI, San Diego, CA).

Although limited e.g. no mutational data were reported for the receptor that could identify or check the influence of amino acids in the binding of ligands, the present approach attempted to rationalize the existing pharmacological data and could be useful for designing original molecules. The total non-bonded interaction energy calculated between the binding site of the receptor and **14** was -24.2 kcal mol^{-1}. This value was only -23.2 and -18.3 kcal mol^{-1} for **6** and **7**, respectively. Although those energy differences should not be over interpreted, they were in agreement with the experimental affinities. The most common molecular

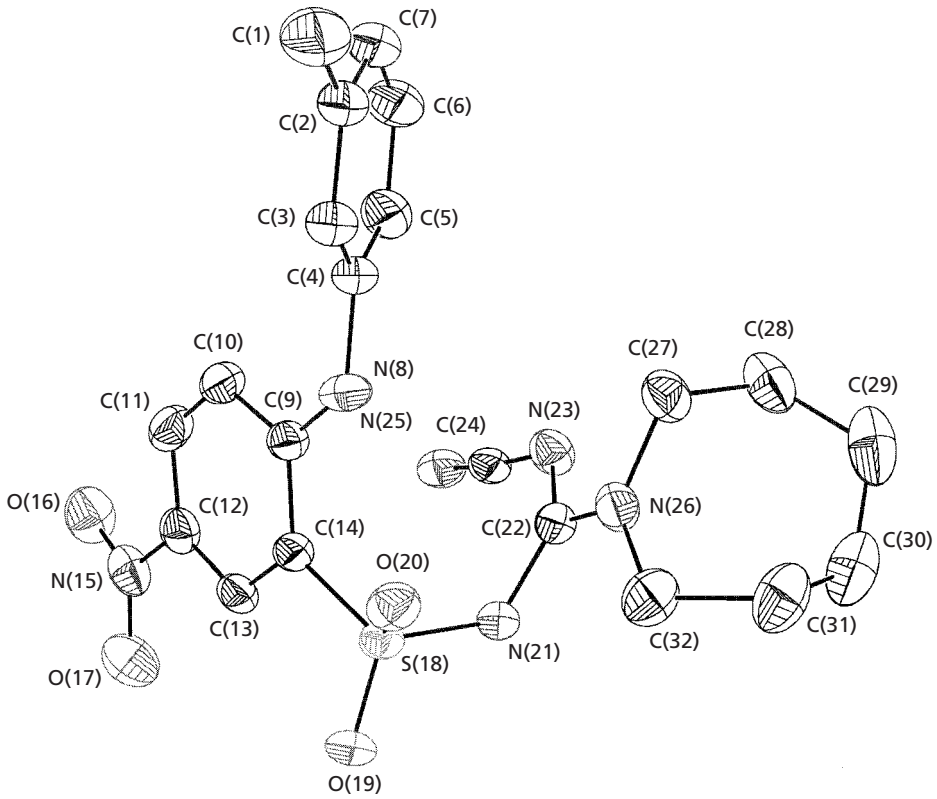


Figure 5 ORTEP diagram of the crystal structure of **14**.

model describing the interactions between the TXA_2 receptor antagonists and their receptor was based on three main interactions (Nicolaï et al 1993; Yamamoto et al 1993; Wouters et al 1999). Firstly, all TXA_2 receptor antagonists such as SQ-29548, seratrodast, sulotroban or tricycle derivatives were postulated to establish an ionic bond between their carboxylate function and the positive charge of Arg 295. Secondly, a second anchoring point to the receptor involved a hydrophobic binding pocket that comprised the side chain of Ser 201 involved in H-bond formation. Thirdly, the TXA_2 receptor antagonists were thought to fill a second hydrophobic pocket. Those two hydrophobic pockets appeared large enough to accommodate different lipophilic substituents of antagonists/ligands (Wouters et al 1999).

Interaction with Arg 295

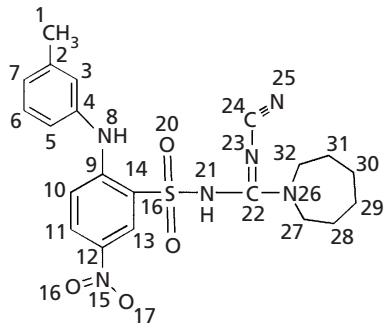
Due to the nitrobenzene ring, the pK_a value of the sulfonylurea **7** (3.90) was lower than that of torasemide (6.68), its pyridine counterpart (Masereel et al 1994). In the pyridine series, we demonstrated that the pK_a of torasemide was higher than that of its sulfonylcyanoguanidine counterpart ($pK_a = 6.00$) (Masereel et al 1995). According to these considerations we postulated that the pK_a of **14** was still lower than that of **7**. Thus, in physiological conditions (pH 7.4), the sulphonamide nitrogen of compounds **6**, **7** and **14** was negatively charged and able to establish an ionic interaction with Arg 295 of segment II. The lack of activity of **15**, deprived of acid function, reinforced this theory.

In our docked structures (Figure 6), all three ligands were placed in such a way that the sulfonamide group could interact with Arg 295 of segment II. Interestingly, the oxygen involved in the interaction was different for the pyridinic analogue (O20 … NH2_Arg 295 = 2.71 Å for **6**) than for the nitrobenzenic analogues (O19 … NH2_Arg 295 = 2.61 Å for **7** and O19 … NH1_Arg 295 = 2.65 Å for **14**). This could be related to the different conformational preferences observed in the crystal structures of ligands.

First hydrophobic pocket

The m-toluyl moiety and aromatic (nitrophenyl or pyridinyl) ring of **6**, **7** or **14** occupied a hydrophobic pocket formed by residues Leu 198, Ser 201, Met 202 on helice V and Leu 262 on helice VI. The absence of the

Table 3 Main torsion angles observed in the crystal structure of **14** and description of the four conformations observed with torasemide and its derivatives.



$\phi_1 = C_9–C_{14}–S_{18}–N_{21}$
$\phi_2 = C_{14}–S_{18}–N_{21}–C_{22}$
$\phi_3 = S_{18}–N_{21}–C_{22}–N_{26}$
$\phi_4 = C_{21}–C_{22}–N_{26}–C_{27}$
$\phi_{4'} = N_{21}–C_{22}–N_{26}–C_{32}$

Conformer	ϕ_1 (°)	ϕ_2 (°)	ϕ_3 (°)	ϕ_4 (°)	$\phi_{4'}$ (°)
crystal	−80.9	+69.2	+78.8	−170.6	+13.7
α	−90	+90	180	180	···
β	+90	+90	180	0	···
γ	+90	+90	180	180	···
δ	+90	+90	0	180	···

nitro function in **6** placed this compound deeper in the hydrophobic pocket. The hydroxyl group of Ser 255 on helice VI pointed into this hydrophobic pocket but was not within binding distance of the nitro (**7** or **14**) nor the pyridinic nitrogen of **6**. In contrast to the model proposed for other TXA_2 receptor antagonists (Yamamoto et al 1993) Ser 201 did not H-bond with any of the antagonists investigated. It is suggested that introduction of an appropriate substituent on the toluyl ring able to interact with this Ser 201 could increase the affinity for the receptor.

Second hydrophobic pocket

A second hydrophobic pocket was formed by residues Leu 161 and Leu 168 of helice IV and Ile 113 of helice III. It accommodated the lateral N′N′-hexamethyleneguanidine or N′-isopropylurea chains of the TXA_2 receptor antagonists under study. The folded conformation of **6** and **14**, stabilized by the intra-molecular stacking of the cyano and aromatic (nitrophenyl or pyridinyl) ring, properly oriented the lateral hexamethylene moiety into the second hydrophobic pocket.

In conclusion, the combination of the pharmacophores of the torasemide derivatives **6** and **7** led to the

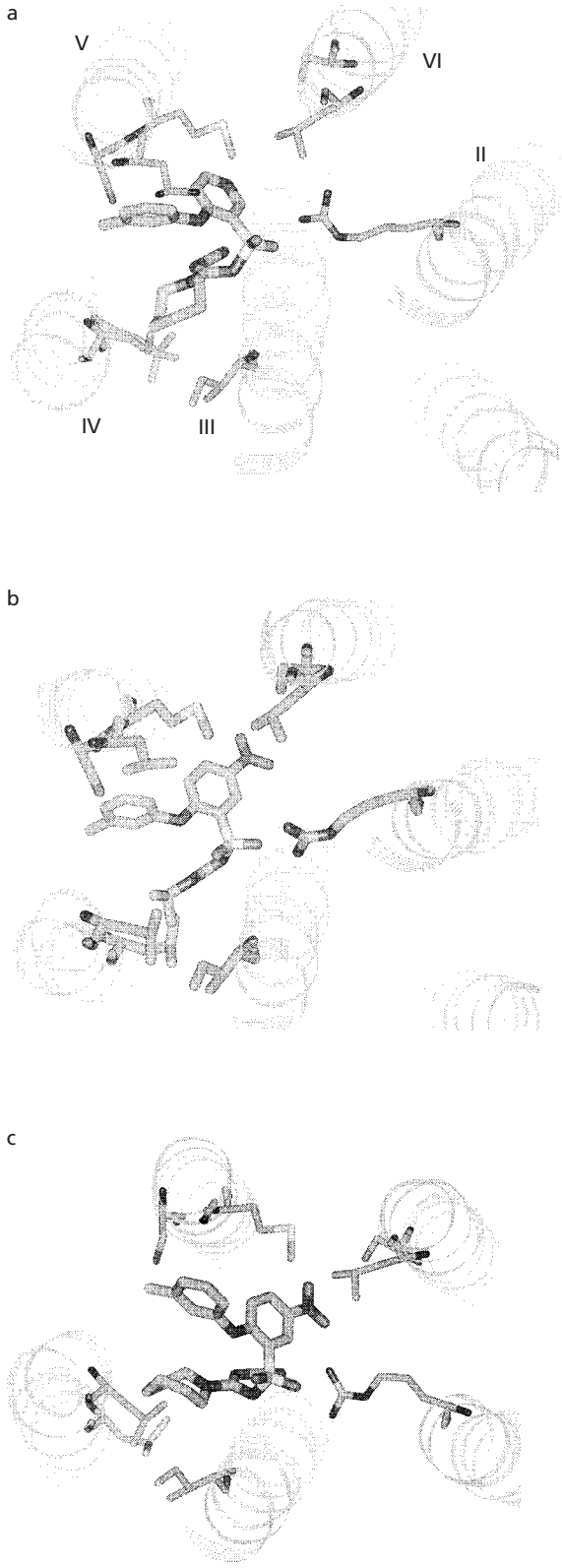


Figure 6 Final models of the complexes between the human TXA_2 receptor and compounds **6** (a), **7** (b) and **14** (c).

design of **14**, a very potent TXA_2 receptor antagonist bearing a TXA_2 synthase inhibitory potency. Its docking in the TXA_2 receptor-binding site led us to propose an interaction model suggesting a modulation to improve the original structure of BM-519 (**14**).

References

Baghwat, S. S., Hamann, P. R., Still, W. C., Bunting, S., Fitzpatrick, F. A. (1985) Synthesis and structure of the platelet aggregation factor thromboxane A_2. *Nature* **315**: 511–513

Coleman, R. A., Humphrey, P. P. A., Kennedy, I., Levy, G. P., Lumley, P. (1981) Comparison of the actions of U-46619, a prostaglandin H_2-analogue, with those of prostaglandin H_2 and thromboxane A_2 on some isolated smooth muscle preparations. *Br. J. Pharmacol.* **73**: 773–778

Cozzi, P., Giordani, A., Menichincherin, M., Pillan, A., Pinciroli, V., Rossi, A., Tonani, R., Volpi, D., Tamburin, M., Ferrario, R., Fusar, D., Salvati, P. (1994) Agents combining thromboxane receptor antagonism with thromboxane synthase inhibition: [[[2-(1H-imidazol-1-yl) ethylidene]amino]oxy] alkanoic acids. *J. Med. Chem.* **37**: 3588–3604

Devillier, P., Bessard, G. (1997) Thromboxane A_2 and related prostaglandins in airways. *Fundam. Clin. Pharmacol.* **11**: 2–18

Dogné, J. M., de Leval, X., Delarge, J., David, J. L., Masereel, B. (2000) New trends in thromboxane and prostacyclin modulators. *Curr. Med. Chem.* **7**: 609–628

Dupont, L., Lamotte, J., Campsteyn, H., Vermeire, M. (1978) Structure cristalline et moléculaire d'un diurétique dérivé de l'alkyl-1[phénylamino-4-pyridyl-3-)sulfonyl]-3 urée: la torasémide ($C_{16}H_{20}N_4O_3S$). *Acta Crystallogr. B* **34**: 1304–1310

Dupont, L., Dideberg, O., Masereel, B., Delarge, J., Schynts, M., Pirotte, B. (1991) Structures du 1-((4-cycloheptylaminopyrid-3-yl)-sulfonyl)3-cyclohexylurée et de l'hydrogénonitrate du 1-((4-cyclo-octylaminopyrid-3-yl)-sulfonyl)3-cyclohexylurée. *Acta Crystallogr. C* **47**: 2152–2156

Dupont, L., Masereel, B., de Tullio, P., Pirotte, B., Delarge, J. (1995) N-Cyano-N′-isopropyl-N″-((4-(3′methylphenylamino)pyrid-3-yl)-sulfonyl)guanidine. *Acta Crystallogr. C* **51**: 505–507

Fiddler, G. I., Lumley, P. (1990) Preliminary clinical studies with thromboxane synthase inhibitors and thromboxane receptor blockers: a review. *Circulation* **81** (Suppl. 1): I69–I78

Ford-Hutchinson, A. W., Girard, Y., Lord, A., Jones, T. R., Cirino, M., Evans, J. F., Gillard, J., Hamel, P., Leveillé, C., Masson, P., Young, R. (1989) The pharmacology of L-670,596, a potent and selective thromboxane/prostaglandin endoperoxide receptor antagonist. *Can. J. Physiol. Pharmacol.* **67**: 989–993

Friedel, H. A., Buckley, M. M. T. (1991) Torasemide. A review of its pharmacological and therapeutic potential. *Drugs* **41**: 81–103

Gresele, P., Deckmyn, H., Arnout, J., Lemmens, J., Janssens, W., Vermylen, J. (1984) BM 13177, a selective blocker of platelet and vessel wall thromboxane receptors, is active in man. *Lancet* **5**: 991–994

Guex, N., Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modelling. *Electrophoresis* **18**: 2714–2723

Guex, N., Peitsch, M. C. (1999) Molecular modelling of proteins. *Immunol. News* **6**: 132–134

Guex, N., Diemand, A., Peitsch, M. C. (1999) Protein modelling for all. *Trends Biochem. Sci.* **24**: 364–367

Hall, R. A., Gillard, J., Guindon, Y., Letts, J., Champion, E., Ethier, D., Evans, J., Ford-Hutchinson, A. W., Fortin, R., Jones, T. R., Lord, A., Morton, H. E., Rokach, J., Yoakim, C. (1987) Pharmacology of L-655,240 (3[1-(4-chlorobenzyl)-5-fluoro-3-methyl-indol-2-yl]-2,2-dimethylpropanoic acid); a potent, selective thromboxane/prostaglandin endoperoxide antagonist. *Eur. J. Pharmacol.* **135**: 193–201

Hantzsch, A., Wolvekamp, M. (1904) Die constitution der sogenannten dithiocyansaüre und persulfocyansäure. *Justus Liebigs Ann. Chem.* **331**: 265–297

Jessup, C. L., Jessup, R., Wayne, M. (1988) ICI192,605, a potent, selective thromboxane A_2 receptor antagonist on smooth muscle. *Br. J. Pharmacol.* **95** (Suppl.): 675P

Johnson, R. J., Nidy, E. G., Aiken, J. W. (1986) Thromboxane A_2 synthase inhibitors. 5-(3-pyridylmethyl)benzofuran-2-carboxylic acids. *J. Med. Chem.* **29**: 1461–1468

Kurokawa, T., Matsumoto, T., Ashida, Y., Sasada, R., Iwasa, S. (1994) Antagonism of the human thromboxane A2 receptor by an anti-asthmatic agent AA-2414. *Biol. Pharm. Bull.* **17**: 383–385

Liel, N., Mais, D. E., Halushka, P. V. (1987) Binding of thromboxane A_2/prostaglandin H_2 agonist [^{3}H]U-46619 to washed human platelets. *Prostaglandins* **33**: 789–797

Masereel, B., Renard, P., Schynts, M., Pirotte, B., de Tullio, P., Delarge, J. (1994) Synthesis and pharmacology of pyrid-3-yl sulfonylureas and sulfonylthioureas as inhibitors of the astrocytic Na^+ $2Cl^-$ K^+ co-transporter. *Eur. J. Med. Chem.* **29**: 527–535

Masereel, B., Dupont, L., Laeckmann, D., Liégeois, J. F., Pirotte, B., de Tullio, P., Delarge, J. (1995) Synthesis and pharmacology of pyrid-3-ylsulfonylcyanoguanidines as diuretics. *Eur. J. Med. Chem.* **30**: 343–351

Masereel, B., Damas, J., Fontaine, J., Varache-Lembège, M., Lacan, F., Nuhrich, A., Delarge, J., Pochet, L., Dogné, J. M. (1999) Thromboxane A_2 receptor antagonism in man and rat by a sulfonylcyanoguanidine (BM-144) and a sulfonylurea (BM-500). *J. Pharm. Pharmacol.* **51**: 695–701

Miki, I., Ishii, A. (1992) Characterization of thromboxane A2/prostaglandin H2 receptors in porcine coronary artery. The inhibitory effect of a novel dibenzoxepin derivative, KW-3635. *Thromb. Haemost.* **67**: 582–584

Nicolaï, E., Goyard, J., Benchetrit, T., Teulon, J. M., Caussade, F., Virone, A., Delchambre, C., Cloarec, A. (1993) Synthesis and structure–activity relationships of novel benzimidazole and imidazo[4,5-b]pyridine acid derivatives as thromboxane A_2 receptor antagonists. *J. Med. Chem.* **36**: 1175–1187

Ogletree, M. L. (1987) Overview of physiological and pathophysiological effects of thromboxane A_2. *Fed. Proc.* **46**: 133–138

Ogletree, M. L., Harris, D. N., Greenberg, R., Haslanger, M. F., Nakane, M. (1985) Pharmacological actions of SQ-29,548, a novel selective thromboxane antagonist. *J. Pharmacol. Exp. Ther.* **234**: 435–441

Romstedt, K. J., Shin, Y., Shams, G., Doyle, K., Tantishaiyakul, V., Clark, M. T., Adejare, A., Hamada, A., Miller, D. D., Feller, D. R. (1993) Halogen-substituted trimetoquinol analogs as thromboxane A2 receptor antagonists in platelets and aorta. *Biochem. Pharmacol.* **46**: 2051–2059

Sheldrick, G. M., Schneider, T. R. (1997) SHELXL: high resolution refinement. In: Sweet, R. M., Carter, C. W. (eds) *Methods in Enzymology*. Orlando, FL: Academic Press, **277**: 319–343

Soyka, R., Heckel, A., Nickl, J., Eisert, W., Müller, T. H.,

Weisenberger, H. (1994) 6,6,-Disubstituted hex-5-enoic acid derivatives as combined thromboxane A_2 receptor antagonists and synthase inhibitors. *J. Med. Chem.* **37**: 26–39

Takeuchi, K., Kohn, T. J., True, T. A., Mais, D. E., Wikel, J. H., Utterback, B. G., Wyss, V. L., Jakubowski, J. A. (1998) Development of dual-acting agents for thromboxane receptor antagonism and thromboxane synthase inhibition. 3. Synthesis and biological activities of oxazolecarboxamide-substituted ω-phenyl-ω-(3-pyridyl)alkenoic acid derivatives and related compounds. *J. Med. Chem.* **41**: 5362–5374

Theis, J. G. W., Dellweg, H., Perzborn, E., Gross, R. (1992) Binding characteristics of the new thromboxane A2/prostaglandin H2 receptor antagonist [3H]BAY U 3405 to washed human platelets and platelets membranes. *Biochem. Pharmacol.* **44**: 495–503

Uchida, T., Kido, H., Yamanaga, K., Okita, M., Watanabe, M. A. (1992) Novel loop-diuretic, torasemide, inhibits thromboxane A_2-induced contraction in the isolated canine coronary artery. *Prostaglandins Leukot. Essent. Fatty Acids* **45**: 121–124

Wouters, J., Durant, F., Masereel, B. (1999) Antagonism of the TXA2 receptor by seratrodast: a structural approach. *Bioorg. Med. Chem. Lett.* **9**: 2867–2870

Yamamoto, Y., Kamiya, K., Terao, S. (1993) Modeling of human thromboxane A_2 receptor and analysis of the receptor-ligand interaction. *J. Med. Chem.* **36**: 820–825