# Synthesis and Pharmacological Evaluation of Novel Nitrobenzenic Thromboxane Modulators as Antiplatelet Agents Acting on Both the Alpha and Beta Isoforms of the Human Thromboxane Receptor

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Thromboxane $A_2$ ($TXA_2$) is an arachidonic acid metabolite involved in pathologies such as stroke, myocardial infarction, and atherosclerosis. Consequently, the design of $TXA_2$ receptor (TP) antagonists remains of great interest in cardiovascular medicine. The actions of $TXA_2$ are mediated by its specific G-protein coupled receptor of which two alternative spliced isoforms, TPα and TP$\beta$, have been described in humans. In this study, we report the synthesis of a series of original *N*-alkyl-*N*′-[2-(cycloalkyl, alkylaryl)-5-nitrobenzenesulfonyl]urea and *N*-alkyl-*N*′-[2-(alkylaryl)-5-nitrobenzenesulfonyl]-*N*′′-cyanoguanidines and outline their pharmacological evaluation using the individual TPα and TP$\beta$ isoforms. Among compounds analyzed, several of them exhibited greater affinity and/or functional activity for either TPα or TP$\beta$. The most promising molecules were also found to be antiplatelet agents. From the present results, structural features involved in isoform selectivity can be proposed, and thereby several lead compounds have been identified for the further development of selective TP isoform antagonists.

## Introduction

The lipid mediator thromboxane $A_2$ ($TXA_2$)[a] plays a key role in several physiologic processes including platelet aggregation as well as vascular and bronchial smooth muscle constriction.[1,2] An overproduction of $TXA_2$ has been associated with many pathological states such as myocardial infarction, thrombosis, unstable angina, pulmonary embolism, septic shock, atherosclerosis, preeclampsia, and asthma.[3] $TXA_2$, a metabolite of arachidonic acid (AA) released mainly by phospholipase (PL)-$A_2$ from membrane phospholipids, is primarily synthesized through the sequential actions of cyclooxygenase (COX) and thromboxane synthase (TXS). COX exists as two main isoforms, COX-1 and COX-2, which are encoded by two separate genes. COX-1 is expressed constitutively in most tissues mediating "housekeeping" functions, whereas COX-2 expression is mainly induced at sites of inflammation by various stimuli.[3] COX-2 is also expressed in a constitutive manner in the brain, the vascular endothelium, and the kidney.[4] $TXA_2$ synthesis mainly occurs in platelets where both COX-1 and TXS are highly expressed.

The actions of $TXA_2$ are mediated by its specific G-protein coupled receptor (GPCR), referred to as the $TXA_2$ receptor or TP.[5] In 1991, Hirata and colleagues reported that the human TP was encoded by a single gene,[6] and in 1994 Raychowdhury et al. identified a second isoform, generated by alternative splicing.[7] The two human TP isoforms, consequently named TPα and TP$\beta$, share the first 328 amino acids but differ exclusively within their carboxy-terminal tails (15 amino acids of the α isoform being replaced by 79 amino acids in the $\beta$ isoform).

In the 1980s, several compounds with differential activities for TPs expressed in platelets or various types of smooth muscle were characterized[8] and none of them selectively bind to TPα or TP$\beta$.

Currently only one compound, 5,8-decadienoic acid, 10-hydroxy-10-[2-(2*Z*)-2-octenylcyclopropyl]-, methyl ester, (5*Z*,8*Z*,-10*S*)- (9CI) (PBT-3), a hepoxilin antagonist, has been described as a preferential TPα antagonist.[9] While the exact role of the TPα and TP$\beta$ isoforms has not been fully elucidated to date, a number of independent investigations highlight potentially important physiological differences between them. For example, Coyle et al. have recently demonstrated that the expression of TPα and TP$\beta$ is under the control of two distinct promoters.[10,11] Ashton et al. have also demonstrated that a specific inhibitor of TP$\beta$ would be useful to enhance postmyocardial infarction revascularization since the stimulation of TP$\beta$ seems to be responsible for vascular endothelial growth factor-induced endothelial cell differentiation and migration.[12] On the other hand, since TPα is the predominant TP isoform expressed in platelets,[13] specific inhibitor(s) of TPα may be beneficial as antiplatelet agent(s). Moreover, it has been established that TPα, but not TP$\beta$, is subject to cross-desensitization of signaling by prostacyclin leading to the proposal that TPα is the predominant TP isoform involved in vascular hemeostasis.[14] The potential benefits of TP receptor/isoform selective antagonists in atherosclerosis have been recently highlighted where it was demonstrated that compounds antagonizing TP receptors reduced

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[a] Abbreviations: $TXA_2$, thromboxane $A_2$; TXS, thromboxane synthase; $PLA_2$, phospholipase $A_2$; AA, arachidonic acid; GPCR, G-protein coupled receptor; COX, cyclooxygenase; TP, thromboxane receptor; PLC, phospholipase C; $IP_3$, inositol 1,4,5-triphosphate.

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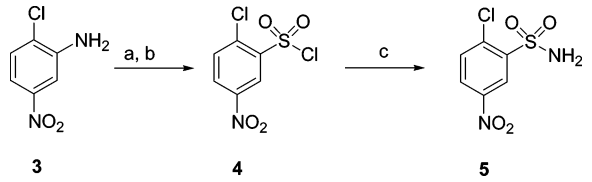
**Scheme 1**[a]




[a] Reagents: (a) $NaNO_2$, HCl; (b) $SO_2$, HOAc, $Cu_2Cl_2$; (c) $NH_4OH$, Δ.

atherosclerotic plaque growth.[15] Several groups have also proposed that the adverse cardiovascular effects of the selective COX-2 inhibitor rofecoxib were due to its reduction of prostacylin synthesis without affecting $TXA_2$ levels, thereby altering the balance between the anti- and prothrombotic actions of prostacyclin and $TXA_2$ within the vasculature.[16]

In the current study, we describe the synthesis of a series of novel nitrobenzenesulfonylureas and nitrobenzenesulfonylcyanoguanidines and outline their pharmacological evaluation as TP receptor antagonists. For the first time, the structure−activity relationships of this large series of compounds for both TPα and/or TP$\beta$ isoforms are discussed. The antiplatelet activity of the most pharmacologically interesting compounds is also described.

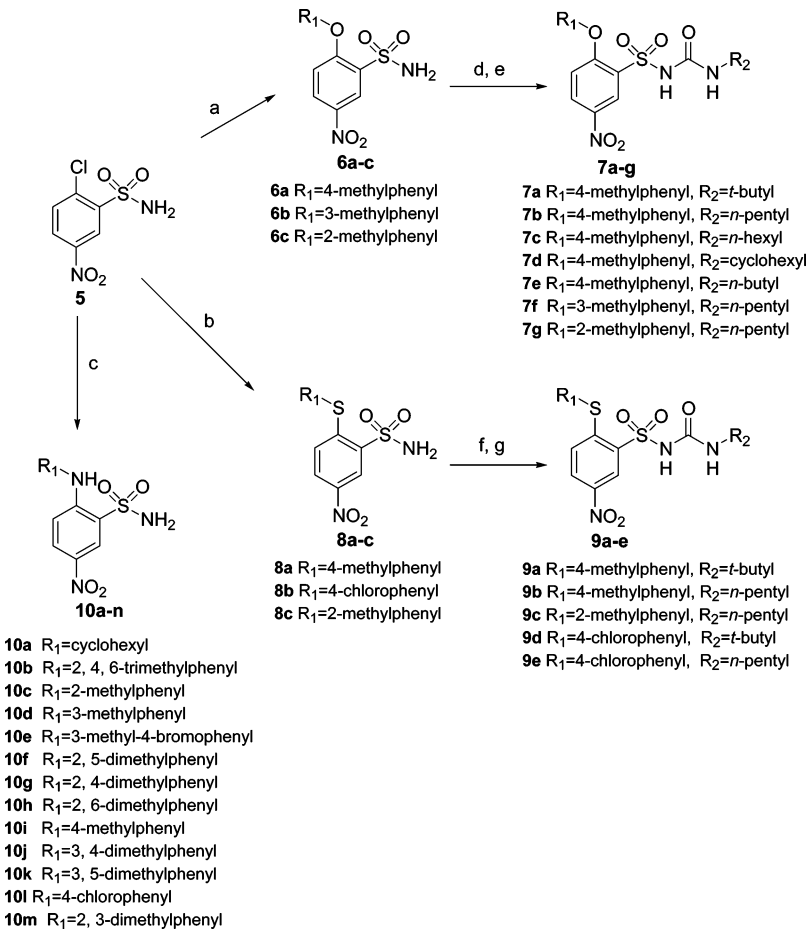
**Chemistry.** The compounds evaluated in this work were synthesized according to the synthesis pathway described in Schemes 1−3. In the first step, which is common to all compounds described, commercially available 2-chloro-5-nitroaniline (**3**) reacted with sodium nitrite in acidic medium, and the resulting solution of diazonium salt was mixed with a solution of sulfur dioxide in acetic acid in the presence of Cu(I) to generate 2-chloro-5-nitrobenzenesulfonyl chloride (**4**) according to the Meerwein variation of the Sandmeyer reaction.[17] Compound **4** was poured into a solution of ammonium hydroxide and gently heated, resulting in the synthesis of 2-chloro-5-nitrobenzenesulfonamide (**5**) which is the common intermediate for the compounds studied (Scheme 1).

The nucleophilic substitution of the chlorine atom in the position para to the nitro group by either amines, phenols, or thiols led to the synthesis of the intermediates **6a**−**c**, **8a**−**c**, and **10a**−**n**, respectively. Deprotonation of the sulfonamide group of compounds **6a**−**c** followed by reaction with the appropriate isocyanate led to the synthesis of compounds **7a**−**g** (Scheme 2).

Intermediates **8a**−**c** are characterized by a sulfur atom as a bridge between the two aromatic rings. These compounds were synthesized by reacting thiophenols, instead of phenols, with compound **5** in the presence of $K_2CO_3$. As with the compounds **6a**−**c**, intermediates **8a**−**c** were deprotonated and reacted with the appropriate isocyanates to afford the formation of compounds **9a**−**e** (Scheme 2).

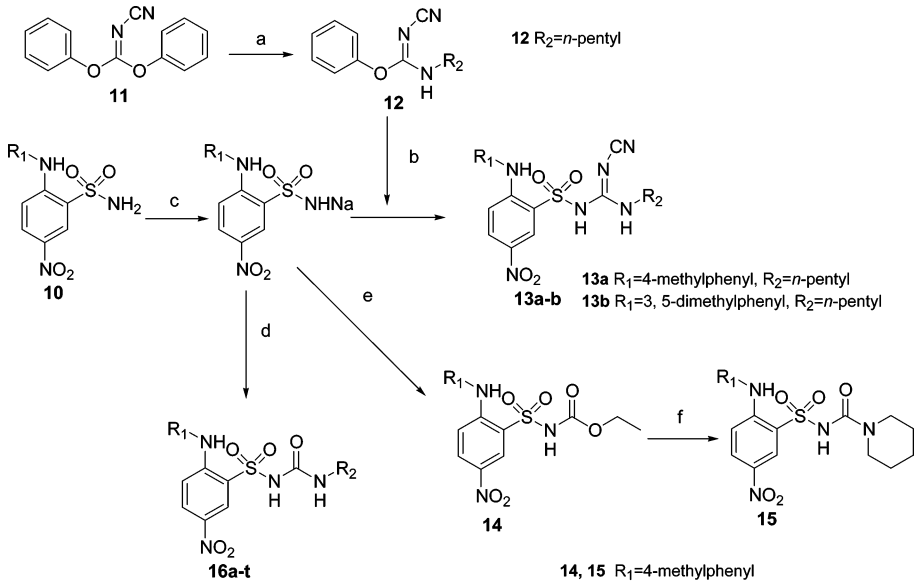
Compounds **10a**−**m** were obtained by reacting the appropriate amines with compound **5**. The sulfonamide group of compounds **10a**−**m** was deprotonated and then submitted to different reaction conditions. For the synthesis of sulfonylcyanoguanidines **13a**−**b**, a reactive synthon was first prepared by direct reaction of diphenyl *N*-cyanoiminocarbonate (**11**) with *n*-pentylamine. This synthon (**12**) directly reacted with the sulfonamidate salts of **10i** and **10m** to generate compounds **13a**−**b** (Scheme 3).

**Scheme 2**[a]




[a] Reagents: (a) $R_1$−ONa, $K_2CO_3$; (b) $R_1$−SH, $K_2CO_3$; (c) $R_1$−$NH_2$, $K_2CO_3$; (d) NaOH; (e) $R_2$−N═C═O; (f) NaOH; (g) $R_2$−N═C═O.

**Scheme 3**[a]




**16a** $R_1$=cyclohexyl, $R_2$=hexyl
**16b** $R_1$=cyclohexyl, $R_2$=heptyl
**16c** $R_1$=cyclohexyl, $R_2$=octyl
**16d** $R_1$=2, 4, 6-trimethylphenyl, $R_2$=*n*-pentyl
**16e** $R_1$=2-methylphenyl, $R_2$=*n*-pentyl
**16f** $R_1$=3-methylphenyl, $R_2$=*n*-pentyl
**16g** $R_1$=3-methyl-4-bromophenyl, $R_2$=*t*-butyl
**16h** $R_1$=2, 5-dimethylphenyl, $R_2$=*n*-pentyl
**16i** $R_1$=2, 4-dimethylphenyl, $R_2$=*n*-pentyl
**16j** $R_1$=2, 6-dimethylphenyl, $R_2$=*n*-pentyl
**16k** $R_1$=4-methylphenyl, $R_2$=benzyl
**16l** $R_1$=4-methylphenyl, $R_2$=cyclohexyl
**16m** $R_1$=3, 4-dimethylphenyl, $R_2$=*n*-pentyl
**16n** $R_1$=3, 5-dimethylphenyl, $R_2$=*n*-pentyl
**16o** $R_1$=3-methyl-4-bromophenyl, $R_2$=*n*-pentyl
**16p** $R_1$=2, 6-dimethylphenyl, $R_2$=*sec*-butyl
**16q** $R_1$=2, 6-dimethylphenyl, $R_2$=*t*-butyl
**16r** $R_1$=4-methylphenyl, $R_2$=*sec*-pentyl
**16s** $R_1$=4-chlorophenyl, $R_2$=*n*-pentyl
**16t** $R_1$=2, 3-dimethylphenyl, $R_2$=*n*-pentyl

[a] Reagents: (a) $R_3-NH_2$, 2-propanol; (b) DMF; (c) NaOH; (d) $R_2-N=C=O$; (e) ethyl chloroformate; (f) piperidine.

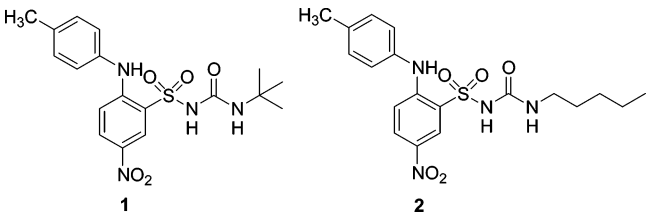


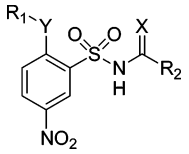

**Figure 1.** Chemical structures of **1** and **2**, two TP receptor antagonists used as references in pharmacological tests.

Compounds **10a**−**m** also reacted, after deprotonation, with appropriate isocyanates to generate the sulfonylureas (**16a**−**t**). Finally, one compound (**15**), bearing a sulfonyl group for which the distal nitrogen atom was fully substituted, was obtained in two steps: (i) reaction of **10i** as the sodium salt with ethyl chloroformate followed by (ii) reaction with piperidine that led to formation of **15** (Scheme 3).

**Pharmacological Evaluation.** The overall aim of this study was to evaluate a series of novel nitro-substituted benzene sulfonamide derivatives as pharmacological antagonists of the TP$\alpha$ and TP$\beta$ isoforms of the human $TXA_2$ receptor. Consequently, we synthesized several analogues of **1** (BM-573) and **2** (BM-613, both structures are presented in Figure 1), two potent TP antagonists characterized by a nitrobenzenic ring bearing a sulfonylurea moiety.[18−22] The binding affinities of each TP isoform for each test compound was assessed through competition binding studies in COS-7 cell lines transiently transfected with respective plasmids encoding TP$\alpha$ or TP$\beta$. Experiments were initially performed at a single concentration (1 nM) of the 35 novel test compounds or reference compounds, **1** and **2**. Competition binding studies were performed with [$^3$H]SQ29,-548 {5-heptenoic acid, 7-[(1*S*,2*R*,3*R*,4*R*)-3-[[2-[(phenylamino)-carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-, (5Z)-(9CI)}, a selective TP antagonist, as the radioligand. It has been previously confirmed that SQ29,548 had no apparent selectivity for either the TP$\alpha$ or TP$\beta$ isoform.[23] Thereafter, those test compounds that displayed the highest affinities for one or both TP isoforms as well as compounds characterized by the greatest differences in affinity for either TP$\alpha$ or TP$\beta$ were selected for a complete concentration−response curve determination with concentrations ranging from $10^{-6}$ to $10^{-11}$ M. Values for the inhibitory concentration $(IC)_{50}$ were obtained from the concentration−response curves and a "selectivity index", defined as the $IC_{50}$ TP$\alpha$/$IC_{50}$ TP$\beta$ ratio, was calculated.

Because binding experiments do not reflect the specific activity and pharmacological profile of a molecule/ligand at its receptor, we also examined the effects of the compounds under study on agonist-induced intracellular signaling by the individual TP$\alpha$ and TP$\beta$ isoforms. To this end, the ability of the compounds to antagonize TP$\alpha$ and TP$\beta$-mediated intracellular calcium mobilization ($[Ca^{2+}]_i$) in response to the $TXA_2$ mimetic

**Table 1.** Displacement of Radiolabeled [$^3$H]SQ29,548 from TP$\alpha$ and TP$\beta$ by Compounds at 1 nM



| compound | $R_1$ | $R_2$ | Y | X | binding affinity (%)[a] TP$\alpha$ | binding affinity (%)[a] TP$\beta$ |
|---|---|---|---|---|---|---|
| **1** | 4-methylphenyl | *tert*-butylamino | NH | O | 49.5 ± 1.6 | 53.6 ± 5.5 |
| **2** | 4-methylphenyl | *n*-pentylamino | NH | O | 39.6 ± 9.1 | 34.2 ± 2.9 |
| **16a** | cyclohexyl | *n*-hexylamino | NH | O | 44.2 ± 16.5 | 42.5 ± 2.3 |
| **16b** | cyclohexyl | *n*-heptylamino | NH | O | 29.5 ± 3.4 | 36.4 ± 11.1 |
| **16c** | cyclohexyl | *n*-octylamino | NH | O | 17.1 ± 3.7 | 24.1 ± 4.4 |
| **16d** | 2,4,6-trimethylphenyl | *n*-pentylamino | NH | O | 27.9 ± 3.0 | 27.5 ± 4.4 |
| **16e** | 2-methylphenyl | *n*-pentylamino | NH | O | 45.3 ± 8.9 | 45.3 ± 3.0 |
| **16f** | 3-methylphenyl | *n*-pentylamino | NH | O | 48.5 ± 3.4 | 35.8 ± 9.2 |
| **16g** | 3-methyl-4-bromophenyl | *tert*-butylamino | NH | O | 28.5 ± 5.4 | 25.0 ± 1.5 |
| **16h** | 2,5-dimethylphenyl | *n*-pentylamino | NH | O | 28.6 ± 3.4 | 33.1 ± 4.9 |
| **16i** | 2,4-dimethylphenyl | *n*-pentylamino | NH | O | 42.3 ± 4.0 | 39.9 ± 4.8 |
| **16j** | 2,6-dimethylphenyl | *n*-pentylamino | NH | O | 36.5 ± 4.7 | 54.0 ± 2.1 |
| **16k** | 4-methylphenyl | benzylamino | NH | O | 32.9 ± 4.5 | 49.3 ± 3.5 |
| **16l** | 4-methylphenyl | cyclohexylamino | NH | O | 31.1 ± 2.9 | 29.0 ± 3.3 |
| **16m** | 3,4-dimethylphenyl | *n*-pentylamino | NH | O | 40.1 ± 8.8 | 48.7 ± 16.9 |
| **16n** | 3,5-dimethylphenyl | *n*-pentylamino | NH | O | 49.7 ± 5.1 | 49.7 ± 11.0 |
| **16o** | 3-methyl-4-bromophenyl | *n*-pentylamino | NH | O | 32.1 ± 5.2 | 49.9 ± 13.0 |
| **16p** | 2,6-dimethylphenyl | *sec*-butylamino | NH | O | 48.4 ± 4.0 | 42.5 ± 6.0 |
| **16q** | 2,6-dimethylphenyl | *tert*-butylamino | NH | O | 46.4 ± 2.2 | 46.3 ± 2.3 |
| **16r** | 4-methylphenyl | *sec*-butylamino | NH | O | 40.7 ± 2.5 | 40.4 ± 6.5 |
| **16s** | 4-chlorophenyl | *n*-pentylamino | NH | O | 48.9 ± 7.7 | 52.7 ± 2.2 |
| **16t** | 2,3-dimethylphenyl | *n*-pentylamino | NH | O | 44.6 ± 5.4 | 39.0 ± 7.8 |
| **7a** | 4-methylphenyl | *tert*-butylamino | O | O | 53.3 ± 5.7 | 44.7 ± 2.1 |
| **7b** | 4-methylphenyl | *n*-pentylamino | O | O | 35.5 ± 2.8 | 48.5 ± 3.8 |
| **7c** | 4-methylphenyl | *n*-hexylamino | O | O | 55.2 ± 2.1 | 46.1 ± 2.1 |
| **7d** | 4-methylphenyl | cyclohexylamino | O | O | 49.8 ± 5.7 | 51.9 ± 3.8 |
| **7e** | 4-methylphenyl | *n*-butylamino | O | O | 55.7 ± 1.7 | 56.0 ± 4.9 |
| **7f** | 3-methylphenyl | *n*-pentylamino | O | O | 20.1 ± 10.3 | 22.8 ± 3.3 |
| **7g** | 2-methylphenyl | *n*-pentylamino | O | O | 35.2 ± 1.6 | 36.8 ± 7.1 |
| **9a** | 4-methylphenyl | *tert*-butylamino | S | O | 47.0 ± 5.6 | 37.7 ± 6.3 |
| **9b** | 4-methylphenyl | *n*-pentylamino | S | O | 38.5 ± 2.7 | 43.0 ± 1.1 |
| **9c** | 2-methylphenyl | *n*-pentylamino | S | O | 33.4 ± 2.2 | 32.7 ± 7.7 |
| **9d** | 4-chlorophenyl | *tert*-butylamino | S | O | 32.5 ± 1.8 | 31.7 ± 3.4 |
| **9e** | 4-chlorophenyl | *n*-pentylamino | S | O | 24.7 ± 7.6 | 21.3 ± 5.7 |
| **13a** | 4-methylphenyl | *n*-pentylamino | NH | N−CN | 25.5 ± 1.8 | 27.2 ± 5.2 |
| **13b** | 3,5-dimethylphenyl | *n*-pentylamino | NH | N−CN | 48.0 ± 6.4 | 53.4 ± 3.2 |
| **15** | 4-methylphenyl | piperidino | NH | O | 46.2 ± 9.2 | 54.2 ± 7.8 |

[a] Expressed as the percentage of displaced [$^3$H]SQ29,548 by our compounds. Results are mean ± standard deviation of at least three determinations ($n \geq 3$). Compounds were evaluated at a final concentration of 1 nM.

U46619 {5-Heptenoic acid, 7-[(1*R*,4*S*,5*S*,6*R*)-6-[(1*E*,3*S*)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-, (5*Z*)- (9CI)} was evaluated in HEK 293 cell lines stably overexpressing either TP$\alpha$ (HEK.TP$\alpha$ cells) or TP$\beta$ (HEK.TP$\beta$ cells). HEK.TP$\alpha$ and/or HEK.TP$\beta$ cells were preloaded with fluorescent dye Fluo-4 and inhibition of the $TXA_2$ mimetic U46619 (1 $\mu$M) stimulation of $[Ca^{2+}]_i$ mobilization was determined in the absence or presence of increasing concentrations of the compounds, using concentrations ranging from $10^{-5}$ M to $10^{-8}$ M. As for the competition binding studies, a selectivity index was determined. Finally, since the TP receptor directly mediates human platelet aggregation, the efficacy of test compounds in an ex vivo human platelet aggregation model was evaluated.

## Results and Discussion

The results obtained with the initial competition-binding studies are presented in Table 1. All compounds evaluated displayed a high affinity for both TP$\alpha$ and TP$\beta$ receptor isoforms. These affinities, expressed as the percentage displacement of [$^3$H]SQ29,548, were all in the nanomolar range and were comparable to that of the reference compounds **1** and **2** (Figure 1) used throughout this study. The affinities of **1** and **2** were in the same range of order of previously published data.[22] The bridging atom (O, S, or NH in the series **7a**−**g**, **9a**−**e**, or **13**, **15**, **16a**−**t**, respectively) between the nitrobenzene and the second ring did not affect the affinity of the molecules for the TP receptors. Additionally, in the "NH" compounds family (**13**, **15**, and **16a**−**t**), a critical loss of affinity was not observed as a result of the disubstitution of the distal nitrogen atom of the urea group or as a result of the replacement of the urea function with a cyanoguanidine isoster moiety. Thereafter, further experiments focused on those compounds that either displayed the highest affinity for the TP receptors or on those that showed significant differences in their TP$\alpha$/TP$\beta$ isoform selectivity.

For compounds **7a**−**e**, characterized by the presence of an oxygen atom as the bridge between two aromatic rings, several concentration−response curves were determined and are summarized in Table 2. Although the affinities of the compounds for both TP$\alpha$ and TP$\beta$ lie within the same range, certain compounds exhibited a significant ratio of selectivity between the two receptor isoforms. For example, compound **7b** exhibited a significant ratio of 4.02 ($p < 0.05$), which was a result of a greater affinity for TP$\beta$. In this family of compounds, it was apparent that the side chain ($R_2$) had an impact on the affinity.

**Table 2.** Estimated $IC_{50}$ Values for Displacement of [$^3$H]SQ29,548 from TPα and TP$\beta$

| compound | binding affinity $IC_{50}$ (nM)[a] TPα | TP$\beta$ | ratio[a] $IC_{50}$ TPα/$IC_{50}$ TP$\beta$ |
|---|---|---|---|
| **1** | 1.05 ± 0.43 | 0.78 ± 0.10 | 1.35 |
| **2** | 2.65 ± 1.59 | 3.53 ± 1.38 | 0.75 |
| **7a** | 1.26 ± 0.74 | 1.77 ± 0.69 | 0.71 |
| **7b** | 2.63 ± 0.94 | 0.65 ± 0.15 | 4.02 |
| **7c** | 0.72 ± 0.08 | 1.58 ± 0.21 | 0.45 |
| **7d** | 1.52 ± 0.70 | 0.79 ± 0.15 | 1.93 |
| **7e** | 0.74 ± 0.02 | 0.70 ± 0.24 | 1.05 |
| **9a** | 0.77 ± 0.33 | 1.74 ± 0.28 | 0.44 |
| **15** | 0.46 ± 0.11 | 0.68 ± 0.11 | 0.68 |
| **16a** | 0.71 ± 0.01 | 1.07 ± 0.26 | 0.67 |
| **16b** | 2.50 ± 1.21 | 2.68 ± 1.06 | 0.93 |
| **16f** | 1.01 ± 0.25 | 4.43 ± 0.47 | 0.23 |
| **16j** | 1.52 ± 0.31 | 1.48 ± 0.35 | 1.03 |
| **16k** | 1.90 ± 0.40 | 1.17 ± 0.12 | 1.62 |
| **16n** | 1.08 ± 0.33 | 0.70 ± 0.43 | 1.55 |
| **16o** | 1.82 ± 0.25 | 0.58 ± 0.27 | 3.11 |
| **16q** | 0.78 ± 0.15 | 0.56 ± 0.20 | 1.40 |

[a] Results expressed as mean ± standard deviation of at least three determinations ($n \geq 3$).

For example in terms of TP isoform affinity, compound **7e** with $R_2$ = *n*-butyl was the most potent compound in this assay and seems to bear the most interesting side chain, among those tested, for these compounds. In terms of the calculated selectivity ratio, the side chain was also found to be involved. On one hand, compounds for which $R_2$ corresponds to *n*-pentyl or cyclohexyl (**7b** and **7d**, respectively) expressed a preferential affinity for TP$\beta$ (Table 2). On the other hand, when $R_2$ corresponds to *tert*-butyl or *n*-hexyl (**7a** and **7c**, respectively), there was either a loss or no change in the selectivity ratio.

Additionally, Table 2 reports the results of the determination of the $IC_{50}$ value of compound **9a**, characterized by a sulfur atom as the intercycle bridge. This compound expressed a nanomalor affinity for the TP receptors in our model. This observation is consistent with our previous statement that the nature of the intercycle bridge (NH, S, or O) did not markedly influence the affinity of the compound for TPα/TP$\beta$.

Compound **15** is characterized by having a NH as the bridge between the two rings and having the distal N urea atom incorporated into a ring. Although the affinity of compound **15** for TPα or TP$\beta$ was not markedly improved compared to the reference compounds **1** and **2** (Table 2), the incorporation of the distal N urea atom into an alicyclic ring appeared to be favorable for the affinity of both isoforms, with no influences on the selectivity.

Finally, with compounds of the **16** family (**16a**−**t**) bearing a NH bridge between the two rings, data on Table 2 indicates that, when the compounds were characterized by a second ring ($R_1$), namely alicyclic, the length of the side chain influenced the affinity. For example, compound **16a** with $R_2$ = *n*-hexyl was characterized by a ∼3-fold higher affinity than **16b** with $R_2$ = *n*-heptyl The results obtained with compound **16f** for which $R_2$ = *n*-pentyl and $R_1$ = *m*-methylphenyl were of particular interest, since it is the most selective compound for TPα isoform. Finally, it is noteworthy that PBT-3, a stable analogue of hepoxilin which is known to be a selective TPα antagonist, was reported to exhibit a selectivity ratio of ∼0.17 in the same assay.[9]

Thereafter, we sought to extend the characterization of the selected high affinity test compounds through functional intracellular signaling studies. Hence, we further examined the properties of the various test compounds through assessment of U46619-induced intracellular calcium ($[Ca^{2+}]_i$ mobilization in HEK293 cell lines stably overexpressing the individual TPα or TP$\beta$ isoforms). Calcium was the obvious second messenger to evaluate since TPα or TP$\beta$ each couple to Gq/phospholipase (PL)C activation, leading to inositol 1,4,5-trisphosphate ($IP_3$)-mediated $[Ca^{2+}]_i$ mobilization from intracellular stores.[24,25] The $[Ca^{2+}]_i$ mobilization was measured with cells loaded with fluorescent dye Fluo-4, whose fluorescence increases upon binding with calcium ion released in response to the $TXA_2$ mimetic U46619. Consequently, the ability of the compounds to antagonize U46619-mediated $[Ca^{2+}]_i$ mobilization was evaluated.

**Table 3.** Estimated $IC_{50}$ Values for the Inhibition of $[Ca^{2+}]_i$ Mobilization Mediated by Either TPα or TP$\beta$ upon Stimulation by U46619 (1 $\mu$M)

| compound | U46619-mediated $[Ca^{2+}]_i$ mobilization (nM)[a] TPα | TP$\beta$ | ratio[a] $IC_{50}$ TPα/$IC_{50}$ TP$\beta$ |
|---|---|---|---|
| **1** | 318.89 ± 202.54 | 53.10 ± 19.38 | 6.01 |
| **2** | 50.04 ± 0.73 | 13.56 ± 1.08 | 3.69 |
| **7a** | 58.34 ± 43.01 | 57.63 ± 3.97 | 1.01 |
| **7b** | 139.32 ± 87.93 | 55.38 ± 4.85 | 2.52 |
| **7c** | 526.42 ± 29.61 | 58.48 ± 0.58 | 9.00 |
| **7d** | 600.71 ± 11.16 | 70.15 ± 5.90 | 8.56 |
| **7e** | 558.04 ± 33.91 | 52.89 ± 2.54 | 10.55 |
| **9a** | 293.54 ± 106.53 | 55.17 ± 3.45 | 5.32 |
| **15** | 64.81 ± 3.54 | 38.52 ± 2.84 | 1.68 |
| **16a** | 81.22 ± 3.50 | 46.17 ± 1.32 | 1.76 |
| **16b** | 92.90 ± 9.03 | 55.55 ± 2.26 | 1.67 |
| **16f** | 60.39 ± 16.07 | 45.53 ± 1.50 | 1.33 |
| **16j** | 80.72 ± 20.39 | 47.10 ± 0.39 | 1.71 |
| **16k** | 64.47 ± 1.84 | 45.01 ± 2.59 | 1.43 |
| **16n** | 537.86 ± 8.18 | 76.50 ± 6.39 | 7.03 |
| **16o** | 126.28 ± 26.07 | 43.19 ± 9.98 | 2.92 |
| **16q** | 56.89 ± 2.35 | 30.78 ± 9.81 | 1.85 |

[a] Results expressed as mean ± standard deviation of at least three determinations ($n \geq 3$).

The results obtained with the test compounds in this assay (Table 3) do not fully correlate with those obtained through competition binding studies (Table 2). This can be explained by the fact that the affinity is not the same property as activity, i.e., that affinity does not necessarily reflect the agonistic or antagonistic properties of a compound at its receptor. Moreover, the mechanism and intensity whereby a compound activates or deactivates a receptor cannot be measured through competition binding studies. Hence, when evaluating a receptor ligand (agonist/antagonist), it is critical to evaluate that agent through a number of independent mechanisms, such as through binding studies and functional studies as outlined herein.

First, it is noteworthy that all compounds assayed exhibited high activity as TPα and TP$\beta$ receptor antagonists. Compound **16q**, one of the most potent compound in this assay, was characterized by a 2,6-dimethylphenylamino group ortho to the sulfonamide group in the aromatic ring. As a result, it is suggestive that a pattern of substitution by methyl groups in the 2- and 6- positions of the second ring may provide an interesting lead for further experimentations. Moreover, in this functional assay, each compound exhibited a better activity on TPα compared with TP$\beta$, except with compound **7a** which possessed activities in the same concentration range. These data also confirmed the fact that the side chain of the molecules could play a role in selectivity. For example, activities on TP$\beta$ of compounds **7b**−**e** were within the same range as compound **7a** characterized by a *tert*-butyl side chain. Nevertheless, their activities on TPα were almost 10-fold less pronounced than that of **7a**. Additionally, it should also be noted that the rank order of potency of **7b**−**e** for TPα did not follow the pattern of the size or steric hindrance of the side chain. Indeed, TPα potency

**Table 4.** Estimated $IC_{50}$ Values for the Inhibition of Platelet Aggregation Induced by U46619 (1 $\mu$M)

| compound | inhibition of platelet aggregation induced by 1 $\mu$M U46619 $IC_{50}$ ($\mu$M)[a] |
|---|---|
| **1** | 0.240 ± 0.013 |
| **2** | 0.278 ± 0.186 |
| **7a** | 0.300 ± 0.003 |
| **7b** | 0.670 ± 0.200 |
| **9a** | 0.800 ± 0.090 |
| **15** | 0.900 ± 0.006 |
| **16q** | 0.090 ± 0.007 |

[a] Results expressed as mean ± standard deviation of at least three determinations (n≥3).

was ranked 4b > 4c ≅ 4e > 4d, indicating that *n*-pentyl > *n*-butyl ≅ *n*-hexyl > cyclohexyl. We could not conclude from these results that the selectivity ratio is directly influenced by the length of the side chain. Nevertheless, it is notable that in this assay the *n*-pentyl side chain was responsible for the best activity in this series compared to other linear side chains.

Finally, we sought to confirm the activity of our most interesting molecules on human platelets by assessment of their ability to inhibit platelet aggregation in response to the $TXA_2$ mimetic U46619. Compounds **7a**, **7b**, **9a**, **15**, and **16q** were selected because of their ability to inhibit U46619-mediated $[Ca^{2+}]_i$ release, affinities, or apparent selectivity in different models. The results obtained are summarized in Table 4. First, compounds evaluated were indeed confirmed to act as TP receptor antagonists inhibiting platelet aggregation in response to 1 $\mu$M U46619 at sub-micromolar concentrations ranging from 0.09 to 0.9 $\mu$M.

It should be highlighted that all the compounds evaluated in the three pharmacological assessment models exhibited promising results in terms of their affinities, as assessed through competition of radioligand binding, measurements of inhibition of $[Ca^{2+}]_i$ mobilization, and inhibition of U46619-induced platelet aggregation. Collective results highlighted compound **16q** as a potential lead of major interest since its $IC_{50}$ for inhibition of platelet aggregation was 0.09 $\mu$M. Finally, it is noteworthy that compound **16q** is one of the most potent compounds on TPα and is also the most potent inhibitor in platelet aggregation studies. These data are consistent with previous findings that TPα is the dominant isoform expressed in human platelets,[13] although this compound was equally active on TP$\beta$ (Table 2).

## Conclusions

We have synthesized and evaluated a series of some 40 novel nitro-substituted benzenesulfonamide derivatives designed as antagonists of the TP/$TXA_2$ receptors. Since there are two TP isoforms in humans, the development of selective compounds for TPα and/or TP$\beta$ is clearly of great clinical interest for human diseases. Hence, we have studied the affinity and activity of our compounds on both TPα and TP$\beta$. All compounds evaluated exhibited very high affinity for both TP receptors, mainly acting in the nanomolar range. Moreover, both receptor ligand binding and inhibition of $[Ca^{2+}]_i$ signaling concentration−response curves have been determined with the most interesting compounds. In these assays, all compounds confirmed their affinity and activity for the TP receptors.

According to the biological data presented herein, we can propose in our series of nitrobenzene-sulfonylureas and -sulfonylcyanoguanidines some structural factors involved in affinity and activity on either TPα or TP$\beta$, which could lead to the development of selective TP receptor antagonists. Finally, the most promising compounds were evaluated on platelet aggregation and confirmed their potent TP receptor antagonism and their interest as antiplatelet agents.

## Experimental Section

**Chemistry.** All commercial chemicals (Sigma-Aldrich, Belgium) and solvents are reagent grade and were used without further purification unless otherwise stated. Compounds **5**, **10d**, **8a**, and **8c** were already described.[26,27] **6c** was commercially available (AmbinterStock Screening Collection). Nevertheless, these compounds were synthesized in our lab according to the method of preparation described below. All reactions were followed by thin-layer chromatography (silica gel $60F_{254}$ Merck) and visualization was accomplished with UV light (254 nm). Elemental analyses (C, H, N, S) were determined on a Carbo Erba EA 1108 and were within ±0.4% of the theoretical values. NMR spectra were recorded either on a Bruker Avance 500 or on a Bruker DRX-400 spectrometer using DMSO-$d_6$ as solvent and tetramethylsilane as internal standard. For $^1H$ NMR spectra, chemical shifts are expressed in $\delta$ (ppm) downfield from tetramethylsilane. The abbreviations d, doublet; t, triplet; m, multiplet; br, broad were used throughout. Infrared spectra were recorded using a Perkin-Elmer FT-IR 1750. All compounds described were recrystallized from hot methanol (40 °C)/$H_2O$ mixture (60/40; 10 mL/100 mg of product) unless otherwise stated.

**General Procedure for the Reaction of 5 with Amines (10).** Compound **5** (0.01 mol) and the appropriate amine (0.05 mol) were dissolved in 3-chlorotoluene (30 mL) and refluxed for 12−96 h. When the reaction was finished (monitored by tcl), the mixture was evaporated under reduced pressure. The residue was dissolved in an aqueous NaOH solution (0.5 N, 10 mL/g of residue). This mixture was extracted with diethyl ether and the aqueous layer was separated and adjusted to pH 1 with 0.5 M hydrochloric acid. The precipitate which appeared was collected by filtration, washed with water and dried (yield: 35−95%).

**General Procedure for the Reaction of 5 with Cresols (6).** Before starting the reaction, the cresolates were obtained from the corresponding cresols (0.05 mol) after their neutralization by 0.06 mol NaOH (in aqueous solution, 10% w/v) in acetone. Evaporation under reduced pressure provided crystals of the cresolates.

Compound **5** (0.01 mol) and the appropriate cresolate (0.05 mol) were dissolved in acetonitrile. The mixture was refluxed and potassium carbonate (0.007 mol) was added. After completion of the reaction monitored by TLC (12−36 h), the solution was acidified and filtered and the filtrate was evaporated under reduced pressure. The crude product was dissolved in methanol and ice was added. The resulting precipitate was collected by filtration (yield: 60−70%).

**General Procedure for the Reaction of 5 with Thiophenols (8).** Compound **5** (0.01 mol) and the appropriate thiophenol (0.05 mol) were dissolved in acetonitrile (30 mL). The mixture was refluxed and potassium carbonate (0.007 mol) was added. When the reaction was finished (0.1−1 h), the solution was acidified and filtered and the filtrate was evaporated under reduced pressure. The resulting crude oil was dissolved in methanol and ice was added. The precipitate was recovered by filtration (yield: 50−75%).

**General Procedure for the Preparation of Sulfonylureas with Isocyanates (1, 2, 7, 9, and 16).** The appropriate sulfonamide (0.01 mol) was dissolved in acetone (30 mL). NaOH (0.01 mol) (10% aqueous sol. w/v) was added. The mixture was gently mixed during 10 min and then was evaporated under reduced pressure. The resulting solid was resuspended in acetone (30 mL) and gently refluxed. The appropriate isocyanate (0.02 mol) was added to the mixture. At the end of the reaction (0.1−1 h), the mixture was evaporated under reduced pressure and the crude product was washed with AcOEt. The solid was collected by filtration and dissolved in an aqueous NaOH solution (0.5 N; 20 mL). The resulting solution was adjusted to pH 1 with hydrochloric acid (12 N), and the solid which precipitated was isolated by filtration (yield 40−60%).

***N*-*tert*-Butyl-*N*′-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (1).** Mp: 126−127 °C. Anal. ($C_{18}H_{22}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (2).** Mp: 145−147 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N*-*tert*-Butyl-*N*′-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7a).** Mp: 162−165 °C. Anal. ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7b).** Mp: 148−150 °C. Anal. ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

***N*-*n*-Hexyl-*N*′-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7c).** Mp: 128−130 °C. Anal. ($C_{20}H_{25}N_3O_6S$) C, H, N, S.

***N*-Cyclohexyl-*N*′-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (7d).** Mp: 170−173 °C. Anal. ($C_{20}H_{23}N_3O_6S$) C, H, N, S.

***N*-*n*-Butyl-*N*′-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7e).** Mp: 207−210 °C. Anal. ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(3-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7f).** Mp: 157−158 °C. Anal. ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

***N*-*n*-Pentyl −*N*′-[2-(2-methylphenoxy)-5-nitrobenzenesulfonyl]urea (7g).** Mp: 150−153 °C. Anal. ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

***N*-*tert*-Butyl-*N*′-[2-(4-methylphenylthio)-5-nitrobenzenesulfonyl]urea (9a).** Mp: 181−183 °C. Anal. ($C_{18}H_{21}N_3O_5S_2$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(4-methylphenylthio)-5-nitrobenzenesulfonyl]urea (9b).** Mp: 149−151 °C. Anal. ($C_{19}H_{23}N_3O_5S_2$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(2-methylphenylthio)-5-nitrobenzenesulfonyl]urea (9c).** Mp: 156−158 °C. Anal. ($C_{19}H_{23}N_3O_5S_2$) C, H, N, S.

***N*-*tert*-Butyl-*N*′-[2-(4-chlorophenylthio)-5-nitrobenzenesulfonyl]urea (9d).** Mp: 139−142 °C. Anal. ($C_{17}H_{18}ClN_3O_5S_2$) C, H, N, S.

***N*-*n*-Pentyl −*N*′-[2-(4-chlorophenylthio)-5-nitrobenzenesulfonyl]urea (9e).** Mp: 131−135 °C. Anal. ($C_{18}H_{20}ClN_3O_5S_2$) C, H, N, S.

***N*-*n*-Hexyl-*N*′-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]-urea (16a).** Mp: 115−116 °C. Anal. ($C_{19}H_{30}N_4O_5S$) C, H, N, S.

***N*-*n*-Heptyl-*N*′-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]-urea (16b).** Mp: 117−118 °C. Anal. ($C_{20}H_{32}N_4O_5S$) C, H, N, S.

***N*-*n*-Octyl-*N*′-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]-urea (16c).** Mp: 93−94 °C. Anal. ($C_{21}H_{34}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(2,4,6-trimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16d).** Mp: 143−145 °C. Anal. ($C_{21}H_{28}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(2-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16e).** Mp: 127−128 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(3-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16f).** Mp: 128−130 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N*-*tert*-Butyl-*N*′-[2-(3-methyl-4-bromophenylamino)-5-nitrobenzenesulfonyl]urea (16g).** Mp: 141−143 °C. Anal. ($C_{18}H_{21}BrN_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(2, 5-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16h).** Mp: 126−128 °C. Anal. ($C_{20}H_{24}N_6O_4S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(2, 4-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16i).** Mp: 127−129 °C. Anal. ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(2, 6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16j).** Mp: 129−131 °C. Anal. ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

***N*-Benzyl-*N*′-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16k).** Mp: 148−150 °C. Anal. **($C_{21}H_{20}N_4O_5S$) C, H, N, S.**. ***N*-Cyclohexyl-*N*′-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16l).** Mp: 166−168 °C. Anal. ($C_{20}H_{24}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(3, 4-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16m).** Mp: 88−90 °C. Anal. ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(3, 5-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16n).** Mp: 122−125 °C. Anal. ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(3-methyl-4-bromophenylamino)-5-nitrobenzenesulfonyl]urea (16o).** Mp: 130−132 °C. Anal. ($C_{19}H_{23}BrN_4O_5S$) C, H, N, S.

***N*-*sec*-Butyl-*N*′-[2-(2,6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16p).** Mp: 97−99 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N*-*tert*-Butyl-*N*′-[2-(2,6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16q).** Mp: 95−97 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N*-*sec*-Butyl-*N*′-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16r).** Mp: 104−106 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(4-Chlorophenylamino)-5-nitrobenzenesulfonyl]urea (16s).** Mp: 124−126 °C. Anal. ($C_{18}H_{21}ClN_4O_5S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(2, 3-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16t).** Mp: 139−142 °C. Anal. ($C_{20}H_{26}N_4O_5S_2$) C, H, N, S.

**Procedure for the preparation of *N*-*n*-Pentyl-*N*′-cyano-*O*-phenylisourea (12).** A mixture of **11** (0.01 mol) and *n*-pentylamine (0.015 mol) in 2-propanol (30 mL) was stirred at room temperature for 10−15 min. The solution was evaporated, and the crude oil was crystallized in cold methanol. Mp: 134−136 °C. $^1$H NMR (DMSO) $\delta$: 0.86 (t, 2H, $J$ = 7 Hz, NH−$CH_2$−$CH_2$−$CH_2$−$CH_2$−$CH_3$); 1.1−1.35 (m, 6H, NH−CH2−$CH_2$−$CH_2$−$CH_2$−$CH_3$); 3.26 (q, 2H, $J$ = 7 Hz, NH−$CH$2-$CH_2$−$CH_2$−$CH_2$−$CH_3$); 7.06−7.41 (m, 5H, $H_{aro}$).

**General Procedure for the Preparation of Nitrobenzenesulfonylcyanoguanidines (13).** The appropriate sulfonamide (0.01 mol) was dissolved in acetone (30 mL). NaOH (0.01 mol) (10% aqueous sol. w/v) was added. The mixture was gently mixed during 10 min and was evaporated under reduced pressure. The solid was resuspended in dimethylformamide (20 mL) at room temperature and the appropriate *N*-alkyl-*N*′-cyano-*O*-phenylisourea was added. The mixture was stirred at RT for 20−24 h. At the end of the reaction, the mixture was evaporated under reduced pressure and the crude oil was suspended in a mixture of methanol and hydrochloric acid (5 N aqueous solution) from which crystals of the desired product appeared.

***N*-*n*-Pentyl-*N*′-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]-*N*″-cyanoguanidine (13a).** Mp: 162−166 °C. Anal. ($C_{20}H_{24}N_6O_4S$) C, H, N, S.

***N*-*n*-Pentyl-*N*′-[2-(3,5-dimethylphenylamino)-5-nitrobenzenesulfonyl]-*N*″-cyanoguanidine (13b).** Mp: 149−151 °C. Anal. ($C_{21}H_{26}N_6O_4S$) C, H, N, S.

**Procedure for the Synthesis of *N*-[2-(4-Methylphenylamino)-5-nitrobenzenesulfonyl]-piperidine-1-carboxamide (15).** Compound **10i** (0.01 mol) was dissolved in acetone. NaOH (0.01 mol) (10% sol. w/v) was added. The mixture was gently mixed during 10 min and was evaporated under reduced pressure. The solid was resuspended in acetone (10 mL). A large excess of ethyl chloroformate (0.03 mol) was added dropwise under vigorous stirring. The mixture was stirred for 15 min, and the solution was evaporated under reduced pressure. The ethyl carbamate obtained was purified by crystallization in methanol. Ethyl 2-(4-methylphenylamino)-5-nitrobenzenesulfonylcarbamate (**14**, 0.01 mol) was dissolved in a mixture of anhydrous toluene (40 mL) and piperidine (0.02 mol). The resulting solution was gently refluxed overnight. At the end of the reaction, the solution was evaporated under reduced pressure, and the residue was dissolved into an aqueous NaOH solution (0.5 N, 20 mL). This mixture was extracted with diethyl ether. The aqueous layer was recovered and adjusted to pH 1 with hydrochloric acid (0.5 N). The precipitate which appeared was recovered by filtration (yield: 65%). Mp: 139−141 °C. $^1$H NMR ($CDCl_3$) $\delta$: 1.60 (b, 6H, piperidine); 2.38 (s, 3H, $CH_3$-4′); 3.39 (s, 4H, piperidine); 7.03 (d, 1H, $J$ = 9 Hz, H-3); 7.17−7.29 (m, 4H, $H_{aro}$′); 8.07 (dd, 1H, $J$ = 9 Hz, $J$ = 2.5 Hz, H-4); 8.75 (d, 1H, $J$ = 2.5

Hz, H-6); 8.85 (s, 1H, Ph-N*H*-Ph); 11.14 (s, 1H, $SO_2$−N*H*-CO-). Anal. ($C_{19}H_{22}N_4O_5S$) C, H, N, S.

**Radioligand Binding Assay.** COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum and 1% of solution containing 10 000 units/mL penicillin G, 10 000 $\mu$g/mL streptomycin, and 25 $\mu$g/mL amphotericin (Cellgro; Mediatech, Herndon, VA). Cells were grown at 37 °C in a humidified atmosphere of 95% $O_2$ and 5% $CO_2$. cDNAs (the cDNA was generously provided by Prof. Perry Halushka, Medical University of South Carolina) for the TP$\alpha$ and TP$\beta$ were subcloned into pcDNA3, the resultant plasmid, pcDNA3:TP$\alpha$ and pcDNA3:TP$\beta$ were introduced into COS-7 cells by the DEAE-dextran/chloroquine method. Forty eight hours posttransfection, cells were harvested by centrifugation at 500*g* for 5 min and washed three times in ice-cold phosphate-buffered saline. Cells were resuspended in buffer containing 25 mM HEPES/125 mM NaCl/10 $\mu$M indomethacin, pH 7.4, and kept on ice for the binding study. Binding reactions were carried out on $5 \times 10^5$ cells in a total volume of 0.2 mL in the above buffer with 10 nM [$^3$H]SQ29,548 (Perkin-Elmer Life and analytical services, Boston, MA) added to all tubes in triplicate, containing various concentrations of studied compounds ($10^{-9}$ for screening assay or $10^{-6}$ to $10^{-11}$ M for competition binding curves) in 1 $\mu$L of ethanol. Additional tubes containing excess unlabeled SQ29,548 (10 $\mu$M) (Cayman Chemical Co, Ann Arbor, MI) were included to assess the extent of nonspecific binding. Binding was allowed to take place for 30 min at 37 °C; free radioligand was removed by rapid vacuum filtration through Whatman (Maidstone, UK) GF/B glass fiber filters prewashed with the cell suspension buffer. The tubes and the filters were rapidly washed with ice-cold 10 mM Tris buffer, pH 7.4 (three times with 3 mL). The radioactivity on the filters containing the ligand−receptor complexes was counted in 10 mL of Ecolite scintillation fluid (ICN, St. Laurent, QC, Canada) in a Beckman (model LS 3800) liquid scintillation counter. The binding experiments were performed on whole cells.

**Calcium Measurements.** HEK.TP$\alpha$ and HEK.TP$\beta$ cell lines, stably overexpressing HA-tagged forms of TP$\alpha$ and TP$\beta$ in human embryonic kidney (HEK) 293 cells have been previously described.[28] HEK 293 cells or their stable cell line equivalents were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Measurement of $[Ca^{2+}]_i$ mobilization either in HEK.TP$\alpha$ or HEK.TP$\beta$ cells was carried out using fluorescent microplate reader Fluoroskan Ascent FL equipped with two dispenser (thermo electron corporation, Finland) according to modified method of Lin et al.[29] Briefly, cells were trypsinized, washed twice with Krebs-HEPES buffer (118 mM, NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 4.2 mM $NaHCO_3$, 11.7 mM D-glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 7.4), and incubated for 1 h with fluorescent dye Fluo-4/AM (5 $\mu$g/mL; Molecular Probes, Invitrogen, Merelbeke Belgium). Cells were then rinsed three times with Krebs-HEPES buffer and 150 $\mu$L of a suspension of cells in that buffer was loaded into each well of a 96-well plate at a density of 150 000 cells/well. Cells were incubated 10 min with various concentrations of the test compound ($10^{-5}$ to $10^{-8}$ M final; 10 $\mu$L) prior to stimulation with U46619 (1 $\mu$M final, 50 $\mu$l). In all cases, compound (1 mM) was diluted in dimethyl sulfoxide (DMSO)/PBS (30/70) prior to further dilution in PBS. Fluorescence emission was read at 538 nM. At the end of each experiment, fluorescence intensities were calibrated for determination of intracellular calcium concentration ($[Ca^{2+}]_i$) values by permeabilizing cells with 1% Triton X-100 to release all the dye (Fmax) and subsequently chelating with 10 mM EGTA ($F_{min}$). Calcium concentrations were calculated using equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, assuming a *K*d of 385 nM for Fluo-4. The results ($IC_{50}$) presented are the concentration required to inhibit 50% of the normal rise of $[Ca^{2+}]_i$ upon stimulation with 1 $\mu$M U46619, determined in the absence of any compounds. The $IC_{50}$s were calculated by nonlinear regression analysis (GraphPad Prism software) from at least three concentration−response curves.

**Human in Vitro Platelet Aggregation.** The antiaggregant potency has been determined according to the turbidimetric Born's method.[30] The blood was drawn from 10 healthy donors of both genders, aged 20−30. The subjects were free from medication for at least 14 days. No significant differences in the results were observed between the donors in our experiments. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described.[26,31] Platelet concentration of PRP was adjusted to $3 \times 10^8$ cells/mL by dilution with PPP. Platelet aggregation of PRP was studied using a double channel aggregometer (Chronolog Corporation, Chicago, IL) connected to a linear recorder as previously described.[32] PRP (294 $\mu$L) was added in a silanised cuvette and stirred (1000 rpm). Each compound was diluted (1 mM) in dimethyl sulfoxide (DMSO)/PBS (30/70) and preincubated in PRP for three minutes at 37 °C before the aggregating agent was added. Platelet aggregation was initiated by addition of a fresh solution of U46619 (1 $\mu$M final). To evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve 6 min after addition of the inducer. The substance concentration preventing 50% of platelet aggregation ($IC_{50}$) induced by U46619 was calculated by nonlinear regression analysis (GraphPad Prism software) from at least three dose−response curves.

**Statistical Analysis.** Results are expressed as the mean ± standard deviation from at least three determinations ($n \geq 3$). Statistical differences between TP isoforms have been determined using unpaired t-test between $IC_{50}$s values. *p* values of less than 0.05 were considered to be significant.

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**Supporting Information Available:** NMR, elemental analysis, melting points, and IR peaks for all compounds presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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