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HALOGEN-SUBSTITUTED TRIMETOQUINOL ANALOGS AS THROMBOXANE A_2 RECEPTOR ANTAGONISTS IN PLATELETS AND AORTA

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Abstract—Trimetoquinol (TMQ) is a non-prostanoid compound that blocks prostaglandin H_2/thromboxane A_2 (TXA_2) receptor-mediated responses initiated by a prostaglandin (PG) H_2 analog, U46619, in human platelets and rat aorta. Ring fluorine-substituted TMQ analogs selectively antagonized PG-dependent human platelet activation induced by U46619, arachidonic acid, collagen, ADP or epinephrine; and were about 300-fold less potent as inhibitors of PG-independent responses mediated by thrombin or bacterial phospholipase C. For each inducer of the PG-dependent pathway, the rank order of inhibitory potency was identical (TMQ > 8-fluoro-TMQ > 5-fluoro-TMQ). Iodine substitution yielded a similar rank order of antagonism against U46619-induced platelet activation (TMQ > 8-iodo-TMQ > 5-iodo-TMQ), and all TMQ analogs inhibited platelet aggregation in whole blood as well as in platelet-rich plasma. Inhibition of specific [^{3}H]SQ 29,548 binding by TMQ analogs was highly correlated with inhibition of functional responses to U46619. Radioligand binding experiments using TMQ analogs with rat platelets showed no interspecies difference in comparison with human platelets. The rank order of inhibitory potencies for the fluorinated (but not iodinated) TMQ analogs changed in rat thoracic aorta with 8-fluoro-TMQ > TMQ ≥ 5-fluoro-TMQ as antagonists of U46619-induced vascular contraction. These findings demonstrate that the primary mechanism of antiplatelet action of TMQ analogs is related to a blockade of TXA_2 receptor sites, and ring-halogenated TMQ analogs distinguish between TXA_2-mediated functional responses in vascular smooth muscle and platelets.

Trimetoquinol (TMQ)‡, a cyclized analog of norepinephrine, exists as two stereoisomers, *S*(−)-TMQ and *R*(+)-TMQ. *S*(−)-TMQ possesses potent β-adrenoceptor activity and this isomer is used for the treatment of moderate asthma [1]. In contrast, *R*(+)- and racemic-TMQ block pharmacological responses to U46619, a prostaglandin H_2/thromboxane A_2 (TXA_2) receptor agonist, in human platelets and in rat thoracic aorta (RTA) [2]. TXA_2 receptor activation may mediate thrombus formation and stenosis in coronary artery disease through platelet aggregation and vasoconstriction [3].

An early report [4] suggested that *R*(+)-TMQ, a nonprostanoid molecule, does not block prostanoid TXA_2 receptors even though this compound inhibited platelet activation by receptor agonists U46619 and U44069 [5]. It has been proposed that TMQ may instead inhibit some transduction process of TXA_2 receptors [6, 7]. Despite the β_2-adrenoceptor effects of *S*(−)-TMQ, antagonism of U46619 by TMQ does not involve α- or β-adrenergic receptors or cAMP accumulation in platelets [8, 9]. The antiplatelet mechanism of TMQ remains controversial [10], and the major emphasis of this paper is to provide evidence linking the efficacy of TMQ analogs to their TXA_2 receptor binding affinities.

Fluorine substitution on the aromatic ring of phenethylamines including epinephrine, norepinephrine and isoproterenol is known to affect their α- and β-adrenergic selectivity [11–13]. We recently prepared two ring-fluorinated TMQ analogs, 5-fluoro-TMQ (5F-TMQ) and 8-fluoro-TMQ (8F-TMQ), and reported that they possessed greater

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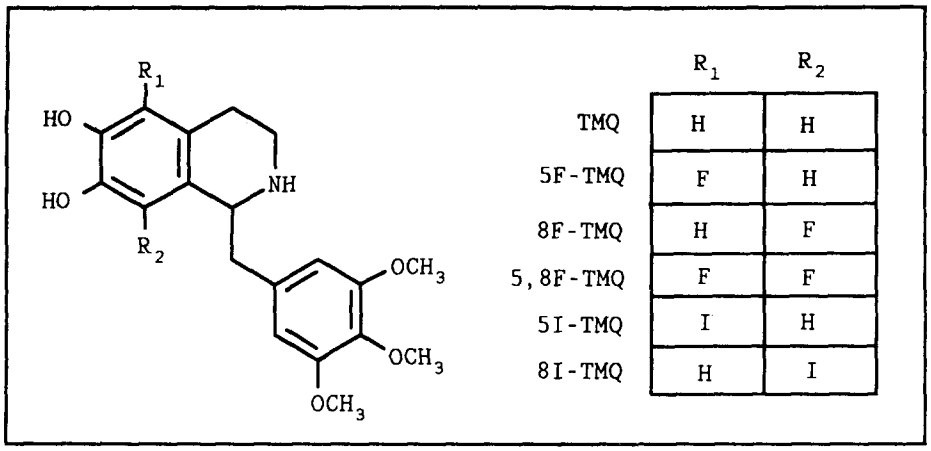
‡ Abbreviations: TMQ, trimetoquinol; 5,8F-TMQ, 5,8-difluoro-trimetoquinol; 5F-TMQ, 5-fluoro-trimetoquinol; 5I-TMQ, 5-iodo-trimetoquinol; 8F-TMQ, 8-fluoro-trimetoquinol; 8I-TMQ, 8-iodo-trimetoquinol; AA, arachidonic acid; EGTA, ethylene glycol-bis-(β-aminoethyl ether)*N*, *N*, *N*′, *N*′-tetraacetic acid; IC_{50}, inhibitor concentration causing 50% inhibition; *m*, correlation slope; P, statistical significance level using a two-tailed test; pA_2, negative log molar antagonist dissociation constant determined from Schild plots; PG, prostaglandin; PGE_1, prostaglandin E_1; pIC_{50}, negative log molar $IC_{50;p}K_a$, negative log molar equilibrium constant for an acid; pK_b, negative log molar antagonist dissociation constant; pK_i, negative log molar K_i, competitive $K_i = IC_{50}/[1 + (L/K_d)]$ where *L* is the radioligand concentration and K_d is the equilibrium dissociation constant of *L*; PLA_2, phospholipase A_2; PLC, bacterial phospholipase C; PRP, platelet-rich plasma; *r*, correlation coefficient; RTA, rat thoracic aorta; SQ 29,548, [1*S*-[1α, 2β(5*Z*), 3β, 4α]]-7-[3-[[2-[(phenylamino)-carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid; TXA_2, prostaglandin H_2/thromboxane A_2; and U46619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$.



	R_1	R_2
TMQ	H	H
5F-TMQ	F	H
8F-TMQ	H	F
5,8F-TMQ	F	F
5I-TMQ	I	H
8I-TMQ	H	I

Fig. 1. Structures and abbreviations of trimetoquinol (TMQ) and ring halogen-substituted analogs.

β_2/β_1-selectivity than TMQ [14]. To date, however, no studies of TXA_2 antagonism by these TMQ analogs have been reported. We have therefore prepared a series of fluorinated TMQ analogs including 5F-TMQ, 8F-TMQ and 5,8-difluoro-TMQ (5,8F-TMQ, Fig. 1) to study their functional selectivity on the TXA_2 receptor systems of human platelets and rat aorta. A comparison of TXA_2 receptor affinities of TMQ analogs on human versus rat blood platelets is also undertaken in this report to determine whether interspecies differences exist for this chemical class of compounds.

To further assess the role of halogen substitution, we also synthesized two iodinated TMQ analogs, 5-iodo-TMQ (5I-TMQ) and 8-iodo-TMQ (8I-TMQ), for comparison with the fluorinated compounds (Fig. 1). Iodine atom substitution provides molecules in which the electronegativity is much lower while the atom size and hydrophobic constant (π) are much greater than for a fluorine atom [15, 16].

MATERIALS AND METHODS

Materials. [^{3}H]SQ 29,548 (34.3 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [^{14}C]Serotonin (58 mCi/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL). Unlabeled U46619 and SQ 29,548 were gifts from the Upjohn Co. (Kalamazoo, MI) and The Squibb Institute for Medical Research (Princeton, NJ), respectively. Stereoisomers of TMQ were provided by Dr. Yoshio Iwasawa (Tanabe Seiyaku Co. Ltd., Osaka, Japan). TMQ and its halogenated analogs were synthesized in our laboratory as previously reported [14, 17]. Other chemicals including bacterial phospholipase C (PLC) from *Clostridium perfringens* were purchased from the Sigma Chemical Co. (St. Louis, MO).

Platelet aggregation and secretion. Human platelet aggregation was monitored turbidometrically in platelet-rich plasma (PRP) using a Payton or Chrono-log aggregometer interfaced to an Apple microcomputer [18]. Secretion of [^{14}C]serotonin was measured in the same experiments as previously described [19]. Aggregation induced by thrombin or PLC was performed using washed platelet suspensions obtanied by centrifugation [20]. U46619-induced aggregation was also investigated as impedance changes in whole blood [21], using a Chrono-log whole-blood aggregometer connected to a microcomputer as above. Special care was taken in whole-blood experiments to add minimal amounts of reagents to avoid temperature-dependent impedance changes. In all experiments, platelets were preincubated with inhibitors 1 min prior to the initiation of aggregation. Aggregation of samples was monitored in the presence of epinephrine (6 min), ADP (6 min), arachidonic acid (4 min), U46619 (4 min), thrombin (4 min) or PLC (7 min). In all PRP samples stimulated with U46619, thrombin or PLC, aspirin (1 mM) was included to block production of endogenous prostaglandins. The minimum inducer concentration that elicited maximal aggregation was employed as the control for each donor. Inhibition was calculated using the final change in sample transmittance except for U46619 data where a change in the rate of aggregation was employed.

Rat thoracic aorta. Male Sprague–Dawley rats weighing 200–250 g were killed by CO_2 released from dry ice. Spirally-cut aortic stips were placed in 10 mL tissue baths containing physiological salt solution, and isometric contractions were measured using a Grass polygraph (model 7C) [2]. Tissues were exposed to phenoxybenzamine (10^{-5} M, 30 min) to block α-adrenergic receptors. Incubations also contained sotalol (3×10^{-5} M) and indomethacin (3×10^{-6} M) to block β-adrenergic receptors and endogenous prostaglandin (PG) synthesis, respectively. Inhibitory values (pK_b) for TMQ analogs were determined from cumulative concentration–response curves of U46619-mediated contractions in the presence or absence of fixed concentrations of TMQ analogs (3, 10, 30 or 100 μM) as described previously [2].

Radioligand binding. For equilibrium binding

experiments, human platelets were obtained from PRP using a method modified from that of Hedberg *et al.* [22]. Platelets were washed three times by suspension and centrifugation (1000 *g*, 3 min) in 50 mM Tris–saline buffer (7.02 g Tris–HCl, 0.67 g Tris base and 9 g NaCl per L, pH 7.2) containing 1 μM prostaglandin E_1 (PGE_1). The first suspension also contained 5 mM ethyleneglycol-bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), and the final stock suspension consisted of 1×10^9 platelets/mL in Tris–saline. Microscopic examination of stock platelet suspensions revealed no signs of activation (shape change) or damage. Rat blood was obtained from the abdominal aorta of 300–350 g male Sprague–Dawley rats under halothane anesthesia, and rat platelets were processed in the same manner as were human platelets.

Platelets (0.1 mL) were added to 0.4 mL of Tris–saline containing 0.5 nM [^{3}H]SQ 29,548 and selected compounds. To increase bound radioactivity, rat platelet incubations contained 5 nm [^{3}H]SQ 29,548. Since, like epinephrine, TMQ analogs slowly oxidize, inhibitor solutions were made fresh or stored frozen in nitrogen-saturated buffer. After a 30-min incubation at 22°, samples were filtered rapidly by vacuum through Whatman GF/B glass fiber filters on a model M12-R Brandel cell harvester (Gaithersburg, MD), and washed continuously for 10 sec with ice-cold Tris–saline. Filters were placed in vials containing 10 mL of an emulsion-type mixture, and the amount of [^{3}H] bound was measured by liquid scintillation spectrometry. Displacement caused by a 50 μM concentration of unlabeled SQ 29,548 was used as the measure of specific binding. Specific binding was partially labile since it decreased from 92% (78% in rat) in freshly prepared suspensions to 80% (73% in rat) after a period of about 1 hr. To avoid oxidative degradation of the radioligand, working solutions were made fresh in N_2-saturated buffer just prior to each run of 12 samples.

Scatchard plots of the specific binding of [^{3}H]SQ 29,548 analyzed by the Ligand computer program [23] indicated the presence of a single binding site in each species with nanomolar K_d (B_{max}, fmol/10^6 platelets) values of 3.1 (0.49) and 3.1 (0.19) for human and rat platelets, respectively. A known prostanoid TXA_2 antagonist, *trans*-13-azaprostanoic acid [24], was included as a positive control for specific displacement of radioligand and showed good agreement between pIC_{50} values (mean ± SEM) for inhibition of [^{3}H]SQ 29,548 binding and U46619-induced aggregation and secretion (5.04 ± 0.21, N = 8; 5.09 ± 0.19, N = 5; 5.07 ± 0.21, N = 4, respectively).

Statistics. Antagonism was quantified as pIC_{50} values (negative log molar 50% inhibitory concentrations) or as negative log molar equilibrium dissociation constants expressed as pK_i, pK_b or Schild plot pA_2 values [25]. Significance levels (P) for correlation coefficients (r) were determined according to Rohlf and Sokal [26].

RESULTS

Functional studies. Racemic-TMQ, 5F-TMQ and 8F-TMQ inhibited human platelet activation by a variety of PG-dependent inducers including U46619, arachidonic acid (AA), collagen, and the second phases of ADP and epinephrine (Table 1). Inhibition data for serotonin secretion was very highly correlated with antagonism of aggregation ($r = 0.97$, $P < 10^{-7}$). For each inducer, the rank order of potency was TMQ > 8F-TMQ > 5F-TMQ. These agonists were chosen to investigate the effects of

Table 1. Inhibition of human platelet aggregation (Agg) and serotonin secretion (Sec) by TMQ and fluorinated analogs (pIC_{50}) in platelet-rich plasma

Inducer		pIC_{50}		
		TMQ	5F-TMQ	8F-TMQ
Prostaglandin-dependent inducers				
ADP	Agg	6.57 ± 0.22	4.82 ± 0.23	5.19 ± 0.19
	Sec	6.70 ± 0.30	4.77 ± 0.18	5.18 ± 0.11
Epinephrine	Agg	5.78 ± 0.13	4.83 ± 0.16	4.96 ± 0.22
	Sec	5.99 ± 0.34	5.12 ± 0.09	5.46 ± 0.24
Collagen	Agg	6.03 ± 0.14	4.29 ± 0.09	5.01 ± 0.10
	Sec	6.23 ± 0.24	4.88 ± 0.04	5.12 ± 0.17
Arachidonic acid	Agg	5.98 ± 0.21	4.11 ± 0.21	4.58 ± 0.13
	Sec	5.90 ± 0.16	4.26 ± 0.24	4.66 ± 0.13
U46619	Agg	6.36 ± 0.13	5.13 ± 0.11	5.41 ± 0.18
	Sec	6.16 ± 0.23	5.16 ± 0.04	5.36 ± 0.10
Prostaglandin-independent inducers				
PLC	Agg	4.27 ± 0.26	3.50 ± 0.29	3.90 ± 0.29
	Sec	4.02 ± 0.12	3.49 ± 0.17	3.66 ± 0.12
Thrombin	Agg	3.02 ± 0.09	3.08 ± 0.09	3.08 ± 0.06
	Sec	<3	<3	<3

Data are means ± SEM from 4–11 donors. Data for ADP and epinephrine are for inhibition of second phase aggregation only. PLC = bacterial phospholipase C.

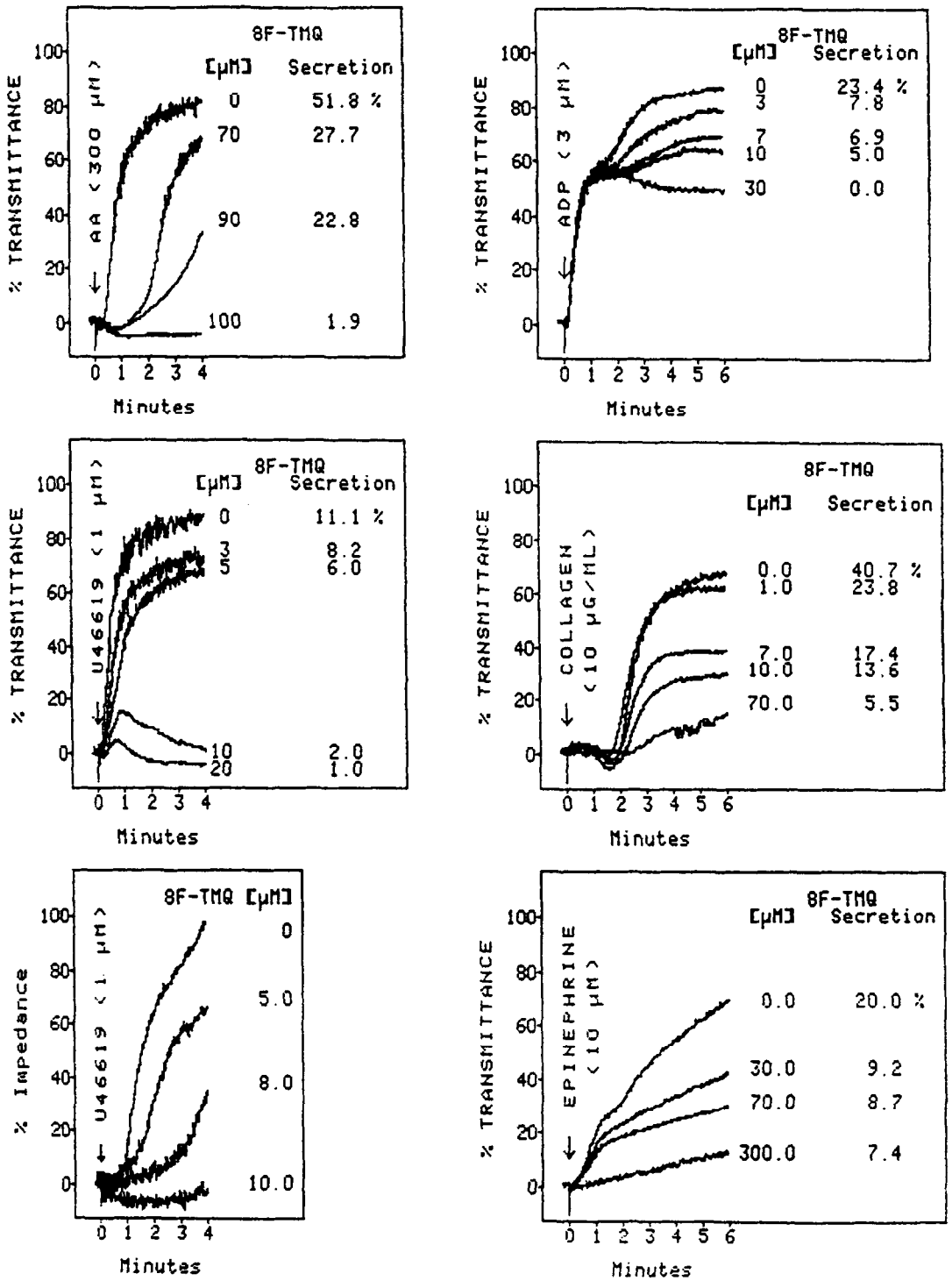


Fig. 2. Superimposed aggregation responses of human platelets to prostaglandin-dependent agonists showing concentration-dependent inhibition by 8F-TMQ. Secretion values represent the per cent of total [^{14}C]serotonin content that was released from each sample. Aggregation was measured as per cent transmittance in platelet-rich plasma or impedance in whole blood. AA = arachidonic acid. Data are representative of 3 experiments.

Table 2. Schild plot analysis of TMQ isomers and halogenated analogs: antagonism of U46619-induced human platelet aggretation (Agg) and secretion (Sec)*

Compound		pA_2	Slope	r
$R(+)$-TMQ	Agg	6.71 ± 0.14	0.84 ± 0.11	0.98 ± 0.01
TMQ	Agg	6.29 ± 0.05	0.84 ± 0.07	0.98 ± 0.01
	Sec	5.71 ± 0.04	1.05 ± 0.06	0.98 ± 0.02
$S(-)$-TMQ	Agg	4.62 ± 0.25	0.95 ± 0.04	0.98 ± 0.01
5F-TMQ	Agg	4.64 ± 0.12	0.94 ± 0.14	0.98 ± 0.01
	Sec	4.46 ± 0.15	1.23 ± 0.23	0.98 ± 0.01
8F-TMQ	Agg	5.05 ± 0.06	1.06 ± 0.06	0.99 ± 0.01
	Sec	4.79 ± 0.14	1.24 ± 0.21	0.98 ± 0.03
5,8F-TMQ	Agg	4.37 ± 0.08	0.78 ± 0.10	0.99 ± 0.00
	Sec	4.13 ± 0.24	0.76 ± 0.13	0.94 ± 0.02
5I-TMQ	Agg	4.26 ± 0.26	1.15 ± 0.16	0.96 ± 0.02
	Sec	4.46 ± 0.31	0.96 ± 0.10	0.97 ± 0.02
8I-TMQ	Agg	5.31 ± 0.35	1.31 ± 0.11	0.98 ± 0.01
	Sec	5.25 ± 0.10	1.21 ± 0.10	0.99 ± 0.01

* Data are means ± SEM of 4–11 experiments. Secretion data were not collected in experiments using the TMQ isomers.

Table 3. Inhibition of U46619-induced human platelet aggregation by TMQ analogs as measured by impedance changes in whole blood

Compound	pIC_{50}	N
$R(+)$-TMQ	6.15 ± 0.22	3
TMQ	5.77 ± 0.05	6
$S(-)$-TMQ	4.48 ± 0.08	3
5F-TMQ	5.07 ± 0.05	3
8F-TMQ	5.31 ± 0.19	5
5I-TMQ	5.11 ± 0.18	5
8I-TMQ	5.13 ± 0.07	6

Values are means ± SEM.

Table 4. Antagonism of U46619-induced contraction or rat thoracic aorta by TMQ and halogenated analogs

Compound	pK_b*	N
TMQ	5.76 ± 0.12	18
5F-TMQ	5.66 ± 0.12	20
8F-TMQ	6.37 ± 0.09	14
5,8F-TMQ	3.98 ± 0.46	4
5I-TMQ	4.31 ± 0.19	4
8I-TMQ	4.84 ± 0.21	4

* $pK_b = -\log [A]/(CR - 1)$ where $[A]$ is the molar antagonist concentration and CR is the concentration ratio (EC_{50} with inhibitor/IC_{50} without). Data are means ± SEM.

TMQ analogs on different sites of the PG pathway [27]. ADP (1–3 μM), epinephrine (1–10 μM) and collagen (10–100 μg/mL) initiate TXA_2 synthesis by activation of phospholipase A_2 (PLA_2). Exogenously added AA (100–300 μM) bypasses the participation of PLA_2, and platelet activation by U46619 (0.5–1.5 μM) acts directly through TXA_2 receptors. For these TMQ analogs, the inhibitory potency and rank order was similar regardless of the inducer employed, suggesting that the site of action is common to all of the agonists. Figure 2 illustrates effects of 8F-TMQ on platelet aggregation and secretion induced by these agonists. Non-specific inhibition did not occur since the primary, PG-independent phase of ADP-induced aggregation was not affected. Although TMQ analogs could inhibit PG-independent platelet aggregation induced by epinephrine (primary phase, Fig. 2), thrombin (0.02 to 0.06 U/mL) and PLC (0.02 to 0.07 U/mL), this was seen only at much higher concentrations (Table 1, Fig. 2). On average, TMQ was >300-fold more potent against inducers of prostaglandin-mediated aggregation.

Further support for TXA_2 receptors as the site of action of TMQ analogs in platelets was obtained using Schild plot analysis. Fixed concentrations of a series of TMQ analogs caused shifts in U46619 concentration–response curves yielding pA_2 values (Table 2). Racemic-TMQ and its fluorinated analogs antagonized U46619-induced platelet aggregation and secretion with a rank order of inhibitory potency of TMQ > 8F-TMQ > 5F-TMQ > 5,8F-TMQ. Likewise, the corresponding iodo analogs exhibited the same relative antiplatelet potency (TMQ > 8I-TMQ > 5I-TMQ). Linear Schild plots ($r = 1.0$) with slope values near unity (1.0) indicate that the compounds acted as competitive inhibitors of U46619 responses; t-tests found no slope value to differ significantly from unity.

Schild plot analysis also revealed that $R(+)$-TMQ and $S(-)$-TMQ were competitive inhibitors of platelet aggregation by U46619, and the rank order of potency was $R(+)$-TMQ > racemic TMQ ≫ $S(-)$-TMQ (Table 2). $R(+)$-TMQ was 123- and 2.6-fold more potent as an antagonist of U46619 than $S(-)$-TMQ and racemic-TMQ, respectively.

The antiaggregatory effects of TMQ analogs were also examined by impedance in whole blood (Table 3). Antagonism of U46619-induced aggregation in whole blood was highly correlated ($r = 0.87$, $P < 0.01$) to data obtained in platelet-rich plasma (Table 2), suggesting that these TMQ analogs are active in a more complex multicellular system and may be efficacious *in vivo*.

TMQ and its halogen-substituted analogs inhibited U46619-induced responses in RTA and human platelets but with differing relative potencies in each system. The rank order of antagonism in RTA (Table 4) was 8F-TMQ > TMQ ≥ 5F-TMQ > 5,8F-TMQ as opposed to TMQ > 8F-TMQ > 5F-TMQ > 5,8F-TMQ in platelets. This change in rank order of potency was not evident with the iodo-substituted analogs. Relative to TMQ, comparisons of inhibitory potencies in RTA (pK_b values) and platelets (pA_2 values) indicated increased selectivities of 8F-, 5F- and 5I-TMQ for antagonism of RTA (Table 5). 8F-, 5F- and 5I-TMQ were 71-, 35- and 4-fold more selective than TMQ for blockade of U46619-mediated aortic contractions versus platelet aggregation. Correlation of the potencies of TMQ analogs against

Table 5. Selective inhibitory activities of halogenated TMQ analogs against U46619-induced responses in human platelets and rat thoracic aorta as compared with TMQ

Compound	Potency ratio*		Tissue selectivity†	Relative selectivity‡
	Platelets	Aorta		
TMQ	1.000	1.000	0.30	1.00
5F-TMQ	0.0224	0.794	10.47	35.48
8F-TMQ	0.0575	4.074	20.89	70.79
5,8F-TMQ	0.0120	0.017	0.41	1.38
5I-TMQ	0.0093	0.036	1.12	3.80
8I-TMQ	0.1047	0.120	0.34	1.15

* Potency compared with TMQ (TMQ IC_{50}/analog IC_{50}).

† Aorta IC_{50}/aggregation IC_{50}. The IC_{50} values were derived from pA_2 values (Table 2) or pK_b values (Table 4). Values >1 were more selective for aorta.

‡ Contraction potency ratio/aggregation potency ratio. Analogs with values >1 were more tissue selective than TMQ.

Table 6. Inhibition of specific [^{3}H]SQ 29,548 radioligand binding by TMQ and halogen-substituted analogs in human and rat platelets

Compound	pK_i*	
	Human	Rat
R(+)-TMQ	6.62 ± 0.17 (8)	6.10 ± 0.10 (5)
TMQ	5.99 ± 0.06 (21)	5.57 ± 0.09 (5)
S(+)-TMQ	3.41 ± 0.10 (10)	3.70 ± 0.14 (6)
5F-TMQ	5.16 ± 0.14 (13)	4.51 ± 0.15 (4)
8F-TMQ	5.50 ± 0.15 (13)	5.07 ± 0.10 (5)
5,8F-TMQ	4.50 ± 0.08 (10)	3.85 ± 0.03 (9)
5I-TMQ	4.49 ± 0.08 (7)	4.56 ± 0.31 (3)
8I-TMQ	5.05 ± 0.23 (11)	4.62 ± 0.09 (4)

* $pK_i = -\log$ molar K_i where K_i = molar $IC_{50}/(1 + [L]/K_d)$. $[L]$ is the radioligand concentration, and K_d is the equilibrium dissociation constant of the radioligand. Values are means ± SEM; the number of experiments is given in parentheses.

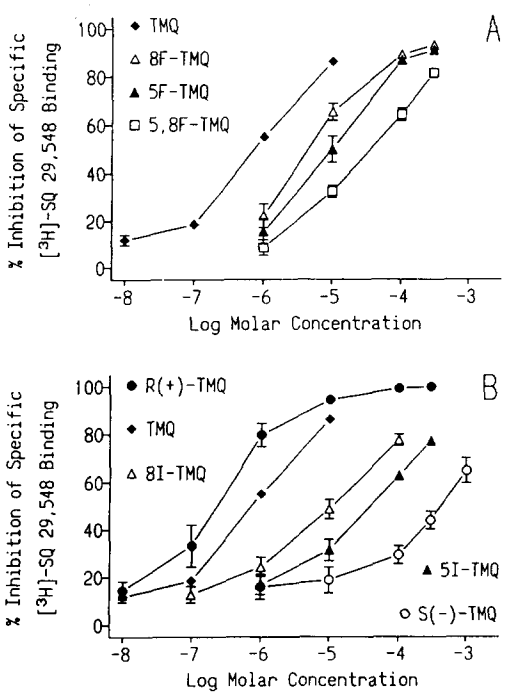


Fig. 3. Inhibitory effects of TMQ and fluorine-substituted analogs (panel A), iodine-substituted analogs (panel B) and optical isomers (panel B) on specific binding of [^{3}H]-SQ 29,548 to intact human platelets. Data are means ± SEM from 6 to 21 determinations per point.

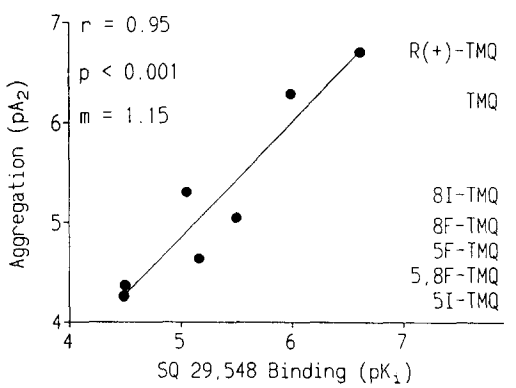


Fig. 4. Correlation of the inhibitory potency of TMQ analogs against platelet aggregation (pA_2) and [^{3}H]SQ 29,548 binding (pK_i). The correlation was significant ($P < 0.001$) with a correlation coefficient (r) of 0.95 and a slope (m) of 1.15.

U46619-induced activity in platelets and aorta was not significant ($r = 0.55$).

Radioligand binding. TMQ, its optical isomers and halogen-substituted analogs inhibited binding of [^{3}H]SQ 29,548 to intact human platelets in a concentration-dependent manner with the same potencies as they blocked platelet function (Fig. 3, Table 6). An exception was *S*(−)-TMQ, which was more effective as an inhibitor of platelet function than radioligand binding, suggesting an additional site of action for this compound. A highly significant positive correlation was found between the inhibition of [^{3}H]SQ 29,548 binding by TMQ analogs and inhibition of platelet aggregation (Fig. 4; $r = 0.95$, $P < 0.001$).

To investigate whether differences in functional antagonism by TMQ in human platelets and rat aorta are due to species differences in TXA_2 receptors, radioligand binding experiments were performed using rat platelets and compared with the results obtained with human platelets (Table 6). With regard to the TXA_2 binding site recognized by [^{3}H]SQ 29,548 and TMQ analogs, no species difference was found. TMQ binding affinities (pK_i)

were highly correlated ($r = 0.95$, $P < 0.001$) in rat and human platelets.

DISCUSSION

Previously, our laboratory reported that TMQ is a selective inhibitor of the TXA_2 agonist U46619 [5, 28]. In the present study, TMQ and halogenated analogs inhibited platelet activation mediated by a variety of PG-dependent agonists with similar inhibitory potencies against each inducer, including U46619. Therefore, the implied common site for antagonism by TMQ analogs is the TXA_2 receptor rather than inhibition of AA release or AA metabolism which differs among the agonists employed. This interpretation is further supported by experiments showing no inhibition of either [^{3}H]AA release or production of the prostaglandin byproduct, malondialdehyde (data not shown).

We have shown previously that U46619 competes with [^{3}H]TMQ for specific binding sites and, conversely, TMQ and fluorinated analogs compete with [^{3}H]U46619 [28–30]. In addition, TMQ blocks other events associated with U46619-induced platelet activation including phosphoinositide turnover, phosphorylation of 20 kDa (myosin light chain) and 40 kDa proteins, dense granule secretion and aggregation [28]. All of these events normally ensue from TXA_2 receptor activation in platelets and their inhibition is consistent with blockade of TXA_2 receptors.

This study provides strong evidence that TMQ analogs are competitive TXA_2 receptor antagonists since highly-specific [^{3}H]SQ 29,548 binding [22] is blocked by TMQ analogs in a concentration-dependent manner. Moreover, the potency of the analogs for inhibition of radioligand binding matched their antiplatelet potency with a highly significant correlation ($r = 0.95$, $P < 0.001$).

An exception was $S(-)$-TMQ, which has more potent antiplatelet effects than its blockage of radioligand binding would predict. However, we have shown previously that $S(-)$-TMQ has a cytoplasmic, PG-independent site of platelet antagonism since it is more effective than $R(+)$-TMQ as an inhibitor of platelet activation induced by PLC, calcium ionophore (A23187) or phorbol esters [28]. This may be related to the β-adrenergic effects of $S(-)$-TMQ. Nevertheless, $R(+)$-TMQ was 123-fold more potent than the $S(-)$-isomer for inhibition of aggregation elicited by U46619, and this inhibitory effect of TMQ is not mediated by adrenergic receptors [8, 9]. Rather, the radioligand binding data presented here indicates that TMQ analogs are TXA_2 receptor antagonists. Although the anti-TXA_2 activity of the halogen-substituted analogs should also reside in their R-enantiomers, only the racemates were available for this study.

TMQ and its analogs represent unusual TXA_2 antagonists in that they are not structurally related to the prostaglandin nucleus. Rather, these compounds resemble side-chain cyclized norepinephrine derivatives that lack a corresponding β-hydroxyl group and contain a large substituent (3,4,5-trimethoxybenzyl group). Accordingly, they have β_2-adrenergic effects in appropriate, non-platelet model systems [14]. The ability of TMQ to interact with both β-adrenergic and TXA_2 receptor systems suggests some homology between the two, although it is important to note that $S(-)$-TMQ stimulates β-adrenergic systems in contrast to $R(+)$-TMQ which antagonizes TXA_2 receptors. As reported here, the stereoselective affinity of TMQ enantiomers for human TXA_2 receptors was in excess of 1500-fold ($R \gg S$).

The fluorine-substituted analogs inhibited responses to U46619 with potencies that did not correlate between human platelets and RTA ($r = 0.55$), and different rank orders of antagonism were observed in each system. Sotalol was included in experiments with RTA to prevent possible β-adrenergic responses. Fluorine atom substitution produces a change in the ionization properties of the lower phenolic hydroxyl group such that 8F- and 5F-TMQ possess lower pK_a values (7.86 and 8.11, respectively) than the parent drug, TMQ ($pK_a = 8.77$) [14]. It appears from the rank order potency data that the interacting TXA_2 receptor sites in RTA (but not platelets) are affected positively by the electronic effect of fluorine atom substitution at the 5- and 8-positions. Overall, the selectivity for blockade of TXA_2 responses in RTA versus platelets shows a favorable correlation with the pK_a-lowering effects (8F-TMQ > 5F-TMQ > TMQ, Table 5). The diminished potency and selectivity of the iodo-TMQ analogs in RTA are consistent with the decreased electronic effects, but increased size and hydrophobicity may also influence the receptor interactions of these analogs.

The differential effects of these halogenated TMQ analogs in human platelets and rat aorta do not appear to be the result of a species difference in TXA_2 receptor binding sites since K_d values for [^{3}H]SQ 29,548 were identical and affinities of TMQ analogs in human and rat were very highly correlated ($r = 0.95$, $P < 0.001$). Hanasaki and Arita [31] have also shown a similarity in human and rat platelets using the radioligand [^{3}H]U46619 with a variety of antagonists. Likewise, functional inhibition by diverse antagonists has been found to be very similar in human and rat platelets [32].

TXA_2 receptors differ pharmacologically in platelets and RTA and have been subclassified as $TXA_{2\alpha}$ (alpha for platelet aggregation) and $TXA_{2\tau}$ (tau for vascular tone) [33–36]. In the absence of species differences, the variable effects of TMQ analogs in platelets and RTA suggest that these compounds may be capable of distinguishing between $TXA_{2\alpha}$ and $TXA_{2\tau}$ receptors. However, the existence of these receptor subclasses has been disputed and remains controversial [32, 37, 38]. Moreover, species differences cannot be ruled out entirely since human and rat platelets are known to respond differently to U46619 [31]. Apparent discrepancies between platelets and RTA may also be due to experimental conditions that may result in differential oxidation, penetration, distribution or metabolism of TMQ analogs.

As TXA_2 antagonists, TMQ analogs have potential as antithrombotic drugs, and experiments in whole blood indicated that TMQ analogs may retain

efficacy *in vivo*. Recently, we have also shown that related TMQ analogs can protect mice from U46619-induced death due to cardiopulmonary thrombosis [39]. Although the TMQ analogs presented here, with the exception of 8F-TMQ in aorta, are less potent that TMQ itself, we have found that replacement of one methoxy group with iodine increases the potency 4-fold in comparison with TMQ [39]. Since this racemic analog has a TXA_2 receptor affinity in the nanomolar range, we are currently preparing stereoisomers for use as nonprostanoid photoaffinity labels.

In summary, TMQ isomers and halogenated analogs blocked U46619-induced human platelet aggregation with the same rank order as they inhibited the specific binding of [^{3}H]SQ 29,548 to platelets. Functional and binding antagonism were highly correlated with the data, indicating that TMQ and its analogs antagonize U46619-induced platelet activation by blockade of TXA_2 receptors. The rank order for functional inhibition by these analogs differed in human platelets and rat aorta. However, the binding affinities to TXA_2 receptors of human and rat platelets were highly correlated, suggesting that differences in functional antagonism are not species dependent.

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