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STEREO-DEPENDENT INHIBITION OF HUMAN PLATELET FUNCTION BY THE OPTICAL ISOMERS OF TRIMETOQUINOL

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Abstract—The stereoisomers of trimetoquinol [1-(3′,4′,5′-trimethoxybenzyl)-6-7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; TMQ] were shown to have potent and selective inhibitory effects on human platelet function *in vitro*. The *R*(+)-isomer was 12.1-, 12.3-, 39.2-, 82.9- and 36.0-fold more effective than the *S*(−)-isomer as an inhibitor of aggregation induced by arachidonic acid (AA), collagen, the epoxymethano-PGH_2 analogs U44069 and U46619, and thromboxane A_2 (TxA_2) respectively. The concentrations of the *R*(+)-isomer that produced 50 percent inhibition (IC_{50}) of platelet aggregation were 4.2, 4.3, 1.4, 0.14 and 0.64 μM using AA, collagen, U44069, U46619, and TxA_2 as respective inducers. The graphical approximation of an inhibitory constant ($K_i = 0.13$ μM) for the effect of TMQ on U46619-induced aggregation suggested that a competitive-like inhibition was operative. In other experiments, platelet aggregation and serotonin release induced by U46619 were inhibited differentially by the TMQ stereoisomers with nearly identical concentration–response curves. In addition, racemic-TMQ blocked the secondary phase of platelet aggregation and serotonin release induced by ADP. These data, together with the ability of the TMQ stereoisomers to selectively inhibit TxA_2-induced aggregation, suggest that TMQ is an inhibitor of endoperoxide or TxA_2 action, e.g. a thromboxane A_2 receptor antagonist.

Trimetoquinol [1-(3′,4′,5′-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; TMQ] causes beta-adrenoceptor stimulation that exhibits stereo-dependence on the *S*(−)-isomer. In addition to beta-receptor-mediated actions, TMQ has been shown to have potent antiaggregatory properties in human platelet preparations [1–4]. The antiaggregatory action is mediated primarily by the *R*(+)-isomer. This stereo-dependence, as initially demonstrated by Dalton and others [1, 2], suggested that the inhibition of aggregation by TMQ was mediated by a mechanism different from that associated with bronchodilation, myocardial stimulation and mobilization of fatty acids. Preliminary reports from our laboratory [3] and that of MacIntyre and Willis [4] have suggested that TMQ is a potent antagonist of prostaglandin endoperoxide and thromboxane A_2-induced aggregation.

To date, the mechanism of the inhibition of platelet function by the TMQ isomers has not been clearly identified. In this report, we describe the inhibitory effects of the stereoisomers of TMQ on platelet aggregation induced by collagen, arachidonic acid, thromboxane A_2 (TxA_2) and the stable epoxymethano prostaglandin endoperoxide analogs, U44069 and U46619. In addition, we report that TMQ is an inhibitor of the release reaction, and of the secondary phase of aggregation induced by ADP. The nature of the inhibition of stable endoperoxide-induced aggregation by TMQ was approximated by graphical methods, and an apparent inhibitory constant (K_i) for this agent is reported.

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METHODS

Collection of blood. Human blood was taken by venipuncture from volunteers who reported being free of aspirin-containing medication for at least 14 days. Whole blood was combined and mixed with 3.8% trisodium citrate (9:1, v/v). Platelet-rich plasma (PRP) was then prepared by centrifugation at 200 *g* for 10 min at room temperature and used within 2 hr of isolation. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 4000 *g* for 10 min. The platelet concentration of the PRP was consistently between 280,000 and 320,000/mm^3 as determined using a Neubauer counting chamber (Spencer, Inc., Buffalo, NY). Platelet aggregation was monitored at 37° by nephelometry in a Chronolog aggregometer (model 330; Havertown, PA) with constant stirring at 1100 rpm. PRP (0.5 ml) was incubated for 3 min at 37° prior to the initiation of aggregation. This time period also served as the incubation interval for modulators of the system. In all experiments, the minimum concentration of inducer that produced irreversible aggregation with each PRP preparation was used. Light transmission through PPP was used to determine a maximum response to antiaggregatory drugs. [^{14}C]Serotonin

was incubated with PRP for 25 min prior to aggregation studies (0.05 μCi/ml PRP). Aliquots of PRP were removed and platelets were caused to aggregate by various agents in the presence or absence of TMQ. Control samples received aliquots of drug vehicle in place of an inducing agent. Samples were immediately centrifuged at 10,000 *g* for 30 sec. Aliquots of the supernatant fraction were transferred to scintillation vials and, following the addition of an emulsion-type scintillation solution (Thrift-Solv, Kew Scientific, Columbus, OH), were examined for radioactivity. ^{14}C was measured on a Beckman liquid scintillation counter (model LS 345, Palo Alto, CA) using external standardization to monitor the extent of quench. Counting efficiencies of not less than 90 percent were obtained. The amount of [^{14}C]serotonin released was calculated by subtracting the activity contained in the control samples from the total activity contained in each drug-modified sample. Maximum release was defined as the radioactivity present in the supernatant fraction after chemical induction of irreversible aggregation without modifiers present. The concentration of [^{14}C]serotonin after maximum aggregation was at least five times greater than the corresponding control samples.

Thromboxane A_2 generation. TxA_2 was generated using the method reported by Chignard and Vergaftig [5]. In this procedure, dog platelets were used to synthesize TxA_2 from exogenously added arachidonic acid. Citrated dog blood was isolated as described previously for human samples. Dog PRP was prepared by centrifugation at 67 *g* for 10 min in a Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, CT) (Sorvall type rotor, SS34). Aliquots of dog PRP (0.5 ml) containing at least 200,000 platelets/mm^3 were incubated with 0.2 mM arachidonic acid with constant stirring at room temperature for 2.0 min. This interval was determined to be optimal for maximum TxA_2 production by Chignard and Vergaftig [5] and in our own experiments where we used maximum aggregation of human PRP as an indicator of activity. After the incubation interval, 0.2 ml of dog PRP was transferred immediately to a cuvette containing 0.5 ml of human PRP. The human PRP was incubated with 0.5 mM indomethacin for 2 min prior to the addition of dog PRP to prevent interference by the arachidonic acid remaining in these samples. The aggregation response of human PRP as a result of stimulation by TxA_2 was monitored as described previously.

Chemicals. [^{14}C]Serotonin (sp. act. 58 mCi/mmole) was purchased from the Amersham Corp. (Arlington Heights, IL) and diluted with 0.05 M potassium phosphate buffer, pH 7.4. Stock solutions of indomethacin (Merck & Co., Inc., Rahway, NJ), arachidonic acid (Nuchek Corp., Elysian, MN), and the epoxymethano prostaglandin endoperoxide analogs, U44069 [(15S)-hydroxy-9α,11α-(epoxymethano)-prosta-5Z-13E-dienoic acid] and U46619 [(15S)-hydroxy-11α,9α-(epoxymethano)-prosta-5Z,13E-dienoic acid] (The Upjohn Co., Kalamazoo, MI), were prepared in absolute ethanol, and working solutions were diluted with phosphate buffer. Collagen and ADP were purchased from the Chrono-log Corp. (Havertown, PA) as aqueous solutions. Racemic-TMQ was synthesized in our laboratories as reported earlier [6]. All compounds were freshly prepared in 0.05 M potassium phosphate buffer, pH 7.4, containing 0.05% metabisulfite.

RESULTS

Initial studies were carried out by examining the concentration dependence of the inhibitory effects of the TMQ isomers on arachidonic acid- and collagen-induced aggregation (Table 1). With each inducer, *R*(+)-TMQ was about 12-fold more effective than the corresponding *S*(−)-isomer as an inhibitor of platelet aggregation. The IC_{50} values for both isomers against each inducer were nearly identical. These data suggest that the inhibitory site of action of TMQ is mediated subsequent to the formation of arachidonic acid.

Since cyclic endoperoxides are the first intermediates generated from arachidonic acid in platelets, we examined the effects of the TMQ isomers on the aggregation induced by the stable epoxymethano PGH_2 analogs, U44069 and U46619 (see Fig. 1). In preliminary experiments, we had found that U44069 was a less potent inducer of platelet aggregation than was U46619. Using the concentrations of the epoxymethano PGH_2 analogs that produced maximum irreversible aggregation responses (2 μM for U46619 and 10 μM for U44069), each of the TMQ isomers

Table 1. Inhibitory activity of the optical isomers of trimetoquinol on aggregation of human platelets induced by arachidonic acid and collagen.

Aggregating agent*	IC_{50} *R*(+)-isomer† (μM)	IC_{50} *S*(−)-isomer† (μM)	Potency ratio‡
Arachidonic acid (10^{-3} M)	4.2 ± 0.61§	47.8 ± 1.1	12.1 ± 1.6 (9.4–14.8)
Collagen (0.12 mg/ml)	4.3 ± 0.78	51.8 ± 6.3	12.3 ± 0.9 (9.9–15.1)

* The minimum concentration of each agent which produced maximum irreversible aggregation was used.

† Inhibitory concentration-50 (IC_{50}); values are the means ±S.E.M. of N = 4.

‡ IC_{50} of *S*(−)-trimetoquinol/IC_{50} of *R*(+)-trimetoquinol; values are the means ±S.E.M. Values in parentheses are the 95 per cent confidence limits of the calculated potency ratios.

§ The IC_{50} value for racemic-trimetoquinol was 4.0 ± 1.0 (μM ±S.E.M. of N = 3).

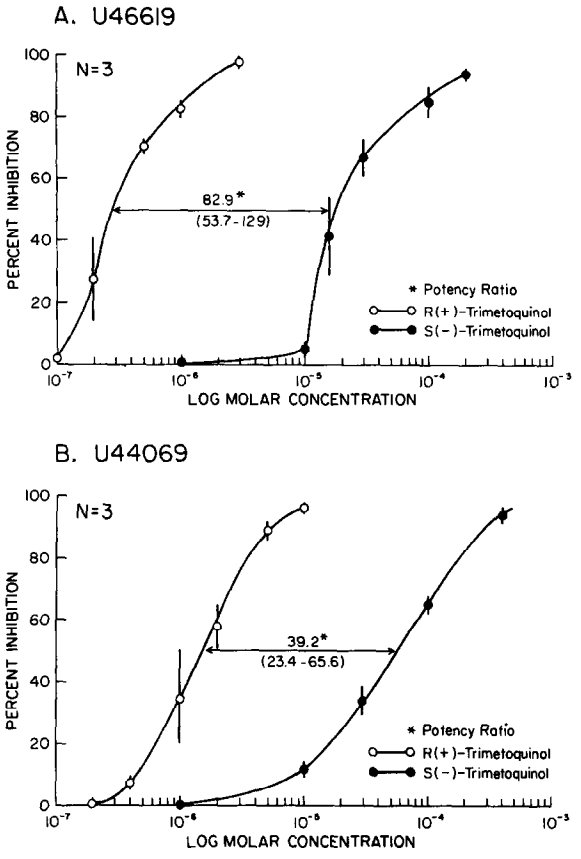

Fig. 1. Inhibition of U46619 (2 μM)- and U44069 (10 μM)-induced aggregation of human platelets by the $R(+)$- (○——○) and $S(-)$- (●——●) isomers of trimetoquinol. The potency ratio within each figure is defined as the IC_{50} for $S(-)$-TMQ/IC_{50} $R(+)$-TMQ. Values in parentheses are the 95 per cent confidence limits of the calculated potency values. Plotted values are the means ±S.E.M. of N = 3.

produced a stereo- and concentration-dependent inhibition (Fig. 1). The potency ratios, calculated for the TMQ isomers, were 82.9 and 39.2 against U46619- and U44069-induced aggregation respectively. IC_{50} Values of 0.14 and 11.2 μM were obtained for the effects of the $R(+)$- and $S(-)$-isomers of TMQ on U46619-induced aggregation, respectively, whereas the corresponding values were 1.42 and 56.2 μM for U44069-induced aggregation. In summary, a marked stereo-dependence of the TMQ inhibition of aggregation induced by the stable endoperoxide analogs was found.

One of the consequences of prostaglandin-mediated stimulation of platelet aggregation is secretion from granules. To assess the effect of TMQ on the release reaction, we simultaneously monitored the release of serotonin and the aggregation of platelets in PRP preparations, using U46619 (Fig. 2) and ADP (Fig. 3) as inducers. With the use of U46619, it could be seen that the inhibitory effects on both responses were stereo-dependent and had nearly identical concentration-response curves (Fig. 2). These data confirm the direct relation that exists between the inhibition of maximum platelet aggregation and of serotonin release by TMQ in the presence of this inducer. Experiments were also done using ADP as an inducer of aggregation and of serotonin release (Fig. 3). Racemic-TMQ blocked the secondary phase of serotonin release induced by ADP (3 μM) at concentrations nearly equivalent to those required by $R(+)$-TMQ for the inhibition of these responses to U46619. Therefore, TMQ is an inhibitor of platelet secretion and has little effect on the ADP-induced primary phase of aggregation (see Fig. 3).

Our data have demonstrated that TMQ is a potent and stereo-dependent inhibitor of U46619-induced aggregation. The nature of the inhibition of U46619-induced aggregation by trimetoquinol was approximated using the graphical method of Dixon [7] (Fig. 4). These experiments suggest that racemic-trimetoquinol inhibits U46619-induced aggregation in a competitive-like manner; the apparent inhibitory constant (K_i) was calculated to be 0.13 μM. This calculated inhibitory constant is in excellent quantitative agreement with the IC_{50} value determined for $R(+)$-TMQ against U46619 (see Fig. 1). We recognize that the estimation of a K_i value uses kinetic analyses designed for enzymatic reactions, and that the relationship between platelet aggregation and changes in light absorbance is very complex; however, we also feel that there is no better alternative at present.

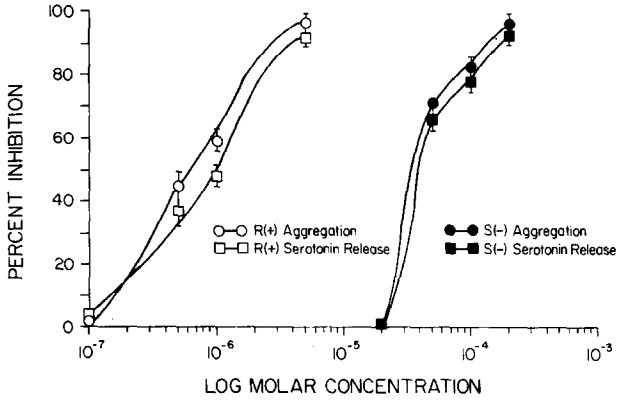

Fig. 2. Concentration-dependent inhibition of U46619 (2 μM)-induced platelet aggregation (○, ●) and [^{14}C]serotonin release (□, ■) by the isomers of trimetoquinol. Plotted values are means ±S.E.M. of N = 3. Key: $R(+)$-trimetoquinol (□, ○), and $S(-)$-trimetoquinol (■, ●).

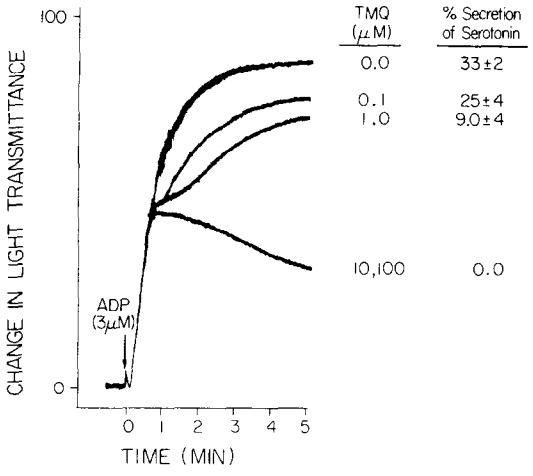

Fig. 3. Concentration-dependent inhibition of ADP (3 μM)-induced serotonin release and secondary phase of aggregation by racemic-trimetoquinol. Representative aggregation tracings of ADP-induced responses in the absence or presence (0.1 to 100 μM) of trimetoquinol are given. Data for serotonin secretion are expressed as a percentage of the net ^{14}C released by 3 μM ADP. Values are the means ±S.E.M. of N = 3.

U46619, the stable endoperoxide analog, is known to produce physiological effects that are more like those of TxA_2 than of PGH_2 [8]. Thus, it may be inferred that the TMQ isomers would also inhibit the aggregatory response to TxA_2. The inhibition of the TxA_2-induced aggregation by TMQ was also stereo-dependent (Fig. 5). The IC_{50} values for *R*(+)- and *S*(−)-TMQ against TxA_2 were 0.64 and 23.2 μM respectively. The calculated potency ratio of the TMQ isomers was 36.0. This value is in favorable agreement with the ratio values determined previously for the stable endoperoxide analogs (Fig. 1).

DISCUSSION

TMQ effectively inhibits platelet aggregation caused by arachidonic acid, collagen, epinephrine, natural endoperoxides (PGG_2 and PGH_2), synthetic epoxymethano endoperoxide analogs (U44069 and U46619), and TxA_2, and it inhibits the secondary phase of aggregation induced by ADP or epinephrine [1–4]. Stereoisomers of agonists or antagonists have been used in a pharmacological approach for the characterization of drug–receptor interactions [9]. In this study, the potency ratio (isomeric activity

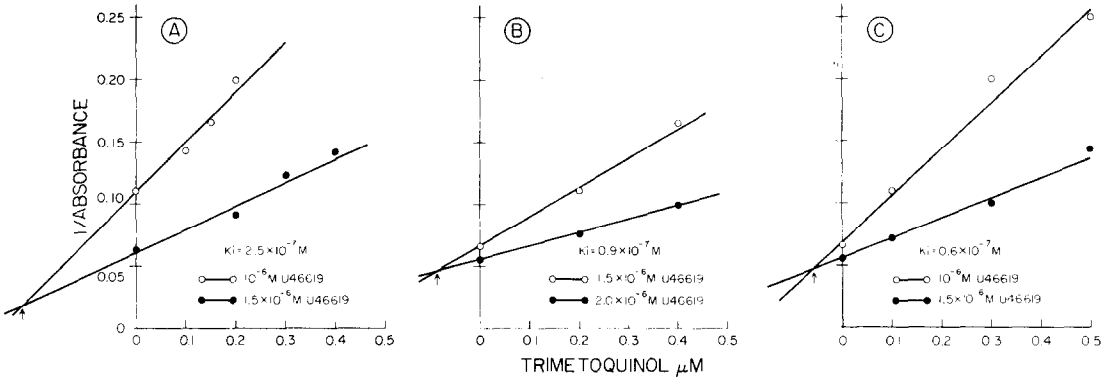

Fig. 4. Estimation of the inhibitory potency (K_i) of racemic-trimetoquinol against U46619-induced aggregation in human platelets. The concentrations of inducer used are given in each panel. Calculated K_i (0.13 ± 0.06) is the mean ±S.E.M. of three estimated K_i values from experiments A, B, and C. Inhibitory potencies were estimated using the method of Dixon [7].

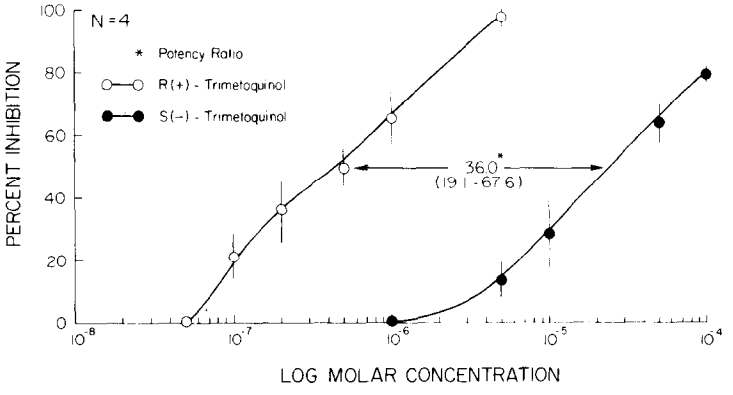

Fig. 5. Inhibition of thromboxane A_2-induced aggregation by the *R*(+)- (○——○) and *S*(−)-(●——●) isomers of trimetoquinol in human platelets. The potency ratio is defined in Fig. 1. Values in parentheses are the 95 per cent confidence limits of the calculated potency values. The generation of thromboxane A_2 in citrated dog plasma was carried out as described in Methods. Plotted values are the means ± S.E.M. of N = 4.

difference) for the stereoisomers of TMQ was determined with several inducers that interact at differing sites in platelets. Our data have shown that there is a high degree of stereo-dependence of the inhibition of arachidonic acid-, collagen-, U46619-, U44069- and TxA_2-induced aggregation. With each inducer, the $R(+)$-isomer of TMQ was the more potent inhibitor of aggregation and possessed IC_{50} values that varied from 0.14 to 4.3 μM. In addition, the potency ratios for the TMQ isomers appear to be separable into two groups. Similar potency ratios were obtained for the isomers of TMQ as inhibitors of collagen- and arachidonic acid- (12-fold) versus U46619-, U44069- and TxA_2- (36- to 83-fold) induced aggregation. These quantitative differences in potency ratios for the TMQ isomers with various inducers may be interpreted in at least two ways: (1) the stereoisomers of TMQ may have more than one inhibitory site of action, and (2) the inducers may modulate the aggregation response through a pathway independent of TxA_2 formation. Nonetheless, the greater potency ratio values and, in particular, the greater inhibitory potency of the $R(+)$-isomer toward the stable endoperoxides and TxA_2, compared to arachidonic acid, are in agreement with an earlier report of MacIntyre and Willis [4] using TMQ. In other experiments, we have shown that each TMQ isomer inhibits both the platelet aggregation response and the serotonin secretion response to U46619 with nearly identical stereo- and concentration-dependence (Fig. 2). Clearly, our studies suggest that both isomers of TMQ are selective inhibitors of platelet function and release reaction at a site subsequent to the formation of endoperoxides and/or TxA_2.

Shtacher *et al.* [2] previously reported that inhibition of the secondary phase of human platelet aggregation induced by ADP was stereo-dependent with respect to TMQ. In these studies, we have demonstrated that TMQ is also very potent as an inhibitor of ADP-induced serotonin release (Fig. 3). These results suggest that TMQ inhibits the release of endogenous ADP which, in turn, is responsible for the secondary phase of aggregation [10].

Our data show that the stereoisomers of TMQ are effective and unique inhibitors of aggregation caused by the endoperoxide PGH_2 analogs or TxA_2 (Figs. 1 and 5). It has also been reported that TMQ is not an inhibitor of thromboxane synthetase activity [4, 11]. Thus, these results suggest to us that TMQ may be an endoperoxide and/or TxA_2 receptor antagonist in platelets. The ability of TMQ to competitively inhibit U46619-induced aggregation strongly supports this view (Fig. 4). However, the question of whether TMQ inhibits TxA_2 or both the endoperoxides and TxA_2 remains unresolved since (1) studies done with TMQ using the endoperoxides as aggregating agents [11] did not reveal the contribution, if any, which endogenously formed TxA_2 (formed from the endoperoxide) made toward the aggregation response, and (2) the stable endoperoxide analogs are thought to more closely mimic PGG_2 and PGH_2 by some [11] and TxA_2 by others [8].

If TMQ is a competitive endoperoxide/thromboxane receptor antagonist, then it represents a novel chemical entity in that TMQ is structurally unrelated to the natural endoperoxides and TxA_2. To date, only prostaglandin analogs (13-azaprostanoic acid, 9α-11α-epoxyiminoprosta-5,13-dienoic acid) have been reported to act as selective TxA_2 antagonists [12, 13]. Finally, since TxA_2 has been suggested to act as an ionophore [14] and, as such, may not interact with a specific receptor, the possibility exists that TMQ may not be a specific thromboxane receptor antagonist but, rather, may inhibit an event in the aggregation scheme subsequent to the action of TxA_2. Our present research is directed toward the investigation of this question.

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