Pulmonary Pharmacology of DT-TX 30 SE, a Potent Selective Combined Thromboxane Synthetase Inhibitor and Receptor Antagonist, in Guinea Pigs

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ABSTRACT—A novel chemical compound, DT-TX 30 SE (E-6-(4-(2-(4-chlorobenzenesulphonylamino)ethyl)phenyl)-6-(3-pyridyl)-hex-5-enoic acid), was studied in various models of guinea pig pulmonary function. The compound was a potent inhibitor (ED₅₀ 0.019 mg/kg, i.v.) of bronchospasm induced by the thromboxane receptor agonist U-46619, indicating thromboxane receptor antagonism. At even lower doses (ED₉₀ 0.0036 mg/kg, i.v.), it blocked arachidonic acid-induced bronchospasm. Interpretation of the latter results as evidence for additional thromboxane synthetase inhibitory activity was supported by the inhibition of arachidonic acid- or bradykinin-induced thromboxane B₂ production in an isolated lung preparation, although prostaglandin E₂ and prostaglandin 6-oxo-F₁₅₂₄₇₉ production measured at the same time were not inhibited. The potency of DT-TX 30 SE was compared with thromboxane receptor antagonists and synthetase inhibitors described in the literature. As a receptor antagonist, DT-TX 30 SE was significantly more potent than BM 13505 and BM 13177 (assessed by antagonism of U-46619-induced bronchospasm), but less potent than SQ 29548, while as a thromboxane synthetase inhibitor, it was significantly more potent than OKY 046 and UK 37248 as assessed by antagonism of arachidonic acid-induced bronchospasm or (OKY 046) inhibition of thromboxane production in isolated lung. The compound was active by the oral route as shown by its ability, at 10 mg/kg, p.o., to significantly reduce the immediate allergic response of sensitized guinea pigs to an ovalbumin aerosol.

Keywords: Thromboxane, Thromboxane synthetase, Inhibitor, Thromboxane receptor antagonist, Lung

Thromboxane A₂ is a short lived lipid mediator whose biological effects in the lung can include bronchospasm and constriction of the vessels of the pulmonary vasculature. It is a potent platelet aggregating agent and may therefore play a role in pulmonary thrombosis (1).

In tissues, thromboxane A₂ is synthesized from an unstable prostaglandin endoperoxide, prostaglandin H₂, by the enzyme thromboxane synthetase. Thromboxane A₂ is rapidly hydrolyzed in aqueous media to more stable inactive metabolites, the most important of which is thromboxane B₂. Prostaglandin H₂ can also be metabolized via alternative routes: non-enzymic conversion to prostaglandin E₂ and prostacyclin synthetase-catalyzed conversion to prostacyclin, another biologically active molecule, many of whose actions (e.g., on platelet aggregation or on smooth muscle) are antagonistic to those of thromboxane A₂ (2). Prostacyclin is itself unstable and rapidly becomes converted to prostaglandin 6-oxo-F₁₅₂₄₇₉ and other more stable but biologically less active metabolites.

Several thromboxane synthetase inhibitors have already been tested clinically for pulmonary diseases. OKY 046 has been reported to provide some benefit in mild to moderate asthma and to reduce bronchial responsiveness to acetylcholine in asthmatic subjects (3, 4).

It has been suggested that one factor that may limit the clinical efficacy of thromboxane synthetase inhibitors is the accumulation, when further metabolism is blocked, of the substrate prostaglandin H₂. Prostaglandin H₂ can bind and activate the thromboxane A₂ receptor almost as well as thromboxane A₂ itself (5, 6). This being so, a molecule that can antagonize the prostaglandin H₂/thromboxane A₂ receptor as well as being a specific thromboxane synthetase inhibitor would be expected to have advantages over a substance that is a thromboxane
The synthesis and characterization of DT-TX 30 SE in in vitro models of platelet function is described elsewhere (7). Here the substance has been characterized for its actions on the lung.

MATERIALS AND METHODS

Test substances

DT-TX 30 SE (E-6-(4-(2-(4-chlorobenzenesulphonyl-amino)ethyl)phenyl)-6-(3-pyridyl)-hex-5-enoic acid) (Fig. 1) was synthesized in the laboratories of Dr. Karl Thomae GmbH, Biberach a.d. Riss, Germany.

BM 13505 and OKY 046 were also synthesized in the laboratories of Dr. Karl Thomae GmbH. BM 13177, CGS 13080 and UK 37248 were synthesized by Dr. E-O. Rent in the laboratories of Boehringer Ingelheim KG, Ingelheim, Germany. SQ 29548 was a gift of Squibb, Princeton, NJ, USA (also obtained commercially from Biomol, Plymouth Meeting, PA, USA). Imidazole was from Janssen, Beerse, Belgium.

In vivo studies of guinea pig bronchospasm

Male Pirbright white guinea pigs (400–500 g) were anesthetized with urethane (1.8 mg/kg, i.p.), injected with a muscle relaxant (gallamine triethiodide 3 mg/kg, i.v.) and ventilated artificially (60 breaths/min) via an indwelling tracheal cannula. The pump stroke volume was 5 ml, and inflation pressure was limited to 10 ml water. Respiratory flow was recorded plethysmographically via a type 0000 pneumotachograph (Fleisch, Lausanne, Switzerland) and a “Baratron” differential pressure transducer (MKS Instruments, Burlington, MA, USA) (The type 0000 Fleisch tube corresponds to a flow range of 0.015 l/sec). Intravenous challenges were via an indwelling jugular vein catheter.

For investigation of thromboxane synthetase inhibition, an aqueous solution of arachidonic acid sodium salt (Sigma, St. Louis, MO, USA) was repeatedly injected i.v. (1 ml/kg) until a dose was found (0.5–2.0 mg/kg) that consistently gave approximately 60% reduction of peak air flow. This dose was then repeated, but 2 min prior to this, DT-TX 30 SE or comparison substance was injected i.v. (1 ml/kg). For investigation of thromboxane receptor antagonism, the procedure was similar, but instead of arachidonic acid, the guinea pigs were challenged with between 2.5 and 25 µg/kg U-46619 (Paesel, Frankfurt am Main, Germany).

Each animal was only tested at one dose level of DT-TX 30 SE or comparison substance. At least three and in some cases four doses of drug were tested with at least five animals at each dose level plus the vehicle (saline) controls. ED₅₀ values with 95% confidence limits were calculated by linear regression.

As a specificity control, the highest dose of DT-TX 30 SE (0.1 mg/kg, i.v.) was also tested against serotonin (20 µg/kg) or histamine (10–20 µg/kg), the histamine challenge being conducted in cervically vagotomized, atropinized (0.5 mg/kg, i.v.) animals.

Studies with isolated lungs

Guinea pigs (660–800 g) were killed by cervical dislocation and the heart and lungs rapidly removed and flushed with a modified Tyrode’s solution of the following composition per liter: 8 g NaCl, 0.2 g KCl, 0.24 g CaCl₂·2H₂O, 0.214 g MgCl₂·6H₂O, 1 g NaHCO₃, 0.576 g NaH₂PO₄·2H₂O, 1 g glucose. The lung was suspended in a humidified chamber maintained at 37°C and ventilated by negative pressure (52 breaths/min, pressure between 0.5 and −16 cmH₂O) using a breathing cycle with 0.4 sec between breaths. Modified Tyrode was perfused at a constant flow rate (0.5 ml/min) via the pulmonary artery and oxygenation ensured by a stream (28 ml/min) of “Carbo-gen” (95% oxygen, 5% carbon dioxide).

The flow of air into and out of the lung was continuously monitored by a “Microbridge” AWM 2100 mass air flow sensor (Honeywell Micro Switch Division, Newtown, CT, USA). Perfusate was collected via a one-way valve for one 2-min interval before and six 2-min intervals after challenge with a 0.1 ml bolus of 3.3 nmol arachidonic acid or 0.02 nmol bradykinin acetate (Serva, Heidelberg, Germany). Volume of perfusate collected during each 2-min period was approximately 1.0 ml. Thromboxane B₂, prostaglandin 6-oxo-F₁₂, and prostaglandin E₂ were estimated in duplicate for each sample by radioimmunoassay using commercially available kits (NEK-024, NEK-025, NEK-020; New England Nuclear, Boston, MA, USA).
The radioimmunoassay of thromboxane B₂ and prostaglandin 6-oxo-F₁₀ was validated by also measuring some of the samples by electron-capture GC-MS as described by Barrow et al. (8).

For the studies with bradykinin, the lungs were challenged twice, DT-TX 30 SE being added to the perfusate of the test (but not the control) lungs for 30 min before until 12 min after the second bradykinin challenge. For studies with arachidonic acid, the lungs were challenged three times, the first challenge being the vehicle used to place the arachidonic acid in solution (7% methanol, 0.1% acetic acid in Tyrode's solution), the second and third challenges being arachidonic acid. DT-TX 30 SE was added to the perfusate 30 min before until 12 min after the third challenge of test (but not control) lungs.

A re-equilibration period of at least 1 hr was allowed between challenges.

For studies with bradykinin, DT-TX 30 SE was tested at 6 dose levels (0.001, 0.01, 0.1, 1, 10 and 100 μM) and comparison substance OKY 046 at 4 dose levels (0.1, 1, 10 and 100 μM) with 4–6 lungs at each dose level. All perfusates were measured by radioimmunoassay, and perfusates from DT-TX 30 SE dose levels 0.01 and 1 μM (plus controls) measured by GC-MS. For the studies using the arachidonic acid challenge, only one dose of DT-TX 30 SE (1 μM) was tested (6 lungs plus 6 control non-substance treated lungs).

Statistical analysis of drug effects was performed by non-parametric methods. The measurements 1, 3, 5, 7, 9 and 11 min after stimulus (arachidonic acid or bradykinin) were summed up for each period for the parameters thromboxane B₂, prostaglandin E₂ and prostaglandin 6-oxo-F₁₀ and averaged for the change of air flow. The effects of DT-TX 30 SE and (where tested) OKY 046 on the four parameters were examined separately for the two substances. The values for the second challenge with arachidonic acid or bradykinin were taken as a percentage of the values for the first challenge with the same stimuli. With respect to this transformation, the statistical evaluation was performed in two steps. In order to look for a dose-response relationship (effect increases/decreases with higher doses of the substances) a Jonckheere test was used (9). Wilcoxon tests calculating exact P-values (permutation test procedure) were added to decide which doses caused different effects compared to the control. Where only two drug doses were examined (bradykinin challenge, GC-MS assay), global effects were tested by the Kruskal-Wallis test. Where only one substance dose was tested (arachidonic acid study), only the Wilcoxon test was applied. In the study with bradykinin challenges, Wilcoxon's test was also used to compare DT-TX 30 SE and OKY 046 within common dose groups. Where the same samples were assayed by two methods (radioimmunoassay, GC-MS), correlation of the test results was made by Pearson's test.

**Studies of the immediate-reaction in allergized guinea pig**

Guinea pigs were sensitized by a single i.p. injection containing 40 mg/kg ovalbumin bound to 2 g/10 ml aluminium hydroxide gel (with saline to 0.5 ml/animal). Then, 6 weeks later, they were challenged for 90 sec with an aerosol of 3% ovalbumin (Technolat type USV 82 nebulizer). Aerosol challenge was preceded by s.c. injection, 30 min before, of an antihistamine (mepyramine hydrochloride 1 mg/kg). Ten minutes later, the animals were killed by cervical dislocation and the lungs rapidly dissected free from the heart, blood vessels and associated tissues. The lung volumes were measured by fluid displacement according to Archimedes' principle, as follows: the lung was held by the trachea for immersion in a beaker of water and the decrease in weight on immersion recorded. The volume measured thus corresponded to that of the lung lobes with trapped air, the bronchi and that part of the trachea (standardly 80% of the total length) which was immersed.

Test drug (or vehicle control) at the appropriate doses was administered orally (1 ml/kg) as an aqueous solution containing 0.5% Tylose at 60 min prior to the aerosol challenge.

**Table 1. In vivo comparison of DT-TX 30 SE with standard thromboxane receptor antagonists (A) and standard thromboxane synthetase inhibitors (B)**

| Substance       | Literature reference | Dose for inhibition of bronchospasm (with 95% confidence limits) mg/kg | Bronchospasm induced by U-46619 | Bronchospasm induced by arachidonic acid |
|-----------------|----------------------|-------------------------------------------------|-------------------------------|-----------------------------------------|
| **A**           |                       |                                                 |                               |                                          |
| SQ 29548        | (20)                 | 0.0011 (0.0008–0.0014)                          | 0.0012 (0.0008–0.0018)         |                                          |
| BM 13505 (daltroban) | (21)          | 0.035 (0.025–0.052)                             | 0.211 (0.013–0.029)           |                                          |
| BM 13177 (sulotroban) | (22)     | 0.052 (0.031–0.135)                             | 0.123 (0.071–0.193)           |                                          |
| **B**           |                       |                                                 |                               |                                          |
| OKY 046 (ozagrel) | (23)             | >10.0                                           | 0.311 (0.210–0.449)           |                                          |
| CGS 13080 (pirmagrel) | (24)         | >3.0                                            | 0.0036 (0.0022–0.0072)        |                                          |
| UK 37248 (dazoxiben) | (25)       | >10.0                                           | 0.35 (0.15–0.64)              |                                          |
| Imidazole       |                       | >10.0                                           | 11.3 (9.0–14.5)               |                                          |
Controls included non-sensitized animals treated with drug or vehicle and exposed to ovalbumin aerosol and sensitised animals exposed to saline aerosol.

For all comparisons, Wilcoxon tests calculating exact P-values (permutation test procedure) were estimated. Because of the plurality of the tests, the significance level was not adjusted, and therefore the P-values should be interpreted in a qualitative manner.

**RESULTS**

**In vivo studies of guinea pig bronchospasm**

DT-TX 30 SE inhibited both arachidonic acid- and U-46619-induced bronchospasm, not only intensity but also duration of bronchospasm being reduced. Compared with standard thromboxane synthetase inhibitors, DT-TX 30 SE was significantly more potent as an inhibitor of...
arachidonic acid-induced bronchospasm than OKY 046 or UK 37248, but did not differ significantly from CGS 13080 (95% confidence limits, Table 1). DT-TX 30 SE differed from all the tested comparison synthetase inhibitors in also being able to block U-46619-induced bronchospasm. In comparison with standard thromboxane receptor antagonists, DT-TX 30 SE was significantly weaker than SQ 29548 but significantly more potent than BM 13505 or BM 13177 (non-overlapping 95% confidence limits).

The effect of DT-TX 30 SE was specific, the drug having, even at 0.1 mg/kg, i.v., no effect on histamine- or serotonin-induced bronchospasm.

DT-TX 30 SE also blocked arachidonic acid-induced systemic hypotension, although the ED$_{30}$ (between 0.005 and 0.01 mg/kg, i.v.) was somewhat higher than that for inhibition of bronchospasm.

Studies with isolated lungs

Arachidonic acid challenge: The injection of 10 µg arachidonic acid caused a marked rise in the production of all three of the arachidonic acid metabolites examined; in comparison with a previous challenge with vehicle, the effect was highly statistically significant (P < 0.0005, Wilcoxon). When a second arachidonic acid challenge was given, thromboxane B$_2$ production differed little from that after the first challenge, but production of prostaglandin E$_2$ and prostaglandin 6-oxo-F$_{1_{\alpha}}$ was somewhat decreased (see Fig. 2). The effect of including 1 µM DT-TX 30 SE in the perfusate shortly before and after the second challenge (6 lungs) was to cause a highly significant (P < 0.005) decrease in thromboxane B$_2$ production; relative to the lungs challenged a second time, but not treated with DT-TX 30 SE (N = 6), the addition of DT-TX 30 SE increased both prostaglandin E$_2$ and prostaglandin 6-oxo-F$_{1_{\alpha}}$, but only the increase in prostaglandin E$_2$ reached statistical significance (P < 0.05). All six lungs in the control group reacted to the second arachidonic acid challenge with bronchospasm (>5% fall in mean respiratory flow over the 12 min period), whereas none of the lungs in the DT-TX 30 SE group showed any arachidonic acid-induced bronchospasm.

Bradykinin challenge: Bradykinin also caused a marked rise in the production of all 3 arachidonic acid metabolites examined, but in contrast to the results with
arachidonic acid, the amounts of the metabolites were more modest, and the pattern of metabolites did not markedly differ between first and second challenges in the absence of test substance (see Fig. 3).

In the analysis of the effects of DT-TX 30 SE, Jonckheere's test showed a highly significant (P < 0.0001) trend of reduction in thromboxane B₂ production (radioimmunoassay measurements) with higher DT-TX 30 SE concentrations. Biologically (median reduction < 25% control thromboxane B₂ production) and statistically (P < 0.05, Wilcoxon) significant reduction was present at concentrations of 0.1 μM and above. The comparison substance OKY 046 also showed the expected decrease in thromboxane B₂ production with higher concentrations (Jonckheere’s test P = 0.0001), but statistically (P < 0.05) significant reduction was only present at dose levels of 10 and 100 μM. At the 1-μM dose level, the difference between DT-TX 30 SE and OKY 046 was statistically significant (P < 0.05, Wilcoxon). Figure 4 represents graphically the individual results at the dose levels used in both the DT-TX 30 SE and OKY 046 studies. Figure 3 compares the time course of arachidonic acid metabolite production in the presence and absence of 1 μM DT-TX 30 SE (radioimmunoassay measurements). GC-MS also showed highly significant (P < 0.01, Wilcoxon) reduction in thromboxane B₂ production by 1 μM DT-TX 30 SE (Fig. 5). Changes in prostaglandin 6-oxo-F₁₅α were not, either by radioimmunoassay or GC-MS measurements, statistically significant (radioimmunoassay, Jonckheere’s test P > 0.05; GC-MS Kruskal-Wallis P > 0.05). There was a reasonable correlation, taking all the studies together (N = 17), between GC-MS and radioimmunoassay measurement (Pearson’s correlation coefficients: thromboxane B₂ measurements, 0.62; prostaglandin 6-oxo-F₁₅α measurements, 0.86). There was a trend for higher doses of either DT-TX 30 SE or OKY 046 to be associated with raised levels of prostaglandin E₂ (Jonckheere’s test P < 0.01). There was a trend (Jonckheere’s test, P < 0.05) for increased doses of DT-TX 30 SE to be associated with reduced bradykinin-induced bronchospasm (reduction in air flow), but the effect was not complete and individual animals, for whom radioimmunoassay and GC-MS both indicated total inhibition of thromboxane B₂ production, still showed some bradykinin-induced bronchospasm.

**Studies of the immediate reaction in allergized guinea pigs**

DT-TX 30 SE, dosed orally 60 min before the allergen aerosol, could significantly decrease the allergen-induced increase in lung relaxation volume (Fig. 6), but the effect was not total. That is, at the doses at which there was a significant difference between the drug treated animals and positive control (sensitized animals + ovalbumin aerosol), there was also a significant (P < 0.05, Wilcoxon) difference from the negative control (non-sensitized animals + ovalbumin, or sensitized animals + saline).

**DISCUSSION**

The studies in guinea pigs challenged with arachidonic acid or the thromboxane receptor agonist U-46619 are in agreement with and confirm in vivo studies on human platelets indicating that DT-TX 30 SE can act as both a thromboxane synthetase inhibitor and a receptor antagonist (7). The dose of DT-TX 30 SE required to inhibit arachidonic acid-induced bronchospasm was significantly (approximately fivefold) lower than the dose required to inhibit U-46619-induced bronchospasm. This ratio is close to the previously observed difference between the
IC50's for thromboxane synthetase inhibition and receptor binding reported in gel filtered human platelets (7). It has previously been suggested that the thromboxane receptors involved in aggregation of washed human platelets and in guinea pig bronchoconstriction belong to a common homogenous population (10).

Fig. 6. Lung relaxation volume following challenge of ovalbumin sensitized (solid lines) and unsensitized (dotted lines) guinea pigs with an ovalbumin aerosol. Test substance (DT-TX 30 SE or 0.5% aqueous Tylose solution, vehicle control) was administered p.o. 60 min before the aerosol. Thick horizontal lines show median values (N=6), together with 25th and 75th percentiles. Asterisked (*) groups are significantly different (P <0.05, Wilcoxon) from the control group without drug treatment. Ovalbumin sensitized animals challenged with saline instead of ovalbumin gave similar results to non-sensitized animals (relaxation volume, median 1.3 ml, N=3).

Interpretation of the in vivo data for inhibition of arachidonic acid-induced bronchospasm as evidence for a thromboxane synthetase inhibitory activity is confirmed by the specific inhibition of arachidonic acid- or bradykinin-induced thromboxane B2 synthesis by relatively low (0.1 or 1 μM) concentrations of DT-TX 30 SE in the isolated lung preparation. Most of the studies here described measured thromboxane B2 by radioimmunoassay, a method whose validity depends on the specificity of the antibodies employed. The correlation between GC-MS and radioimmunoassay measurements suggests the radioimmunoassay results are valid. That the thromboxane B2 measurements made by radioimmunoassay were consistently somewhat higher than those made by GC-MS may be explained by a contribution to the radioimmunoassay results from structurally related alternative metabolites of thromboxane A2 in guinea pig lungs such as 15-oxo-13,14 dehydrothromboxane B2, 11-dehydrothromboxane B2 or 2,3-dinor-thromboxane B2 (11). Since levels of these thromboxane metabolites are likely to vary in a similar way after drug treatment as those of thromboxane B2 itself, cross-reactivity of the radioimmunoassay antibody with these metabolites does not invalidate the conclusions of the study relating to DT-TX 30 SE.

The proportion of arachidonic acid collected as metabolites was relatively small (2.3% of the perfused dose), a situation that probably results from ready incorporation of free fatty acids into membrane lipids. This and the relative proportion of the three metabolites (prostaglandin 6-oxo-F1α > thromboxane B2 > prostaglandin E2) reflects the observations of other workers (12–15).

Inhibition of thromboxane synthesis was associated with increased prostaglandin E2 synthesis, but as prostaglandin 6-oxo-F1α synthesis was, even in the absence of drug, relatively large in relation to thromboxane synthesis, and varied from challenge to challenge, it is not...
possible to estimate from these data to what extent inhibition of prostaglandin H₂ conversion to thromboxane A₂ was accompanied by metabolism of the unconverted prostaglandin H₂ by the alternative route to prostacyclin (and thus prostaglandin 6-oxo-F₁₂₀₆). What was clear was the inhibition of arachidonic acid metabolism to thromboxane A₂ by DT-TX 30 SE was specific, so that the ratio of prostaglandin 6-oxo-F₁₂₀₆ to thromboxane B₂ (and therefore presumably the ratio of prostacyclin to thromboxane A₂) was greatly increased in the presence of DT-TX 30 SE concentrations of 0.1 μM and above.

In comparison with the thromboxane receptor antagonists reported in the literature, the in vivo studies with U-46619 showed that DT-TX 30 SE had slightly greater potency than BM 13505 and BM 13177, but was somewhat weaker than SQ 29548. Comparison with standard thromboxane synthetase inhibitors (inhibition of arachidonic acid-induced bronchospasm) showed that it had a potency similar to CGS 13080 and significantly better than UK 37248 or OKY 046. The difference between the ED₅₀’s of DT-TX 30 SE and OKY 046 was about 80-fold, which compares with a difference of 100-fold in the concentrations of these drugs required to inhibit thromboxane B₂ production in the isolated lung.

To show the biological activity of DT-TX 30 SE in a pulmonary disease model, the effect of the drug against the immediate allergen reactions in guinea pigs was tested. Other thromboxane synthetase inhibitors have previously been reported to reduce the immediate allergic reaction in this species (16, 17). The parameter measured, increase in lung relaxation volume, is a simple method for measuring the immediate reaction in conscious animals, which has been used in a number of laboratories (18, 19). Since the relaxation volume is a static measurement, airway closure (in which contraction of the bronchial musculature is likely to play an important role) is the only mechanism that can account for the increase in this parameter. The histamine component of the immediate response was excluded by pretreating the animals with mepyramine. DT-TX 30 SE was active in this model. We are cautious in extrapolating these results from guinea pig to humans; the study does, however, suggest that DT-TX 30 SE is orally active and reaches the lung in pharmacologically active amounts.

The combined profile of oral activity, very potent and specific thromboxane synthetase inhibitory activity and potent thromboxane receptor antagonism, suggests that this compound alone, or in combination with other drugs, may prove a useful tool for elucidating the role of thromboxane in pulmonary disease.

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