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# TRIAZOLOBENZODIAZEPINES: A NEW CLASS OF STIMULATORS OF TISSUE-TYPE PLASMINOGEN ACTIVATOR SYNTHESIS IN HUMAN ENDOTHELIAL CELLS

TEAKE KOOISTRA,* KARIN TOET, CORNELIS KLUFT, PHILIP F. VONVOIGTLANDER,† MICHAEL D. ENNIS,† JAMES W. AIKEN,† JOYCE A. BOADT† and LAURENCE A. ERICKSON†

IVVO-TNO Gaubius Laboratory, Leiden, The Netherlands and †Upjohn Laboratories, Kalamazoo, MI, U.S.A.

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**Abstract**—In our search for compounds that can stimulate endogenous fibrinolysis, we have found that certain triazolobenzodiazepines enhance the production of tissue-type plasminogen activator (t-PA) by vascular endothelial cells maintained *in vitro*, with no or even a lowering effect on plasminogen activator inhibitor type-1 (PAI-1) production. The most active compounds tested, U-34599, U-46195 and U-51477, were studied in more detail and showed a time- and dose-dependent increase in the production of t-PA by human umbilical vein endothelial cells. At optimal stimulatory concentrations (about 10 $\mu$M), the three compounds stimulated t-PA expression about 2-fold after 24 hr and maximally about 4-fold after 48 hr of incubation; this maximal increase in t-PA synthesis was sustained at prolonged incubations of 72 or 96 hr. The triazolobenzodiazepine effects on t-PA production were accompanied by parallel increases in t-PA mRNA levels, without marked changes in PAI-1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA concentrations. Numerous analogues of the three lead compounds were then tested to determine the relationship between benzodiazepine structure and the ability to stimulate t-PA production. No positive correlation was found between the ability of the various triazolobenzodiazepines to stimulate t-PA production and their affinity for the benzodiazepine receptor. In agreement with this, no specific binding of [$^3$H]flunitrazepam, a specific ligand for benzodiazepine receptors, to endothelial cell membrane preparations was observed. Thus, it is unlikely that the triazolobenzodiazepines act through central-type benzodiazepine receptors to stimulate t-PA production. Similarly, no evidence was found for the presence of peripheral-type benzodiazepine receptors on endothelial cell membranes. The ability of the benzodiazepines to stimulate t-PA production, however, appeared to be related to their platelet-activating factor (PAF) antagonist activity. Despite this finding, several non-benzodiazepine PAF antagonists did not stimulate t-PA production. While the precise mechanism of action is not yet clear, selected benzodiazepine analogues possessing PAF antagonist activity stimulate the production of t-PA by endothelial cells *in vitro*.

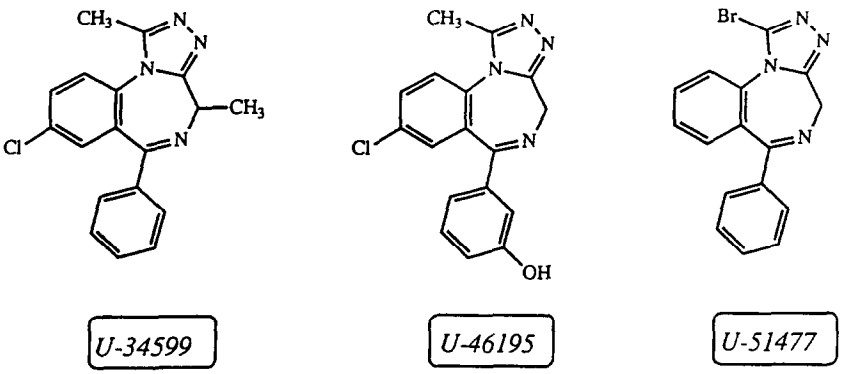
Several clinical studies have demonstrated an inverse correlation between blood fibrinolytic activity and the risk of thromboembolic disease [1–4]. Factors that may enhance endogenous fibrinolytic activity would therefore provide a potential therapeutic and preventive approach to intravascular thrombosis. The net fibrinolytic activity in blood is the resultant of the relative concentrations of the plasminogen activators, tissue-type plasminogen activator (t-PA‡) and urokinase-type plasminogen activator, and of the plasminogen activator inhibitor type-1 (PAI-1). Vascular endothelial cells represent a direct target for modulating the fibrinolytic activity of the blood since they synthesize and release t-PA and PAI-1.

The use of cultured human endothelial cells provides us with an experimental model to study modulation of endogenous fibrinolysis in an *in vitro* system [5]. A variety of compounds have been shown to stimulate t-PA synthesis in cultured human endothelial cells, including the vasoactive agents, thrombin and histamine [6–8], short-chain fatty acids like butyrate [9], protein kinase C activators such as phorbol 12-myristate 13-acetate [10–12] and retinoids [13, 14]. Endothelial cells are able to increase their PAI-1 production on stimulation with bacterial lipopolysaccharide [8, 15–17], the cytokines, interleukin-1 [16–19], tumour necrosis factor-$\alpha$ [17] or lymphotoxin [17], transforming growth factor $\beta$ [20] and thrombin [7, 8, 21]. Only with thrombin is the increase in PAI-1 synthesis accompanied by enhanced t-PA synthesis, whereas t-PA synthesis does not change or may even decrease with the other PAI-1 synthesis stimulating compounds.

Of the compounds shown to modulate t-PA and/or PAI-1 expression in cultured human endothelial cells, only retinoids seem physiologically relevant in stimulating endogenous fibrinolytic activity, but these compounds face problems of side effects, particularly teratogenicity. In a search for other

* Corresponding author: Dr T. Kooistra, Gaubius Laboratory IVVO-TNO, P.O. Box 430, 2300 AK Leiden, The Netherlands. Tel. (31)71 181450; FAX (31)71 181904.

‡ Abbreviations: t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor type-1; EC, endothelial cells; DMSO, dimethyl sulphoxide; CM, conditioned media; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAF, platelet-activating factor.




Scheme 1. Structure of triazolobenzodiazepines.

compounds that may stimulate endogenous fibrinolysis, we have found that certain benzodiazepines stimulate the production of t-PA by cultured human endothelial cells, without increasing PAI-1 synthesis. The availability of various benzodiazepine analogues enabled us to study further the relationship between benzodiazepine structure and the ability to stimulate t-PA production, and the mechanism of action of the benzodiazepines with respect to enhanced t-PA expression.

## MATERIALS AND METHODS

*Materials.* Triazolobenzodiazepines and derivatives were provided by the Upjohn Co. (Kalamazoo, MI, U.S.A.). The structure of the three triazolobenzodiazepines with the highest t-PA stimulating activity, U-34599, U-46195 and U-51477, is shown in Scheme 1. Stock solutions of the triazolobenzodiazepines and analogues (3 mg/mL) were prepared in dimethyl sulphoxide (DMSO) and preserved at −20°. Immediately before use, the test compounds were diluted in incubation medium to the final test concentration. The concentration of DMSO in the incubation medium did not exceed 0.1% (v/v), unless stated otherwise.

t-PA (>99% two-chain) was purified from Bowes-melanoma-cell culture medium as described by Kluft *et al.* [22]. The activity is expressed as international units (IU), using the first international standard of the World Health Organisation, code 83/517, as a standard [23]. Our t-PA preparation has a specific activity of 500,000 IU/mg.

Enzyme immunoassay kits for determination of human t-PA antigen and PAI-1 antigen ("Imulyse") were obtained from Biopool (Umeå, Sweden). Other materials used in the methods described below have been specified in detail in the relating references.

Deoxycytidine 5[$\alpha$-$^{32}$P]triphosphate was from Amersham, International plc (Bucks, U.K.).

*Cell culture experiments.* Endothelial cells (EC) were isolated from human umbilical cord veins using collagenase by a technique similar to that described by Jaffe *et al.* [24]. Cells were grown in fibronectin-coated dishes in M199 medium supplemented with 20 mM HEPES, 10% (v/v) human serum, 10% (v/v) newborn calf serum (heat-inactivated), 150 $\mu$g endothelial cell growth supplement/mL [25], 100 IU of penicillin/mL and 100 $\mu$g of streptomycin/mL at 37° in a 5% $CO_2$ atmosphere, as described [26]. The medium was replaced every 2–3 days. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 1:3.

For the experiments, confluent cultures were used at the second or third passage, and cells were always re-fed the day before the experiment with incubation medium, namely M199 medium supplemented with 20 mM HEPES, 10% (v/v) human serum and penicillin/streptomycin. Conditioned media (CM) were obtained by incubating cells in 5-cm$^2$ dishes at 37° for 24 or 48 hr with 1 mL incubation medium containing the appropriate concentration of the test compound or stock solvent [final concentration maximally 0.1% (v/v)]. The media were refreshed every 24 hr. CM were centrifuged for 2 min in a Beckman Microfuge to remove cells and cellular debris, and samples were frozen at −20° until use. Cells were washed at 37° three times with phosphate-buffered saline (0.15 M NaCl/10 mM $Na_2HPO_4$/1.5 mM $KH_2PO_4$, pH 7.4) and were used for isolation of RNA.

*Assays.* t-PA antigen and PAI-1 antigen determinations were made using enzyme immunoassays. The enzyme immunoassay for human PAI-1 antigen permits detection of active and "latent" (inactive) forms of PAI-1, whereas t-PA–PAI-1 complexes are detectable with about a 10-fold lower efficiency, according to the manufacturer.

PAI-1 activity was determined by the method of Verheijen *et al.* [27]: samples were titrated with increasing amounts of t-PA, followed by spectrophotometric measurement of the residual t-PA activity. One unit of PAI-1 is defined as the amount of inhibitor that neutralizes one IU of t-PA activity.

*RNA hybridization.* Total RNA was extracted from endothelial cells as described by Chomczynski and Sacchi [28]. RNA samples were dissolved in $H_2O$. The RNA concentration in each sample was determined spectrophotometrically. Equal amounts of RNA from the different dishes were analysed for their t-PA, PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content by northern blot hybridization. With northern blotting,

RNA samples were subjected to gel electrophoresis in formaldehyde agarose gels, as outlined by Maniatis *et al.* [29]. After electrophoresis, RNA was transferred to Hybond N according to the instructions of the manufacturer. Prehybridization and hybridization were performed at 60° [1 mM EDTA; 7% sodium dodecyl sulphate (SDS), 0.5 M $NaH_2PO_4$–$Na_2HPO_4$; pH 7.2] (modified from Church and Gilbert [30]). DNA fragments to be used as a probe were isolated from low melting agarose [29]. Hybridization was usually performed with 1 ng/mL of probe labelled by random prime labelling to approximately $2 \times 10^8$ cpm/μg DNA [29].

The filters were washed at a stringency of 0.2 × standard saline citrate (SSC) and 1% SDS for 2 × 15 min at 60° [29]. The membranes were subsequently exposed to an Amersham Hyperfilm-MP film with an intensifying screen at −80°. For the quantification of the relative amounts of mRNA, densitometry was used. In short, a scan of the bands was made on a CS 910 Shimadzu scanner and the areas under the peaks were integrated and plotted with the aid of a United Technology Packard data processor.

*cDNA probes.* The following cDNA fragments were used as probes in the hybridization experiments: a 1.9 kb Bgl II fragment of the human t-PA cDNA [31], a 2.5 kb EcoRI fragment of a human PAI-1 cDNA of the 3.1 kb transcript [32], and a 1.2 kb PstI fragment of a rat GAPDH cDNA [33] provided by Dr R. Offringa, which is commonly used as an international standard probe [34]. The cDNA fragments were radiolabelled using the random primer method (Multiprime, Amersham, Houten, The Netherlands).

## RESULTS

### *Effect of various triazolobenzodiazepines on t-PA and PAI-1 synthesis in cultured human EC*

Of the various triazolobenzodiazepines tested, the three compounds shown in Scheme 1, U-34599, U-46195 and U-51477, were the most active ones in stimulating t-PA synthesis in cultured human EC. Figure 1 illustrates a representative experiment in which EC were incubated with various concentrations of the three triazolobenzodiazepines for two periods of 24 hr; after the first incubation period, the CM were collected for analysis and replaced by fresh media containing the appropriate triazolobenzodiazepine concentration. In general, the three compounds induced a concentration- and time-dependent increase in t-PA synthesis, with maximal stimulatory effects being seen at concentrations of 3 μg/mL (U-34599 and U-46195) to 10 μg/mL (U-51477) after 48 hr of incubation. At higher triazolobenzodiazepine concentrations cells became detached from the matrix, which was accompanied by a fall in t-PA production. At optimal stimulatory concentrations, the different triazolobenzodiazepines showed about equal efficacy to stimulate t-PA expression: about 2-fold stimulation after 24 hr and about 4-fold stimulation after 48 hr. Prolonged incubations, 72 hr or 96 hr, showed a sustained 4-fold increase in t-PA production (results not shown).

For comparison, we also studied the effect of triazolobenzodiazepines on the synthesis of the inhibitor, PAI-1 and urokinase. As seen in Fig. 1, the three compounds concentration- and time-dependently decreased PAI activity in the medium. At concentrations that were optimal at stimulating t-PA synthesis PAI activities fell to 50–80% of control values after 24 hr of incubation, and to about 40% after 48 hr of incubation. Since only a small portion (<5%) of PAI-1 present in 24 hr EC CM is in an active form [35, 36], we also analysed the CM for the total amount of PAI-1 secreted by the EC by measuring PAI-1 antigen. Apart from the highest test concentration used (30 μg/mL), PAI-1 antigen levels did not fall below 80% of control values, even after 48 hr of incubation. Thus, the marked decreases in PAI-1 activity may at least partly be explained by complexing of active PAI-1 molecules with the increased levels of t-PA in the CM.

None of the triazolobenzodiazepines tested showed an induction of urokinase expression.

### *Effect of triazolobenzodiazepines on t-PA and PAI-1 mRNA levels*

To analyse these triazolobenzodiazepine effects further, RNA was harvested from U-51477-treated EC and control EC, and subjected to northern hybridization using t-PA, PAI-1 and GAPDH cDNA probes. As shown in Fig. 2, EC incubated for 24 or 48 hr in the presence of 3 μg/mL U-51477 contained about 2-fold and 4-fold higher levels of t-PA mRNA, respectively, than control cells, but showed little if any change in mRNA levels for PAI-1 as compared to the internal standard GAPDH.

### *Mechanism of action of triazolobenzodiazepines on t-PA synthesis*

Benzodiazepines are known to bind to benzodiazepine receptors [37–39] and to block PAF action [40]. To learn more about the mechanism by which triazolobenzodiazepines could stimulate t-PA expression, we have compared the ability of numerous analogues of the above triazolobenzodiazepines to stimulate t-PA synthesis with their binding to benzodiazepine receptors and their ability to block PAF. Although conclusions drawn from a comparable rank order would be only inferential, it could readily lead to more refined experiments to test more rigorously the suggested hypothesis.

No positive correlation between benzodiazepine receptor binding activity and the ability of the triazolobenzodiazepines to stimulate t-PA production was found (Table 1). In addition, no specific binding to EC membranes was found using [$^3$H]flunitrazepam (a specific ligand for central-type benzodiazepine receptors) or Ro 5-4864 (a ligand for peripheral-type benzodiazepine receptors) as ligand (data not shown). Thus, it is unlikely that the benzodiazepines act through central-type or peripheral-type benzodiazepine receptors to stimulate t-PA production. The ability of the benzodiazepines to stimulate t-PA production, however, appeared to be related to their PAF antagonist activity (Table 1). Despite this finding, several non-benzodiazepine PAF (receptor) antagonists (BN

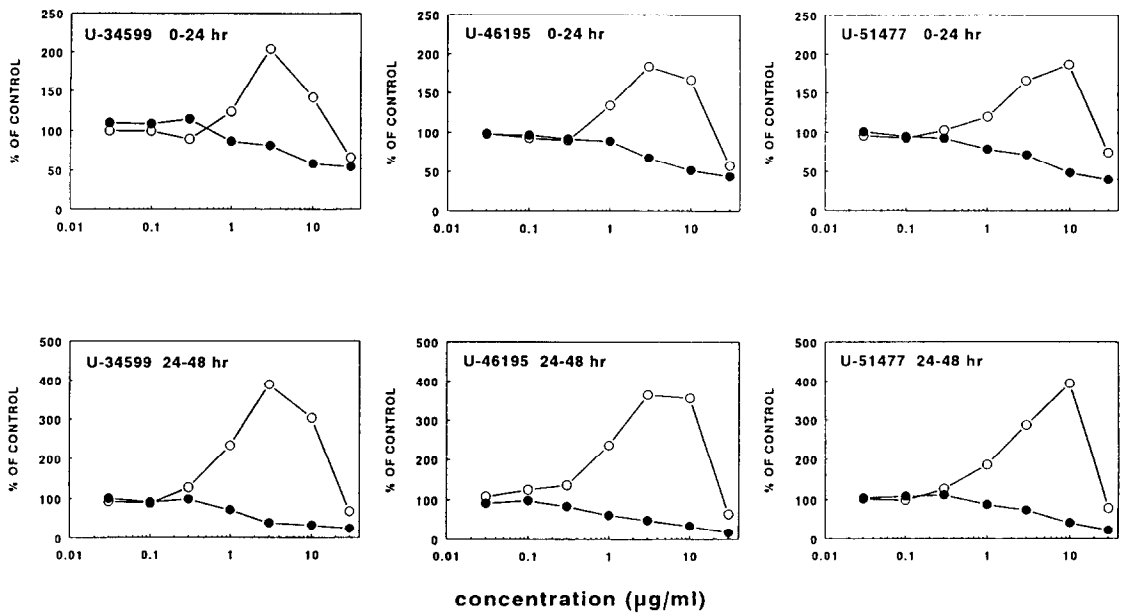



Fig. 1. Effect of triazolobenzodiazepines on t-PA antigen and PAI-1 activity production in cultured human endothelial cells. Human umbilical vein endothelial cells were incubated for two periods of 24 hr with differing concentrations of U-34599, U-46195 or U-51477, and the conditioned media were analysed for t-PA antigen (○) and PAI-1 (●) activity as described in Materials and Methods. Results are means of duplicate incubations of a representative experiment; the data are expressed as percentage values of controls. The vehicle (DMSO) had no effect on t-PA or PAI-1 synthesis at the concentrations used.

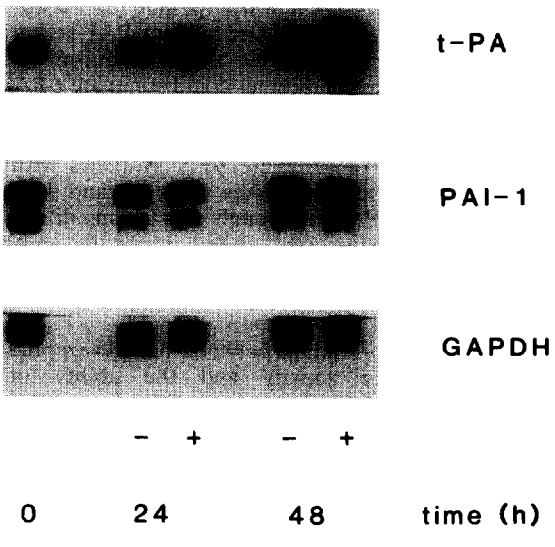



Fig. 2. Autoradiogram showing effect of U-51477 on t-PA, PAI-1 and GAPDH mRNA content in human umbilical vein endothelial cells. Total RNA was isolated from confluent cultures of endothelial cells incubated in the absence (−) or presence (+) of 3 μg/mL U-51477 for the indicated time periods. mRNA levels were estimated by northern blot analysis, shown as autoradiograms.

52021, U-58599, U-66985E, U-80271, U-83381 and U-83382) did not stimulate t-PA production.

*Structure–activity relationships*

In order to clarify further the structural prerequisites for t-PA stimulating activity, we tested a number of analogues (provided by the Upjohn Co.) for their ability to stimulate t-PA synthesis in EC. The results indicate that the methyl-group (U-34599, U-46195) or the bromo-group (U-51477) at the 1-position is possibly important for the stimulating effect of the triazolobenzodiazepines, since the introduction of a more bulky group at the 1-position, including an ethyl group, abolished the stimulatory effect. On the other hand, replacement of the methyl group by a hydrogen atom also suppressed the t-PA enhancing effect. Substitution of the second methyl group in U-34599 by an amino group or a hydroxyl group only slightly diminished the stimulatory action of U-34599. Deletion of the hydroxyl group of U-46195 completely abolished the stimulating effect, whereas substitution by a methoxy group partially quenched the effect of U-46195 on t-PA production. The molecular variations we examined in U-51477 (mainly the introduction of new groups) generally diminished the effect on t-PA expression. Triazolo "benzodiazepine fragments" and open ring triazolo and imidazolo benzodiazepines (benzophenones) related to nizofenone (claimed to be effective in subarachnoid haemorrhage [41]) were ineffective in stimulating t-PA synthesis.

Table 1. Relationship between t-PA stimulatory effects of triazolobenzodiazepines, their affinity for the benzodiazepine receptor and their PAF antagonist activity

| Compound | [$^3$H]FNZ* $K_i$ (nM) | PAF antagonist activity† | Stimulation of t-PA synthesis‡ |
|---|---|---|---|
| U-46195 | 1.0 | 4.75 | 3.9 |
| U-34599 | 34 | 1.9 | 4.2 |
| U-51477 | 78 | 1.0 | 3.0 |
| U-45699 | 950 | 1.0 | 1.8 |
| Triazolam | 0.80 | 1.0 | 1.2 |
| Alprazolam | 3.1 | 0.5 | 1.9 |
| U-31805 | 73 | 0.2 | 1.3 |
| U-40125 | 5.8 | 0.2 | 1.0 |
| Clonazepam | 0.94 | Inactive | 1.0 |
| Diazepam | 5.9 | Inactive | 1.1 |
| U-40129E | 1700 | Inactive | 1.1 |

* $K_i$ for [$^3$H]flunitrazepam ([$^3$H]FNZ) binding to the cerebral cortex (V. H. Sethy, personal communication).

† Activity ratio (triazolam = 1), human platelets (R. R. Gorman, personal communication).

‡ Endothelial cells were incubated for two periods of 24 hr with 3 μg/mL of triazolobenzodiazepine; results are means of duplicate incubations and expressed as -fold stimulation relative to t-PA synthesis in control incubations.

## DISCUSSION

The endogenous fibrinolytic system has been implicated in the etiology of cardiovascular disease. Specifically, the lack of a sufficient fibrinolytic capacity has been noted in patient populations with thrombosis [1–4]. Thus, a low fibrinolytic potential represents a target for a new therapeutic approach in the treatment of thrombosis and myocardial infarction. Stimulation of the fibrinolytic capacity in plasma would be a powerful means to improve the morbidity and mortality associated with myocardial infarction. This has led to an interest in the mechanism(s) involved in the regulation of plasma fibrinolytic activity. Cultured human endothelial cells have been shown to represent a useful model system in finding compounds that might stimulate endogenous fibrinolysis in humans and allow detailed studies of regulatory mechanisms of t-PA and PAI-1 synthesis [5].

The present experiments indicate that selected triazolobenzodiazepine analogues possessing PAF antagonist activity can stimulate t-PA synthesis in cultured human endothelial cells, with no or even a lowering effect on PAI-1 production. The most potent compounds, U-34599, U-41695 and U-51477 (see Scheme 1), were studied in more detail, and showed a time- and concentration-dependent stimulatory effect on t-PA synthesis. At optimal stimulatory concentrations (~10 μM) these three compounds stimulated t-PA expression about 2-fold after 24 hr and maximally about 4-fold after 48 hr (Fig. 1). These increases in t-PA synthesis followed similar increases in t-PA mRNA levels (Fig. 2), while no significant changes in PAI-1 mRNA levels were seen. The results obtained with the triazolobenzodiazepines are comparable with the data reported for retinoids [13], both with respect to the stimulatory effect on t-PA expression and the unchanged PAI-1 expression.

The potential future usefulness of triazolobenzodiazepines in stimulating endogenous fibrinolytic activity would depend on their pharmacokinetic properties, particularly with respect to reaching the desired target tissue, i.e. the endothelium, and without undesirable side effects. Since benzodiazepines belong to one of the most widely used classes of drugs in therapy due to their anxiolytic, anticonvulsant and sedative properties, much is known about their pharmacology. Besides their interaction with specific recognition sites located in the brain, the "central" benzodiazepine receptors [37–39], benzodiazepines bind to membranes prepared from various tissues containing the so-called peripheral-type benzodiazepine recognition site(s)/receptors [37]. It is now generally assumed that the specific high affinity binding sites in the brain are the receptors by which the benzodiazepines exert their pharmacologically and clinically relevant actions. The physiological role of the peripheral-type benzodiazepine receptors has remained unknown, although they have been implicated in a number of cellular phenomena, e.g. protooncogene expression [42]. In our studies, no positive correlation between benzodiazepine receptor binding activity and the ability of the triazolobenzodiazepines to stimulate t-PA production was observed (Table 1). In accordance with this, no specific binding of [$^3$H]flunitrazepam, a ligand for benzodiazepine receptors, to endothelial cell membrane preparations was observed. Binding studies using the ligand Ro 5-4864 showed no specific binding to endothelial cell membrane preparations, indicating the absence of peripheral-type benzodiazepine receptors on the endothelium. Thus, it is unlikely that the triazolobenzodiazepines act through benzodiazepine receptors to stimulate t-PA production.

A possible lead to the basic mechanism of action of triazolobenzodiazepines was suggested by the

relationship between the ability of the triazolobenzodiazepines to stimulate t-PA production and their PAF antagonist activity (Table 1). However, several non-benzodiazepine PAF antagonists did not stimulate t-PA production. Thus, the regulatory pathway by which the triazolobenzodiazepines exert their t-PA stimulatory action is not understood at present. Selected substitutions or deletions in the chemical structures of the lead compounds clarified many of the structural prerequisites for optimal t-PA stimulatory activity. However, we found no compounds more potent than our lead compounds.

Further research should now be directed at testing the triazolobenzodiazepines in *in vivo* thrombosis models to evaluate whether or not this class of compounds represent a reasonable therapeutic agent for the prevention or treatment of acute or subacute intravascular thrombosis.

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