Antagonistic Activity of Etizolam on Platelet-Activating Factor 
In Vivo Experiments

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Abstract—The ability of etizolam, 6-(o-chlorophenyl)-8-ethyl-1-methyl-4H-s-triazolo[3,4-c]thieno[2,3-e][1,4]diazepine (Y-7131), an anti-anxiety drug, to inhibit platelet-activating factor (PAF)-induced reactions was investigated in experimental animals in vivo. Etizolam (0.01–0.3 mg/kg, i.v.) dose dependently inhibited PAF (0.3 μg/kg, i.v.)-induced bronchoconstriction (Konzett and Rössler's method) in guinea pigs, but even at doses as large as 3 mg/kg, i.v., it had no effect on bronchoconstriction induced by histamine, serotonin, acetylcholine, arachidonic acid, bradykinin, angiotensin I or leukotriene D₄. Etizolam (0.1–1 mg/kg, i.v.) also dose-dependently reversed PAF (1 μg/kg, i.v.)-induced hypotension in anesthetized rats. Injection of PAF into the tail veins of mice produced lethal shock within 10–30 min. Etizolam (0.1–3 mg/kg, i.v. and 1–10 mg/kg, p.o.) protected against the lethal effect of PAF (75 μg/kg, i.v.) in a dose-dependent manner. These results indicate that etizolam specifically inhibits the action of PAF in vivo.

Platelet-activating factor (PAF-acether, 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) is a phospholipid originally described as a mediator of anaphylaxis, which is released by rabbit circulating basophils (1). It has since been found to be synthesized and released by a variety of cells including macrophages (2), basophils (1), neutrophils (3, 4), platelets (5), mast cells (6), eosinophils (7) and endothelial cells (8). PAF is considered to be an important mediator of inflammation and allergic reactions (9).

Several PAF antagonists have been reported (10). CV-3988, an analogue of PAF, is one of these compounds which inhibits PAF-induced reactions in vivo and in vitro (11, 12). Recently, the psychotropic triazolobenzodiazepine drugs alprazolam and triazolam have been shown to potently inhibit PAF-induced changes in the shape, aggregation and secretion of washed human platelets (13). Their effect was specific for PAF activation, in as much as they failed to inhibit the response of human platelets to adenosine diphosphate, thrombin, epinephrine, collagen, arachidonic acid and the calcium ionophore A 23187.

6-(o-Chlorophenyl)-8-ethyl-1-methyl-4H-s-triazolo[3,4-c]thieno[2,3-e][1,4]-diazepine (Y-7131, etizolam, Depas®) is one of the thienodiazepine derivatives with a triazole ring synthesized by Tahara et al. (14). Etizolam has the typical pharmacological and biochemical properties of other anti-anxiety drugs (15, 16). It is used clinically as a potent and characteristic anti-anxiety drug which is different from the benzodiazepines.

This paper demonstrates that etizolam is a potent and specific inhibitor of the action of PAF in experimental animals in vivo.

Materials and Methods

1. Animals: Female Hartley guinea pigs, male Lewis rats (Seiwa Institute of Experimental Animals, Fukuoka, Japan) and male ICR mice (Charles River, Kanagawa, Japan) were used. All animals were housed at constant temperature (23±2°C) and relative humidity (55±5%), and they were allowed free access to food and water.
2. Materials: Etizolam (Y-7131), triazolam, BW755C and indomethacin were synthesized by the chemical division of our laboratory. Dexamethasone was purchased from Sigma Chemical Co. All test drugs were suspended in 0.5% methylcellulose solution for oral administration. Etizolam and triazolam were dissolved in propylene glycol for intravenous administration. Agents used were platelet-activating factor (PAF, Serdary Research Lab.), histamine dihydrochloride (His, Nakarai), serotonin creatinine sulfate (5-HT, Sigma), acetylcholine chloride (Ach, Sigma), arachidonic acid sodium salt (AA, Sigma), bradykinin triacetate (BK, Sigma), angiotensin I acetate salt (Ang I, Sigma) and leukotriene D4 (LTD4, Paesel GmbH & Co.). A stock solution of PAF was made in ethanol and stored at -20°C. The solution was diluted in 0.9% saline and placed on ice for use. All other agents were dissolved in 0.9% saline before use.

3. Bronchoconstriction in guinea pigs: Resistance to lung inflation was measured by the overflow method of Konzett and Rossler (17), modified by the use of an air pressure transducer for electrical recording. Female Hartley guinea pigs (350-450 g) were anaesthetized with urethane (1.5 g/kg, i.p.). An endotracheal tube was inserted in the trachea and connected to an air pump (Matsushita Denko Co., Ltd.) with an exhaust relay of 50 strokes/min (PK-0305-NC, Takasago Electric, Inc.). The animals were artificially ventilated at a constant volume (3-4 ml). Tracheal pressure was recorded with a side-arm of the cannula connected to a pressure transducer (MFP-1, Nihon Kohden Kogyo Co., Ltd.) with an exhaust relay of 50 strokes/min (PK-0305-NC, Takasago Electric, Inc.). The animals were artificially ventilated at a constant volume (3-4 ml). Tracheal pressure was recorded with a side-arm of the cannula connected to a pressure transducer (MFP-1, Nihon Kohden Kogyo Co., Ltd.). Test drugs or drug vehicle were administered i.v. 2 min prior to the i.v. injection of PAF (0.03-1 µg/kg), His (2.5 µg/kg), 5-HT (10 µg/kg), Ach (10 µg/kg), AA (0.4 mg/kg), BK (4 µg/kg), Ang I (25 µg/kg) or LTD4 (10 µg/kg). All compounds were injected through the cannulated jugular vein. The peak bronchoconstriction of each animal was determined as the percentage of the maximal pressure obtained by clamping off the trachea at the end of each experiment. The inhibitory effect of the drugs was expressed as follows:

\[ \% \text{ of inhibition} = (1 - t/c) \times 100 \]

where t and c are the average peak bronchoconstrictions for the test and control groups, respectively.

4. PAF-induced hypotension in rats: The test was carried out according to the method described by Terashita et al. (18). Male Lewis rats (250–300 g) were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). The mean arterial blood pressure was measured with a pressure transducer (MPU-0.5, Nihon Kohden Kogyo Co., Ltd.) connected to a polyethylene catheter placed in the right carotid artery. The left jugular vein was cannulated with a polyethylene catheter for i.v. administration of PAF and drugs. The drugs were given when the blood pressure reached a minimum, 3 min after the PAF injection. Blood pressure changes were observed for 10 min after the PAF injection.

5. PAF-induced mortality in mice: Male ICR mice (25–30 g) were used. PAF (10–100 µg/kg) solution was administered i.v. in a lateral tail vein. All animals were observed for at least 24 hr after the PAF (75 µg/kg) injection. Results were given as 24 hr survival rates. Drugs were given i.v. 15 min before the PAF injection or p.o. 3 hr before the PAF injection.

6. Statistical analysis: Statistical significance was evaluated by analysis of one-way layout. LD50 and 95% confidence limits were obtained by the probit method.

Results

1. Effect on bronchoconstriction in guinea pigs: PAF (0.03–1 µg/kg, i.v.) produced a dose-related bronchoconstriction (ED50=0.3 µg/kg) that peaked at 1 min. The PAF concentration (0.3 µg/kg) producing 40–60% maximal bronchoconstriction was chosen for evaluating the drug effects. Etizolam (0.01–0.3 mg/kg) and triazolam (0.01–0.3 mg/kg), administered i.v. 2 min before the PAF injection, significantly inhibited the PAF-induced bronchoconstriction in a dose-dependent manner (Fig. 1), with ED50 values of 0.022 and 0.066 mg/kg, respectively. At 0.3 mg/kg, etizolam completely inhibited the bronchoconstriction, and this effect persisted for at least 30 min (Fig. 2). However, even at 3 mg/kg, i.v., it
was inactive against the bronchoconstriction caused by His, 5-HT, Ach, AA, BK, Ang I and LTD4, with inhibition percentages of -3%, 4%, 3%, 18%, 9%, 29% and 24%, respectively. Etizolam tended to inhibit the Ang I and LTD4-induced bronchoconstriction, but these results were not statistically significant (N=5).

2. Reversal of PAF-induced hypotension in rats: PAF (1 μg/kg) markedly reduced blood pressure, reaching a nadir 1 min after the injection, followed by a slow recovery to normal which took more than 10 min. Etizolam (0.1–1 mg/kg, i.v.) quickly reversed this hypotension in a dose-dependent manner (Fig. 3). The reversed blood pressure 1 min after the drug injection, expressed as the percentage of the PAF-induced blood pressure reduction, was 31%, 54% and 70% for 0.1, 0.3 and 1 mg/kg, respectively.

3. Effect on PAF-induced mortality in mice: Tail vein injections of 10–100 μg/kg PAF into ICR mice produced lethal shock dose-dependently, with an LD50 value of 28 μg/kg and 95% confidence limits of 20–35 μg/kg (Fig. 4). Although all groups of animals were observed for at least 24 hr after the PAF injection, death occurred within 10–30 min with very few exceptions. At 75 μg/kg, i.v., PAF caused almost complete mortality within 24 hr (Fig. 4). Accordingly, this dose was chosen for the subsequent experiments. At this dose, animals showed general depression, labored respiration and cardiac depression prior to death.
Etizolam (0.1–3 mg/kg, i.v. and 1–10 mg/kg, p.o.) protected against the lethality of PAF in a dose-dependent manner (Table 1). Oral administration of dexamethasone (20 mg/kg) 3 hr before the PAF injection also caused complete protection. Indomethacin (10 mg/kg, p.o.) was ineffective, while the oral administration of a dual inhibitor of lipoxygenase and cyclooxygenase, BW755C (100 mg/kg), caused good protection (Table 1).

**Discussion**

Intravenous PAF induces a platelet dependent bronchoconstriction in guinea pigs (19). This bronchoconstriction is little affected by cyclooxygenase inhibitors alone (19, 20), but lipoxygenase inhibitors and the leukotriene antagonist FPL-55712 are effective (21). Recently, Desquand et al. (22) reported that a selective PAF antagonist, BN 52021 (1 mg/kg, i.v. and 10 mg/kg, p.o.), inhibited the bronchoconstriction, the hematocrit increase and the accompanying thrombopenia and leukopenia induced by PAF. Their experiments showed that PAF-induced bronchoconstriction is useful for demonstrating the systemic activity of novel PAF antagonists.

In recent years, the psychotropic triazolo-benzodiazepine drugs alprazolam and triazolam have been shown to be potent and specific inhibitors of the PAF activation of human platelets (13). Our results (Fig. 1) showed that one of their analogues, etizolam, like triazolam, inhibited the PAF-induced bronchoconstriction, but at a relatively low

![Graph showing mortality induced by various i.v. doses of PAF in mice. LD50 value and 95% confidence limits were 28 µg/kg and 20–35 µg/kg, respectively (n=10–14).](image)

**Table 1. Effect of etizolam and standard drugs on PAF-induced mortality in mice**

<table>
<thead>
<tr>
<th>Compound</th>
<th>mg/kg</th>
<th>Route</th>
<th>No. Survivors</th>
<th>Survival rate (%)</th>
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<tr>
<td></td>
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<td>No. Tested</td>
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<tr>
<td>Vehicle</td>
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<td>i.v.</td>
<td>0/10</td>
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<tr>
<td>Etizolam</td>
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<td>9</td>
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<td></td>
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<td></td>
<td>3/10</td>
<td>30</td>
</tr>
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<td></td>
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<td>75</td>
</tr>
<tr>
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<td>3</td>
</tr>
<tr>
<td>Etizolam</td>
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<td></td>
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Test drugs were given i.v. 15 min before PAF (75 µg/kg) injection or p.o. 3 hr before PAF injection.
dose range (0.01–0.3 mg/kg, i.v.). The efficacy of etizolam was 3 times that of triazolam. Thus, the effect of etizolam may be due to an antagonism of PAF. The fact that even at a dose of 3 mg/kg, i.v., etizolam failed to block bronchoconstriction induced by His, 5-HT, Ach, AA, BK, Ang I and LTD₄ indicates that it does not inhibit the PAF-induced bronchoconstriction via nonselective bronchodilation or an antagonist of LTD₄.

In the rat, PAF induces severe hypotension, but causes neither bronchoconstriction nor thrombopenia. Although the precise mechanism of the hypotensive action of PAF is not completely known, peripheral vasodilation and α-adrenergic antagonism probably contribute to the action (23). Furthermore, Terashita et al. (11) suggest that neither β-adrenergic, histaminergic and cholinergic receptor mediation nor cyclooxygenase products of arachidonic acid may be involved in the PAF-induced hypotension, because neither blocking these receptors nor administering indomethacin had any effect on the hypotension.

Etizolam also markedly reversed the hypotension induced by PAF (Fig. 4), an effect similar to that of CV-3988 as described by Terashita et al. (18).

PAF-induced mortality in the mouse is a useful model for the in vivo evaluation of steroids which protect against anaphylactic shock (24), lipoxygenase inhibitors, and leukotriene antagonists (25) as well as PAF antagonists. Since mouse platelets, like those of the rat, are insensitive to PAF (26), this model may operate by some kind of platelet-independent mechanism. Young et al. (25) showed that ICR mice were sensitive to PAF-induced mortality. Accordingly, we used ICR mice to evaluate the anti-PAF effect of etizolam. As shown in Table 1, single oral or intravenous doses of etizolam inhibited the lethal effect of PAF in a dose-dependent manner.

The inactivity of indomethacin suggests that cyclooxygenase products of arachidonic acid do not mediate PAF toxicity. In contrast, dexamethasone and the lipoxygenase inhibitor BW755C were highly protective against PAF induced sudden death, confirming the results described by Young et al. (25). These results also suggest that leukotrienes formed via the 5-lipoxygenase pathway play a major role in PAF-induced death in mice.

Takehara et al. (27) reported that etizolam inhibited PAF-induced aggregation and chemotaxis of polymorphonuclear leucocytes, but did not inhibit similar reactions induced by formyl-methionyl peptide (FMLP) and zymosan-activated serum (ZAS). In contrast, the 5-lipoxygenase inhibitor nordihydroguaiaretic acid inhibited both reactions induced not only by PAF but also those by FMLP and ZAS. Their results suggest that etizolam has almost no effect on 5-lipoxygenase pathways, and they also indicate that the inhibitory effect of etizolam on PAF-induced death may be due to an antagonism of PAF at PAF receptor sites.

There is little or no direct evidence for a role of PAF under various clinical conditions due to the lack of available PAF antagonists. Since etizolam is now used clinically as an anti-anxiety drug, it could be used for investigating the role of PAF in humans.

The above findings indicate that etizolam specifically inhibits the action of PAF in vivo, so that it may be useful in treating disorders caused by PAF.

References


