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A SPECIFIC, PHOTOLABILE AND IRREVERSIBLE ANTAGONIST(L662,025) OF THE PAF-RECEPTOR

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SUMMARY: PAF (0.2 μM) induced maximal platelet aggregation in human PRP and [^{3}H]-PAF (1 - 5 nM) binding to platelet membrane preparations had Kd value of 3.8 nM and B_{max} of 200 fmoles/mg of protein. Without UV irradiation, a synthetic azido tetrahydrofuran derivative L662,025 was a reversible and competitive PAF-receptor antagonist with IC_{50} values of 5.6 $\pm$ 0.3 μM (platelet aggregation) and 1.0 $\pm$ 0.25 μM (receptor binding). Photolysis of L662,025 in the presence of PRP produced an irreversible inhibition of platelet aggregation and specific binding of [^{3}H]-PAF (1 nM). L662,025 did not affect collagen- or ADP-induced human platelet aggregation before or after photolysis. It is a new probe that can be used to identify and characterize the PAF-receptor.

Platelet-activating factor (PAF) is a highly potent ether-linked phospholipid (1-O-alkyl-2-acetyl-sn-glycerol-3-phosphoryl-choline) which activates platelets as well as modulates the function of leukocytes, and other target cells. Many biological actions of PAF have been attributed to its interaction with a specific membrane recognition site coupled to phosphatidyl-inositol metabolism (1, 2, 3). Specific PAF receptors have been identified in a variety of cells by radioreceptor studies followed by Scatchard analyses of the data (4, 5). A number of reversible PAF-receptor antagonists have recently been developed (6, 7, 8).

Data on the purification and characterization of the PAF-receptor protein have been published in the last five years (9, 10, 11, 12). The use of affinity chromatography and radiolabeled irreversible ligand is an essential pre-requisite for characterizing a highly purified receptor. In this communication, we report the biochemical properties of a novel photoactivable antagonist, L662,025 (an azido derivative of

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substituted trans-2,5-diaryltetrahydrofuran) which may be used as a probe for characterizing the PAF-receptor.

MATERIALS

[^{3}H]-PAF (specific activity = 40.1 Ci/mmole) and Aquasol-2 (scintillation cocktail) were purchased from NEN (Boston, MA). L662,025 was a gift from Dr. M. N. Chang and K. L. Thompson (Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.). Adenosine diphosphate (ADP) was purchased from Sigma Chemicals (St. Louis, MO) and collagen was from Chron-log (Havertown, PA).

STATISTICS

Results are presented as the mean $\pm$ standard error of the mean (s.e.m.). Statistical differences between results were determined using the Student 't'-test for paired and unpaired data as appropriate. A probability (P) value of 0.05 or less was considered significant.

METHODS

(i) Platelet Aggregation

Blood was drawn from healthy donors (who had not ingested aspirin-like drugs or steroids in the previous 10 days) into a plastic syringe containing 3.8% w/v trisodium citrate (10 : 1 v/v) and centrifuged at room temperature to obtain platelet-rich plasma (PRP). The platelet count was adjusted to 5 x 10^8 platelets/ml. Platelet aggregation was measured at 30°C turbidometrically using a dual-channel aggregometer (Chrono-log, PA, Model 340 Vs) coupled to a pen recorder. PAF-receptor antagonists were added to the cuvettes 2 min before the injection of PAF, ADP or collagen and aggregation allowed to proceed for 5 min.

(ii) Radioreceptor Studies

Platelet membranes were prepared as described by Hwang et al (4) using sucrose density gradients. The binding of [^{3}H]-PAF to membrane fraction was carried out in a 1 ml reaction mixture containing 100 μg of membrane protein, [^{3}H]-PAF (0.5 - 5 nM) with or without unlabeled PAF_{C-16} (1000-fold) and a solution of $MgCl_2$ 10 mM, EDTA 2 mM and Tris-HCl 10 mM (pH 7.4). The reaction was incubated at 0°C for 1 hour. The free and bound ligand were separated by a filtration technique. The difference between total amount of bound [^{3}H]-PAF in the absence and presence of excess unlabeled PAF_{C-16} was defined as specific binding of the radiolabeled ligand. In a set of experiments, [^{3}H]-PAF (1 nM) was incubated with different concentrations of L662,025 and the effect of the antagonist on the specific binding was expressed as percent inhibition of the control.

(iii) Photolysis

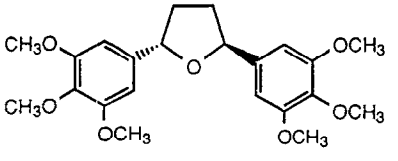
PRP and platelet membrane preparations (1 ml) in siliconized quartz test tubes were incubated with L662,025 (1-20 μM) and exposed to UV light supplied by a medium mercury lamp (SP-200) for 1 - 15 min at 4°C or room temperature. $CuSO_4.5H_2O$ (5% w/v) was used as a chemical filter to exclude infra-red light which may denature the proteins. In some experiments, platelet

membrane preparations exposed to UV light were washed by centrifugation at 14,000 x g for 5 min (three times) before radioreceptor studies were carried out.

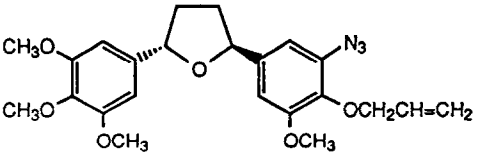
RESULTS

L662,025 is an azido derivative of trans-2,5-aryltetrahydrofuran (Fig. 1). Table 1 shows the IC_{50} (concentration of an antagonist required to produce 50% inhibition of maximal aggregation induced by PAF) values of selected tetrahydrofuran derivatives in human PRP. The relative order of potency is L659,989 > L653,150 > L662,025 $\geq$ L652,731. None of these compounds had any effect on ADP (1 - 5 μM) or collagen (1 - 5 μg/ml) produced platelet aggregation (data not shown).

In order to determine the appropriate wavelength for the photoactivation, UV spectra were recorded after treating an ethanolic solution of L662,025 with UV light for various times. Following irradiation, the UV absorption of the azido compound increased between the wavelength of 260 and 300 nm. Thus, subsequent photoaffinity-coupling experiments were performed by irradiating PRP and platelet membranes with UV light of 260 - 320 nm wavelength for 15 min.



L-652,731



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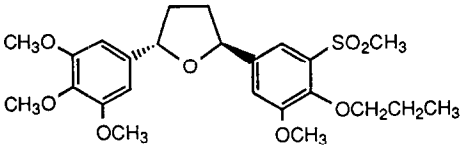
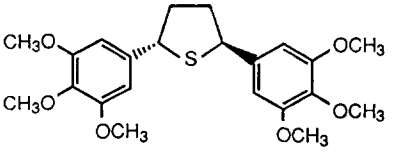


L-659,989



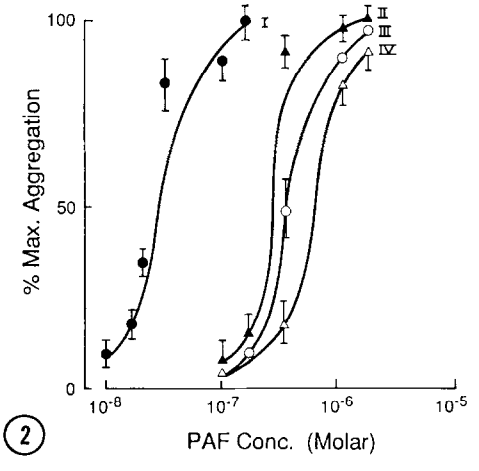
L-653,150

Figure 1. Chemical structures of tetrahydrofuran derivatives.

TABLE 1: IC_{50} values for tetrahydrofuran derivatives in inhibiting PAF (0.2 μM) induced platelet aggregation in PRP (n = 5)

Antagonist	IC_{50} (μM)
L659,989	0.8 ± 0.1
L653,150	4.4 ± 0.3
L662,025	5.6 ± 0.3
L652,731	6.0 ± 0.5

In platelet aggregation experiments, L662,025 (6 - 20 μM) prior to photoactivation, displaced the responses of the platelets to PAF (0.01 - 0.2 μM) to the right (Fig. 2). The asymptote was produced by increasing PAF concentration. In order to demonstrate the covalent linkage of L662,025 to the PAF-receptor, platelet aggregation induced by PAF was assessed following exposure of PRP and the azido compound to UV light for 15 min (Fig. 3). Increasing PAF concentration to 5.0 μM did not



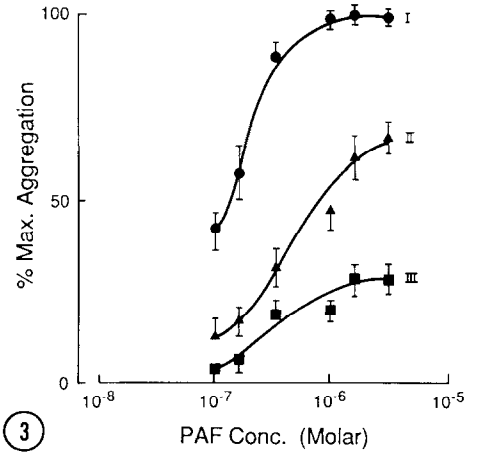


Figure 2. Dose - Percent maximum aggregation curves for PAF in the absence (I) and presence of L662,025 6 μM (II), 10 μM (III) and 20 μM (IV) before photoactivation (control). Vertical bars are mean ± s.e. (n = 5).

Figure 3. Effect of L662,025 6 μM (I), 10 μM (II) and 20 μM (III) on PAF-induced human platelet aggregation after photoactivation (260 - 320 nM) for 15 min. The control curve (without L662,025) for PAF was the same as shown in Figure 2.

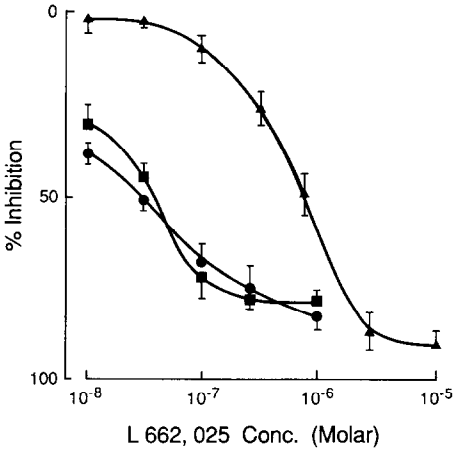


Figure 4. Platelet membrane binding on [^{3}H]-PAF (1 nM, 6 x 10^4 CPM) in the presence of L662,025 before photolysis (▲——▲), after photolysis without (●——●) and with (■——■) washing three times by centrifugation at 14,000 x g for 5 min. Results show mean of 3 experiments.

produce platelet aggregation above the EC_{50} value in the presence of L662,025 (20 μM). In contrast, PAF still produced maximum platelet aggregation in PRP irradiated with either L652,731, L659,989 or L653,150, which are not photoactive.

The binding of [^{3}H]-PAF (1 nM) to human platelet membrane preparations exhibited a single binding site with Kd value of 3.8 nM and B_{max} of 200 fmoles/mg of protein (Scatchard plot not shown). [^{3}H]-PAF was displaced by L662,025 (Fig. 4) with an IC_{50} value of 1.0 $\pm$ 0.25 μM. Photolysis of platelet membranes with L662,025 (at 4^{o}C) produced an even greater inhibition which was not removed by repeated washing (by centrifugation at 14,000 x g for 5 min, 3 times). In experiments where either L662,025, L652,731 or L659,989 and platelet membrane preparations were washed without exposure to UV light, the antagonist did not inhibit [^{3}H]-PAF binding. The specific bindings of [^{3}H]-PAF (1 nM) to human platelet membranes before and after UV irradiation were 142 $\pm$ 15 and 122 $\pm$ 17 fmoles/mg of protein, respectively.

DISCUSSION

In the last five years, a number of competitive and reversible PAF-receptor antagonists have been developed. These include PAF analogues e.g. CV-3988, ONO-6248, SR-63441 (13, 14, 15), kadsurenone (7), extract (BN 52021) from Ginkgo biloba (3), derivatives of tetrahydrofuran lignans (6, 16) and a host of other synthetic compounds. This report presents the biochemical

properties of a photolabile reversible PAF-receptor antagonist, L662,025, which upon photoactivation irreversibly inhibits PAF-induced platelet aggregation and binding of [^{3}H]-PAF to platelet membrane preparations.

Before photolysis, L662,025 produced a concentration dependent inhibition of PAF-induced aggregation of human platelets with a parallel shift of the dose-response curve to the right and reproduction of the maximum effect of the control by increasing PAF concentration. Following photolysis of human platelets with the azido compound (20 μM), the asymptote of the concentration-percent maximum aggregation curve was dropped below the EC_{50} value of PAF and was not reversed by increasing the ligand concentration. L662,025 had no effect on ADP- or collagen-induced platelet aggregation before or after photolysis. This indicates that L662,025 is a specific PAF antagonist.

Since PAF binds to a specific recognition site on cells or tissue membranes to elicit its biological action, we have assessed the effect of L662,025 on the binding of [^{3}H]-PAF to human platelet membrane preparations. The photoactivable antagonist produced a dose-related inhibition of [^{3}H]-PAF binding to membrane fractions both before and after exposure to UV light. Repeated (three times) washing of the photolyzed platelet membranes incubated with L662,025 did not alter the effect of the azido compound on [^{3}H]-PAF receptor binding. In contrast, centrifugation at 14,000 x g for 5 min (at least three times) removed the effect of L659,989, L652,731 and L662,025 on the binding of [^{3}H]-PAF to human platelet membranes. In fact, we observed a significant increase in binding of [^{3}H]-PAF to the washed membrane preparations.

The structure-activity relationships of kadsurenone and diaryltetrahydrofuran derivatives indicated that both allyl and n-propyl substituents have comparable activity-enhancing effects (8). This observation has led to the preparation of [^{3}H]dihydro-kadsurenone, by tritiation of the allyl side-chain, as a PAF-receptor ligand (17). Similarly the allyloxy group in L662,025 may provide a convenient site for the introduction of tritium labels to form a [^{3}H]dihydro derivative of L662,025 as a radiolabeled irreversible probe of the PAF-receptor.

These results indicate that L662,025 is a specific and high affinity ligand of the PAF-receptor and binds covently to the receptor following photoactivation. The azido compound is a new

probe that could be used in the identification and characterization of the PAF-receptor.

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REFERENCES

1. Snyder, F (1985) Medicinal Research Review, 5, 107-140.

2. Hanahan, D.J. (1986) Ann. Rev. Biochem., 55, 483-509.

3. Braquet P., Shen, T.Y., Touqui, L. and Vargaftig, B.B. (1987) Pharmacol. Rev., 39, 97-145.

4. Hwang, S-B., Lee, C-S. C, Cheah, M.J. and Shen, T.Y. (1983) Biochem., 22, 4756-4763.

5. Valone, F.H. and Goetzl, E.J. (1983) Immun., 48, 141-149.

6. Biftu, T., Gamble, N.F., Doebber, T.W., Hwang, S.-B., Shen, T.Y., Snyder, J., Spunger, J.P. and Stevenson, R. (1986) J. Med. Chem., 23, 1917-1921.

7. Shen, T.Y., Hwang, S-B., Chang, M.N., Doebber, T.W., Lam, M.H., Wu, M.S., Wang, X., Han, G.O. and Li, R.Z. (1985) Proc. Natl. Acad. Sci. U.S.A., 82, 672-676.

8. Shen, T.Y., Hwang, S-B., Doebber, T.W. and Robbins, J.C. (1987) In: Platelet-Activating Factor and Related Lipid Mediators, edited by F. Snyder, Plenum Press, New York, 1987, pp 153-190.

9. Valone, F.H. (1984) Immun., 52, 169-174.

10. Nishihira, J., Ishibashi, T., Imai, Yol and Muramatsu, T. (1985) Tohoku J. Exp. Med., 147, 145-152.

11. Chau, L.Y. and Jil, Y.L. (1988) Biochim. Biophys. Acta, 970, 103-112.

12. Shen, T. Y., Hussaini, I., Hwang, S.-B. and Chang, M.N. (1988) Taipei Conference on Prostaglandin and Leukotriene Research, 60p 569.

13. Terrashita, Z., Tsuchima, S., Yoshiota, Y. Normura, J., Inada, Y. and Nishikoda, S. (1983) Life Sciences, 32, 1975-1982.

14. Winslow, C.M., Gubler, H.U., Delillo, A.K., Davies, F.J., Frisch, G.E., Tomesch, J.C. and Saunders, R.N. (1986) Second International Conference on platelet-activating factor and structurally related alkyl ether lipids, Gatlinburg, Tennessee, October, 33p.

15. Miyamoto, T., Ohno, H., Tano, T., Okada, T., Hamanaka, N. and Kawasaki, A. (1984) Third International Conference on Inflammation, Paris, Sept. 513-519.

16. Ponpidom, M.M., Hwang, S-B., Doebber, T.W., Acton, J.J., Leinweber, F.J., Klevans, L. et al. (1988) Biochem. Biophys. Res. Comm., 150, 1213-1220.

17. Hwang, S-B., Lam, M-H. and Chang, M.N. (1986) J. Biol. Chem., 261, 13720-13726.