# Tachykinin Antagonists Screening from Microbial Origin

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Data from several studies suggest that tachykinins may play an important role in the pathophysiology of airway diseases, especially asthma. We had a screening program for selective tachykinin antagonists of microbial products origin. In the previous papers we described the discovery and characterization of WS9326, FK224[1,2] and actinomycin D[3] as tachykinin antagonists. We screened them for activity in a receptor binding assay to tachykinin receptors on guinea-pig lung membrane and for inhibition of the neurokinin A (NKA)-induced guinea-pig tracheal contractile response. In this paper we report on cyclosporin C (WF3484) as an inhibitor of substance P (SP)-induced platelet aggregation, and on the receptor binding properties of cyclosporin C, actinomycin D, WS9326 and FK224 for human tachykinin receptor subtypes in transfected mammalian cells.

We discovered the phenomenon that SP or NKA can induce the platelet aggregation *in vitro*. By application of this phenomenon, we started to search for selective inhibitors of SP-induced platelet aggregation *in vitro*. In the course of our screening for SP-induced platelet aggregation inhibitors, we have isolated WF3484 from the cultured broth of *Nectria* sp. No. 3484. The structure elucidation studies of WF3484 demonstrated that WF3484 is identical with cyclosporin C. Cyclosporin C is a well-known immunosuppressive agent. However, tachykinin antagonistic activity of the compound is not known. Here, we wish to describe the isolation of WF3484, its identification as cyclosporin C, and show the unique anti-tachykinin (SP, NKA) activities of the compound.

*Nectria* sp. No. 3484 was cultivated in four 500-ml Erlenmeyer flasks each containing 120 ml of a medium containing (% w/v): soluble starch 3, glucose 1, pharmamedia 1, dried yeast 1, peptone 0.5, corn steep liquar 0.5 and $CaCO_3$ 0.3. These flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) for 4 days at 25°C and then transfered to the production medium. Production of WF3484 was examined in a 30-liter jar fermentor containing 20 liters of production medium containing (% w/v) soluble starch 3, sucrose 1, wheat germ 1, dried yeast 0.5 and $CaCO_3$ 0.2. The fermentation was carried out at 25°C for 4 days under aeration of 20 liters/minutes, back pressure of 1.0 kg/cm$^2$ and agitation speed of 250 rpm. The fermentation broth (19 liters) was extracted with acetone (19 liters) and the extract was filtered. The extract (38 liters) was diluted to 48 liters with 10 liters of water and passed through a Diaion HP-20 column (4 liters). The column was washed with 10 liters of 50% acetone and adsorbent was eluted with 10 liters of 90% acetone. The eluate was concentrated under reduced pressure to an aqueous solution. The solution was extracted with ethyl acetate (1 liter). The organic layer was washed with 0.5 liter of water two times. The organic layer was concentrated under reduced pressure and the ethyl acetate extract was subjected to column chromatography on silica gel (Kiegel gel 60, Merck). The column was washed with chloroform and active compound was eluted with chloroform - methanol (20 : 1). The eluate was concentrated to give 490 mg of WF3484 as an amorphous powder. WF3484 is soluble in methanol, ethanol, acetone, ethyl acetate and diethyl ether and chloroform and it is insoluble in *n*-hexane. The molecular formula was determined to be $C_{62}H_{111}N_{11}O_{13}$ by FAB-MS and elementary analysis. The molecular formula of WF3484 is consistent with it being cyclosporin C. The physico-chemical properties (data not shown) of WF3484 are in good agreement with the reported data for cyclosporin C[4]. Finally WF3484 as identified as cyclosporin C by direct comparison with our authentic specimen of cyclosporin C.

Platelet aggregation was measured by the turbidimetric technique of BORN and CROSS[5] in NKK Hema tracer (Niko Bioscience Inc.). In brief, blood was collected from the central ear arteries of male Japanease White rabbit (male 11 week; 1.5 ~ 2.0 kg body weight). The blood was prevented from coagulation with 1 volume of 3.8% sodium citrate to 9 volumes of blood. Platelet rich plasma (PRP) was prepared by centrifugation of the blood at $150 \times g$ for 10 minutes at room temperature. For most experiments PRP-saline/inhibitor mixtures (0.25 ml PRP plus 0.02 ml saline/inhibitor) were incubated in an aggregometer with stirring (1,000 rpm) at 37°C for 2 minutes, and then the aggregating agent (0.03 ml) was added. Platelet aggregation was measured turbidimetrically by recording changes in the light transmission of PRP during aggregation. Activities of inhibitors were expressed as $IC_{50}$ values *i.e.* concentration required to inhibit the platelet aggregation responses by 50%. $2 \times 10^{-6}$ M ADP (Sigma), $2 \times 10^{-6}$ M SP (Peptide Institute, Inc.) or $2 \times 10^{-6}$ M NKA (Peptide Institute, Inc.) were used as aggregation inducers. [$^3$H]SP binding to guinea-pig lung membranes and SP and NKA-induced guinea-pig tracheal constriction were carried out as described previously[1,2]. As shown in Fig. 1 SP produced a platelet aggregation in a dose dependent manner and stable aggregation occured at levels above $4 \times 10^{-7}$ M SP. NKA also produced the platelet aggregation in a dose dependent manner (data not shown). WF3484 (cyclosporin C) exhibited the inhibitory activities for the platelet aggregation induced by SP ($2 \times 10^{-6}$ M) and NKA ($2 \times 10^{-6}$ M) with the $IC_{50}$ values $5.0 \times 10^{-7}$ M and

Fig. 1. Rabbit platelet aggregation induced by substance P.

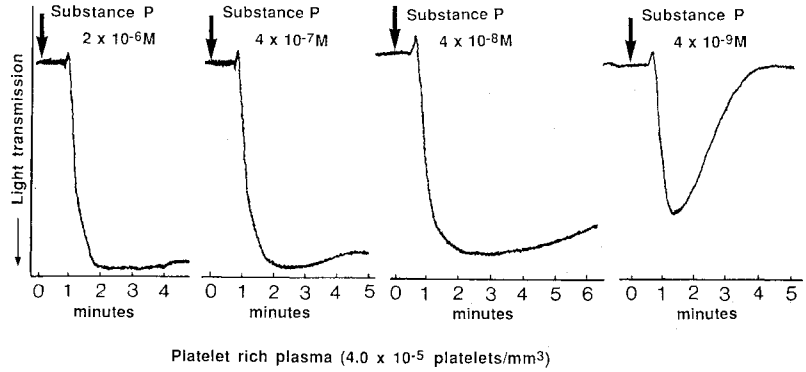


Table 1. The $IC_{50}$ (M) values of cyclosporin C, actinomycin D, WS9326A and FK224 on various *in vitro* experimental models.

| | Agonist | Cyclosporin C | Actinomycin D | WS9326A | FK224 |
|---|---|---|---|---|---|
| Platelet aggregation | SP (2X10$^{-6}$M) | 5.0 X 10$^{-7}$ | > 6.2 X 10$^{-6}$ | > 7.7 X 10$^{-6}$ | 3.2 X 10$^{-7}$ |
| | NKA (2X10$^{-6}$M) | 1.7 X 10$^{-6}$ | > 6.2 X 10$^{-6}$ | > 7.7 X 10$^{-6}$ | 9.6 X 10$^{-7}$ |
| | ADP (2X10$^{-6}$M) | > 1.6 X 10$^{-4}$ | > 6.2 X 10$^{-6}$ | > 7.7 X 10$^{-6}$ | > 7.7 X 10$^{-6}$ |
| Receptor binding assay (guinea-pig lung receptor) | SP (1X10$^{-9}$M) | 4.1 X 10$^{-7}$ | > 5.0 X 10$^{-6}$ | 3.6 X 10$^{-6}$ | 1.0 X 10$^{-7}$ |
| Tracheal contraction | SP (1X10$^{-8}$M) | no effect | > 1.0 X 10$^{-5}$ | 9.7 X 10$^{-6}$ | 2.6 X 10$^{-6}$ |
| | NKA (1X10$^{-9}$M) | no effect | 1.8 X 10$^{-6}$ | 3.5 X 10$^{-6}$ | 1.3 X 10$^{-6}$ |

Table 2. Selectivities and affinities of antagonists for binding to human tachykinin receotor subtypes.

| | $IC_{50}$(M) | | | |
|---|---|---|---|---|
| Agonist | Cyclosporin C | Actinomycin D | WS9326A | FK224 |
| SP (1.0 x 10$^{-10}$M) | 7.47 x 10$^{-7}$ | > 5.00 x 10$^{-4}$ | 2.00 x 10$^{-6}$ | 1.23 x 10$^{-7}$ |
| NKA (1.0 x 10$^{-10}$M) | 1.89 x 10$^{-6}$ | 3.01 x 10$^{-6}$ | 5.71 x 10$^{-7}$ | 2.37 x 10$^{-7}$ |

$1.7 \times 10^{-6}$ M, respectively. On the other hand WF3484 (cyclosporin C) was inactive against for the platelet aggregation induced by $2 \times 10^{-6}$ M of ADP (Table 1).

The transfection and functional expression of cDNA clones for single receptor subtypes in the separate cell lines are useful for characterization of tachykinin antagonist. Chinese hamster ovary (CHO) (dhfr$^-$) cells that were transfected with the human $NK_1$ and $NK_2$ receptors and stably expressing cell lines were established for radioligand binding studies. Clonal cells expressing the human $NK_1$ receptor have been described previously[6]. A clonal cell line expressing the human $NK_2$ receptor was established according to the procedures described previously[6]. Clonal cells were maintained in a minimal essential medium lacking ribonucleosides and deoxyribonucleosides and supplemented with 10% dialyzed fetal bovine serum. Membrane preparation of receptor-expressing cells was performed as described previously[7]. Displacement binding experiments were carried out by using [$^{125}$I]Bolton-Hunter SP and [$^{125}$I]NKA as described[8]. Cell membranes (0.4~15 $\mu$g/ml) were incubated with 0.1 nM [$^{125}$I]Bolton-Hunter SP and 0.1 nM [$^{125}$I]NKA in the absence and presence of various concentrations of tachykinin peptides and antagonists. All experiments were carried out at least twice in duplicate. The nonspecific binding was defined as the binding activity in the presence of 3.2 $\mu$M of the corresponding unlabeled tachykinin and was subtracted from the total binding activity for determination of the specific binding. The radioactivity was counted in a gamma counter (Packard Auto Gamma Model 5650). The results obtained in these experiments are summarized in Table 2. Cyclosporin C was active against human NK-1 and NK-2 receptors, although the affinity for human NK-1 receptor is slightly higher than that for the human NK-2 receptor. FK224 was a selective NK-1, NK-2 dual

antagonist, and was equipotent for both the guinea-pig and human receptors. On the contrary actinomycin D showed highly selective for guinea-pig and human NK-2 receptors.

In Tables 1 and 2, the various anti-tachykinin activities we obtained with these fermentation products are compared. Cyclosporin C exhibited about a 3-fold difference between platelet aggregation inhibition and binding affinity for human NK-1 and NK-2 receptors. FK224 has a higher affinity for human NK-1 and NK-2 receptors and it has been reported that FK224 acts as NK-1,2 dual receptor antagonist *in vitro* and *in vivo*[2,9,10]. Present data demonstrated that actinomycin D is highly selective for the human NK-2 receptor and it has been reported that actinomycin D act as NK-2 specific receptor antagonist *in vitro* and *in vivo*[3]. Cyclosporin C was a potent inhibitor of platelet aggregation induced by SP and NKA and also was active against guinea-pig NK-1 receptor. As shown in Table 2, cyclosporin C was an NK-1, NK-2 dual receptor antagonist. However, against our expectation, cyclosporin C has no effect on tracheal constrictions produced by SP and NKA. We tried receptor binding for SP using platelet membrane fractions, and observed a small amount of specific binding of SP to the platelet membranes. So we could not confirm the half-maximal concentration ($IC_{50}$) of cyclosporin C to inhibit specific binding of platelet SP receptors. Detailed mechanisms and actions for the tachykinin inhibition of cyclosporin C are to be studied further. However, it was observed in the present investigation that platelets express the tachykinin receptors on the platelet cell surface.

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