Functional characterization of the rat multispecific organic anion transporter OAT1 mediating basolateral uptake of anionic drugs in the kidney

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Received 23 September 1998

Abstract The functional characteristics of rat organic anion transporter OAT1 were investigated using *Xenopus laevis* oocytes. Uptake of *p*-aminohippurate (PAH) by the oocytes expressing OAT1 was markedly inhibited by glutarate, α-ketoglutarate and probenecid, moderately inhibited by folate and methotrexate, but not inhibited by taurocholate or tetraethylammonium. Methotrexate and folate were transported by OAT1, but probenecid, a typical inhibitor of organic anion transporter, was not transported. Inhibition of PAH uptake by aliphatic dicarboxylates with various alkyl chain lengths was maximal at 5 (glutarate) and 6 (adipate) carbon atoms. OAT1-mediated PAH uptake was markedly inhibited by phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate and mezerein, but not by 4α-phorbol 12,13-didecanoate. The inhibitory effect of PMA was attenuated in the presence of staurosporine, suggesting that OAT1 is regulated by protein kinase C. These results suggest that the substrate recognition of OAT1 is comparable to that of renal basolateral organic anion transporter, and the transport activity is regulated by protein kinase C.

Key words: Organic anion transporter; OAT1; Renal tubular secretion; *Xenopus* oocyte; Protein kinase C; *p*-Aminohippurate

1. Introduction

Renal excretion of various organic anions is mediated by organic anion transport systems expressed in the proximal tubules [1,2]. Using isolated proximal tubules [3–5], membrane vesicles isolated from the kidney cortex [6–11] and cultured epithelial cells derived from opossum kidney (OK) [12–15], basolateral uptake of organic anions has been shown to be mediated by organic anion/dicarboxylate exchangers and apical extrusion of organic anions has been shown to be mediated by Cl^-/organic anion exchange systems and/or membrane potential sensitive systems. Recently, cDNAs encoding basolateral organic anion transporters were isolated from rat (OAT1 [16] and ROAT1 [17]) and winter flounder (fROAT) [18] by functional expression cloning. The amino acid sequences of OAT1 and ROAT1 are identical and are homologous to NKT (94% identity), which is an OAT1 isoform isolated from mouse kidney [19]. However, the substrate recognition and regulatory mechanisms of these cloned organic anion transporters are not well characterized.

It is generally believed that protein kinase C regulates functions of various enzymes, receptors and transporters [20]. We have reported previously that basolateral uptake of organic anions in OK cells is regulated by protein kinase C and not by protein kinase A [13]. However, the precise mechanisms responsible for the regulation of basolateral uptake of organic anion remains unclear. In the present study, we investigated the functional characteristics of OAT1 using *Xenopus* oocytes, and obtained further information on the substrate recognition and regulatory mechanisms of OAT1.

2. Materials and methods

2.1. Materials

p-[*glycyl*-^{14}C]Aminohippurate (PAH) (1.6 GBq/mmol), [^{3}H]taurocholate (74.0 GBq/mmol) and [1-^{14}C]tetraethylammonium bromide (185 MBq/mmol) were obtained from Du Pont-New England Nuclear Research Products (Boston, MA, USA). [^{3}H]Methotrexate (285.0 GBq/mmol) and [3′,5′,7,9-^{3}H]folate (1.52 GBq/mmol) were from Amersham International (Buckinghamshire, UK). [^{14}C]Levofloxacin (1.07 GBq/mmol) and unlabeled levofloxacin were kindly supplied by Daiichi Seiyaku (Tokyo, Japan), and [^{14}C]grepafloxacin (1.17 GBq/mmol) and unlabeled grepafloxacin were from Otsuka Pharmaceutical (Tokushima, Japan). Probenecid, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), mezerein, and 4α-phorbol 12,13-didecanoate (4α-PDD) were purchased from Sigma (St. Louis, MO, USA), and unlabeled methotrexate, folate and staurosporine were from Wako Pure Chemical Industries (Osaka, Japan). Taurocholate, *p*-aminohippurate and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were from Nacalai Tesque (Kyoto, Japan). All other chemicals used were of the highest purity available.

2.2. Reverse transcription (RT)-PCR and cDNA sequencing

Because the nucleic acid sequence of OAT1 was not available at the beginning of this study, degenerate PCR primers were designed and synthesized based on the nucleotide sequence of NKT [19]. Sense strand with a *Sal*I site, 5′-CCGTCGACATGGCCTTCAATGACCTCCT-3′ (corresponding to amino acids 1–7); antisense strand with a *Not*I site, 5′-CCGCGGCCGCTCAGAGTCCGTTCTTCTCTT-3′ (corresponding to amino acids 540–545). One μg of poly(A)$^+$ RNA from rat kidney obtained by methods previously reported [21] was reverse-transcribed with Superscript II reverse transcriptase (Gibco-BRL Life Technologies, Gaithersburg, MD, USA) at 42°C for 50 min and then heated at 70°C for 15 min. The synthesized cDNA was used for subsequent PCR with a set of primers (1 μM) according to the following profile: 95°C for 45 s, 60°C for 45 s, 72°C for 5 min, 30 cycles. The PCR products were separated by electrophoresis through 1% agarose gels, and size-selected DNA fragments (approx. 1650 bp) were extracted using a DNA purification matrix (Bio-Rad Laboratories, Richmond, CA, USA). The purified PCR products were cut with *Sal*I and *Not*I (New England Biolabs, Beverly, MA, USA), ligated into pSPORT1 cut with the same enzymes, and transformed into

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Abbreviations: PAH, *p*-aminohippurate; RT-PCR, reverse-transcription polymerase chain reaction; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate

PII: S0014-5793(98)01328-3

competent *Escherichia coli* DH5α (Gibco-BRL). Both strands of the subcloned cDNA inserts were sequenced with a fluorescence 373A DNA sequencer (Applied Biosystems) and cDNA clones with base sequences 94% identical with NKT were isolated.

2.3. Screening of cDNA library

An oligo(dT)-primed directional rat kidney cDNA library used previously for the cDNA cloning of peptide transporters [21,22], organic anion transporters [23] and organic cation transporters [24] was screened by hybridization in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.2% SDS, 10 μg/ml salmon sperm DNA at 42°C with a PCR clone labeled with [α-^{32}P]dCTP (111 TBq/mmol; Amersham). A positive clone (rat OAT1) with a 2.2-kb insert was isolated and subcloned into pSPORT1 cut with *Sal*I and *Not*I. The complete sequence of the clone was determined on both strands using synthetic oligonucleotide primers. It was 2217 bp long and deduced amino acid sequence from the open reading frame of the nucleotide sequence was identical to that of rat OAT1.

2.4. Functional expression of OAT1 in Xenopus laevis oocytes

Aliquots of 25 ng of capped RNA transcribed in vitro from *Not*I-linearized OAT1 cDNA with T7 RNA polymerase were injected into *Xenopus* oocytes. Injected oocytes were maintained in modified Barth's medium at 18°C for 3 days. Functional expression of OAT1 was analyzed by measuring uptake of substrates in groups of oocytes injected with 50 nl of water or RNA, as described previously [21,24]. Oocytes were incubated for specified periods at 25°C in uptake buffer (in mM: 100 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH 7.4)) containing [^{14}C]PAH, except where otherwise noted. In the experiments of protein kinase C regulation, oocytes were preincubated with active or inactive phorbol esters and/or inhibitor for specified periods, before incubation with [^{14}C]PAH. Stock solutions of phorbol esters and staurosporine were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO was not more than 0.1%. At the end of the uptake periods, oocytes were washed three times in 1.5 ml of ice-cold uptake buffer (pH 7.4), solubilized in 10% SDS solution and then radioactivity was determined in 5 ml of ACSII (Amersham) by liquid scintillation counting. Four oocytes were used for each uptake experiment.

2.5. Detection of probenecid by high-performance liquid chromatography

Oocytes incubated with probenecid were homogenized in 0.5 ml of extraction solution (20 mM potassium phosphate (pH 5.4)/methanol/isopropanol, 6:3:1). The homogenate was centrifuged at 13 000 rpm for 10 min (Eppendorf 5415, Hamburg, Germany) and the supernatant was filtered through a Millipore filter (SJGVL, 0.22 μM). Probenecid in the filtrate was determined with a LC-10AS high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an UV spectrophotometric detector (SPD-6A, Shimadzu) under the following conditions: column, TSK-gel ODS 80TM, 4.6 mm inside diameter×150 mm (Tohso, Tokyo, Japan); mobile phase, 20 mM potassium phosphate (pH 5.4)/methanol/isopropanol, 6:3:1; flow rate 1.0 ml/min; wavelength, 254 nm; temperature, 40°C.

3. Results

3.1. Effects of various organic ions on PAH uptake by Xenopus oocytes expressing OAT1

When the oocytes injected with 25 ng of OAT1 RNA were incubated with 25 μM [^{14}C]PAH, uptake of PAH linearly increased during the periods examined (15–120 min). To clarify the substrate specificity of OAT1, effects of various organic ions (1 mM) on [^{14}C]PAH (25 μM) uptake by OAT1-expressing oocytes were examined (Fig. 1). When the oocytes expressing rat OAT1 were incubated with [^{14}C]PAH in the presence of various organic ions for 60 min, [^{14}C]PAH uptake by these oocytes was markedly inhibited by unlabeled PAH, glutarate, α-ketoglutarate and probenecid, moderately inhibited by folate and methotrexate, but not inhibited by taurocholate or tetraethylammonium. The pyridonecarboxylic acid antibacterial drugs levofloxacin and grepafloxacin slightly inhibited [^{14}C]PAH uptake.

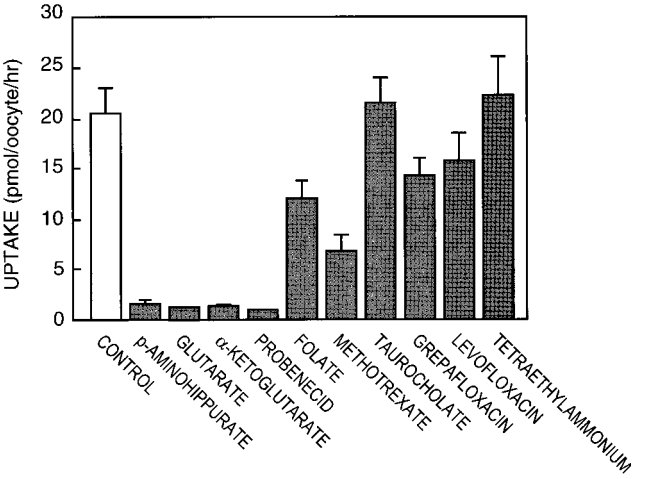

Fig. 1. Effect of various organic ions on PAH uptake by *Xenopus* oocytes injected with water or OAT1 RNA. Uptake was assayed for 1 h at 25°C in buffer containing 25 μM [^{14}C]PAH with (shaded column) or without (open column) 1 mM unlabeled PAH, glutarate, α-ketoglutarate, probenecid, folate, methotrexate, taurocholate, grepafloxacin, levofloxacin or tetraethylammonium. Each column represents the mean ± S.E. of four experiments.

3.2. Uptake of various organic ions by Xenopus oocytes expressing OAT1

When incubated with [^{14}C]PAH, [^{3}H]folate or [^{3}H]methotrexate for 60 min, uptake was stimulated in OAT1-expressing oocytes compared to water-injected oocytes, suggesting that folate and methotrexate as well as PAH are transported by OAT1 (Fig. 2A). However, OAT1 did not transport [^{3}H]taurocholate, [^{14}C]grepafloxacin, [^{14}C]levo-

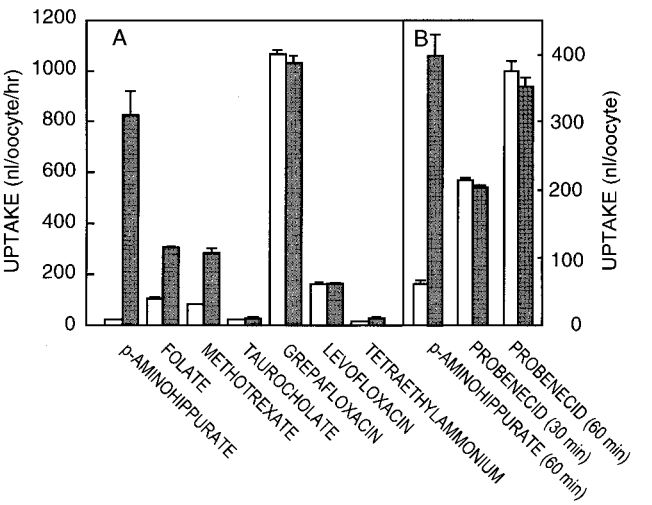

Fig. 2. Uptake of various organic ions by *Xenopus* oocytes injected with water or OAT1 RNA. A: Uptake was assayed for 1 h at 25°C in buffer containing 25 μM [^{14}C]PAH, 150 nM [^{3}H]folate, 500 nM [^{3}H]methotrexate, 1 μM [^{3}H]taurocholate, 5 μM [^{14}C]grepafloxacin, 5 μM [^{14}C]levofloxacin, and 100 μM [^{14}C]tetraethylammonium. Water (open column), RNA (shaded column). B: Uptake was assayed for indicated periods at 25°C in buffer containing 50 μM probenecid. Water (open column), RNA (shaded column). Uptake of each compound is expressed as uptake clearance. Each column represents the mean ± S.E. of four experiments.

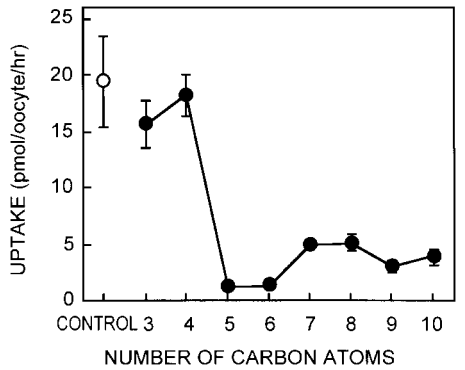

Fig. 3. Effect of dicarboxylates with various carbon chain lengths on PAH uptake by *Xenopus* oocytes injected with OAT1 RNA. Uptake was assayed for 1 h at 25°C in buffer containing 25 μM [^{14}C]PAH with (●) or without (○) 1 mM dicarboxylates (HOOC-$(CH_2)_X$-COOH) with 3 (malonate), 4 (succinate), 5 (glutarate), 6 (adipate), 7 (pimelate), 8 (suberate), 9 (azelate) or 10 (sebacate) carbon atoms. Each column represents the mean ± S.E. of four experiments.

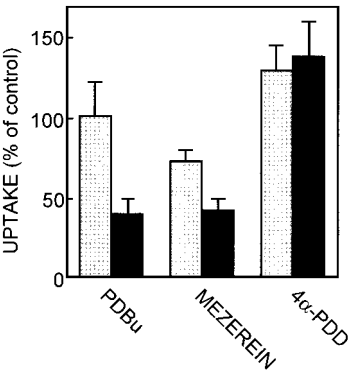

Fig. 5. Effect of protein kinase C activators on PAH uptake by *Xenopus* oocytes injected with OAT1 RNA. Oocytes were incubated for 60 min with 10^{-6} M (shaded column) or 10^{-7} M (closed column) of phorbol esters. After washing the oocytes, uptake was assayed for 1 h at 25°C in buffer containing 25 μM [^{14}C]PAH. Control value of PAH uptake by OAT1-expressing oocytes was 26.7 ± 4.4 pmol/oocyte/h. Each column represents the mean ± S.E. of four experiments. PDBu, phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate.

floxacin or [^{14}C]tetraethylammonium. Furthermore, probenecid, a typical inhibitor of organic anion transporter in the kidney, was not transported by OAT1 (Fig. 2B).

3.3. Effects of aliphatic dicarboxylates with various carbon chain lengths on PAH uptake by Xenopus oocytes expressing OAT1

Fritzsch et al. [25] as well as the present authors [12] have reported on characteristic inhibition of the basolateral PAH transport by various dicarboxylates using in situ stopped-flow microperfused tubules or cultured renal epithelial cell line OK, respectively. In the present study, we investigated the effects of aliphatic dicarboxylates with various carbon chain lengths on PAH uptake by OAT1-expressing oocytes. As illustrated in Fig. 3, the inhibitory effects of aliphatic dicarboxylates were maximal at 5 and 6 carbon atoms. In contrast, aliphatic dicarboxylates with 3 and 4 carbon atoms did not substantially inhibit PAH uptake. Inhibition became weaker for molecules with 7 or 8 carbon atoms, and then stronger for longer dicarboxylates.

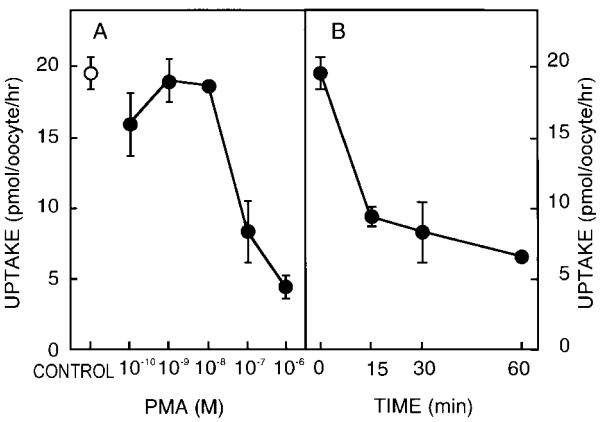

Fig. 4. Dose (A) and time (B) dependence of PMA effect on PAH uptake by *Xenopus* oocytes injected with OAT1 RNA. A: Oocytes were incubated for 30 min with (●) or without (○) PMA (10^{-10}–10^{-6} M). After washing the oocytes, uptake was assayed for 1 h at 25°C in uptake buffer containing 25 μM [^{14}C]PAH. B: Oocytes were incubated for various periods in the presence of 10^{-7} M PMA. After washing the oocytes, uptake was assayed for 1 h at 25°C in buffer containing 25 μM [^{14}C]PAH. Each point represents the mean ± S.E. of four experiments.

3.4. Effects of PMA on PAH uptake by Xenopus oocytes expressing OAT1

Next, we examined the effects of protein kinase C modulators on PAH uptake by OAT1-expressing oocytes. As illustrated in Fig. 4A, 30 min incubation with PMA (10^{-7} to 10^{-6} M), a representative activator of protein kinase C, resulted in marked decreases in PAH uptake by OAT1-expressing oocytes, suggesting that protein kinase C is involved in the regulation of OAT1. Fig. 4B shows a time-course of PMA effects on PAH uptake by OAT1-expressing oocytes. Incubation of the oocytes with 10^{-7} M PMA for 15 min resulted in marked decreases in PAH uptake by OAT1-expressing oocytes (48% of control), and longer incubation periods resulted in larger decreases in PAH uptake.

3.5. Specificity of PMA effect on PAH uptake by Xenopus oocytes expressing OAT1

To clarify the involvement of protein kinase C in the regulation of OAT1, the effects of various protein kinase C activators on PAH uptake by OAT1-expressing oocytes were examined (Fig. 5). Like PMA, active phorbol esters PDBu and mezerein inhibited PAH uptake at 10^{-6} M. In contrast, 4α-PDD (10^{-6} and 10^{-7} M), an inactive phorbol ester, did not affect PAH uptake. Fig. 6 shows the effect of staurosporine, a potent inhibitor of protein kinase C, on PMA-induced inhibition of PAH uptake by OAT1-mediated PAH transport. Oocytes were preincubated with various concentrations (10^{-10} to 10^{-6} M) of staurosporine for 30 min and then treated with 10^{-7} M PMA. The inhibitory effect of PMA on PAH uptake by OAT1-expressing oocytes was almost completely blocked by staurosporine (10^{-8} to 10^{-6} M), suggesting this inhibitory effect is mediated by protein kinase C and not by non-specific interactions of PMA with other pathways.

4. Discussion

Recently, we have reported cDNA cloning of another or-

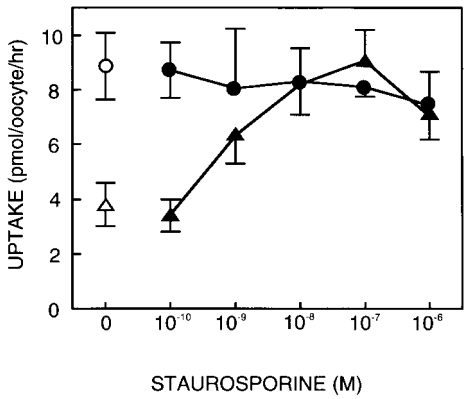

Fig. 6. Effect of staurosporine on PMA-induced inhibition of PAH uptake by *Xenopus* oocytes injected with OAT1 RNA. Oocytes were exposed to various concentrations of staurosporine (10^{-10}–10^{-6} M) for 30 min (●,▲), followed by an additional 60-min incubation in the absence (○,●) or presence (△,▲) of PMA (10^{-7}). After washing the oocytes, uptake was assayed for 1 h at 25°C in buffer containing 25 μM [^{14}C]PAH. Each point represents the mean ± S.E. of four experiments.

ganic anion transporter, OAT-K1, which is expressed predominantly in the kidney [23]. OAT-K1 stimulated the transport of methotrexate and folate, but not that of PAH. Immunological studies using anti-peptide antibody confirmed that OAT-K1 is predominantly expressed in the brush-border membranes of renal tubules [27]. Therefore, it is likely that OAT1 and OAT-K1 at least in part regulate tubular secretion of methotrexate at the basolateral and brush-border membranes, respectively.

In the present study, probenecid was not transported by OAT1 (Fig. 2B), although probenecid markedly inhibited the transport of PAH by OAT1 (Fig. 1). These results suggest that probenecid is recognized, but not translocated by OAT1, and that other transporters might contribute to tubular secretion of probenecid. In addition, taurocholate, a representative substrate of organic anion transporter in the liver, did not inhibit PAH uptake (Fig. 1) and was not transported by OAT1 (Fig. 2A). These and previous results suggest the existence of multiple organic anion transporters with different substrate specificities mediating tubular secretion of various organic anions.

The inhibitory effects of various dicarboxylates with 3–10 carbon-chain lengths on PAH uptake by OAT1-expressing oocytes showed a characteristic pattern similar to that of basolateral PAH transport in rat kidney [25,26] and the cultured renal epithelial cell line, OK [12] (Fig. 3). These results suggest that OAT1 is the dominant organic anion transporter responsible for PAH transport in renal basolateral membranes.

Previously, we reported that basolateral uptake of organic anion in OK cells is downregulated by various active phorbol esters [13]. Because this downregulation was not blocked by treatment with protein synthesis or cytoskeleton inhibitors, it was deduced that the organic anion transporter in basolateral membranes of OK cells was regulated by direct phosphorylation of the transporter protein. In the present study, we demonstrated that OAT1 was also inhibited by treatment with active phorbol esters for 15 min, and this decrease was blocked by pretreatment with staurosporine, suggesting that OAT1 is inhibited by protein kinase C. Five putative phosphorylation sites for protein kinase C have been found in the deduced amino acid sequence of OAT1 [16,17], suggesting that OAT1 protein may be phosphorylated by protein kinase C, resulting in the decrease of transport activity. However, the possibility that membrane expression of OAT1 was decreased by protein kinase C could not be excluded.

In conclusion, OAT1 recognizes a wide variety of organic anions and is regulated by protein kinase C. The present results suggest that functional characteristics of OAT1 are comparable to those of the basolateral PAH transporter in the kidney.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research (B), a Grant-in-Aid for Scientific Research on Priority Areas (No. 296) from the Ministry of Education, Science, Sports, and Culture of Japan, Grants-in-Aid from the Japan Health Sciences Foundation, Grants-in-Aid from the Yamada Science Foundation, and by a grant from the Japan Research Foundation for Clinical Pharmacology.

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