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Active Intestinal Absorption of Fluoroquinolone Antibacterial Agent Ciprofloxacin by Organic Anion Transporting Polypeptide

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RUNNING TITLE

Oatp-mediated absorption of ciprofloxacin

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ABSTRACT

Fluoroquinolone antimicrobial drugs are efficiently absorbed after oral administration despite of their hydrophilicity, implying an involvement of carrier-mediated transport in their membrane transport process. We have shown that several fluoroquinolones are substrates of organic anion transporter polypeptides OATP1A2 expressed in human intestine derived Caco-2 cells. In the present study, to clarify the involvement of OATP in intestinal absorption of ciprofloxacin, we investigated a contribution of Oatp1a5, which is expressed at the apical membranes of rat enterocytes, to intestinal absorption of ciprofloxacin in rats. Intestinal membrane permeability of ciprofloxacin was measured by *in situ* and vascular perfused closed loop method. Disappearance and absorbed amount of ciprofloxacin from the intestinal lumen were markedly increased in the presence of 7,8-benzoflavone, a breast cancer resistance protein inhibitor, and ivermectin, a P-glycoprotein inhibitor, while it was significantly decreased in the presence of these inhibitors in combination with naringin, an Oatp1a5 inhibitor. Furthermore, The Oatp1a5-mediated uptake of ciprofloxacin was saturable with K_m value of 140 μM, and naringin inhibited the uptake with IC_{50} value of 18 μM by *Xenopus* oocytes expressing Oatp1a5. Naringin reduced the permeation of ciprofloxacin from mucosal-to-serosal side, with IC_{50} value of 7.5 μM by Ussing-type chamber method. The estimated IC_{50} values were comparable. These data suggested that Oatp1a5 was partially responsible for the intestinal absorption of ciprofloxacin. In conclusion, intestinal absorption of ciprofloxacin could be affected by influx

transporters, such as Oatp1a5 as well as the efflux transporters such as P-gp and Bcrp.

KEY WORDS

Ciprofloxacin; intestinal absorption; Oatp; P-gp; Bcrp

Introduction

Fluoroquinolones are antimicrobial drugs in clinical use, which are effective for treatment of a wide range of bacterial and fungal infections. Since most of fluoroquinolones are orally administrated to patients, their bioavailability is an important determinant for their pharmacological activity. Early pharmacokinetic research has shown that some fluoroquinolones are efficiently absorbed from small intestines despite their hydrophilicity [1]. For example, the bioavailability of ciprofloxacin was reported to be 63-84% [1,2]. Relatively high intestinal absorption might be explained by an involvement of carrier-mediated transport of ciprofloxacin across intestinal epithelial cells. Indeed, previous reports have suggested that fluoroquinolones including ciprofloxacin are transported by several classes of membrane transporters; organic anion transporter 3 (OAT3/*SLC22A8*) [3], and efflux pumps such as p-glycoprotein (P-gp/*ABCB1*) and breast cancer resistance protein (BCRP/*ABCG2*) [4-7]. Our recent study has shown that ciprofloxacin and levofloxacin can be transported by organic anion transporting polypeptide 1A2 (OATP1A2/*SLCO1A2*) in the human intestinal model Caco-2 cells and *Xenopus* oocytes expressing OATP1A2 [8].

OATP1A2 is a solute carrier transporter that belongs to OATP/SLCO family, and functions as an influx transporter for a wide variety of anionic compounds in a Na^+ independent manner [9, 10]. Since OATP1A2 is expressed in brain, liver, and to a lesser extent kidney, testis and intestines [9,11], it likely contributes to pharmacokinetics of drugs. In intestine, OATP/Oatp

molecules including OATP1A2 contribute to intestinal absorption of several drugs [12-18]. In addition to OATP1A2, apically-expressed OATP2B1 in intestinal epithelial cells has been also extensively studied and shown to be involved in the intestinal absorption of various compounds such as fexofenadine and pravastatin [11,19]. More importantly, clinical studies indicated that these intestinal OATPs are putative sites for drug-fruit juice (e.g. grapefruit, orange and apple juices) interactions, resulting in alteration of pharmacokinetics of their substrate drugs such as atenolol, celiprolol, fexofenadine and talinolol [20-26]. When ciprofloxacin was orally administered with orange juice, a significant decrease of its bioavailability was observed [27]. These observations indicate that intestinal absorption of ciprofloxacin is mediated by membrane transporters such as OATPs, while the absorption mechanism is poorly understood.

In the present study, to clarify a mechanism of intestinal absorption of ciprofloxacin, we studied whether the drug is transported by rat Oatp1a5 (*Slco1a5*) or not and evaluated effect of naringin, which is a OATP/Oatp inhibitor in the constituent of grapefruit juice [28], since Oatp1a5 is expressed at the apical membranes of rat small intestinal epithelial cells [29] and has been the most extensively studied as the rat intestinal uptake transporter [30-32]. We present here Oatp1a5 at least plays a role in intestinal absorption of ciprofloxacin by means of *in situ* and *in vitro* rat intestinal membrane permeation method and *Xenopus* oocytes expressing Oatp1a5, manifesting a putative mechanism in which fruit juice affects its oral bioavailability.

Materials and Methods

Materials

Ciprofloxacin, naringin, 7,8-benzoflavone, ivermectin and [^{3}H]estrone-3-sulfate, ammonium salt (1702 GBq/mmol) were purchased from Tokyo Chemical Industry (Tokyo, Japan), Kanto Chemical (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (St. Louis, MO), and PerkinElmer Inc. (Boston, MA), respectively. All other compounds and reagents were obtained from Nakalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO).

Uptake experiments using Xenopus laevis oocyte

The coding region of *Oatp1a5* gene was *in vitro* transcribed using mMESSAGE mMACHINE kit (Ambion, Austin, TX), and then a 50 nL of RNA solution containing 25 ng of transcribed RNA was directly injected to defoliculated *Xenopus* oocytes. In general, uptake experiment was conducted as previously described [33]. Briefly, injected oocytes were placed for 3 days in modified Barth's solution (MBS, 88 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$ and 10 mM HEPES adjusted to pH 7.4 with NaOH) at 18ºC, and then used for uptake experiments. Uptake of ciprofloxacin (20 μM)

was measured at room temperature in the MBS at pH 7.4. Uptake was terminated by the addition of ice-cold MBS. Oocytes were washed 3 times with ice-cold MBS.

Uptake results were shown by cell-to-medium ratio (microliters per oocyte), which was obtained by dividing the cellular uptake amount by the initial concentration of test compound in the uptake medium. Cell-to-medium ratio represents the volume of cleared extracellular transport medium. Oatp1a5-mediated uptake rate was obtained by subtracting the uptake of water-injected oocytes from that of Oatp1a5 cRNA-injected oocytes. For inhibition study, 2, 5, 10, 20 and 50 μM of naringin were used. Kinetic parameters were established by means of nonlinear least-squares analysis using Kaleida Graph (Synergy Software, Reading, PA). The Michaelis-Menten constant (K_m) of ciprofloxacin uptake mediated by Oatp1a5 and its maximal velocity (V_{max}) were obtained by fitting raw data to the following equation (1):

$$v = V_{max} \times C/(K_m + C) \qquad (1)$$

where v and C are the initial uptake rate and concentration of substrate, respectively. All data were expressed as means ± S.E.M., and statistical analysis was performed by the use of Student's t test with $p < 0.05$ as the criterion of significance.

The half-inhibitory concentration (IC_{50}) value of inhibitor to ciprofloxacin uptake was calculated by the following equation (2), where % of control and I are observed % of control uptake, the inhibitor concentration used, respectively.

% of Control = $IC_{50}/(IC_{50}+ I) \times 100$ (2)

In situ intestinal closed loop experiment

Male Wistar rats (220 ± 20 g body weight) were housed three per cage with free access to commercial chow and tap water, and were maintained on a 12 h dark/light cycle in an air-controlled room (temperature, 24.0 ± 1°C; humidity, 55 ± 5%). All animal experimentation was carried out in accordance with the guide of Tokyo University of Science's Institutional Animal Care and Use Committee. The permeability of rat intestinal membrane was evaluated by an *in situ* intestinal closed loop method. Male Wistar rats fasted overnight were anesthetized with pentobarbital. The abdominal cavity was opened and an intestinal loop (length: 10 cm) was made at the jejunum (2-12 cm form the ligament of Treitz) and ileum (2-12 cm from the caecum) by cannulation with silicone tubing (i.d., 3 mm). The intestinal contents were removed by slow infusion of saline and air. The test solution (phosphate-buffered solution, adjusted to pH 7.4) containing ciprofloxacin (10 μM) in the absence or the presence of inhibitors was introduced into the intestinal loop and both ends of the loop were ligated. For P-gp, Bcrp and Oatp inhibitor, ivermectin (10 μM), 7,8-benzoflavone (10 μM), naringin (500 μM) was used, respectively. After a certain period (usually 20 min), the luminal solution in the loop was collected. The permeability of ciprofloxacin was evaluated in terms of the percentage of dose

absorbed, calculated by subtracting the remaining amount of ciprofloxacin from the administered amount. The following equation (3) was used to calculate the apparent permeability (P_{app}):

$$P_{app} = k_a \times V/2\pi rl \qquad (3)$$

where k_a is the first-order absorption rate constant of ciprofloxacin estimated from the percentage of the dose absorbed during the defined period, V is the volume of ciprofloxacin solution introduced to the loop, and r and l represent the radius and length of the used segment of intestine, respectively. The length was 10 cm, and the values of the radius of each intestinal segment reported by Fagerholm et al. [34] were used (0.18 cm for jejunum and ileum).

Preparation of the vascular perfused intestinal loop

A small intestinal loop (ileum, about 10 cm) was isolated from male Wistar rats (220 ± 20 g body weight) and its blood vessels were perfused as described [35]. Briefly, in addition with *in situ* closed loop study, the superior mesenteric artery and vein were cannulated with polyethylene tubes (i.d., 0.5 mm). The cannulated intestinal segment was isolated from other portions and suspended in a serosal bath containing 100 mL of Krebs-Henseleit bicarbonate buffer solution (KHBB, 118 mM NaCl, 1.18 mM KCl, 1.18 mM KH_2PO_4, 1.27 mM $CaCl_2$, 4.47 mM $MgSO_4$, 24.88 mM $NaHCO_3$, and 10 mM glucose adjusted to pH 7.4 with NaOH) at 37^{o}C. Single-pass

perfusion of the blood vessel was started just after the intestine was isolated and continued throughout the experiment. KHBB containing BSA (3%) was used as the vascular perfusate at a rate of 0.5 mL/min and was gassed with O_2:CO_2 = 95:5 before and during the transport experiments [36]. The vascular perfusate was collected at 0-5, 5-10, 10-15, and 15-20 min. The permeability of ciprofloxacin evaluated by disappearance from intestinal lumen and by absorbed amount in vascular perfusate was estimated following equation (3).

Ussing-type chamber experiment

Rat intestinal tissue sheets were prepared as described previously [37]. Briefly, male Wistar rats (220 ± 20 g body weight) were anesthetized with pentobarbital and a segment of ileum was removed. The tissue was rapidly stripped from serosal muscle layers, and mounted vertically in a Ussing-type chamber having an exposed area of 0.5 cm^2. The volume of bathing solution on each side was 5.0 mL, and the temperature was maintained at 37°C. The transport buffer solution (125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM glucose, and 25 mM HEPES adjusted to pH 7.4 with Tris) was gassed with O_2:CO_2 = 95:5 before and during the experiments. In the inhibition study, the inhibitors were added to both mucosal and serosal chambers. Transport was estimated in terms of permeability coefficient (in unit of centimeter per minute), which was obtained by dividing the flux by the initial concentration of ciprofloxacin (10 μM) on the donor side. For inhibition study, 1, 3, 10,

30, 100 and 500 μM of naringin was used. The flux was obtained from the slope of the linear portion of plots of permeated amount against time per unit area of membrane against time.

Analytical methods

The concentration of ciprofloxacin in all samples was quantified with an HPLC. The HPLC system consisted of a constant-flow pump (PU-980; Japan Spectroscopic Co., Ltd., Tokyo, Japan), a fluorescence detector (FP-1520; Japan Spectroscopic Co., Ltd., Tokyo, Japan), integrator (807-IT; Japan Spectroscopic Co., Ltd., Tokyo, Japan), and an automatic sample injector (AS-950; Japan Spectroscopic Co., Ltd., Tokyo, Japan). A TSK-gel ODS 80-Ts (C_{18}. 150 mm × 4.6 mm, 5 mm, Tosoh Co., Ltd., Tokyo, Japan) was used as the analytical column, and the mobile phase was composed of 30 mM sodium phosphate in water and acetonitrile. The assay was linear over the concentration range used in the present study. The calibration curve for ciprofloxacin was prepared in a concentration range from 5 nM to 100 nM. The correlation coefficient was always better than 0.998. The intra-day coefficient of variation (CV) and the corresponding inter-day CV of the assay were less than 2% and 5%, respectively.

RESULTS

Absorption of ciprofloxacin by in situ closed loop method

At first, rat *in situ* closed loop method using rat jejunum and ileum was performed in order to evaluate absorptive transporters of ciprofloxacin. As shown in Fig. 1, the permeability of ciprofloxacin (10 μM) in rat jejunum and ileum was 1.15 ± 0.143 × 10^{-5} cm/sec and 1.14 ± 0.154 × 10^{-5} cm/sec, respectively. The permeability in the presence of ivermectin (10 μM), an inhibitor of P-gp, and 7,8-benzoflavone (10 μM), an inhibitor of Bcrp, was increased to 1.41 ± 0.03 × 10^{-5} cm/sec in jejunum and 2.28 ± 0.02 × 10^{-5} cm/sec in ileum. When an OATP inhibitor naringin was added in the presence of ivermectin or 7,8-benzoflavone, the permeability was significantly decreased to 0.650 ± 0.28 × 10^{-5} cm/sec in jejunum and 0.603 ± 0.16 × 10^{-5} cm/sec in ileum, respectively.

Absorption of ciprofloxacin by vascular perfusion method

P_{app} of ciprofloxacin in rat ileum was measured with or without vascular perfusion by means of closed loop method to confirm the absence of nonspecific effect of vascular perfusion on the intestinal permeability. The permeability of ciprofloxacin (10 μM) in rat ileum without and with vascular perfusion was 1.14 ± 0.15 × 10^{-5} cm/sec and 1.20 ± 0.30 × 10^{-5} cm/sec,

respectively (Table 1). Because no significant difference was observed in P_{app} between both conditions in all conditions, it was confirmed that vascular perfusion caused no effect on the intestinal permeability of ciprofloxacin during the experimental period. As shown in Fig. 2, cumulative amount of ciprofloxacin appeared in the vascular lumen after addition to intestinal lumen was increased 1.7-fold from 302 ± 55.7 pmol/20 min to 518 ± 95.0 pmol/20 min in the presence of 7,8-benzoflavone (10 μM) and ivermectin (10 μM) in perfusate. Addition of naringin (500 μM) with ivermectin and 7,8-benzoflavone, the cumulative amounts of ciprofloxacin significantly decreased to 139 ± 43.3 pmol/20 min.

Intestinal permeability of ciprofloxacin by Ussing-type chamber method

To characterize the naringin sensitive portion of ciprofloxacin absorption, effect of naringin on permeation of ciprofloxacin was studied by the Ussing-type chamber method. The permeation of ciprofloxacin (10 μM) in the serosal-to-mucosal direction was higher than that in the opposite direction (Fig. 3). Naringin reduced the mucosal-to-serosal permeation of ciprofloxacin with an IC_{50} value of 7.48 ± 1.94 μM (Fig. 4). Such reduction of permeation of ciprofloxacin was observed only in the direction from apical to serosal permeation but not in serosal-to-mucosal permeation (Fig. 3).

Characterization of ciprofloxacin uptake by Xenopus laevis oocytes expressing Oatp1a5

To clarify whether ciprofloxacin is a substrate for Oatp1a5 and to investigate inhibitory effect of naringin on Oatp1a5-mediated uptake of ciprofloxacin, the uptake of ciprofloxacin by *Xenopus laevis* oocytes expressing Oatp1a5 was examined. The time course of ciprofloxacin uptake by oocytes injected with Oatp1a5 cRNA or water is shown in Fig. 5A. The uptake of ciprofloxacin by Oatpt1a5-expressing oocytes was significantly higher than that by water-injected oocytes ($p < 0.05$). Since the Oatp1a5-specific uptake of ciprofloxacin increased linearly up to 30 min, the initial uptake rate was evaluated as an index of uptake in the following studies. Fig. 5B shows the concentration dependence of the Oatp1a5-mediated ciprofloxacin uptake. The K_m and V_{max} values were 136 ± 21.8 μM and 31.1 ± 1.80 pmol/oocyte/30 min, respectively. The uptake of ciprofloxacin by Oatp1a5-expressing oocytes was not changed from pH 5.0 to 7.0 (Fig. 6). Naringin inhibited the Oatp1a5-mediated uptake of ciprofloxacin with IC_{50} value of 17.8 ± 7.64 μM (Fig. 7).

Discussion and Conclusion

Fluoroquinolones are efficiently absorbed from small intestines despite their hydrophilicity, implying an involvement of carrier-mediated transport in their absorption process. We have already demonstrated that several fluoroquinolones are transported by OATP1A2, which is one of human OATP molecules expressed in Caco-2 cells [8]. Accordingly, we hypothesized that OATP is responsible for the intestinal absorption of fluoroquinolones and in the present study, we examined intestinal absorption of ciprofloxacin, as a model compound of fluoroquinolones, in rats by means of *in vitro* and *in situ* experimental techniques, especially focusing on rat Oatp1a5, since it was shown to be expressed at the apical membrane of rat intestinal epithelial cells [29].

In this study, naringin was selected as an inhibitor for Oatp1a5, because it was reported to inhibit OATP/Oatp-mediated transport of talinolol, pravastatin and pitavastatin [14-18]. However, recent investigations have shown that naringin has a significant inhibitory effect on not only Oatp1a5 but also P-gp and Bcrp [14,15,20,28,38,39]. Since ciprofloxacin is a substrate of P-gp and Bcrp [4,5,7,37,40], ivermectin (an inhibitor of P-gp) [41] and 7,8-benzoflavone (an inhibitor of Bcrp) [39] were simultaneously used with naringin in order to evaluate absorptive transport of ciprofloxacin. Meanwhile, ivermectin (10 μM) and 7,8-benzoflavone (10 μM) did not inhibit [^{3}H]estrone-3-sulfate uptake by oocytes expressing Oatp1a5, indicating that both ivermectin and 7,8-benzoflavone do not affect Oatp1a5-mediated transport at the present condition (data not shown).

To clarify the impact of influx transporters in intestinal absorption of ciprofloxacin, rat intestinal permeability of ciprofloxacin was measured by the *in situ* closed loop method (Fig. 1). In the presence of ivermectin and 7,8-benzoflavone, the permeability with naringin was decreased by 70% of that in the absence of naringin, suggesting that influx transporters susceptible to naringin are significantly involved in intestinal absorption process of ciprofloxacin. In addition, this phenomenon was also observed in vascular perfused study combined with *in situ* closed loop method (Fig. 2), demonstrating that inhibitory effect of naringin on intestinal influx transporter decreases *in vivo* plasma concentration of ciprofloxacin. The permeability obtained by absorbed ciprofloxacin into vascular vessels was lower than that by disappeared ciprofloxacin form small intestine (table 1). The permeability by disappeared ciprofloxacin from small intestine involve absorbed, adsorbed and metabolized amount in epithelial cells. Taki et al. [36] showed that 60-80% of Metkephamid was eliminated from rat upper jejunum, while absorbed amount of Metkephamid was only 0.3-1.2%. They discussed that these differences may be caused by well-metabolized characteristics of Metkephamid in rat upper jejunum. But, considering that ciprofloxacin is relatively stable [42], the differences may be caused by basolateral transport in rat ileum. In addition to *in vitro* study (Fig. 5), these findings imply that Oatp1a5 at least contributes to the intestinal absorption of ciprofloxacin in rats. However, efflux transporters such as P-gp and Bcrp may decrease ciprofloxacin transport from being absorbed simultaneously.

Because K_m value of 136 μM (Fig. 5) in the Oatp1a5-mediated transport of ciprofloxacin is close to solubility limit of ciprofloxacin, approximately 500 μM, Oatp1a5 is considered to

function effectively in rat intestinal absorption of ciprofloxacin. In order to prove the involvement of Oatp1a5 in intestinal absorption of ciprofloxacin, Ussing-type chamber method using rat ileum is performed (Figs. 3 and 4). Because secretary transport of ciprofloxacin was not inhibited by 500 μM of naringin on Fig. 3, we consider that this ciprofloxacin transport by P-gp and Bcrp is not inhibited by the bellow 500 μM of naringin. So, we carried out the Ussing Chamber without ivermectin and 7,8-benzoflavone. Naringin reduced the absorptive permeation of ciprofloxacin, with IC_{50} value of 7.48 μM (Fig. 4). This value involves more than apical uptake. Assuming that the absorptive permeation is depending on apical uptake, the value is comparable to the IC_{50} value of 17.8 μM obtained by oocytes expressing Oatp1a5 (Figs. 4 and 7). Because similar IC_{50} values were reported in Oatp1a5-mediated transport of talinolol (12.7 μM) and pravastatin (30.4 μM) by Oatp1a5-expressing oocytes, this accordance suggested that Oatp1a5 contributes to the intestinal permeation of ciprofloxacin [14,16]. However, we are unable to rule out a possibility of an involvement of other influx transporters such as Oatp2b1 in the ciprofloxacin absorption, while the localization of Oatp2b1 in the intestinal epithelial cells has yet to be determined [30,32]. Our previous data suggested that Oatp1a5-mediated transport of estrone 3-sulfate, Compound A and pravastatin observed pH-dependency [13,16]. But that of ciprofloxacin did not represent pH-dependency. Our previous data suggested that OATP1A2 mediated uptake of levofloxacin was not observed pH dependency, but that of tebipenem pivoxil was observed pH-dependency [8,43]. pH-dependency by OATP/Oatp transport may be dependent on substrate.

Decreased amounts of ciprofloxacin permeability by naringin represent absorptive transporters expression of ciprofloxacin such as Oatp1a5, suggesting that the expression of the transporters in ileum is higher than that in jejunum. Since it is reported that Oatp1a5 expression is higher in lower part of small intestine than in upper part of the small intestine [29,44], this may also support the involvement of Oatp1a5 in intestinal absorption of ciprofloxacin.

On the other hand, both OATP1A2 and OATP2B1 are expressed at apical membranes of human intestinal epithelial cells [11,19]. Interestingly, expression level of OATP2B1 was reported to be much higher than that of OATP1A2 in human small intestine [45]. Imanaga et al. [26] demonstrated that OATP2B1-mediated absorption of fexofenadine is subject to the genotype of *OATP2B1* c.1457C>T and apple juice in human clinical study. Therefore, OATP2B1-mediated transport may substantially contribute to intestinal absorption of drugs in human. Neuhofel et al. [27] reported that oral bioavailability of ciprofloxacin with orange juice was decreased compared to water. Several investigations have indicated that OATP/Oatp-mediated absorption of drugs is a putative site of drug-fruit juice interactions [25,46]. Since orange juice contains OATP/Oatp inhibitors such as naringin and hesperidine, observed ciprofloxacin-orange juice interaction supports that ciprofloxacin is absorbed by OATP transporter [20,47].

In conclusion, the present study demonstrates that Oatp1a5 has several roles in the intestinal absorption of ciprofloxacin in rats, although other influx and/or efflux transporters may not be excluded for intestinal absorption of ciprofloxacin. Because most of fluoroquinolones are

efficiently absorbed from small intestines and show relatively high bioavailability, our present study suggests that OATP/Oatp at least contributes to intestinal absorptive process of fluoroquinolones. Furthermore, our findings should be useful to predict drug-fruit juice interaction in the intestinal absorption process.

Conflict of Interest

We declare no conflict of interest.

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Table 1

Disappeared and absorptive permeability of ciprofloxacin in various conditions

Drug medium	*in situ* closed Loop	Vascular perfused loop (disappeared)	Vascular perfused loop (absorbed)
CPFX (10 μM)	1.14 ± 0.154	1.20 ± 0.304	0.334 ± 0.0511
+ ivermectin (10 μM) + 7,8-benzoflavone (10 μM)	2.28 ± 0.227†	1.89 ± 0.195†	0.535 ± 0.0979
+ ivermectin (10 μM) + 7,8-benzoflavone (10 μM) + naringin (500 μM)	0.655 ± 0.248*	1.07 ± 0.130*	0.106 ± 0.0477*

All data are presented as mean ± S.E.M. (n=3-5). † and * indicates a significant difference from the permeability of ciprofloxacin without any inhibitors and with ivermectin and 7,8-benzoflavone in each method

Figure Legends

Fig. 1. Effect of Inhibitors of Oatps, P-gp and Bcrp on Ciprofloxacin Disappearance from Rat Jejunum and Ileum by *In Situ* Loop Method.

Intestinal Permeability of 10 μM ciprofloxacin was obtained by disappearance from rat ileum loop at pH 7.4 and 37^{o}C. Closed and open bars represent permeability of 10 μM ciprofloxacin without and with 10 μM ivermectin and 10 μM 7,8-benzoflavone, respectively. Dotted bars represent permeability of ciprofloxacin with 500 μM naringin in addition to 10 μM ivermectin and 10 μM 7,8-benzoflavone. Each result represents the mean ± S.E.M. (n=3-6). † and * indicates a significant difference from the permeability of ciprofloxacin without any inhibitors and with ivermectin and 7,8-benzoflavone.

Fig. 2. Absorption of Ciprofloxacin by *In Vitro* Loop Method with Vascular Perfusion in Rat Ileum.

Absorbed amount was calculated from the recovered amount of ciprofloxacin in vascular outflow sample at pH 7.4 and 37^{o}C. Closed and open circles represent 10 μM ciprofloxacin without and with 10 μM ivermectin and 10 μM 7,8-benzoflavone. Triangles represent absorbed amount of ciprofloxacin with 500 μM naringin in addition to 10 μM ivermectin and 10 μM 7,8-benzoflavone. Each result represents the mean ± S.E.M. (n=3-6). * indicates a significant difference from absorbed amount of ciprofloxacin with ivermectin and

7,8-benzoflavone.

Fig. 3. Effect of Naringin on Absorptive and Secretory Transports of Ciprofloxacin by Ussing-type Chamber Method in Rat Ileum.

Mucosal-to-serosal and serosal-to-mucosal permeation of 10 μM ciprofloxacin was calculated from sample in receiver-side by Ussing-type chamber method in rat ileum at pH 7.4 and 37^{o}C. Circle and triangle symbols represent transported ciprofloxacin without and with 500 μM naringin, respectively. Open and closed symbols represent serosal-to-mucosal and mucosal-to-serosal transport of ciprofloxacin, respectively. Each result represents the mean ± S.E.M. (n=3-5) and * indicates a significant differences from mucosal-to-serosal transport of ciprofloxacin in the absence of naringin.

Fig. 4. Concentration Dependent Effect Naringin on Absorptive Permeability of Ciprofloxacin by Ussing-type Chamber Method in Rat Ileum.

Mucosal-to-serosal transport of ciprofloxacin was measured in the presence of 1-500 μM naringin at pH 7.4 and 37^{o}C. Each result represents the mean ± S.E.M. (n=3-6).

Fig. 5. Characterization of Uptake of Ciprofloxacin by Oatp1a5 Expressing Oocytes.

Uptake of 20 μM ciprofloxacin by *Xenopus* oocytes injected with cRNA of Oatp1a5 or water was measured at pH 7.4 and room temperature. (A) Time-dependence of uptake of

ciprofloxacin by Oatp1a5 expressing oocytes was performed. Closed and open circles represent Oatp1a5-expressing and water-injected oocytes, respectively. Uptakes are expressed as cell-to-medium ratio. Each result represents the mean ± S.E.M. (n=10-13) and * indicates a significant difference from the uptake by water-injected oocytes ($p < 0.05$). (B) Concentration-dependent uptake of ciprofloxacin by Oatp1a5 expressing oocytes was performed. Uptake of ciprofloxacin was measured for 30 min at pH 7.4 and room temperature. Oatp1a5 mediated uptake was obtained by subtraction of the uptake by water-injected oocytes from these by Oatp1a5-expressing oocytes. Concentrations of ciprofloxacin are 5, 20, 50, 200, and 500 μM. Each result represents the mean ± S.E.M. (n=4-8).

Fig. 6. pH-Dependency of Ciprofloxacin Uptake by Oatp1a5 Expressing Oocytes.

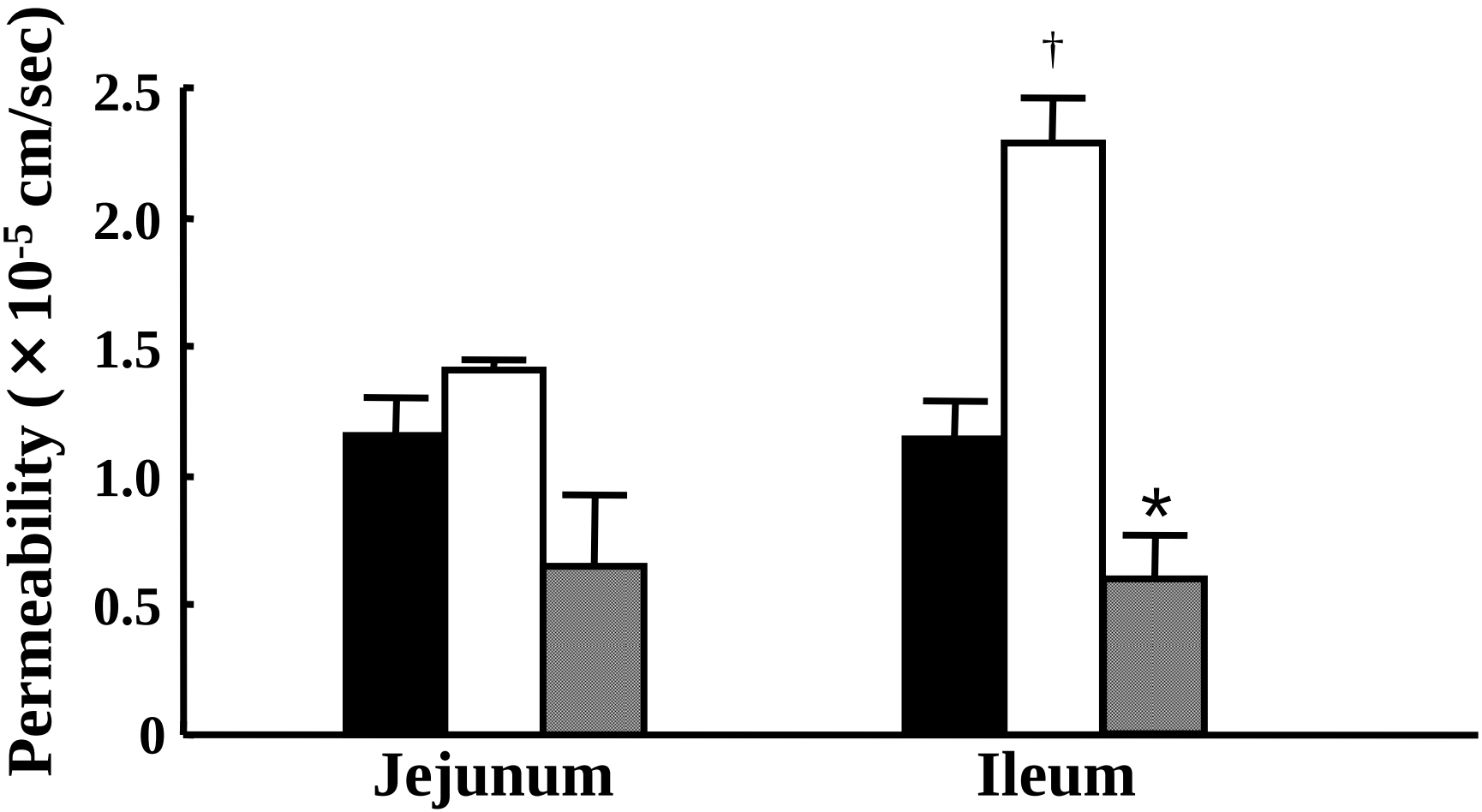
Uptake of 20 μM ciprofloxacin by *Xenopus* oocytes injected with cRNA of rat Oatp1a5 or water was measured for 30 min at pH 5.0, 6.0 and 7.0, and room temperature. Closed and open circles represent Oatp1a5-expressing and water-injected oocytes, respectively. Uptakes are expressed as cell-to-medium ratio. Each result represents the mean ± S.E.M. (n=6-10).

Fig. 7. Concentration Dependent Effect of Naringin on Ciprofloxacin Uptake by Oatp1a5 Expressing Oocytes.

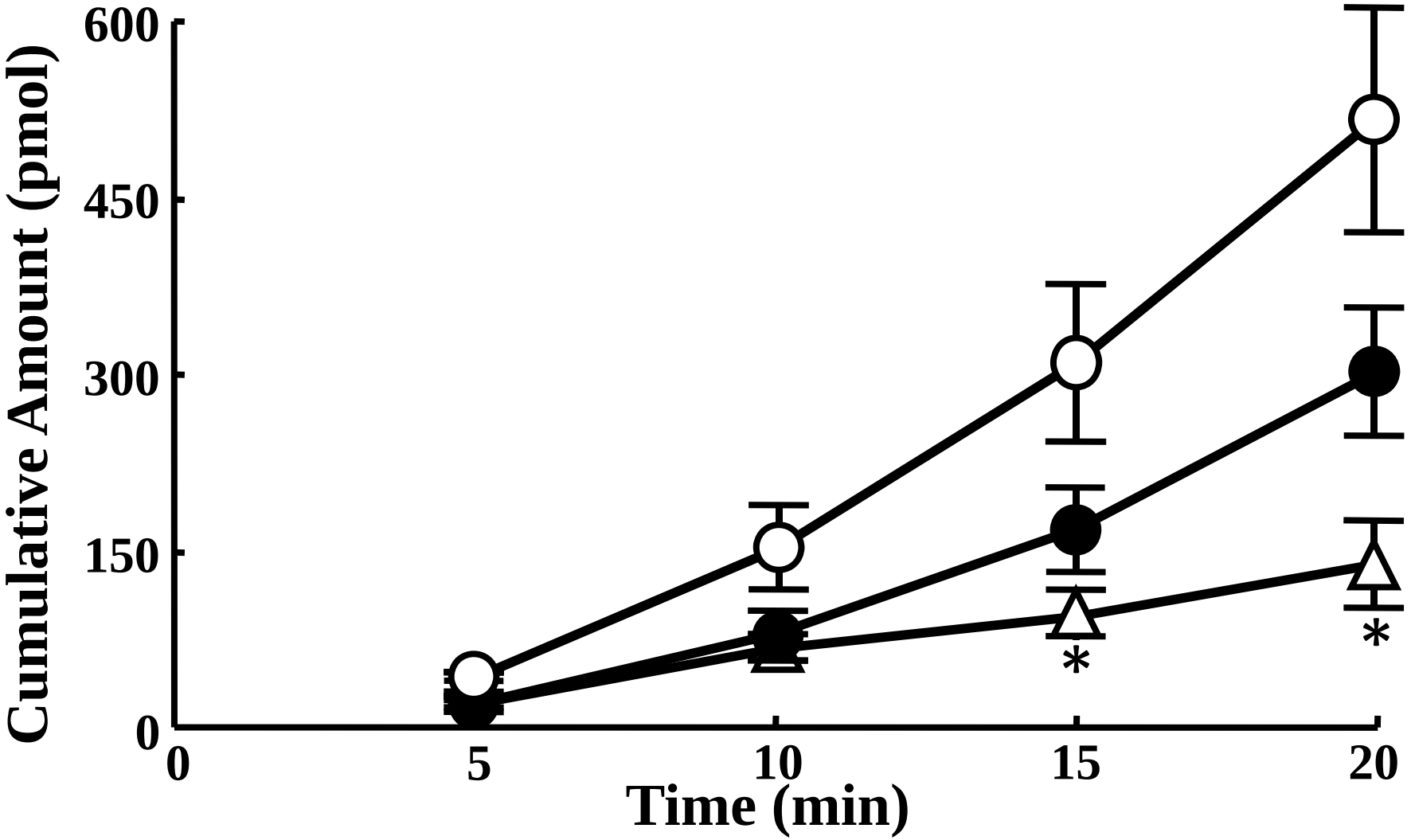
Uptake of 20 μM ciprofloxacin by *Xenopus* oocytes injected with cRNA of rat Oatp1a5 or water was measured for 30 min at pH 7.4 and room temperature. Results are shown as

percentage of control uptake measured in the absence of naringin after subtracting the uptake by water-injected oocytes from that by rat Oatp1a5 cRNA-injected oocytes. Concentrations of naringin are 2, 5, 10, 20 and 50 μM. Each result represents the means ± S.E.M. (n=6-10).

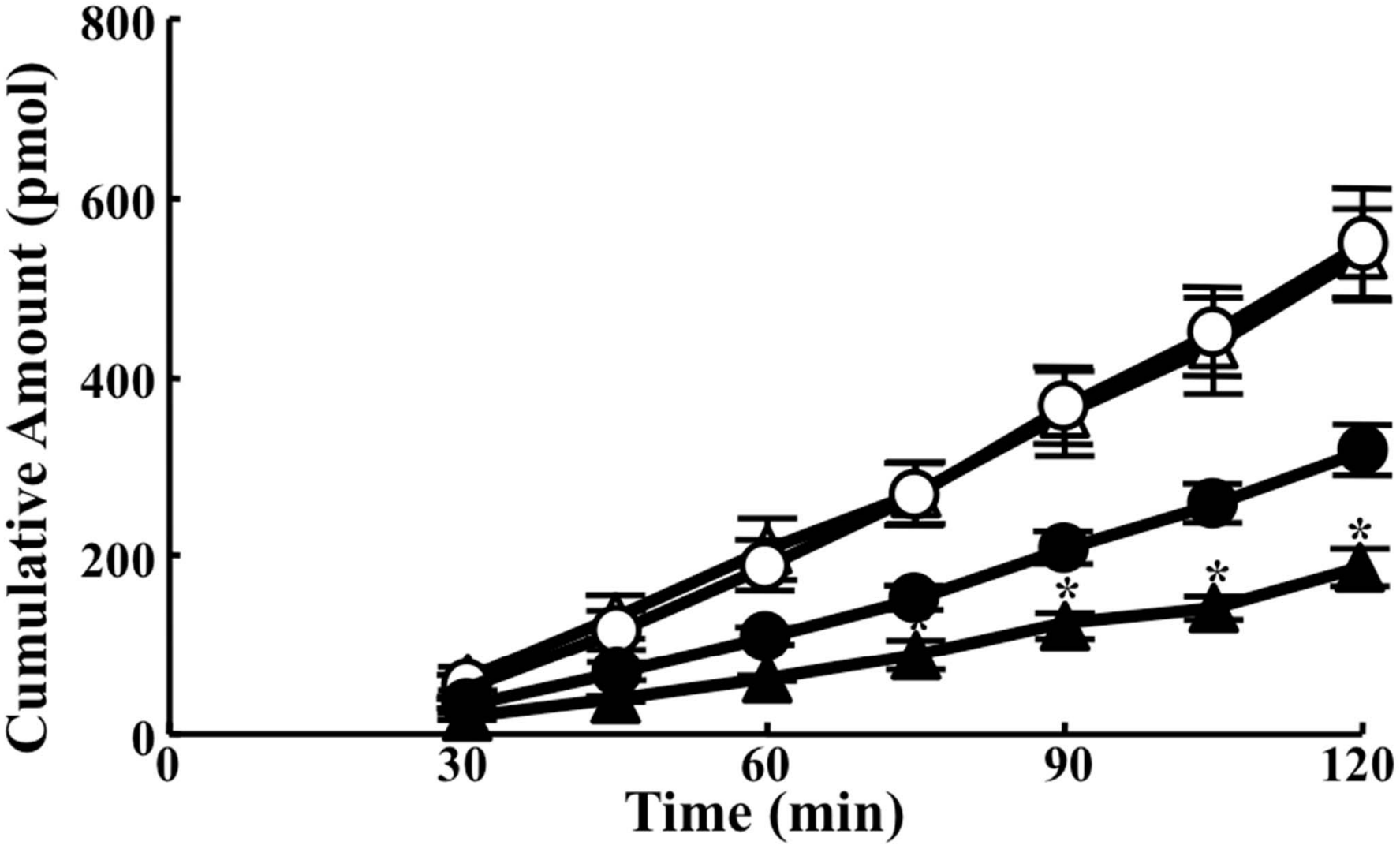
Hiroshi Arakawa Figure 1



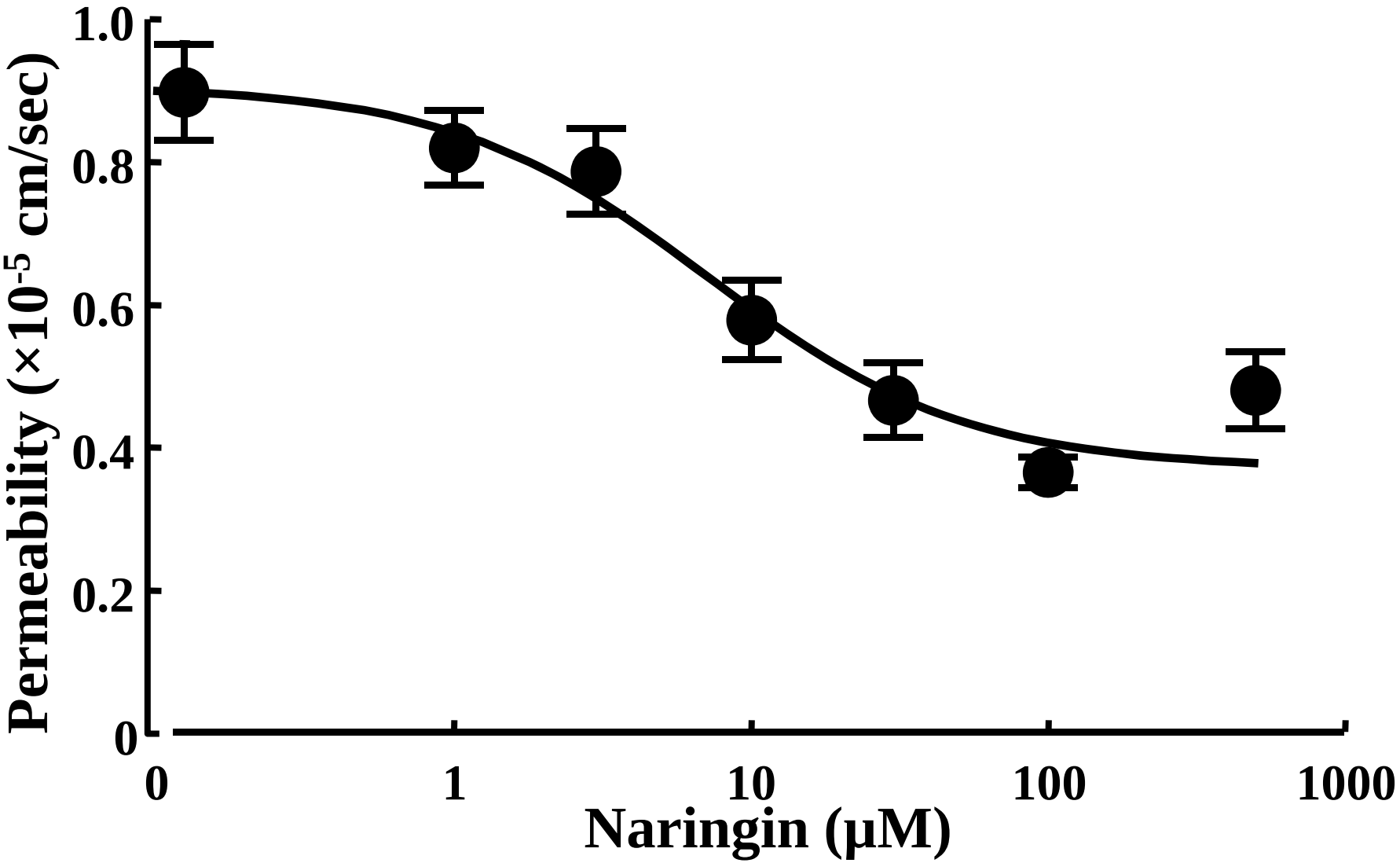
Hiroshi Arakawa Figure 2



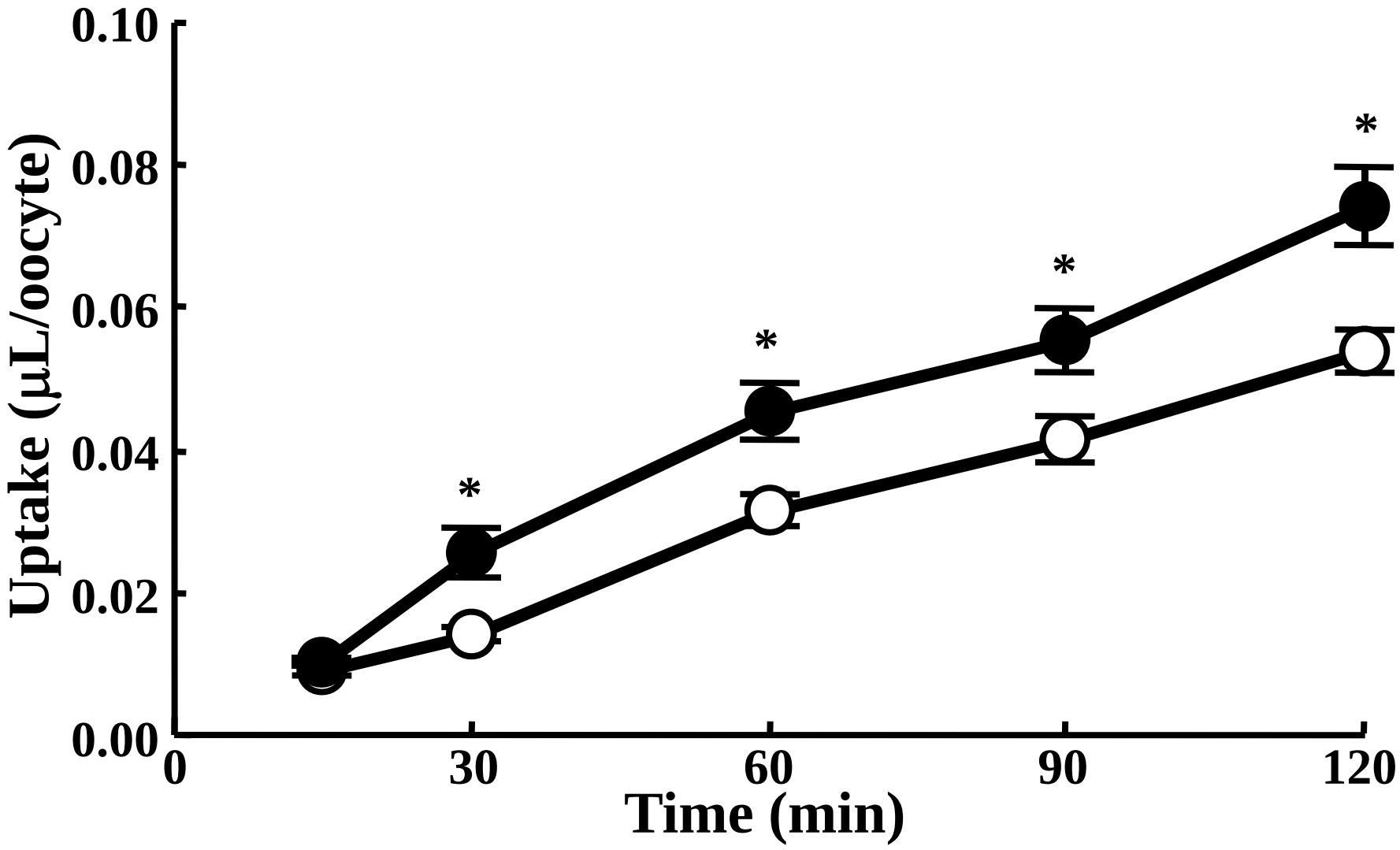
Hiroshi Arakawa Figure 3



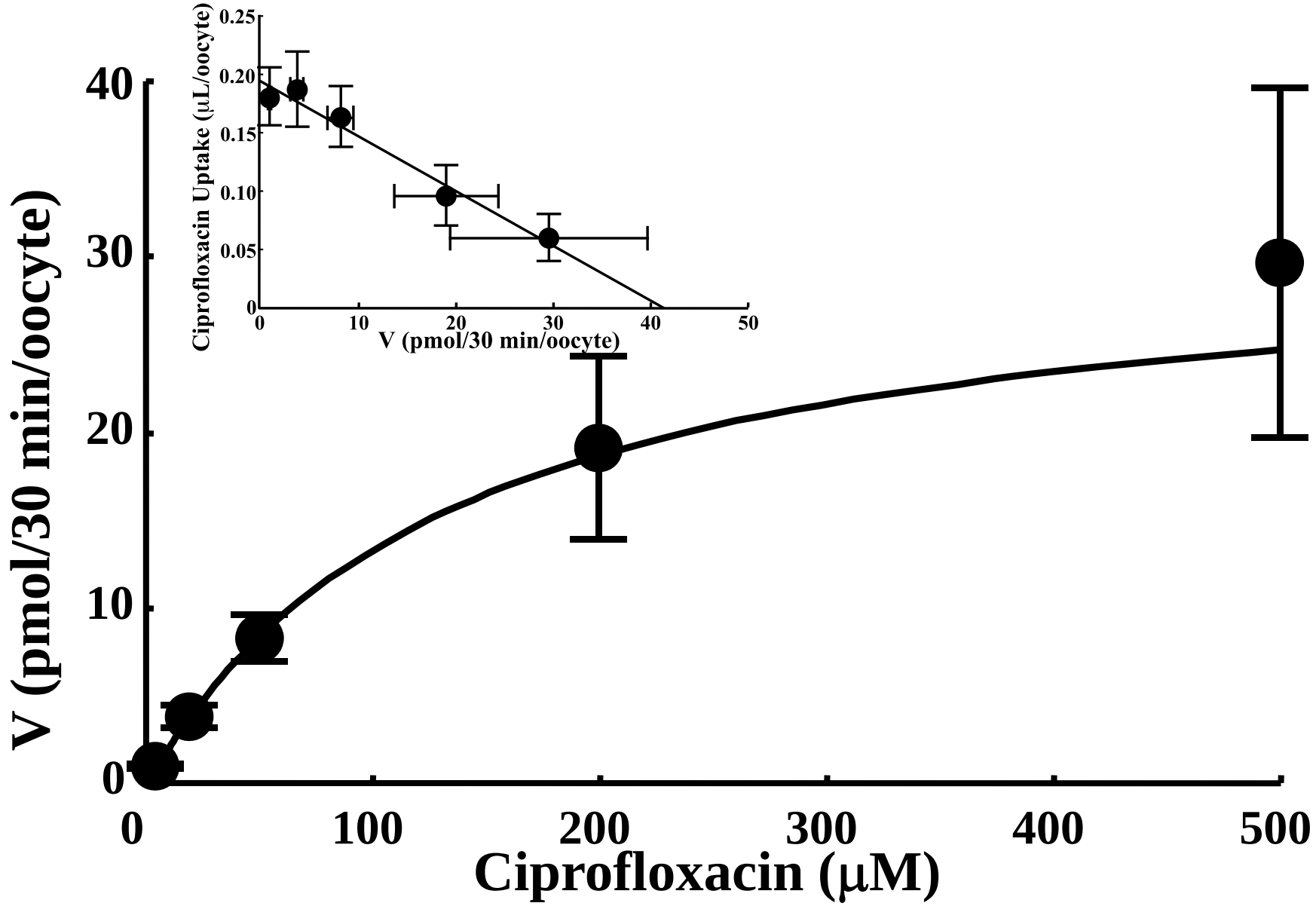
Hiroshi Arakawa Figure 4



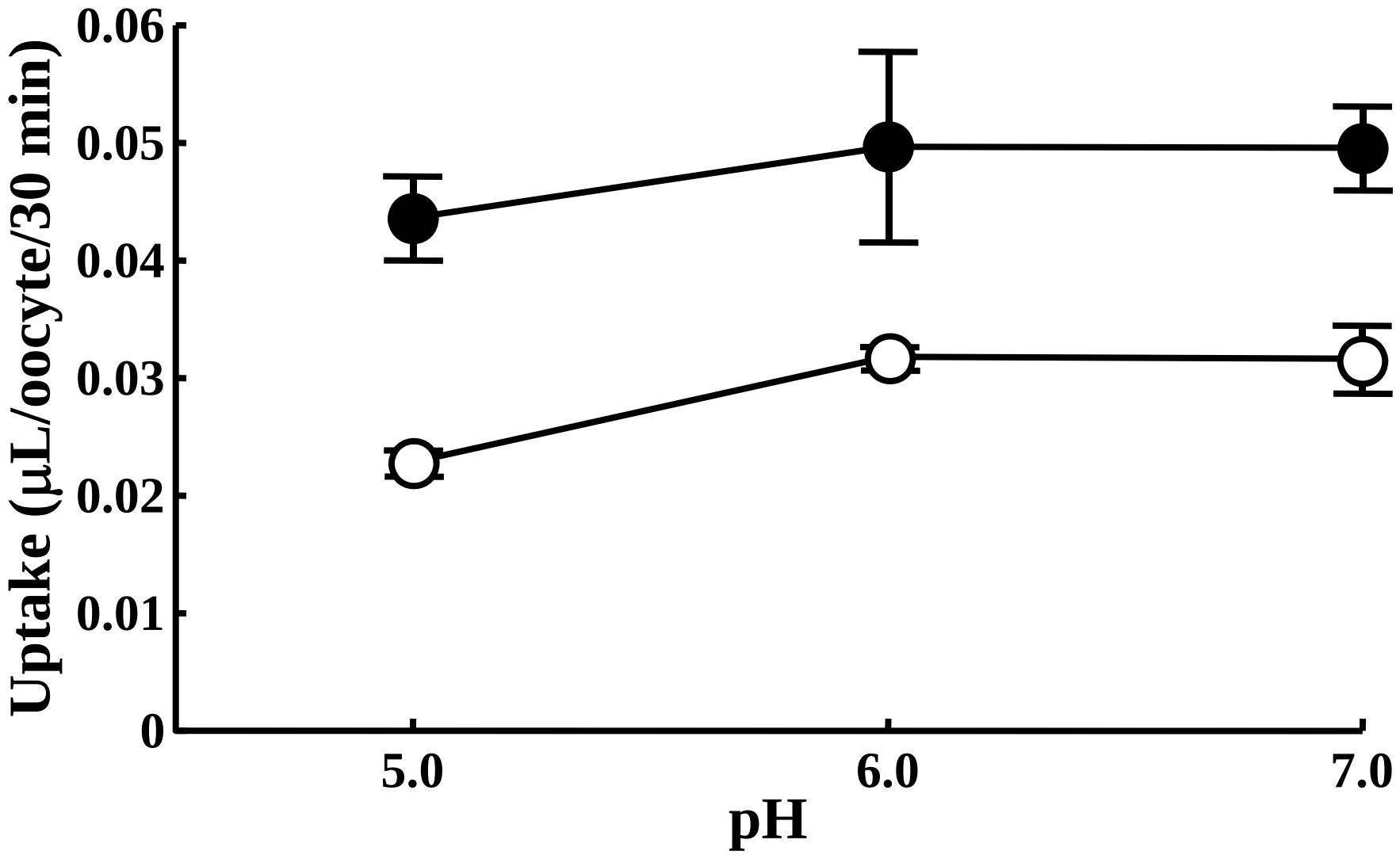
Hiroshi Arakawa Figure 5 (A)



Hiroshi Arakawa Figure 5 (B)



Hiroshi Arakawa Figure 6



Hiroshi Arakawa Figure 7