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Inhibitory effect of JTP-59557, a new triazole derivative, on intestinal phosphate transport in vitro and in vivo

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Abstract

JTP-59557 [(−)-4-(2-*tert*-Butyl-4,5-dichlorophenyl)-5-(5-trifluoromethylpyridin-2-ylsulfanyl)-4H-[1,2,4]triazol-3-ol] showed an inhibitory effect on Na^+-dependent inorganic phosphate (Pi) transport in intestinal brush border membrane vesicles with an IC_{50} value of 0.40 μM in rabbit and with an IC_{50} of 0.19 μM in rat, without affecting Na^+-independent Pi and Na^+-dependent D-glucose transport activities. In Chinese hamster ovary (CHO) cells expressing human type IIb Na/Pi cotransporter (type IIb), JTP-59557 decreased human type IIb-mediated Pi uptake with an IC_{50} of 0.12 μM. In rabbit intestinal brush border membrane vesicles, JTP-59557 behaved as a noncompetitive inhibitor with respect to Pi. In an in vivo study, single administration of JTP-59557 significantly decreased the intestinal Pi absorption rate, when either Pi solution or laboratory chow was given to rats. In this report, we show that JTP-59557 is a potent, selective, stereospecific, noncompetitive inhibitor of intestinal Na/Pi cotransporters including type IIb, and it may represent a new class of intestinal Pi absorption inhibitor.

Keywords: JTP-59557; Type IIb Na/Pi cotransporter; Intestinal Na^+-dependent Pi transport; Pi absorption

1. Introduction

In chronic renal failure, inorganic phosphate (Pi) retention may lead to secondary hyperparathyroidism and uremic bone disease and progression to end-stage renal disease (Slatopolsky and Bricker, 1973). The current therapy for hyperphosphatemia in patients with chronic renal failure consists of dietary Pi restriction in combination with administration of Pi binders, but each therapy has practical problems (Malluche and Monier-Faugere, 2000). Dietary restriction of Pi is limited by poor patient compliance. Aluminum-containing Pi binders were used in the past, but are currently rarely used because aluminum accumulation has serious, toxic effects on bone and brain. Calcium-based binders have largely replaced aluminum, but the use of these is limited by the excessive amounts of calcium absorbed, which can lead to hypercalcemia and soft tissue calcification. Sevelamer hydrochloride, a newer nonabsorbed Pi binder that is aluminum- and calcium-free, has been developed, but reported to cause serious adverse digestive symptoms of the intestine. Thus, new and safe inhibitors for intestinal Pi absorption are needed.

Intestinal Pi absorption occurs by both Na^+-dependent and Na^+-independent processes mainly in the proximal small intestine. The intestinal Na^+-dependent Pi transport has been characterized in use by in vivo intestines, in vitro tissues, and brush border membrane vesicles in various species of mammals (Cross et al., 1990; Danisi and Murer, 1991). The Na^+-dependent component is secondary active and mediated by Na/Pi cotransporter proteins, whereas Na^+-independent transport occurs by an unknown passive mechanism.

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Since 1991, three families (type I, type II, and type III) of Na/Pi cotransporters have been identified. In particular, type II Na/Pi cotransporters (type IIa, type IIb, and type IIc) are expressed in polarized cells and involved in intestinal and/or renal Pi absorption (Werner et al., 1998; Forster et al., 2002). Type IIb Na/Pi cotransporter (type IIb), which has been cloned from human, mouse, and rat (Feild et al., 1999; Xu et al., 1999; Hilfiker et al., 1998; Hashimoto et al., 2000), is thought to be responsible for intestinal Na^+-dependent Pi transport for the following reasons: first, the type IIb protein is located in the brush border membrane of enterocytes (Hilfiker et al., 1998); second, type IIb-mediated Pi transport characteristics (K_m value, pH-dependence) are similar to those of Na^+-dependent Pi transport in intestinal brush border membrane vesicles (Hilfiker et al., 1998; Feild et al., 1999); third, the expression level of type IIb protein is correlated with the transport capacity of intestinal Na^+-dependent Pi transport, when Pi absorption is modulated by various factors (dietary phosphorus, 1,25-dihydroxy vitamin D_3, glucocorticoid) and during ontogeny (Hattenhauer et al., 1999; Katai et al., 1999a; Arima et al., 2002).

There are some Pi-containing compounds that have been reported as intestinal Na^+-dependent Pi transport inhibitors. In in vitro brush border membrane vesicles studies, phosphonoformic acid and 2′-phosphophloretin showed selective and competitive inhibition with respect to Pi, with IC_{50} values of 1 mM and 60 nM, respectively (Loghman-Adham et al., 1987; Peerce and Clarke, 2002). Oral administration of phosphonoformic acid shows the hypophosphatemic effect in normal and uremic rats, and 2′-phosphophloretin also shows a similar effect in normal rats (Loghman-Adham et al., 1993; Brooks et al., 1997; Peerce and Clarke, 2002); however, their contributions to the inhibition of intestinal Pi absorption in vivo remain unknown.

Here, we report that JTP-59557, a new triazole derivative, is a potent, selective, stereospecific, noncompetitive inhibitor of intestinal Na/Pi cotransporters including type IIb, and shows direct inhibition of intestinal Pi absorption in vivo.

2. Materials and methods

2.1. Materials

JTP-59557 [(−)-4-(2-*tert*-Butyl-4,5-dichlorophenyl)-5-(5-trifluoromethylpyridin-2-ylsulfanyl)-4H-[1,2,4] triazol-3-ol] and JTP-59564 [(+)-4-(2-*tert*-Butyl-4,5-dichlorophenyl)-5-(5-trifluoromethylpyridin-2-ylsulfanyl)-4H-[1,2,4] triazol-3-ol] were synthesized by Japan Tobacco Inc (Takatsuki, Japan). Because rotation around the bond between triazole and phenyl was restricted due to steric hindrance of *tert*-Butyl, two stable axially chiral isomers (atropisomers) existed (Fig. 1). These were resolved into JTP-59557 and JTP-59564 (absolute configurations were not determined). [^{32}P]orthophosphate and [^{33}P]orthophosphate were purchased from Amersham Biosciences (Piscataway, USA), D-[6- ^{3}H(*N*)]glucose from PerkinElmer Life Sciences Inc (Boston, USA). Phosphonoformic acid and phlorizin, a Na/glucose cotransporter inhibitor, were purchased from Sigma-Aldrich (St. Louis, USA). For in vitro assays, JTP-59557, JTP-59564, and phlorizin were dissolved in dimethyl sulfoxide, phosphonoformic acid in distilled water.

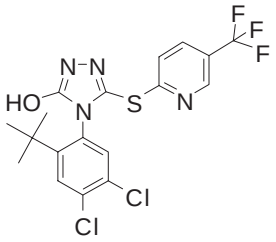

Fig. 1. Chemical structure of JTP-59557 and JTP-59564. Rotation around the bond between triazole and phenyl is restricted due to steric hindrance of *tert*-Butyl. Therefore, two stable axially chiral isomers (atropisomers) were resolved into JTP-59557 and JTP-59564 (absolute configurations were not determined).

2.2. Animals

Male Japanese White rabbits (Kitayama Labes, Nagano, Japan) and male Sprague-Dawley (IGS) rats (Charles River Japan, Yokohama, Japan) were used. Animals were housed in a room with controlled light (on from 8 A.M. to 8 P.M.), temperature (23±2 °C), and humidity (55±10%) and allowed free access to standard laboratory chow and water. All procedures related to the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee of Japan Tobacco Inc.

2.3. Preparation of intestinal brush border membrane vesicles

Male Japanese White rabbits weighing 2.6–3.4 kg were anesthetized with intravenous injection of pentobarbital, and the duodenum and proximal jejunum (first 100 cm below pylorus) were rapidly removed and washed with ice-cold saline. The intestinal mucosa was scraped with glass microscope slides onto an ice-cold glass plate. Brush border membrane vesicles were prepared by magnesium precipitation and differential centrifugation according to the procedure of Danisi et al. (1984) with some modifications. Briefly, fresh mucosa was homogenized twice with Polytron homogenizer (Kinematica AG, Littau, Switzerland) at 1100 g for 20 s. $MgCl_2$ was added up to a final concentration of 10 mM. After the mixture was left for 20 min, it was centrifuged at 3000 g for 15 min. The supernatant was centrifuged at 19,000 g for 40 min. The pellet was resuspended and homogenized with a Polytron homogenizer at 1100 g for 20 s in 300 mM mannitol, 20 mM HEPES–Tris (pH 7.5). After the mixture was centrifuged at 22,500 g for 30 min, the final pellet was suspended in 300 mM mannitol and 20 mM HEPES–Tris (pH 7.5) by passage through a 25-gauge needle.

Rat intestinal brush border membrane vesicles were prepared by calcium precipitation and differential centrifugation, according to the procedure of Tsuji et al. (1987). After male Sprague-Dawley (IGS) rats weighing 310–450 g were decapitated, the proximal jejunum were rapidly removed and washed with ice-cold saline. The intestinal mucosa was scraped with glass microscope slides onto an ice-cold glass plate. The final pellet was suspended in 270

mM mannitol and 10 mM Tris–HEPES (pH 7.5) by passage through a 25-gauge needle.

Protein concentration of brush border membrane vesicles suspension was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, USA). Rabbit intestinal brush border membrane vesicles suspension with the final protein concentration of 10 mg/ml was stored at −80 °C until use. Rat brush border membrane vesicles suspension with the final protein concentration of 10 mg/ml was used on the day of preparation. The purity of the preparation was assessed by enrichment of brush border membrane vesicles/mucosa homogenate ratio of alkaline phosphatase activity, which was 12.7-fold in rabbit and 12.0-fold in rat.

2.4. Transport assays in intestinal brush border membrane vesicles

The transport of radiolabeled Pi or radiolabeled D-glucose was measured by a rapid-filtration technique (Hopfer et al., 1973). The uptake was started by adding 90 μl of the assay solution containing either vehicle (control) or test compound to 10 μl of brush border membrane vesicles suspension (100 μg protein). The reaction was carried out at room temperature and terminated at the desired time by adding 2 ml of ice-cold stop solution. The mixture was then transferred to a premoistened nitrocellulose filter (pore size 0.45 μm, Millipore, Bedford, USA) maintained under a vacuum, and the filter was rinsed 6 times with 1 ml of ice-cold stop solution. The radioactivity on the filter was measured with a liquid scintillation counter (Wallac 1410, Wallac Oy, Turku, Finland).

In Pi transport assay, the composition of assay solution was 0.1 mM KH_2PO_4, 100 mM NaCl or 100 mM Choline-Cl, 100 mM mannitol, 20 mM HEPES–Tris (pH 6.8) containing [^{33}P]orthophosphate, and stop solution containing 100 mM Choline-Cl, 100 mM mannitol, 20 mM $MgSO_4$, 5 mM KH_2PO_4, and 20 mM HEPES–Tris (pH 7.4).

In D-glucose transport assay, the composition of assay solution was 0.1 mM D-glucose, 100 mM NaSCN or 100 mM KSCN, 100 mM mannitol, 10 mM HEPES–Tris (pH 7.4) containing D-[6-^{3}H(*N*)]glucose, and stop solution: 150 mM NaCl, 0.3 mM phlorizin, and 10 mM HEPES–Tris (pH 7.4).

Na^+-dependent Pi or D-glucose transport activity could be calculated by subtracting the transport value in the absence of Na^+ from that in the presence of Na^+.

2.5. Construction of expression vector and stable transfection

According to previously published sequence information (Feild et al., 1999; GenBank accession no. AF111856), the following primers were designed for PCR amplification of human type IIb Na/Pi cotransporter (type IIb): 5′-TCCATCCCAGCACCTGCGGA-3′ (first forward primer), 5′-ATCCCTGACAATCTGGGGCGT-3′ (first reverse primer), 5′-AAGAATTCACCATGGCTCCCTGGCCTGAAT-3′ (second forward primer), 5′-TTGCGGCCGCCTACAAGGCCGTGCATTCGGT-3′ (second reverse primer). PCR amplification of human type IIb sequence from human lung cDNA library (Clontech, Palo Alto, USA) was conducted for 20 cycles (98 °C for 15 s, 65 °C for 2 s, and 74 °C for 35 s), yielding a product that was 2.1k bp in length. The human type IIb PCR product was cloned into the EcoRV site of the cloning vector pBluescriptIIKS(+) (Stratagene, LaJolla, USA). By dideoxy sequencing, the cDNA was verified to include one different base (1859G in GenBank accession no. AF111856–AF111859A) and then was isolated by NotI and XbaI digestion. The isolated insert was ligated into the mammalian expression vector pIT106-1 (Japan Tobacco Inc.), which was made by insertion of the multi cloning site and neomycin-resistant gene into the mammalian expression vector pEF-BOS (Mizushima and Nagata, 1990).

Chinese hamster ovary (CHO) cells were cultured at 37 °C in a 95% air–5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. CHO cells expressing human type IIb were created by transfection of pIT106-1/human type IIb into CHO cells by Trans IT Polyamine Transfection Reagents (Invitrogen, Carlsbad, USA). Transfected cells were selected in the growth medium described above additionally containing 1 mg/ml G418 (Sigma-Aldrich). Approximately 50 colonies were picked and expanded, and then tested for Pi uptake.

2.6. Pi uptake assay by CHO cells stably expressing human type IIb Na/Pi cotransporter

Pi uptake was measured in CHO cells transfected stably with vector containing human type IIb or non-transfected (wild-type). The cells were plated in a 96-well plate at 3×10^4 cells/well and incubated overnight. Immediately after the medium was replaced with 50 μl of [^{33}P]orthophosphate-free assay solution containing either vehicle (control) or test compound, Pi uptake was initiated by addition of 50 μl of assay solution containing [^{33}P]orthophosphate. After the desired incubation time at room temperature, the assay solution was aspirated and cells were rinsed 3 times with 150 μl of ice-cold stop solution. Cells in each well were mixed with MICROSCINT™20 (PerkinElmer), and the radioactivity was determined in a liquid scintillation counter (Topcount, Packard, CT, USA). The composition of assay solution was 0.1 mM KH_2PO_4, 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 14 mM Tris–HCl (pH 7.4), and stop solution: 137 mM NaCl, and 14 mM Tris–HCl (pH 7.4). Protein concentration of cells per well was determined using a BCA protein assay kit (Pierce Biotechnology).

The human type IIb-mediated Pi uptake could be calculated by subtracting Pi uptake value in wild-type cells from that in type IIb-expressing cells.

2.7. Measurement of intestinal Pi absorption in rats

Male Sprague-Dawley (IGS) rats weighing 180–270 g fasted overnight were used. The animals were divided into 3–4 groups; one group being used as vehicle (0.5% methylcellulose)-treated control while the remaining groups received JTP-59557 suspension. They were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The abdomen was opened, and the intestine was ligated at the pylorus. Immediately after intraduodenal administration of the compound (5 ml/kg), saline (0.9% NaCl) solution containing NaH_2PO_4 (1.3 mM) and [^{32}P]orthophosphate was administered in the same way (5 ml/kg). At various times after administration, rats were bled from either peripheral (tail) or portal vein. Radioactivity of 100 μl serum was measured in a liquid scintillation counter (Wallac 1410).

2.8. Measurement of residual phosphorus in rat intestinal lumen

Male Sprague-Dawley (IGS) rats weighing 280–350 g fasted overnight were used. The animals were divided into two groups;

one group being used as vehicle (0.5% methylcellulose)-treated control while the other group received JTP-59557 (30 mg/kg) suspension. The compound was administered orally (5 ml/kg) just before rats were fed 5 g of AIN-93G diet (Reeves et al., 1993) containing 0.15% inorganic and 0.15% organic phosphorus. At 1 h after oral administration, rats were decapitated and the residual luminal contents of stomach, small intestine, and cecum were collected. The residual contents were then heated at 105 °C overnight to remove water, and dry weight was measured. The dry contents were ashed at 550 °C for 6 h and the ashes were heated at 100 °C for 1 h with 10 ml of 5N HNO_3. Extracted Pi was measured using a standard molybdate assay (Taussky and Shorr, 1953) and the amount of phosphorus was calculated.

2.9. Data analysis and statistics

Values are expressed as mean ± S.E.M. In brush border membrane vesicles studies, percent inhibition of the test compound in Na^+-dependent component (Pi or D-glucose transport) was calculated according to the formula

$$[1 - (100\mathrm{NaT} - 0\mathrm{NaT})/(100\mathrm{NaV} - 0\mathrm{NaV})] \times 100(\%)$$

100NaT: adding test compound in assay solution containing 100 mM NaCl or NaSCN
0NaT: adding test compound in assay solution containing 100 mM Choline-Cl or KSCN
100NaV: adding vehicle in assay solution containing 100 mM NaCl or NaSCN
0NaV: adding vehicle in assay solution containing 100 mM Choline-Cl or KSCN.

In Pi uptake assay by CHO cells, percent inhibition of the test compound in human type IIb-mediated Pi uptake was calculated according to the formula

$$[1 - (\mathrm{IIbT} - \mathrm{wildT})/(\mathrm{IIbV} - \mathrm{wildV})] \times 100(\%)$$

IIbT: adding test compound in CHO cells expressing human type IIb
wildT: adding test compound in wild-type CHO cells
IIbV: adding vehicle in CHO cells expressing human type IIb
wildV: adding vehicle in wild-type CHO cells.

In in vivo studies, the differences between vehicle- and compound-treated groups were analyzed by analysis of variance (ANOVA) followed by Dunnett's test or by *F*-test followed by Student *t*-test.

3. Results

3.1. Transport assays in intestinal brush border membrane vesicles

In rabbit intestinal brush border membrane vesicles, Na^+-dependent Pi transport, which was calculated by subtracting Pi transport in 0Na from that in 100Na, increased linearly up to 2 min (data not shown), and so, the reaction time (2 min) was used to quantify the rate of transport in the studies that followed. JTP-59557 inhibited the Na^+-dependent Pi transport in a concentration-dependent manner with an IC_{50} of 0.40 ± 0.20 μM and did not affect the Na^+-independent component. In contrast, JTP-59564, a stereoisomer of JTP-59557, showed no inhibition at as much as 10 μM (Fig. 2A, B).

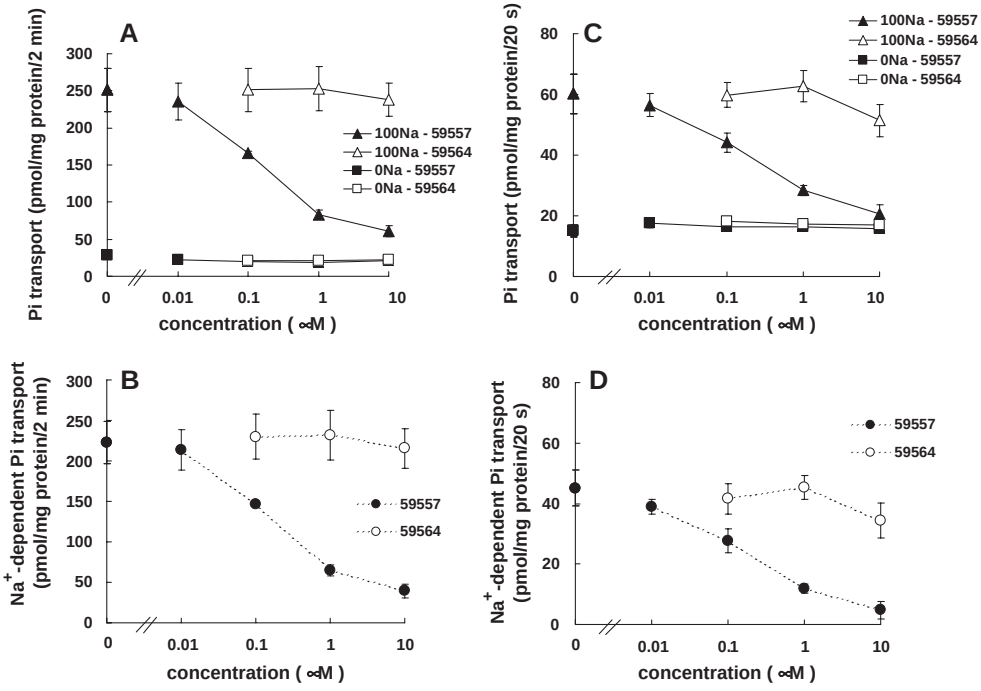

Fig. 2. Effects of JTP-59557 and JTP-59564 on Pi transport in rabbit and rat intestinal brush border membrane vesicles. Transport was initiated by addition of assay solution containing either 100 mM NaCl (100Na) or 100 mM Choline-Cl (0Na) to brush border membrane vesicles suspension. The reaction time was 2 min in rabbit (A, B), and 20 s in rat (C, D) brush border membrane vesicles. Na^+-dependent Pi transport was calculated by subtracting Pi transport in 0Na from that in 100Na (B, D). Each value represents the mean ± S.E.M. of triplicate determinations and independent three experiments.

In the control study, we confirmed that phosphonoformic acid inhibited 54% of Na^+-dependent component at 1 mM and 84% at 3 mM (Table 1). To confirm the specificity, we evaluated effects of the test compounds on Na^+-dependent D-glucose transport activity. The initial value of Na^+-dependent D-glucose transport was 1068.6±90.0 pmol/mg protein/10 s. Phlorizin, a Na/glucose cotransporter inhibitor, inhibited 30% (3 μM) and 56% (10 μM) of Na^+-dependent D-glucose transport, respectively. In contrast, JTP-59557 showed no inhibition at 10 μM (Table 1).

In rat intestinal brush border membrane vesicles, Na^+-dependent Pi transport at 20 s was evaluated as transport rate, because it increased linearly up to 20 s (data not shown). JTP-59557 inhibited the Na^+-dependent Pi transport in a concentration-dependent manner with an IC_{50} of 0.19±0.01 μM without affecting the Na^+-independent component. JTP-59564 showed only 24% inhibition at as much as 10 μM (Fig. 2C, D). Phosphonoformic acid inhibited 58% (1 mM) and 81% (3 mM) of the Na^+-dependent component, respectively (Table 1). The effects of JTP-59557 and phlorizin on Na^+-dependent D-glucose transport are shown in Table 1. The initial value of Na^+-dependent D-glucose transport was 639.9±67.6 pmol/mg protein/10 s. Phlorizin inhibited 68% (3 μM) and 86% (10 μM) of Na^+-dependent D-glucose transport, respectively. In contrast, JTP-59557 showed no inhibition at 10 μM.

3.2. Pi uptake by human type IIb Na/Pi cotransporter

In the time–course study, human type IIb-mediated Pi uptake increased linearly up to 20 min (data not shown), and so, the uptake time (10 min) was used to evaluate the effect of test compound. At this uptake time, the Pi uptake activity was increased 5-fold in type IIb-expressing cells (13.3±1.3 nmol/mg protein) compared with wild-type cells (2.7±0.2 nmol/mg protein). JTP-59557 inhibited human type IIb-mediated Pi uptake in a concentration-dependent manner with an IC_{50} of 0.12±0.03 μM, but JTP-59564 did not (Fig. 3A, B). In the control study, phosphonoformic acid inhibited 21% at 1 mM and 53% at 3 mM, respectively (Table 1).

3.3. Kinetic analysis in rabbit intestinal brush border membrane vesicles

To determine the inhibition mode of JTP-59557 for Na^+-dependent Pi transport, transport measurement was performed with or without test compounds at various external Pi concentrations in rabbit intestinal brush border membrane vesicles. Kinetic analysis using Lineweaver–Burk double-reciprocal plots demonstrated that JTP-59557 showed noncompetitive inhibition on Na^+-dependent Pi transport due to reduction of V_{max}, and not to a change in K_m. In the control study, phosphonoformic acid showed a competitive mode of inhibition with respect to Pi (Fig. 4).

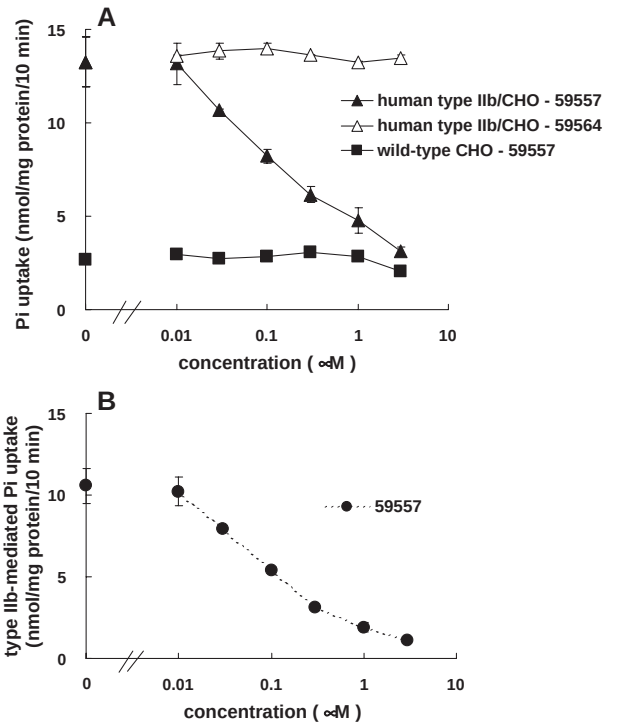

Fig. 3. Effects of JTP-59557 and JTP-59564 on Pi uptake by CHO cells stably expressing human type IIb Na/Pi cotransporter (type IIb) and by wild-type CHO cells. The Pi uptake values were measured at the uptake time (10 min) (A, B). The type IIb-mediated Pi uptake could be calculated by subtracting Pi uptake in wild-type CHO cells from that in type IIb-expressing CHO cells (B). Each value represents the mean±S.E.M. of triplicate determinations and 3–4 independent experiments.

3.4. Pi absorption in rat ligated intestine

After intraduodenal administration of radiolabeled Pi solution, peripheral (tail) vein serum radioactivity increased linearly up to at least 30 min (data not shown). Fig. 5A shows that JTP-59557 significantly decreased peripheral vein serum radioactivity

Table 1
Inhibitory effects of phosphonoformic acid on Na^+-dependent Pi transport, and JTP-59557 and phlorizin on Na^+-dependent D-glucose transport in in vitro assays

		Rabbit BBMV		Rat BBMV		Human type IIb
		Phosphate	D-glucose	Phosphate	D-glucose	Phosphate
Phosphonoformic acid	1 mM	53.5±1.2	NT	57.6±7.2	NT	20.8±2.0
	3mM	83.6±1.7	NT	81.1±1.4	NT	52.8±0.5
Phlorizin	3 μM	NT	29.8±2.3	NT	68.0±1.5	NT
	10 μM	NT	55.5±1.8	NT	86.1±0.6	NT
JTP-59557	1 μM	69.2±6.9	1.8±1.3	73.4±0.7	4.8±2.1	82.8±3.5
	10 μM	81.0±5.5	0.5±2.4	90.5±4.8	5.3±1.5	NT

Each value represents percent inhibition from controls (with vehicle) and the mean±S.E.M. of triplicate determinations and 3–4 experiments. BBMV is an abbreviation of brush border membrane vesicles, and type IIb is type IIb Na/Pi cotransporter. NT means 'not tested'.

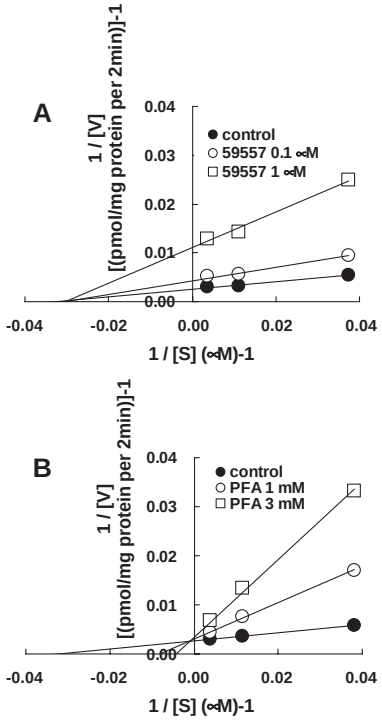

Fig. 4. Mode of inhibition of JTP-59557 and phosphonoformic acid on Na^+-dependent Pi transport in rabbit intestinal brush border membrane vesicles determined using Lineweaver–Burk double-reciprocal plot. Transport assay was done for 2 min using assay solutions containing various external Pi concentrations, without or with 0.1 or 1 μM JTP-59557 (A), without or with 1 or 3 mM phosphonoformic acid (PFA) (B). Each value represents the mean of triplicate determinations.

in a dose-dependent manner at 15 min. To confirm that JTP-59557 has a direct inhibitory effect on intestinal Pi absorption and to avoid overestimation caused by the effect of absorbed JTP-59557 on the renal Pi handling and Pi metabolism in various peripheral tissues, we evaluated whether JTP-59557 inhibited portal vein serum radioactivity. In the time–course study, portal vein serum radioactivity increased linearly up to 15 min, followed by plateau to 30 min (data not shown). JTP-59557 significantly decreased portal vein serum radioactivity at 15 min, similar to its effect on peripheral vein serum radioactivity (Fig. 5B).

3.5. Residual phosphorus in rat intestinal lumen

We evaluated whether JTP-59557 had the inhibitory effect on intestinal phosphorus absorption after normal rats were fed 5 g of AIN-93G diet containing 0.15% inorganic and 0.15% organic phosphorus. All rats started to eat immediately after feeding, and completely finished the chow provided within 30 min. At 1 h after oral administration of the compound, the contents in gastrointestinal tract were collected. There were no differences between the vehicle-treated and JTP-59557-treated groups in dry weight of residual luminal contents in stomach, small intestine, and cecum (Fig. 6A). JTP-59557 significantly increased residual phosphorus concentration (residual phosphorus per dry weight of residual contents) in lumen of small intestine and cecum, without affecting that in lumen of stomach (Fig. 6B).

4. Discussion

In severe renal failure and end-stage renal disease, one possible therapeutic target to control hyperphosphatemia is the development of compounds that inhibit renal and/or intestinal Na/Pi cotransporters, and further, intestinal Pi absorption inhibitor is thought to be more useful than renal Pi reabsorption inhibitor because of serious loss of renal function (Edwards, 2002). Thus we have evaluated inhibitory effects of various compounds on Na^+-dependent Pi transport in a rabbit intestinal brush border membrane vesicles assay system, and finally selected the racemic mixture that could be resolved into JTP-59557 and JTP-59564, and determined the activity of these two chiral compounds.

In brush border membrane vesicles from rabbit and rat small intestine, the property of Pi or D-glucose transport (initial values, time–course, the effect of known inhibitors) is consistent with that in previous reports (Berner et al., 1976; Kessler et al., 1978; Danisi et al., 1984; Loghman-Adham et al., 1987; Shirazi-Beechey et al., 1988). JTP-59557 inhibited Na^+-dependent Pi transport in a concentration-dependent manner with an IC_{50} value of 0.19–0.40 μM. Specificity of JTP-59557 inhibition for the Na^+-dependent Pi transport was confirmed, because JTP-59557 did not affect the Na^+-independent Pi transport component and the Na^+-dependent D-glucose transport activity at higher concentration. Further,

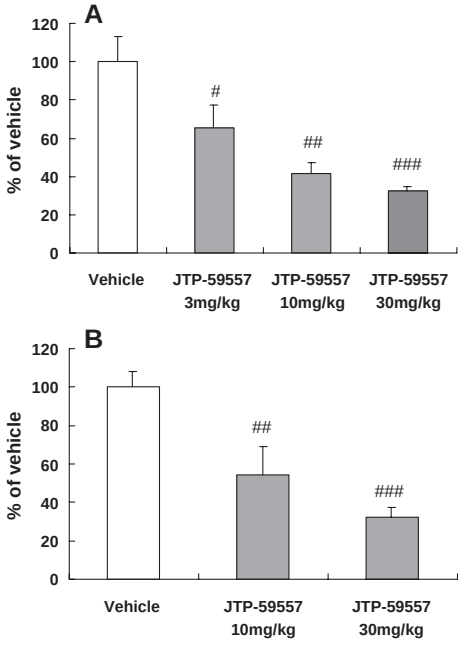

Fig. 5. Effect of JTP-59557 on Pi transport in rat ligated intestine. Immediately after intraduodenal administration of the compound, saline solution containing NaH_2PO_4 and [^{32}P]orthophosphate was administered in the same way. Rats were bled from tail vein (A) or portal vein (B) at 15 min and radioactivity of 100 μl serum was measured. The serum radioactivity with administration of vehicle was taken as 100%. Each value represents the mean ± S.E.M. of five rats (A), 5–7 rats (B). #$P<0.05$, ##$P<0.01$, ###$P<0.001$ compared with vehicle-treated group by analysis of variance (ANOVA) followed by Dunnett's test.

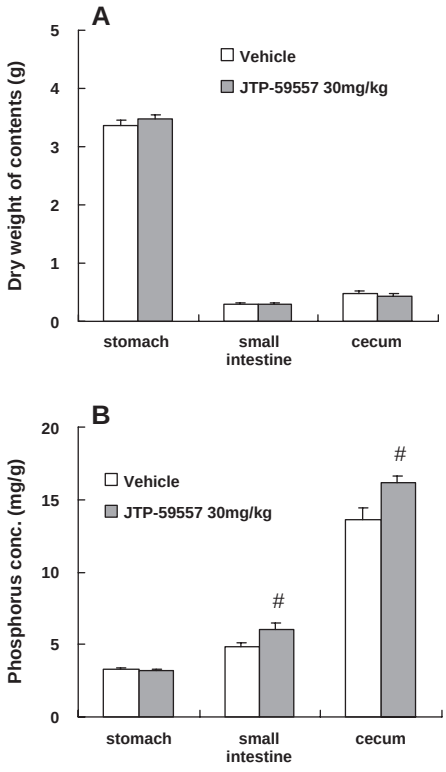

Fig. 6. Effect of JTP-59557 on residual luminal phosphorus contents after chow consumption in normal rats. The compound was administered orally just before rats were fed AIN-93G diet. At 1 h after oral administration, the residual luminal contents of stomach, small intestine, and cecum were collected. The dry weight of each was measured (A). The phosphorus concentration (mg/g) means residual luminal phosphorus (mg)/dry weight of residual luminal contents (g) (B). Each value represents the mean ± S.E.M. of ten rats. #$P<0.05$ compared with vehicle-treated group by F-test followed by Student t-test.

we established CHO cells stably expressing human type IIb Na/Pi cotransporter. The human type IIb-mediated Pi uptake activity was 5-fold higher than Pi uptake by wild-type CHO cells, and showed high activity at lower pH (data not shown) as described previously (Feild et al., 1999; Xu et al., 2002). JTP-59557 decreased the human type IIb-mediated Pi uptake in a concentration-dependent manner with an IC_{50} value of 0.12 μM, without affecting Pi uptake in wild-type CHO cells. In rabbit intestinal brush border membrane vesicles, JTP-59557 showed noncompetitive mode of inhibition with respect to external Pi. To our knowledge, this is the first report regarding a noncompetitive inhibitor for intestinal Na^+-dependent Pi transport. Usually, Na^+-dependent Pi transport assay in brush border membrane vesicles is performed at an external Pi concentration of approximately 100 μM, which is around the K_m value. However, endogenous Pi concentration in intestinal lumen in vivo is thought to be at least over 1 mM, and so a larger amount of competitive inhibitors may be needed for the inhibition of physiological Pi transport in the intestinal brush border membrane. In view of the needed amount, noncompetitive inhibitors appear to be more useful than competitive inhibitors.

In contrast to JTP-59557, the activity of JTP-59564 for the inhibition of Na^+-dependent Pi transport is less potent, suggesting that the stereospecific interaction between JTP-59557 and Na/Pi cotransporter protein including type IIb is important for the inhibition of Na^+-dependent Pi transport function. The stereoscopic conformation between triazole and phenyl of these compounds may be associated with the activity of the inhibition.

In previous reports, some compounds that decrease intestinal Na^+-dependent Pi transport in vitro have been reported regarding in vivo effects. Phosphonoformic acid has been shown to inhibit Na/Pi cotransport competitively with respect to external Pi in intestinal and renal brush border membrane vesicles (Loghman-Adham et al., 1987; Szczepanska-Konkel et al., 1986), but its activity is relatively weak (IC_{50} ~1 mM). Phosphonoformic acid reduced serum Pi levels when administered intraperitoneally or orally in normal rats and orally in uremic rats (Loghman-Adham et al., 1993; Brooks et al., 1997), but these hypophosphatemic effects appeared to be caused mainly by increase of renal Pi excretion. Thus, it is not confirmed whether the inhibitory effect of phosphonoformic acid on intestinal brush border membrane Na/Pi cotransport shown in vitro can lead to reduction of intestinal Pi absorption and serum Pi levels in vivo. Recently, 2′-phosphophloretin, a phosphate ester of phloretin, has been shown to inhibit Na^+-dependent Pi uptake in rat, rabbit, and human intestinal brush border membrane vesicles (Peerce and Clarke, 2002; Peerce et al., 2003). Inhibition of brush border membrane Na^+-dependent Pi uptake by 2′-phosphophloretin required external Na^+ and was competitive with respect to external Pi. In vivo, 2′-phosphophloretin reduced plasma Pi levels in normal rats in a concentration-dependent manner. The effects of 2′-phosphophloretin on fecal and urinary Pi excretion in vivo, which they did not report, might elucidate whether 2′-phosphophloretin inhibits renal Pi reabsorption and how much its inhibitory effect on intestinal Pi absorption contributes to the reduction of plasma Pi levels. Niceritrol, a nicotinic acid derivative, is an anti-hyperlipidaemia agent for which administration to patients undergoing dialysis caused a reduction of serum Pi levels (Shimoda et al., 1996). Although it remains unknown whether Niceritrol directly inhibits Na/Pi cotransport function or decreases the expression level of Na/Pi cotransporter proteins, repeated oral administration of Niceritorol to normal rats reduced intestinal brush border membrane Na^+-dependent Pi transport activity and increased fecal Pi excretion, without affecting serum Pi levels and urinary Pi excretion (Kuboyama et al., 1999; Katai et al., 1999b). Unfortunately, because of critical side effects, administration of Niceritorol is not a better therapeutic approach to hyperphosphatemia, but these effects have suggested that the inhibition of intestinal Na/Pi cotransport function might be useful in the therapy of hyperphosphatemia.

In the present study, JTP-59557 inhibited peripheral and portal vein serum radioactivity in a dose-dependent manner after intraduodenal administration of radiolabeled Pi sol-

ution to normal rats. Further, after feeding chow to normal rats, JTP-59557 significantly increased residual luminal phosphorus per dry weight of contents in small intestine and cecum, without affecting that in stomach. JTP-59557 did not affect the movement of diet in gastrointestinal lumen, because there were no differences between vehicle-treated and JTP-59557-treated group in dry weight of residual luminal contents in all three regions. Since organic phosphorus in the food is generally resolved into Pi by intestinal alkaline phosphatase and is absorbed, these in vivo effects suggest that inhibition of brush border membrane Pi uptake by JTP-59557 leads to increase of residual luminal phosphorus and decrease of intestinal Pi absorption rate. In the future, it will be necessary to examine whether JTP-59557 dosed repeatedly has the effects on serum Pi levels, fecal/renal Pi excretion, and Pi metabolism in various tissues in normal and uremic animals (Kuboyama et al., 1999; Nagano et al., 2001; Sanai et al., 1989; Loghman-Adham et al., 1993).

In conclusion, we showed that JTP-59557 is a potent and a specific inhibitor of type IIb Na/Pi cotransporter, intestinal Na^+-dependent Pi transport in vitro, and intestinal Pi absorption in vivo. These results suggest that JTP-59557 may represent a new class of intestinal Pi absorption inhibitor.

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