Original Paper

Effect of Known Inhibitors of Ion Transport on Pendrin (SLC26A4) Activity in a Human Kidney Cell Line

Emanuele Bernardinelli Roberta Costa Charity Nofziger Markus Paulmichl
Silvia Dossena

Institute of Pharmacology and Toxicology, Paracelsus Medical University, Salzburg, Austria

Key Words

Pendrin • Kidney • Airways • Hypertension • Asthma • Niflumic acid • Tenidap

Abstract

Background/Aims: Pendrin is a $\text{Cl}^-/\text{I}^-/\text{HCO}_3^-$ exchanger playing a fundamental role in controlling blood pressure and airway function, therefore representing an attractive target for the treatment of hypertensive states and respiratory distresses. A review of the literature regarding the ability of some compounds (namely several known inhibitors of ion transport) to block pendrin activity revealed discordant findings. These incongruous findings may be due, in part, to the concentration of compound and/or the nature of the model system used in the study. **Methods:** Pendrin activity was evaluated by measuring pendrin-dependent iodide influx following overexpression of the transporter in a human kidney cell line, in the presence of selected test compounds or the respective vehicles. **Results:** Pendrin activity was significantly hampered by 0.1 mM 5-nitro-2-[(3-phenylpropyl)amino]benzoic acid (NPPB), niflumic acid and tenidap, but was resistant to 0.1 mM 4, 4'-diisothiocyano-2, 2'-stilbene-disulfonic acid (DIDS), furosemide and probenecid. **Conclusions:** The results of the present study indicate that clinically effective non-steroidal anti-inflammatory drugs (niflumic acid and tenidap) directly inhibit pendrin activity.

© 2016 The Author(s)
Published by S. Karger AG, Basel

Introduction

Pendrin (solute carrier family 26, member A4; SLC26A4) is an electroneutral, Na^+ -independent $\text{Cl}^-/\text{I}^-/\text{HCO}_3^-$ exchanger of which malfunction leads to forms of sensorineural autosomal recessive syndromic (Pendred syndrome; OMIM ID_274600) [1] or non-syndromic deafness (DFNB4; OMIM ID_600791) [2], with and without thyroid involvement, respectively. Besides being expressed in the inner ear and thyroid, pendrin is also found in the kidney and airways.

In the kidney, pendrin is localized on the apical membrane of B and non-A, non-B intercalated cells of the cortical collecting duct (CCD) and connecting tubule (CNT) [3-5]. The first well characterized function of pendrin in the kidney was the secretion of HCO_3^- and the control of acid-base balance [3, 6]. Later, a consistent body of evidence pointed to a significant

role of pendrin in the conservation of Cl^- [7, 8] and regulation of blood pressure [9, 10]. Differences in blood pressure between pendrin null and wild type mice, although not evident in basal conditions, became apparent following dietary NaCl restriction or aldosterone infusion [7, 11]. In these settings, pendrin null mice were unable to appropriately conserve NaCl and, as a consequence of reduced abundance [12], channel surface density and activity [13] of the epithelial sodium channel (ENaC), a major determinant of the aldosterone-dependent, amiloride-sensitive salt reabsorption in the distal nephron, developed hypotension. In addition, pendrin governs an electroneutral, thiazide-sensitive NaCl reabsorption in tandem with the sodium-driven chloride/bicarbonate exchanger/solute carrier family 4, member A8 (NDCBE/SLC4A8) [14]. Accordingly, mice overexpressing pendrin develop salt-sensitive hypertension when exposed to a high-salt diet [15]. The role of pendrin in regulating blood pressure is further evidenced in pendrin/sodium-chloride cotransporter (NCC) double knockout mice, as they display severe salt wasting, sharp increases in urine output and profound volume depletion [16]. Overall, these evidences suggest that pendrin gene ablation may be protective against the development of hypertension [9], and that pendrin could be a promising target for diuretic therapy [17-19].

In the airways, increased pendrin expression has been linked to respiratory diseases including bronchial asthma, chronic obstructive pulmonary disease (COPD), rhinovirus infection, rhinitis and chronic rhinosinusitis [20, 21]. Pendrin expression is upregulated in murine models of asthma [22] via an interleukin (IL)-4/13-dependent pathway [23, 24], leading to increased mucus production and neutrophilic infiltration [25]. In addition, pendrin-driven Cl^- reabsorption can reduce the thickness of the airway surface liquid, therefore worsening airway inflammation and obstruction [26]. Accordingly, pendrin gene transcription was increased in endobronchial biopsies from asthmatic patients [27]. In addition, pendrin was upregulated by IL-17A in human bronchial epithelial cells [28] and lungs of *Bordetella pertussis* infected mice [29]. The emerging evidence supporting a role of pendrin in the pathogenesis of inflammatory and infectious lung disease indicates that this molecule could be a potential novel therapeutic target for the treatment of respiratory distresses [20, 21].

Because pendrin inhibition may represent a novel strategy in the treatment of various disease states, notable effort was devoted to the identification of pendrin blockers. In the absence of an experimentally determined structure [30], structure-based drug design was inapplicable in the difficult task of identifying new pendrin inhibitors. Indeed, very recently, selective pendrin inhibitors, belonging to the tetrahydropyrazolopyridine and pyrazolothiophenesulfonamide classes of compounds, were identified by a high-throughput screen of ~36,000 synthetic small molecules following pendrin expression in Fischer rat thyroid cells. Among those, PDS_{inh}-A01 increased airway surface liquid volume in IL-13-treated airway cell cultures from both healthy subjects and patients affected by cystic fibrosis [31].

A review of the literature regarding the ability of some compounds (namely several known inhibitors of ion transport) to block pendrin activity revealed discordant findings (Table 1). These incongruous findings may be due, in part, to the concentration of compound and/or the nature of the model system used in the study. Here we show the functional screening of relatively low concentrations (0.1 mM) of 22 small molecules on pendrin-driven iodide influx following overexpression of human pendrin in a human kidney cell line, performed with the aims of (i) shedding light on previous conflicting results and (ii) confirming previous findings obtained with different techniques and/or model cell systems.

Materials and Methods

Plasmids

The open reading frame (ORF) of human pendrin from normal thyroid tissue was cloned into the pTARGET (Promega Corporation) vector, originally provided by Prof. P. Beck-Peccoz, University of Milan,

Table 1. Compounds tested on pendrin activity. The established target(s) known to be blocked by each compound is (are) reported together with the previously reported (when known) effect on pendrin and the effect observed in the present study. The concentration tested in the present or previous studies was 0.1 mM, except where otherwise specified. The concentration of CFTR-inhibitor 172 was 1.6 μ M, suggested by previous reports leading to maximal reduction in the open probability of CFTR [88]. The concentration of eplerenone was 10 μ M, due to poor solubility. CFTR, cystic fibrosis transmembrane conductance regulator; COX, cyclooxygenase; DIDS, 4, 4'-diisothiocyano-2, 2'-stilbene-disulfonic acid; DNDS, 4, 4'-dinitrostilbene-2, 2'-disulfonate; DRA, down-regulated in adenoma; ENaC, epithelial sodium channel; KCNJ1, potassium channel, inwardly rectifying subfamily J, member 1; LOX, lipoxygenase; NCC, sodium chloride cotransporter; NKCC2, sodium potassium chloride cotransporter 2; NPPB, 5-nitro-2-[(3-phenylpropyl)amino] benzoic acid; OAT1, organic anion transporter 1; PLA2, phospholipase A2; SLC26A3, solute carrier family 26, member A3

Test compound	Conventional target	Effect on pendrin	
		Previous reports	Present study
Diuretics and antihypertensive agents			
Acetazolamide	Carbonic anhydrase [80]	No effect [49]	No effect
Amiloride	ENaC [81, 82]	No effect [49]	No effect
Bumetanide	NKCC2 [82]	No effect [49]	No effect
Chlorothiazide	NCC [83, 84]	No effect [49]	No effect
Eplerenone	Aldosterone antagonist [82, 85]	-	No effect (10 μM)
Furosemide	NKCC2 [82, 86]	No effect [49]	No effect
		59% inhibition (1 mM) [47]	39% inhibition (1 mM)
Hydrochlorothiazide	NCC [83, 84]	No effect [49]	No effect
Hydroflumethiazide	NKCC2 [82]	No effect [49]	No effect
Indapamide	NCC (?) [84]	No effect [49]	No effect
Methazolamide	Carbonic anhydrase [87]	No effect [49]	No effect
Torsemide	NKCC2 [82]	-	No effect
Triamterene	ENaC [82]	No effect [49]	No effect
Spironolactone	Aldosterone antagonist [82, 85]	No effect [49]	No effect
Chloride channels and anion transport blockers			
CFTR-inhibitor 172	CFTR [88]	No effect [49]	No effect (1.6 μM)
DIDS	Anion transport [89-91]	No effect [49]	No effect
		No effect (0.5 mM) [50]	48% inhibition (1 mM)
		No effect (0.5 mM) [44]	
		88% inhibition (0.5 mM) [51]	
		Full inhibition (0.5 mM) [52]	
		62% inhibition (1 mM) [47]	
DNDS	Anion transport [92]	-	No effect
NPPB	Chloride channels [75, 90]	No effect [49]	33% inhibition
		59% inhibition [44]	
Probenecid	OAT1 [93]	No effect [49]	No effect
		37% inhibition (1 mM) [47]	No effect (1 mM)
Previously identified inhibitors of SLC26A3 (DRA)			
Glybenclamide	KCNJ1 [94]	No effect [49]	No effect
Hydroxycinnamate		No effect [49]	No effect
		No effect (1 mM) [50]	
Niflumic acid	COX, PLA2 [91, 95]	Inhibition (0.25 mM) [25]	64% inhibition
		Inhibition [44, 49]	
Tenidap	COX [64], LOX [65] (?)	49 % inhibition [50]	30% inhibition

Italy. The plasmid encoding for the enhanced yellow fluorescent protein (EYFP) H148Q:I152L (an EYFP mutant with substantially improved sensitivity for iodide [32]) was obtained by site-directed mutagenesis of the pEYFPN1 vector (Clontech) and is heretofore referred to as pEYFP H148Q:I152L N1. All plasmid inserts were sequenced prior to use in experiments (Microsynth AG, Switzerland).

Cell culture and transient transfection

Human embryonic kidney (HEK) 293 Phoenix [33] cells were cultured in Minimum Essential Eagle Medium (Sigma-Aldrich, Austria) supplemented with 10% fetal bovine serum (Lonza), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM pyruvic acid (sodium salt). The cells were maintained at 37°C, 5% CO₂, 95% air and 100% humidity. Subcultures were routinely established every second to third day by seeding the cells into 100 mm diameter Petri dishes following trypsin/ethylenediaminetetraacetic acid (EDTA) treatment.

For functional tests, cells were seeded into black 96-multiwells, grown overnight and transfected with a total amount of 0.2 µg/well of plasmid DNA. For western blots, cells were seeded into 6-well plates, grown overnight and transfected with a total amount of 2 µg/well of plasmid DNA. The calcium phosphate co-precipitation method was used for transient transfection into cells.

Western blot

Cells were transiently transfected with the pTARGET plasmid bearing the cDNA of pendrin or with an empty pTARGET vector (control). 72 hours after transfection cells were collected and centrifuged at 216 x g for 15 minutes at 4°C. The extraction of the total membrane protein fraction, including both plasma membrane proteins and membrane proteins from cellular organelles, was obtained with the Plasma Membrane Extraction Kit (MBL, USA), according to the manufacturer's instructions. Total cellular membranes proteins were solubilized with 100 mM dithiothreitol and 8% sodium dodecyl sulphate (SDS) and boiled for 5 minutes before loading on a 7.5% polyacrylamide gel. Separated proteins were transferred on polyvinylidene difluoride membranes (Immunoblot PVDF Membranes, BIO-RAD, Germany) by applying a constant voltage (75 V) for 2 hours at 4°C. The membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 (TBST), cut horizontally in two parts and incubated overnight at 4°C with the primary antibodies (rabbit anti-pendrin, polyclonal, raised against amino acids 586-780 of human pendrin, sc-50346, Santa Cruz Biotechnology, 1:500, or rabbit anti-calreticulin, ab4, Abcam, 1:1,000) in 3% nonfat dry milk in phosphate buffered saline (PBS). Membranes were successively washed thrice in TBST, incubated for 1 hour at room temperature with infrared dye-conjugated secondary antibodies (goat anti-rabbit IRDye 800CW conjugated, LI-COR, USA, 1:20,000 in 3% nonfat dry milk in PBS) and washed thrice in TBST. Detection of the signal of immunocomplexes was obtained with the ODYSSEY infrared imaging system (LI-COR). Blot images were densitometrically analyzed with ImageJ 1.46r software.

Pendrin functional test

For testing the function of pendrin in the presence of test compounds and the respective vehicles, the influx of iodide was measured in pendrin-overexpressing and control cells. Cells were co-transfected with 0.1 µg of pEYFP H148Q:I152L N1 plasmid and 0.1 µg of pTARGET plasmid bearing the cDNA of pendrin. Control cells were co-transfected with pEYFP H148Q:I152L N1 and the empty pTARGET vectors. The functional test was performed 48 hours after transient transfection as already described [34-37], with adaptations allowing for the use of a multiplate reader [38-41]. As high transfection efficiency is an essential prerequisite, HEK 293 Phoenix cells, in which transfection efficiencies of ~90% are obtained, are particularly suitable for this approach. In short, cells were washed from the culture medium and bathed in 70 µl of high chloride solution (in mM: KCl 2, NaCl 135, CaCl₂ 1, MgCl₂ 1, D-glucose 10, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) 20, 308 mOsm/KgH₂O with mannitol, pH 7.4) containing 0.3 mM of the test compound or the respective vehicle. After measuring the fluorescence intensity (1 measurement/sec for 3 sec), 140 µl of high iodide solution (in mM: KCl 2, NaI 135, CaCl₂ 1, MgCl₂ 1, D-glucose 10, HEPES 20, 308 mOsm/KgH₂O with mannitol, pH 7.4) were injected into each well to obtain a final concentration 0.1 mM for the test compound, except where otherwise specified (Table 1). Then, the fluorescence intensity was measured again (1 measurement/sec for 16 sec). Since pendrin can act as a chloride/iodide exchanger [34, 42, 43], the presence of iodide in the extracellular medium induces an iodide flux into the cytoplasm of pendrin-expressing cells. Iodide is a much better quencher of EYFP H148Q:I152L fluorescence than chloride; therefore, an increase in intracellular iodide leads to a decrease of EYFP H148Q:I152L fluorescence [32], which reflects pendrin transport efficiency in pendrin-overexpressing cells. Fluorescence variations in control cells (not overexpressing pendrin) reflect the activity of anion channels/transporters endogenously expressed in HEK 293 Phoenix cells. Experiments were performed at room temperature.

Fig. 1. Pendrin expression levels in control and transiently transfected HEK 293 Phoenix cells. (a) representative immunoblotting on total cellular membrane extracts from control and pendrin transfected HEK 293 Phoenix cells. Control cells were transfected with an empty vector. 1, 2 and 3 in the upper panel indicate the non-glycosylated, partially glycosylated and maturely glycosylated forms of pendrin, respectively. The lower panel corresponds to the signal of the housekeeping protein calreticulin. (b) the expression levels of pendrin were quantified by densitometry and normalized to calreticulin. n = 4, ***: p < 0.001, two-tailed, unpaired Student's t test.

Fluorescence intensity was detected with the VICTOR™ X3 Multilabel Plate Reader (Perkin Elmer) equipped with a liquid dispenser and the following filters: excitation: F485 (excitation center wavelength (CWL): 485 nm, bandwidth: 14 nm), emission: F535 (emission CWL: 535 nm, bandwidth: 25 nm). The background fluorescence measured in cells transfected with 0.2 µg of the pTARGET empty vector was subtracted from all other fluorescence measurements of the same 96-well plate. Data were expressed as % fluorescence variations (ΔF%). The transport activity of pendrin expressing or control cells in the presence of the test compound was statistically compared to the transport activity of pendrin expressing or control cells in the presence of the appropriate vehicle from a same 96-well plate.

Salts and chemicals

4, 4'-diisothiocyano-2,2'-stilbene-disulfonic acid (DIDS; D3514), 4, 4'-dinitrostilbene-2, 2'-disulfonic acid (DNDS; S347523), 5-nitro-2-[(3-phenylpropyl)amino]benzoic acid (NPPB; N4779), acetazolamide (A6011), amiloride (A7410), bumetanide (B3023), CFTR inhibitor-172 (C2992), chlorothiazide (C4911), eplerenone (E6657), furosemide (F4381), glybenclamide (G0639), hydrochlorothiazide (H4759), hydroflumethiazide (H8760), hydroxycinnamic acid (545287), indapamide (I1887), methazolamide (VETRANAL™ 30711), niflumic acid (N0630), probenecid (P8761), spironolactone (S3378), tenidap (PZ0196), torsemide (T3202) and triamterene (T4143) were purchased from Sigma-Aldrich (Austria). Stock solutions (0.1 M) were prepared in an appropriate organic solvent and stored at -20 °C in the dark. All salts and chemicals used were of *pro analysis* grade.

Statistical Analysis

All data were expressed as arithmetic means ± S.E.M. For statistical analysis, GraphPad Prism software (version 4.00 for Windows, GraphPad Software, San Diego, California, USA) was used. Significant differences between data sets were tested by one way Analysis of Variance (ANOVA) with Bonferroni's multiple comparison ad hoc post-test or unpaired Student's t test, as appropriated. Statistically significant differences between data sets were assumed at p < 0.05; (n) corresponds to the number of independent measurements.

Results

Expression levels of pendrin in total cellular membrane protein extracts of control and pendrin transfected cells were evaluated by western blot (Fig. 1). In control cells, pendrin expression was virtually undetectable, in agreement with previous findings [41, 44]. Following transfection, a significant increase in pendrin protein expression was observed.

Fig. 2. Pendrin activity is resistant to known diuretics. Representative original recording of the intracellular fluorescence intensity (left) and percentage of fluorescence decrease ($\Delta F\%$, right) determined over the experimental period (19 sec) in HEK 293 Phoenix cells transfected with pendrin (SLC26A4, white bars) or in control cells (control, black bars) in the presence of 0.1 mM (a) acetazolamide (ACTZ; $n = 30$), (b) amiloride ($n = 24$), (c) bumetanide (BMT; $n = 24$), or (d) hydrochlorothiazide (HCTZ; $n = 24$) or the corresponding vehicle. Data were collected from 4-5 independent experiments. ***: $p < 0.001$, n.s.: not statistically significant, one-way Anova with Bonferroni's multiple comparison post-test.

Fig. 3. Pendrin activity is resistant to DIDS and sensitive to NPPB. Representative original recording of the intracellular fluorescence intensity (left) and percentage of fluorescence decrease ($\Delta F\%$, right) determined over the experimental period (19 sec) in HEK 293 Phoenix cells transfected with pendrin (SLC26A4, white bars) or in control cells (control, black bars) in the presence of 0.1 mM (a) DIDS ($n = 48$) or (b) NPPB ($n = 24$), or the corresponding vehicle. Data were collected from 8 and 4 independent experiments for a and b, respectively. ***: $p < 0.001$, n.s.: not statistically significant, one-way Anova with Bonferroni's multiple comparison post-test.

Bands between 100 and 130 kDa represent the non-glycosylated, partially glycosylated and maturely glycosylated forms of pendrin and correspond to that described in previous reports [45, 46].

The compounds tested on pendrin activity (divided in three categories as follows: established diuretics and hypertensive agents, blockers of anion transport and previously identified inhibitors of solute carrier family 26, member A3/down-regulated in adenoma, SLC26A3/DRA) are represented in Table 1. The outcome of the functional test in the presence of compounds representative of each of the aforementioned categories is represented in Fig. 2-4. Addition of iodide to the extracellular solution invariably led to a marked decrease in the fluorescence of the iodide sensor (EYFP H148Q;I152L) in vehicle-exposed pendrin transfected cells (SLC26A4 + vehicle, Fig. 2-4). This effect was significantly different ($p < 0.001$) from that measured in cells transfected with the iodide sensor alone and exposed to the same vehicle (control + vehicle, Fig. 2-4), thereby confirming that pendrin does transport iodide [47, 48], most likely acts as a Cl^-/I^- anion exchanger in this system [34] and that the vehicles used in the present study do not significantly interfere with pendrin activity. The fluorescence variations detected in control cells exposed to a given test compound did not differ significantly from those measured in control cells exposed to the corresponding vehicle, indicating that endogenous anion transport was not affected. The only exception to this was the use of 1 mM probenecid (see below).

Fig. 4. Pendrin activity is sensitive to niflumic acid and tenidap. Representative original recording of the intracellular fluorescence intensity (left) and percentage of fluorescence decrease ($\Delta F\%$, right) determined over the experimental period (19 sec) in HEK 293 Phoenix cells transfected with pendrin (SLC26A4, white bars) or in control cells (control, black bars) in the presence of 0.1 mM (a) niflumic acid (NFA; $n = 12$) or (b) tenidap ($n = 48$), or the corresponding vehicle. Data were collected from 2 and 8 independent experiments for a and b, respectively. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, n.s.: not statistically significant, one-way Anova with Bonferroni's multiple comparison post-test.

Pendrin activity is not affected by thiazides and commonly used diuretics

Thirteen diuretics were selected among classes with established targets in distinct segments of the nephron, specifically (i) the carbonic anhydrase in the proximal tubule, (ii) the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) in the thick ascending limb, (iii) NCC in the distal convoluted tubule, (iv) ENaC in the CCD and CNT and (v) the aldosterone pathway in the distal nephron (Table 1). None of them significantly altered pendrin-dependent iodide influx (Fig. 2 and Table 1) when used at a concentration of 0.1 mM. Increasing the concentration of furosemide to 1 mM significantly hampered ($\sim 39\%$) pendrin activity ($p < 0.001$, $n = 42$).

Pendrin activity is resistant to DIDS but affected by NPPB

Five established inhibitors of chloride channels and anion exchangers were tested on pendrin activity (Table 1). When used at a concentration of 0.1 mM, only NPPB led to a significant (33%) inhibition of pendrin-dependent iodide influx (Fig. 3b and Table 1). Increasing the concentration of DIDS to 1 mM significantly blocked ($\sim 48\%$) pendrin activity ($p < 0.001$, $n = 42$), while 1 mM probenecid altered the anion transport in control cells ($p < 0.001$, $n = 42$) with no effect on pendrin-dependent iodide influx. However, 1 mM DIDS also significantly inhibited ($\sim 23\%$; $p < 0.001$, $n = 10$) voltage-dependent, potassium-selective channels endogenously expressed in HEK 293 Phoenix cells (data not shown).

Pendrin activity is affected by niflumic acid and tenidap

Four compounds previously reported to inhibit the activity of SLC26A3/DRA were tested (Table 1) on pendrin activity. Of them, niflumic acid and tenidap led to a significant

(64 and 30%, respectively) inhibition of pendrin-dependent iodide influx (Fig. 4a and b and Table 1).

Discussion

Six known inhibitors of ion transport (DIDS, furosemide, niflumic acid, NPPB, probenecid and tenidap) were previously reported as capable of inhibiting pendrin activity, but some (DIDS, furosemide, NPPB, probenecid) led to discordant results and were therefore re-assayed in the present study.

Incongruous effects of some compounds on pendrin transport activity may arise from differences in the concentration used in each individual study. The concentration of most of the compounds tested in the present work (0.1 mM) was based on previous studies [44, 49, 50]. Although this exceeds the IC_{50} for the respective conventional targets (Table 1), it was chosen with the aim of unequivocally excluding ineffective substances. In an initial report, relatively high concentrations (1 mM) of DIDS lead to a 62% inhibition of radiolabelled chloride uptake in pendrin-expressing *Xenopus* oocytes [47]. In later studies, DIDS was found either to almost completely inhibit (0.5 mM) pendrin activity [51, 52] or to be ineffective (0.1–0.5 mM) [44, 49, 50]. In the present study, 0.1 mM DIDS and the closely related DNDS did not block pendrin activity (Table 1 and Fig. 3a), in agreement with historical observations on β -intercalated cell dependent HCO_3^- secretion, that was found to be DIDS insensitive [53, 54]. Increasing the DIDS concentration to 1 mM led to a ~48% inhibition of pendrin activity (Table 1), but also significantly affected potassium-selective currents, and is therefore considered to be largely unspecific.

Similarly to DIDS, furosemide and probenecid (1 mM) were initially reported to inhibit radiolabelled chloride uptake in pendrin-expressing *Xenopus* oocytes by 59% and 37%, respectively [47], and then later showed to be ineffective [49]. In the present study, neither 0.1 mM furosemide nor probenecid inhibited pendrin-driven iodide influx (Table 1). Increasing the furosemide concentration to 1 mM led to a ~39% inhibition of pendrin activity, while 1 mM probenecid affected the endogenous anion transport in the cell utilized for the functional tests, without affecting pendrin-dependent iodide influx (Table 1).

In a former study, we showed that 0.1 mM NPPB, a known inhibitor of anion channels that is not expected to inhibit anion exchangers, significantly blocked pendrin-induced chloride uptake [44], a finding that could not be reproduced on SCN^- transport [49]. Here we found that 0.1 mM NPPB leads to a ~33% inhibition of pendrin-driven iodide influx (Table 1 and Fig. 3b).

Among the SLC26 family members, SLC26A3/DRA is the closest paralog of pendrin with the two proteins showing 44% identity and 60% similarity [55]. Therefore, we verified if some established inhibitors of SLC26A3/DRA (glybenclamide, hydroxycinnamate, niflumic acid and tenidap, Table 1) were also effective against pendrin activity. Of those, 0.1 mM glybenclamide was reported to significantly affect SLC26A3/DRA activity [56], while hydroxycinnamate was relatively weaker [57]. However, in the present study, neither glybenclamide nor hydroxycinnamate inhibited pendrin activity (Table 1), in agreement with other reported observations [49, 50].

The anti-inflammatory drug niflumic acid is known to exert its action *via* cyclooxygenase inhibition [58], but it is also involved in the modulation of chloride channels, such as ClC-Ka and ClC-Kb [59], and was shown to efficiently block SLC26A3/DRA activity [57, 60]. Niflumic acid led to a ~64% inhibition of pendrin-driven iodide influx (Table 1 and Fig. 4a), therefore confirming our former findings obtained with radiolabelled chloride uptake [44]. Noteworthy, screening a library of 2,000 compounds later confirmed that niflumic acid decreased pendrin-dependent SCN^- transport [49]. Niflumic acid also inhibited pendrin-induced iodide uptake and Mucin 5AC production in stably transfected NCI H292 cells [25]. As previously mentioned, in the respiratory epithelium, pendrin expression is enhanced upon stimulation of the IL-4/13 pathway [23] and exacerbates the pathological

manifestations of respiratory distresses [20]. Pendrin sensitivity to niflumic acid is in agreement with findings showing that this drug could ameliorate IL-13-induced asthma features such as airway hyperresponsiveness, goblet cell hyperplasia and degranulation and eosinophil accumulation [61, 62], although inhibition of the calcium-activated chloride channel/chloride channel regulator mCLCA3 (mouse homolog of hCLCA1) was postulated to be the underlying mechanism in those studies.

Tenidap is an anti-rheumatic, anti-inflammatory drug effective against rheumatic arthritis [63]. Its action is related to the inhibition of cyclooxygenases 1 and 2 [64], as well as lipoxygenase 5 [65], but it is also known to modulate a number of membrane transporters and channels, including the rat kidney $\text{Na}^+/\text{HCO}_3^-$ cotransporter (rkNBC) [66] and the inwardly rectifying K^+ channel Kir2.3 [67]. Tenidap was shown to efficiently block SLC26A3/DRA activity in *Xenopus* oocytes [57] and HEK 293 cells [56], as well as pendrin activity in *Xenopus* oocytes [50]. Here we confirm that tenidap also blocks pendrin activity following expression in human kidney cells (Table 1 and Fig. 4b).

Pendrin activity was found to be resistant to thiazides (Table 1). This finding is particularly relevant when considering that pendrin expression is upregulated in a setting of NCC gene ablation [16, 68] or impeded NCC activation [69], conditions that can be regarded as analogous to the state resulting from chronic thiazide treatment. The resistance of pendrin to thiazides is consistent with the development of thiazide diuretics resistance following prolonged therapy [70].

As previously mentioned, the functional test utilized here evaluates the Cl^-/I^- exchange activity of pendrin [34-37]. The Cl^-/I^- exchange activity, in general, reflects the $\text{Cl}^-/\text{HCO}_3^-/\text{OH}^-$ exchange activity [71]; when differences between the Cl^-/I^- and $\text{Cl}^-/\text{HCO}_3^-$ exchange efficiency were observed, they were considered to be “moderate”, and unlikely of pathophysiological relevance [72]. In addition, in the kidney, pendrin plays a role in I^- absorption, thus contributing to I^- balance [73, 74]. Therefore, the Cl^-/I^- exchange activity can be used as a reliable tool to measure pendrin transport efficiency. In addition, inhibitors of anion transport can lead to intracellular acidification [75], therefore indirectly affecting pendrin $\text{Cl}^-/\text{HCO}_3^-$ activity by limiting the availability of intracellular HCO_3^- . The Cl^-/I^- exchange transport mode does not affect the intracellular pH and therefore does not rely on intracellular HCO_3^- [34]. Therefore, measuring the Cl^-/I^- exchange is a suitable approach for testing pendrin activity in the presence of possible decreases in intracellular pH. Moreover, it should be considered that intracellular pH variations can intrinsically modify the transport kinetics of pendrin, independently of transmembrane concentration gradients, with increased activity at low intracellular pH [52]. This could have led to underestimates of the inhibition percentages reported in Table 1.

As previously mentioned, fluorescence % variations measured in control cells (not overexpressing pendrin) exposed to the tested substances (with the only exception of 1 mM probenecid) did not significantly differ from those measured in control cells exposed to the respective vehicles; therefore, an effect of NPPB, tenidap and niflumic acid on anion channels/transporters endogenously expressed in HEK 293 Phoenix cells can be excluded.

Theoretically, the inhibitory effect of NPPB, tenidap or niflumic acid observed in the present study may reflect an inhibition of an iodide influx through endogenous chloride channels (such as CIC2 [76]) that may be activated following pendrin overexpression. This effect would not be apparent in control cells that do not overexpress pendrin. However, previous studies revealed that pendrin overexpression does not activate chloride currents [77]. Therefore, an effect of the aforementioned compounds on endogenously expressed ion channels seems unlikely.

To conclude, we confirmed that human pendrin activity in human kidney cells was inhibited by the anti-inflammatory drug niflumic acid, the anti-rheumatic drug tenidap and the anion channel inhibitor NPPB. Further studies are warranted to verify if niflumic acid (a drug currently in use in clinical practice) and tenidap (a drug that was used in clinical trials [78, 79]) could represent useful adjuncts in the treatment of respiratory distresses and/or hypertensive states.

Acknowledgments

We sincerely thank Elisabeth Mooslechner for her expert secretarial assistance.

Disclosure Statement

The authors have no conflict of interests to declare.

References

- 1 Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis AD, Sheffield VC, Green ED: Pendred syndrome is caused by mutations in a putative sulphate transporter gene (*PDS*). *Nat Genet* 1997;17:411-422.
- 2 Li XC, Everett LA, Lalwani AK, Desmukh D, Friedman TB, Green ED, Wilcox ER: A mutation in *PDS* causes non-syndromic recessive deafness. *Nat Genet* 1998;18:215-217.
- 3 Royaux IE, Wall SM, Karniski LP, Everett LA, Suzuki K, Knepper MA, Green ED: Pendrin, encoded by the Pendred syndrome gene, resides in the apical region of renal intercalated cells and mediates bicarbonate secretion. *Proc Natl Acad Sci U S A* 2001;98:4221-4226.
- 4 Kim YH, Kwon TH, Frische S, Kim J, Tisher CC, Madsen KM, Nielsen S: Immunocytochemical localization of pendrin in intercalated cell subtypes in rat and mouse kidney. *Am J Physiol Renal Physiol* 2002;283:F744-F754.
- 5 Wall SM, Hassell KA, Royaux IE, Green ED, Chang JY, Shipley GL, Verlander JW: Localization of pendrin in mouse kidney. *Am J Physiol Renal Physiol* 2003;284:F229-F241.
- 6 Amlal H, Petrovic S, Xu J, Wang Z, Sun X, Barone S, Soleimani M: Deletion of the anion exchanger *Slc26a4* (pendrin) decreases apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger activity and impairs bicarbonate secretion in kidney collecting duct. *Am J Physiol Cell Physiol* 2010;299:C33-C41.
- 7 Wall SM, Kim YH, Stanley L, Glapion DM, Everett LA, Green ED, Verlander JW: NaCl restriction upregulates renal *Slc26a4* through subcellular redistribution: role in Cl^- conservation. *Hypertension* 2004;44:982-987.
- 8 Verlander JW, Kim YH, Shin W, Pham TD, Hassell KA, Beierwaltes WH, Green ED, Everett L, Matthews SW, Wall SM: Dietary Cl^- restriction upregulates pendrin expression within the apical plasma membrane of type B intercalated cells. *Am J Physiol Renal Physiol* 2006;291:F833-F839.
- 9 Wall SM: The Role of Pendrin in Blood Pressure Regulation. *Am J Physiol Renal Physiol* 2015;2015 Nov 4:ajprenal.00400.2015.
- 10 Wall SM, Lazo-Fernandez Y: The role of pendrin in renal physiology. *Annu Rev Physiol* 2015;77:363-378.
- 11 Verlander JW, Hassell KA, Royaux IE, Glapion DM, Wang ME, Everett LA, Green ED, Wall SM: Deoxycorticosterone upregulates PDS (*Slc26a4*) in mouse kidney: role of pendrin in mineralocorticoid-induced hypertension. *Hypertension* 2003;42:356-362.
- 12 Kim YH, Pech V, Spencer KB, Beierwaltes WH, Everett LA, Green ED, Shin W, Verlander JW, Sutliff RL, Wall SM: Reduced ENaC protein abundance contributes to the lower blood pressure observed in pendrin-null mice. *Am J Physiol Renal Physiol* 2007;293:F1314-F1324.
- 13 Pech V, Wall SM, Nanami M, Bao HF, Kim YH, Lazo-Fernandez Y, Yue Q, Pham TD, Eaton DC, Verlander JW: Pendrin gene ablation alters ENaC subcellular distribution and open probability. *Am J Physiol Renal Physiol* 2015;309:F154-F163.
- 14 Eladari D, Chambrey R, Picard N, Hadchouel J: Electroneutral absorption of NaCl by the aldosterone-sensitive distal nephron: implication for normal electrolytes homeostasis and blood pressure regulation. *Cell Mol Life Sci* 2014;71:2879-2895.
- 15 Jacques T, Picard N, Miller RL, Riemyndy KA, Houillier P, Sohet F, Ramakrishnan SK, Busst CJ, Jayat M, Corniere N, Hassan H, Aronson PS, Hennings JC, Hubner CA, Nelson RD, Chambrey R, Eladari D: Overexpression of pendrin in intercalated cells produces chloride-sensitive hypertension. *J Am Soc Nephrol* 2013;24:1104-1113.
- 16 Soleimani M, Barone S, Xu J, Shull GE, Siddiqui F, Zahedi K, Amlal H: Double knockout of pendrin and Na-Cl cotransporter (NCC) causes severe salt wasting, volume depletion, and renal failure. *Proc Natl Acad Sci USA* 2012;109:13368-13373.

- 17 Amlal H, Soleimani M: Pendrin as a novel target for diuretic therapy. *Cell Physiol Biochem* 2011;28:521-526.
- 18 Soleimani M: A novel target for diuretic therapy. *Iran J Kidney Dis* 2012;6:419-425.
- 19 Denton JS, Pao AC, Maduke M: Novel diuretic targets. *Am J Physiol Renal Physiol* 2013;305:F931-F942.
- 20 Nofziger C, Dossena S, Suzuki S, Izuhara K, Paulmichl M: Pendrin function in airway epithelia. *Cell Physiol Biochem* 2011;28:571-578.
- 21 Sala-Rabanal M, Yurtsever Z, Berry KN, Brett TJ: Novel Roles for Chloride Channels, Exchangers, and Regulators in Chronic Inflammatory Airway Diseases. *Mediators Inflamm* 2015;2015:497387.
- 22 Kuperman DA, Lewis CC, Woodruff PG, Rodriguez MW, Yang YH, Dolganov GM, Fahy JV, Erle DJ: Dissecting asthma using focused transgenic modeling and functional genomics. *J Allergy Clin Immunol* 2005;116:305-311.
- 23 Nofziger C, Vezzoli V, Dossena S, Schonherr T, Studnicka J, Nofziger J, Vanoni S, Stephan S, Silva M, Meyer G, Paulmichl M: STAT6 Links IL-4/IL-13 Stimulation With Pendrin Expression in Asthma and Chronic Obstructive Pulmonary Disease. *Clin Pharmacol Ther* 2011;90:399-405.
- 24 Vanoni S, Nofziger C, Dossena S, Soyal SM, Patsch W, Plevani P, Duschl A, Paulmichl M: The human pendrin promoter contains two N4 GAS motifs with different functional relevance. *Cell Physiol Biochem* 2013;32:238-248.
- 25 Nakao I, Kanaji S, Ohta S, Matsushita H, Arima K, Yuyama N, Yamaya M, Nakayama K, Kubo H, Watanabe M, Sagara H, Sugiyama K, Tanaka H, Toda S, Hayashi H, Inoue H, Hoshino T, Shiraki A, Inoue M, Suzuki K, Aizawa H, Okinami S, Nagai H, Hasegawa M, Fukuda T, Green ED, Izuhara K: Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease. *J Immunol* 2008;180:6262-6269.
- 26 Nakagami Y, Favoreto S Jr, Zhen G, Park SW, Nguyenvu LT, Kuperman DA, Dolganov GM, Huang X, Boushey HA, Avila PC, Erle DJ: The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surface liquid, and increases airway reactivity and inflammation in an asthma model. *J Immunol* 2008;181:2203-2210.
- 27 Yick CY, Zwinderman AH, Kunst PW, Grunberg K, Mauad T, Dijkhuis A, Bel EH, Baas F, Lutter R, Sterk PJ: Transcriptome sequencing (RNA-Seq) of human endobronchial biopsies: asthma versus controls. *Eur Respir J* 2013;42:662-670.
- 28 Adams KM, Abraham V, Spielman D, Kolls JK, Rubenstein RC, Conner GE, Cohen NA, Kreindler JL: IL-17A induces Pendrin expression and chloride-bicarbonate exchange in human bronchial epithelial cells. *PLoS One* 2014;9:e103263.
- 29 Scanlon KM, Gau Y, Zhu J, Skerry C, Wall SM, Soleimani M, Carbonetti NH: Epithelial anion transporter pendrin contributes to inflammatory lung pathology in mouse models of *Bordetella pertussis* infection. *Infect Immun* 2014;82:4212-4221.
- 30 Sharma AK, Zelikovic I, Alper SL: Molecular dynamics simulations of the STAS domains of rat prestin and human pendrin reveal conformational motions in conserved flexible regions. *Cell Physiol Biochem* 2014;33:605-620.
- 31 Haggie PM, Phuan PW, Tan JA, Zlock L, Finkbeiner WE, Verkman AS: Inhibitors of pendrin anion exchange identified in a small molecule screen increase airway surface liquid volume in cystic fibrosis. *FASEB J* 2016.
- 32 Galletta LJ, Haggie PM, Verkman AS: Green fluorescent protein-based halide indicators with improved chloride and iodide affinities. *FEBS Lett* 2001;499:220-224.
- 33 DiCiommo DP, Duckett A, Burcescu I, Bremner R, Gallie BL: Retinoblastoma protein purification and transduction of retina and retinoblastoma cells using improved alphavirus vectors. *Invest Ophthalmol Vis Sci* 2004;45:3320-3329.
- 34 Dossena S, Rodighiero S, Vezzoli V, Bazzini C, Sironi C, Meyer G, Furst J, Ritter M, Garavaglia ML, Fugazzola L, Persani L, Zorowka P, Storelli C, Beck-Peccoz P, Botta G, Paulmichl M: Fast fluorometric method for measuring pendrin (SLC26A4) Cl⁻/I⁻ transport activity. *Cell Physiol Biochem* 2006;18:67-74.
- 35 Fugazzola L, Cirello V, Dossena S, Rodighiero S, Muzza M, Castorina P, Lalatta F, Ambrosetti U, Beck-Peccoz P, Botta G, Paulmichl M: High phenotypic intrafamilial variability in patients with Pendred syndrome and a novel duplication in the *SLC26A4* gene: clinical characterization and functional studies of the mutated SLC26A4 protein. *Eur J Endocrinol* 2007;157:331-338.

- 36 Pera A, Dossena S, Rodighiero S, Gandia M, Botta G, Meyer G, Moreno F, Nofziger C, Hernandez-Chico C, Paulmichl M: Functional assessment of allelic variants in the *SLC26A4* gene involved in Pendred syndrome and nonsyndromic EVA. *Proc Natl Acad Sci USA* 2008;105:18608-18613.
- 37 Dror AA, Politi Y, Shahin H, Lenz DR, Dossena S, Nofziger C, Fuchs H, Hrabe de AM, Paulmichl M, Weiner S, Avraham KB: Calcium oxalate stone formation in the inner ear as a result of an *Slc26a4* mutation. *J Biol Chem* 2010;285:21724-21735.
- 38 Dossena S, Nofziger C, Brownstein Z, Kanaan M, Avraham KB, Paulmichl M: Functional characterization of pendrin mutations found in the Israeli and Palestinian populations. *Cell Physiol Biochem* 2011;28:477-484.
- 39 Dossena S, Bizhanova A, Nofziger C, Bernardinelli E, Ramsauer J, Kopp P, Paulmichl M: Identification of allelic variants of pendrin (SLC26A4) with loss and gain of function. *Cell Physiol Biochem* 2011;28:467-476.
- 40 Procino G, Milano S, Tamma G, Dossena S, Barbieri C, Nicoletti C, Ranieri M, Di Mise A, Nofziger C, Svelto M, Paulmichl M, Valenti G: Co-regulated pendrin and aquaporin 5 expression and trafficking in type-B intercalated cells under potassium depletion. *Cell Physiol Biochem* 2013;32:184-199.
- 41 de Moraes V, Bernardinelli E, Zocal N, Fernandez J, Nofziger C, Castilho AM, Sartorato EL, Paulmichl M, Dossena S: Reduction of cellular expression levels is a common feature of functionally affected pendrin (SLC26A4) protein variants. *Mol Med* 2016 Jan 4.
- 42 Yoshida A, Hisatome I, Taniguchi S, Sasaki N, Yamamoto Y, Miake J, Fukui H, Shimizu H, Okamura T, Okura T, Igawa O, Shigemasa C, Green ED, Kohn LD, Suzuki K: Mechanism of iodide/chloride exchange by pendrin. *Endocrinology* 2004;145:4301-4308.
- 43 Shcheynikov N, Yang D, Wang Y, Zeng W, Karniski LP, So I, Wall SM, Muallem S: The *Slc26a4* transporter functions as an electroneutral $\text{Cl}^-/\text{I}^-/\text{HCO}_3^-$ exchanger: role of *Slc26a4* and *Slc26a6* in I^- and HCO_3^- secretion and in regulation of CFTR in the parotid duct. *J Physiol* 2008;586:3813-3824.
- 44 Dossena S, Vezzoli V, Cerutti N, Bazzini C, Tosco M, Sironi C, Rodighiero S, Meyer G, Fascio U, Furst J, Ritter M, Fugazzola L, Persani L, Zorowka P, Storelli C, Beck-Peccoz P, Botta G, Paulmichl M: Functional characterization of wild-type and a mutated form of SLC26A4 identified in a patient with Pendred syndrome. *Cell Physiol Biochem* 2006;17:245-256.
- 45 Yoon JS, Park HJ, Yoo SY, Namkung W, Jo MJ, Koo SK, Park HY, Lee WS, Kim KH, Lee MG: Heterogeneity in the processing defect of SLC26A4 mutants. *J Med Genet* 2008;45:411-419.
- 46 Lee K, Hong TJ, Hahn JS: Roles of 17-AAG-induced molecular chaperones and Rma1 E3 ubiquitin ligase in folding and degradation of Pendrin. *FEBS Lett* 2012;586:2535-2541.
- 47 Scott DA, Wang R, Kreman TM, Sheffield VC, Karniski LP: The Pendred syndrome gene encodes a chloride-iodide transport protein. *Nat Genet* 1999;21:440-443.
- 48 Gillam MP, Sidhaye AR, Lee EJ, Rutishauser J, Stephan CW, Kopp P: Functional characterization of pendrin in a polarized cell system. Evidence for pendrin-mediated apical iodide efflux. *J Biol Chem* 2004;279:13004-13010.
- 49 Pedemonte N, Caci E, Sondo E, Caputo A, Rhoden K, Pfeffer U, Di CM, Bandettini R, Ravazzolo R, Zegarar-Moran O, Galletta LJ: Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: role of pendrin and anion channels. *J Immunol* 2007;178:5144-5153.
- 50 Reimold FR, Heneghan JF, Steward AK, Zelikovic I, Vandorpe DH, Shmukler BE, Alper SL: Pendrin function and regulation in *Xenopus* oocytes. *Cell Physiol Biochem* 2011;28:435-450.
- 51 Soleimani M, Greeley T, Petrovic S, Wang Z, Amlal H, Kopp P, Burnham CE: Pendrin: an apical $\text{Cl}^-/\text{OH}^-/\text{HCO}_3^-$ exchanger in the kidney cortex. *Am J Physiol Renal Physiol* 2001;280:F356-F364.
- 52 Azroyan A, Laghmani K, Crambert G, Mordasini D, Doucet A, Edwards A: Regulation of pendrin by pH: dependence on glycosylation. *Biochem J* 2011;434:61-72.
- 53 Ko SB, Luo X, Hager H, Rojek A, Choi JY, Licht C, Suzuki M, Muallem S, Nielsen S, Ishibashi K: AE4 is a DIDS-sensitive $\text{Cl}^-/\text{HCO}_3^-$ exchanger in the basolateral membrane of the renal CCD and the SMG duct. *Am J Physiol Cell Physiol* 2002;283:C1206-C1218.
- 54 Godinich MJ, Jennings ML: Renal chloride-bicarbonate exchangers. *Curr Opin Nephrol Hypertens* 1995;4:398-401.
- 55 Mount DB, Romero MF: The SLC26 gene family of multifunctional anion exchangers. *Pflugers Arch* 2004;447:710-721.

- 56 Lamprecht G, Baisch S, Schoenleber E, Gregor M: Transport properties of the human intestinal anion exchanger DRA (down-regulated in adenoma) in transfected HEK293 cells. *Pflugers Arch* 2005;449:479-490.
- 57 Chernova MN, Jiang L, Shmukler BE, Schweinfest CW, Blanco P, Freedman SD, Stewart AK, Alper SL: Acute regulation of the SLC26A3 congenital chloride diarrhoea anion exchanger (DRA) expressed in *Xenopus* oocytes. *J Physiol* 2003;549:3-19.
- 58 Kim BM, Maeng K, Lee KH, Hong SH: Combined treatment with the Cox-2 inhibitor niflumic acid and PPARgamma ligand ciglitazone induces ER stress/caspase-8-mediated apoptosis in human lung cancer cells. *Cancer Lett* 2011;300:134-144.
- 59 Picollo A, Liantonio A, Babini E, Camerino DC, Pusch M: Mechanism of interaction of niflumic acid with heterologously expressed kidney CLC-K chloride channels. *J Membr Biol* 2007;216:73-82.
- 60 Musch MW, Arvans DL, Wu GD, Chang EB: Functional coupling of the downregulated in adenoma Cl⁻/base exchanger DRA and the apical Na⁺/H⁺ exchangers NHE2 and NHE3. *Am J Physiol Gastrointest Liver Physiol* 2009;296:G202-G210.
- 61 Nakano T, Inoue H, Fukuyama S, Matsumoto K, Matsumura M, Tsuda M, Matsumoto T, Aizawa H, Nakanishi Y: Niflumic acid suppresses interleukin-13-induced asthma phenotypes. *Am J Respir Crit Care Med* 2006;173:1216-1221.
- 62 Kondo M, Nakata J, Arai N, Izumo T, Tagaya E, Takeyama K, Tamaoki J, Nagai A: Niflumic acid inhibits goblet cell degranulation in a guinea pig asthma model. *Allergol Int* 2012;61:133-142.
- 63 Breedveld F: Tenidap: a novel cytokine-modulating antirheumatic drug for the treatment of rheumatoid arthritis. *Scand J Rheumatol Suppl* 1994;100:31-44.
- 64 Moore PF, Larson DL, Otterness IG, Weissman A, Kadin SB, Sweeney FJ, Eskra JD, Nagahisa A, Sakakibara M, Carty TJ: Tenidap, a structurally novel drug for the treatment of arthritis: antiinflammatory and analgesic properties. *Inflamm Res* 1996;45:54-61.
- 65 Blackburn WD Jr, Heck LW, Loose LD, Eskra JD, Carty TJ: Inhibition of 5-lipoxygenase product formation and polymorphonuclear cell degranulation by tenidap sodium in patients with rheumatoid arthritis. *Arthritis Rheum* 1991;34:204-210.
- 66 Ducoudret O, Diakov A, Muller-Berger S, Romero MF, Fromter E: The renal Na-HCO₃-cotransporter expressed in *Xenopus laevis* oocytes: inhibition by tenidap and benzamil and effect of temperature on transport rate and stoichiometry. *Pflugers Arch* 2001;442:709-717.
- 67 Liu Y, Liu D, Printzenhoff D, Coghlan MJ, Harris R, Krafte DS: Tenidap, a novel anti-inflammatory agent, is an opener of the inwardly rectifying K⁺ channel hKir2.3. *Eur J Pharmacol* 2002;435:153-160.
- 68 Vallet M, Picard N, Loffing-Cueni D, Fysekidis M, Bloch-Faure M, Deschenes G, Breton S, Meneton P, Loffing J, Aronson PS, Chambrey R, Eladari D: Pendrin regulation in mouse kidney primarily is chloride-dependent. *J Am Soc Nephrol* 2006;17:2153-2163.
- 69 Grimm PR, Lazo-Fernandez Y, Delpire E, Wall SM, Dorsey SG, Weinman EJ, Coleman R, Wade JB, Welling PA: Integrated compensatory network is activated in the absence of NCC phosphorylation. *J Clin Invest* 2015;125:2136-2150.
- 70 Knepper MA: Systems biology of diuretic resistance. *J Clin Invest* 2015;125:1793-1795.
- 71 Dossena S, Nofziger C, Tamma G, Bernardinelli E, Vanoni S, Nowak C, Grabmayer E, Kossler S, Stephan S, Patsch W, Paulmichl M: Molecular and functional characterization of human pendrin and its allelic variants. *Cell Physiol Biochem* 2011;28:451-466.
- 72 Choi BY, Stewart AK, Madeo AC, Pryor SP, Lenhard S, Kittles R, Eisenman D, Kim HJ, Niparko J, Thomsen J, Arnos KS, Nance WE, King KA, Zalewski CK, Brewer CC, Shawker T, Reynolds JC, Butman JA, Karniski LP, Alper SL, Griffith AJ: Hypo-functional SLC26A4 variants associated with nonsyndromic hearing loss and enlargement of the vestibular aqueduct: genotype-phenotype correlation or coincidental polymorphisms? *Hum Mutat* 2009;30:599-608.
- 73 Matsushima Y, Muto S, Taniguchi J, Imai M: Mechanism of iodide transport in the rabbit cortical collecting duct. *Clin Exp Nephrol* 2006;10:102-110.
- 74 Kim YH, Pham TD, Zheng W, Hong S, Baylis C, Pech V, Beierwaltes WH, Farley DB, Braverman LE, Verlander JW, Wall SM: Role of pendrin in iodide balance: going with the flow. *Am J Physiol Renal Physiol* 2009;297:F1069-F1079.
- 75 Brown CD, Dudley AJ: Chloride channel blockers decrease intracellular pH in cultured renal epithelial LLC-PK1 cells. *Br J Pharmacol* 1996;118:443-444.

- 76 Domingue JC, Ao M, Sarathy J, George A, Alrefai WA, Nelson DJ, Rao MC: HEK-293 cells expressing the cystic fibrosis transmembrane conductance regulator (CFTR): a model for studying regulation of Cl⁻ transport. *Physiol Rep* 2014;2:pil:e12158.
- 77 Dossena S, Maccagni A, Vezzoli V, Bazzini C, Garavaglia ML, Meyer G, Furst J, Ritter M, Fugazzola L, Persani L, Zorowka P, Storelli C, Beck-Peccoz P, Botta G, Paulmichl M: The expression of wild-type pendrin (SLC26A4) in human embryonic kidney (HEK 293 Phoenix) cells leads to the activation of cationic currents. *Eur J Endocrinol* 2005;153:693-699.
- 78 Wylie G, Appelboom T, Bolten W, Breedveld FC, Feely J, Leeming MR, Le L, X, Manthorpe R, Marcolongo R, Smolen J: A comparative study of tenidap, a cytokine-modulating anti-rheumatic drug, and diclofenac in rheumatoid arthritis: a 24-week analysis of a 1-year clinical trial. *Br J Rheumatol* 1995;34:554-563.
- 79 Ayral X, Mackillop N, Genant HK, Kirkpatrick J, Beaulieu A, Pippingskiold P, Will RK, Alava S, Dougados M: Arthroscopic evaluation of potential structure-modifying drug in osteoarthritis of the knee. A multicenter, randomized, double-blind comparison of tenidap sodium vs piroxicam. *Osteoarthritis Cartilage* 2003;11:198-207.
- 80 BELSKY H: Use of a new oral diuretic, diamox, in congestive heart failure. *N Engl J Med* 1953;249:140-143.
- 81 McDonald FJ, Snyder PM, McCray PB Jr, Welsh MJ: Cloning, expression, and tissue distribution of a human amiloride-sensitive Na⁺ channel. *Am J Physiol* 1994;266:L728-L734.
- 82 Tamargo J, Segura J, Ruilope LM: Diuretics in the treatment of hypertension. Part 2: loop diuretics and potassium-sparing agents. *Expert Opin Pharmacother* 2014;15:605-621.
- 83 Monroy A, Plata C, Hebert SC, Gamba G: Characterization of the thiazide-sensitive Na⁺-Cl⁻ cotransporter: a new model for ions and diuretics interaction. *Am J Physiol Renal Physiol* 2000;279:F161-F169.
- 84 Tamargo J, Segura J, Ruilope LM: Diuretics in the treatment of hypertension. Part 1: thiazide and thiazide-like diuretics. *Expert Opin Pharmacother* 2014;15:527-547.
- 85 Frishman WH, Stier CT Jr: Aldosterone and aldosterone antagonism in systemic hypertension. *Curr Hypertens Rep* 2004;6:195-200.
- 86 Garay RP, varez-Guerra M, Alda JO, Nazaret C, Soler A, Vargas F: Regulation of renal Na-K-Cl cotransporter NKCC2 by humoral natriuretic factors: relevance in hypertension. *Clin Exp Hypertens* 1998;20:675-682.
- 87 Lindskog S: Structure and mechanism of carbonic anhydrase. *Pharmacol Ther* 1997;74:1-20.
- 88 Taddei A, Folli C, Zegarra-Moran O, Fanen P, Verkman AS, Galletta LJ: Altered channel gating mechanism for CFTR inhibition by a high-affinity thiazolidinone blocker. *FEBS Lett* 2004;558:52-56.
- 89 Jessen F, Sjöholm C, Hoffmann EK: Identification of the anion exchange protein of Ehrlich cells: a kinetic analysis of the inhibitory effects of 4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid (DIDS) and labeling of membrane proteins with 3H-DIDS. *J Membr Biol* 1986;92:195-205.
- 90 Zhang JY, Wu F, Gu XM, Jin ZX, Kong LH, Zhang Y, Zhou JJ, Gao F: The Blockade of Transmembrane Cl⁻ Flux Mitigates I/R-Induced Heart Injury via the Inhibition of Calpain Activity. *Cell Physiol Biochem* 2015;35:2121-2134.
- 91 Shen B, Fu J, Guo J, Zhang J, Wang X, Pan X, Chen M, Zhou Y, Zhu M, Du J: Role of Na⁺-K⁺-2Cl⁻ Cotransporter 1 in Phenylephrine-Induced Rhythmic Contraction in the Mouse Aorta: Regulation of Na⁺-K⁺-2Cl⁻ Cotransporter 1 by Ca²⁺ Sparks and KCa Channels. *Cell Physiol Biochem* 2015;37:747-758.
- 92 Barzilay M, Cabantchik ZI: Anion transport in red blood cells. II. Kinetics of reversible inhibition by nitroaromatic sulfonic acids. *Membr Biochem* 1979;2:255-281.
- 93 Takeda M, Narikawa S, Hosoyamada M, Cha SH, Sekine T, Endou H: Characterization of organic anion transport inhibitors using cells stably expressing human organic anion transporters. *Eur J Pharmacol* 2001;419:113-120.
- 94 Gribble FM, Reimann F: Sulphonylurea action revisited: the post-cloning era. *Diabetologia* 2003;46:875-891.
- 95 Jabeen T, Singh N, Singh RK, Sharma S, Somvanshi RK, Dey S, Singh TP: Non-steroidal anti-inflammatory drugs as potent inhibitors of phospholipase A2: structure of the complex of phospholipase A2 with niflumic acid at 2.5 Angstroms resolution. *Acta Crystallogr D Biol Crystallogr* 2005;61:1579-1586.