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RESEARCH PAPER

Structure–activity relationships of bumetanide derivatives: correlation between diuretic activity in dogs and inhibition of the human NKCC2A transporter

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BACKGROUND AND PURPOSE

The N-K-Cl cotransporters (NKCCs) mediate the coupled, electroneutral movement of Na^+, K^+ and Cl^- ions across cell membranes. There are two isoforms of this cation co-transporter, NKCC1 and NKCC2. NKCC2 is expressed primarily in the kidney and is the target of diuretics such as bumetanide. Bumetanide was discovered by screening ~5000 3-amino-5-sulfamoylbenzoic acid derivatives, long before NKCC2 was identified in the kidney. Therefore, structure–activity studies on effects of bumetanide derivatives on NKCC2 are not available.

EXPERIMENTAL APPROACH

In this study, the effect of a series of diuretically active bumetanide derivatives was investigated on human NKCC2 variant A (hNKCC2A) expressed in *Xenopus laevis* oocytes.

KEY RESULTS

Bumetanide blocked hNKCC2A transport with an IC_{50} of 4 μM. There was good correlation between the diuretic potency of bumetanide and its derivatives in dogs and their inhibition of hNKCC2A ($r^2 = 0.817$; $P < 0.01$). Replacement of the carboxylic group of bumetanide by a non-ionic residue, for example, an anilinomethyl group, decreased inhibition of hNKCC2A, indicating that an acidic group was required for transporter inhibition. Exchange of the phenoxy group of bumetanide for a 4-chloroanilino group or the sulfamoyl group by a methylsulfonyl group resulted in compounds with higher potency to inhibit hNKCC2A than bumetanide.

CONCLUSIONS AND IMPLICATIONS

The *X. laevis* oocyte expression system used in these experiments allowed analysis of the structural requirements that determine relative potency of loop diuretics on human NKCC2 splice variants, and may lead to the discovery of novel high-ceiling diuretics.

Abbreviations

NKCC, Na-K-Cl cotransporter; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; TAL, thick ascending limb

Tables of Links

TARGETS
Transporters
NKCC1, Na-K-Cl co-transporter (SLC12A2)
NKCC2 (SLC 12A1)
OAT, organic anion transporter

LIGANDS
Bumetanide

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Bumetanide is a widely used, highly potent loop diuretic that inhibits Na^+ and Cl^- reabsorption by the thick ascending limb (TAL) of the loop of Henle by blocking the Na^+-K^+-Cl^- cotransporter (NKCC) isoform located in the apical membrane of these epithelial cells, that is, NKCC2 (Haas and Forbush, 1998). While NKCC2 is expressed primarily in the kidney, the second bumetanide-sensitive NKCC isoform, NKCC1, is expressed in many tissues and plays a major role in the regulation of intracellular Cl^- concentration (Markadieu and Delpire, 2014). Although NKCC2 is encoded by a single gene, differential splicing results in the generation of three full-length splice variants (NKCC2A, NKCC2B, NKCC2F), which differ in their location along the TAL of Henle and their transport characteristics (Castrop and Schnermann, 2008; Alvarez-Leefmans, 2012). NKCC2A is the dominant isoform in humans (Carota *et al.*, 2010) and was therefore used in the present study.

The molecular identification of NKCC1 and NKCC2 was made in 1994 (Delpire *et al.*, 1994; Gamba *et al.*, 1994; Xu *et al.*, 1994), that is, long after clinical approval of bumetanide. At the time of development of bumetanide in the 1960s, its exact target in the kidney was not known. Bumetanide was discovered as a result of systematic structure–activity studies of a large series of diuretically active 3-amino-5-sulfamoylbenzoic acid derivatives (Feit, 1971) and was followed by several series of compounds related both in chemical structure and diuretic profile (Nielsen and Feit, 1978; Feit, 1981; 1990). In other words, the synthesis of sulfamoyl benzoic acid diuretics was done long before their cellular and molecular mechanisms of action were elucidated (Feit, 1981). Rodents metabolize bumetanide and its derivatives too rapidly to allow any meaningful assessment of their diuretic potency (Olsen, 1977; Töpfer *et al.*, 2014). Consequently, dogs were used to screen large series of diuretically active aminobenzoic acid compounds, which eventually led to discovery of bumetanide (Feit, 1971; 1981; 1990; Frey, 1975; Nielsen and Feit, 1978). The potency of bumetanide exceeded that of the clinically established loop diuretics furosemide and ethacrynic acid in humans as well as dogs (Cohen, 1981), indicating that the dog is a valuable translational model for structure–activity studies on loop diuretics acting via inhibition of renal NKCC2.

In 1979, Frizzell *et al.* proposed that the diuretic effect of loop diuretics was mediated by inhibition of a co-transport of Na^+ plus Cl^-. This prompted Palfrey *et al.* (1980) to study the effect of various aminobenzoic acid derivatives, including bumetanide and some of its derivatives, on a cation co-transport system in avian erythrocytes, resulting in a good correlation between diuretic potency in the dog assay and their inhibition of cation cotransport in this model system. This cation co-transport in erythrocytes was later shown to be mediated by NKCC1 (Flatman and Creanor, 1999). To our knowledge, a similar correlation analysis is not available for mammalian NKCC2 transporters, the molecular targets of bumetanide and other loop diuretics in the apical membrane of TAL of Henle epithelial cells. This prompted us to perform such an analysis with bumetanide and several of its derivatives previously synthesized by Peter W. Feit. Bumetanide derivatives were chosen on the basis of their diuretic activity in dogs, covering a wide range of diuretic potencies and structural modifications (Figure 1). To determine the inhibitory profile of bumetanide and its derivatives on human NKCC2 splice variant A (hNKCC2A) in an isolated system, NKCC2 activity was assayed in *Xenopus* oocytes heterologously expressing hNKCC2A.

Methods

Chemistry

The bumetanide derivatives tested in this study are illustrated in Figure 1. With the exception of BUM13 and BUM13Ox, all samples of bumetanide derivatives used for the present *in vitro* experiments were from the batches originally used for determining diuretic activity in the dog assay (see below). The synthesis of these compounds has been described previously by one of us (PWF): PF-2178, Palfrey *et al.* (1980); PF-1712, Feit (1971); PF-1659, Feit (1971); PF-1962, Feit *et al.* (1974); HH-562, Feit *et al.* (1970) and Feit (1971); PF-1573, Feit (1971); PF-1574, Feit (1971); PF-1730, Feit and Nielsen (1976).

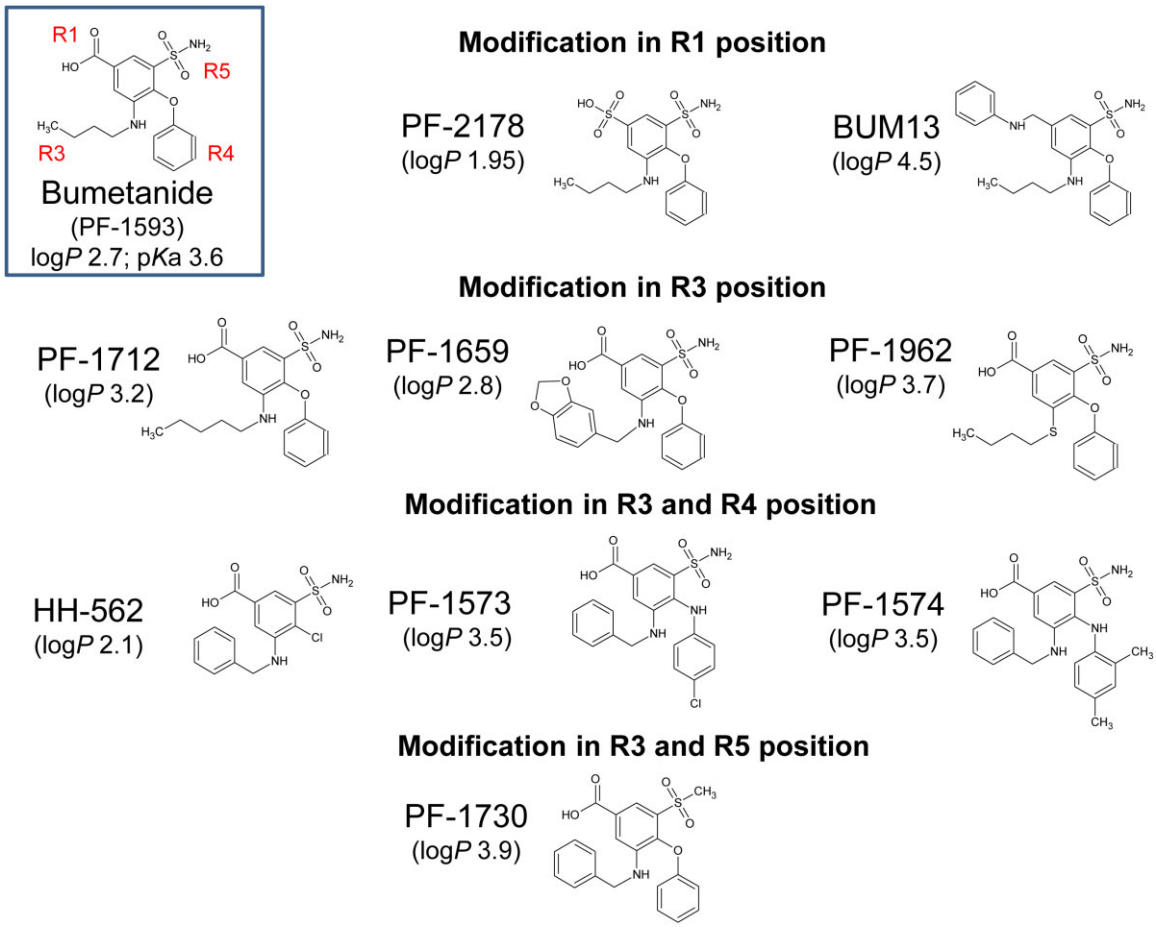


Figure 1

Structures of bumetanide derivatives and their code numbers and, for comparison, bumetanide (PF-1593). For all compounds, lipophilicity (log*P*) is indicated. Furthermore, the acidic dissociation constant, p*Ka*, of the carboxylic group is shown for bumetanide. Bumetanide derivatives had similar p*Ka* values, except PF-2178 (0.7) and BUM13 (7.0).

Synthesis of BUM13 was first described by Nielsen and Feit (1978), but it was resynthesized by one of us (TE) both as free base and as oxalate (BUM13Ox) to enhance solubility. These compounds were analysed routinely for C, H, Cl, N and S contents. Furthermore, infrared and NMR spectroscopy were applied. The purity of the compounds was furthermore checked by thin-layer chromatography as detailed elsewhere (Feit *et al.*, 1970; 1974; Feit, 1971; Feit and Nielsen, 1976). The characterization of BUM13 and BUM13Ox included ^{1}H HMR, ^{13}C NMR, MS and combustion analysis. All compounds were of high purity of ≥95%. Bumetanide (≥98% purity) was obtained from Sigma-Aldrich (Copenhagen, Denmark).

Lipophilicity (log*P*) and acidic dissociation constant (p*Ka*) of the bumetanide derivatives were calculated by the Molecular Operating Environment (MOE 2012.10; Chemical Computing Group Inc., Montreal, QC, Canada). For comparison, log*P* and p*Ka* of bumetanide were calculated by the same software and verified by published experimental approaches (Orita *et al.*, 1976).

For testing the inhibitory effect of bumetanide and its derivatives on hNKCC2A, drugs had to be dissolved before addition to the test medium used for the transport experiments. However, some of the bumetanide derivatives, particularly BUM13 and BUM13Ox, HH-562, PF-1573 and PF-1730, were difficult to dissolve in vehicles that could subsequently be added to the medium used for the *in vitro* NKCC2 assay (see below), so that various preliminary experiments with different solvents were performed. The procedure finally used for the different compounds was as follows. Up to 10 mM stock solutions was prepared freshly with DMSO (≥98% purity) (for bumetanide, PF-2178, PF-1712, PF-1659, PF-1962 and PF-1574), absolute ethanol (for HH-562 and PF-1573), Tween®80 (polyoxyethylene 80 sorbitan monooleate; Merck-Schuchardt, München, Germany) and absolute ethanol (1:9; for BUM13 and BUM13Ox), or polyethylene glycol 400 (Rotipuran® Ph. Eur.; Sigma-Aldrich) and ethanol (1:1; for PF-1730). The stock solutions were added to the test medium used for the transport assays, resulting in final drug concentrations of 1, 10 and 100 μM, with respective vehicles included as required to obtain comparable solvent concentrations in all compared conditions (≤1%). The experiments were carried out no later than 3 h after preparation of the drug. Because BUM13 and its oxalate salt BUM13Ox did not differ in solubility or biological activity, only the term BUM13 will be used in this paper.

Oocyte preparation and NKCC2 protein expression

All animal care and experimental procedures complied with the European Community guidelines for the use of experimental animals. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 31 animals were used in the experiments described here.

Oocytes from *Xenopus laevis* were obtained from our own frogs (Nasco, Fort Atkinson, WI, USA, or National Centre for Scientific Research, Rennes, France) or purchased from Ecocyte Bioscience, Castrop-Rauxel, Germany. The surgical removal and preparation of defolliculated oocytes was performed essentially as previously described (Fenton *et al.*, 2010). Human NKCC2A in the expression vector pTLN (obtained from Dr H. Castrop, NIH, Bethesda, MD, USA) was linearized downstream from the poly-A segment, and *in vitro* transcribed using SP6-mMessage mMachine according to manufacturer's instructions (Ambion, Austin, TX, USA). cRNA was then extracted with MEGAclear (Ambion) and microinjected into defolliculated *X. laevis* oocytes (50 ng RNA/oocyte). The oocytes were kept in Kulori medium (in mM: 90 NaCl, 1 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4) for 5–6 days at 19°C before experiments.

NKCC2 activity assay

To activate NKCC2 before the uptake experiments, hNKCC2A-expressing oocytes or uninjected control oocytes were pre-incubated 30 min at room temperature in a K^+-free solution containing (in mM: 5 choline chloride, 95 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.4, 207 mOsm) 5–15 oocytes per well. To measure K^+ influx, oocytes were exposed to an isosmotic test solution in which KCl was substituted for choline chloride and 2–3 $\mu Ci \cdot mL^{-1}$ $^{86}Rb^+$ (NEZ072; PerkinElmer, Germany) included as a tracer for K^+. Osmolarities of the test media were verified by using an automatic osmometer type 15 (Löser; Berlin, Germany). Bumetanide (0.03–100 μM) (B3023, Sigma-Aldrich), its derivatives (1–100 μM), or control vehicle (≤1% relevant drug solvent) was added to the test solution. The uptake assay was performed at room temperature with mild agitation for 5 min, which we have demonstrated to be within the linear phase of K^+ uptake (data not shown, and Zeuthen and MacAulay, 2012). The influx experiments were terminated by rapid washing three times in ice-cold $^{86}Rb^+$-free assay solution after which the oocytes were individually dissolved in 200 μL 10% sodium dodecyl sulfate in scintillation vials. The radioactivity present was determined by liquid scintillation β-counting with Ultima Gold XR scintillation liquid (6013199; PerkinElmer, Waltham, MA, USA) using a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer). hNKCC2A-mediated K^+ uptake was assessed as ([$flux_{NKCC2\text{-}expressing\ oocytes}$ in the presence of x μM drug]-[$flux_{uninjected\ oocytes}$ in the presence of x μM drug]) in order to correct for endogenous NKCC activity.

Data analysis

Sigmoidal curves were constructed for determination of the IC_{50} value for bumetanide and its derivatives on hNKCC2A using GraphPad Prism 6.0, assuming the curves would reach complete inhibition [going from 100% to 0 according to a dose–response inhibition curve with log(inhibitor) vs. response, variable slope; Y = 100/(1 + 10∧((LogIC50-X)*HillSlope))]. As we only had access to limited amounts of the bumetanide derivatives, these drugs were only tested in two to four concentrations, that is, in the range from 1 to 100 μM. With this limited number of data points, we only assigned an *estimation* of the IC_{50} values to these data. To distinguish these IC_{50} values from the ones obtained from a full data set, the estimated values are referred to as eIC_{50}. eIC_{50} values (or IC_{50} in case of bumetanide) were obtained from each individual experiment and averaged across all experiments (at least three) with the given drug to obtain the average eIC_{50} ± SEM. Although different batches of oocytes express NKCC2 to different levels, we did not observe any trends towards batch-specific differences in the eIC_{50}s and the difference in expression level should thus not affect the obtained values. All representative experiments are shown with mean ± SD whereas pooled experiments and IC_{50} values are presented as means ± SEM.

Results

Inhibitory potency of bumetanide in the hNKCC2A assay

To obtain an experimental system in which to determine the transport activity of NKCC2, and the inhibitory potency of a range of diuretics on hNKCC2A activity, with minor contamination of endogenous NKCC activity, we employed the *X. laevis* oocyte expression system. Heterologous expression of hNKCC2A in *Xenopus* oocytes increased the $^{86}Rb^+$ uptake 6.3 ± 0.4-fold (n = 37) of that of the uninjected oocytes (a representative experiment is illustrated in Figure 2A). Bumetanide displayed dose-dependent inhibition of $^{86}Rb^+$ uptake in NKCC2-expressing oocytes (Figure 2A); at 100 μM, the $^{86}Rb^+$ uptake was reduced to background levels, as previously observed (Zeuthen and MacAulay, 2012). Bumetanide caused, in addition, a reduction of the $^{86}Rb^+$ uptake in the uninjected oocytes (Figure 2A), which is compatible with the low levels of endogenous NKCC expressed by *Xenopus* oocytes (Suvitayavat *et al.*, 1994). For determination of the IC_{50} for hNKCC2A (see Methods), the $^{86}Rb^+$ uptake in uninjected oocytes was, for all bumetanide concentrations, established in parallel and subsequently subtracted from the $^{86}Rb^+$ uptake obtained in the NKCC2-expressing oocytes (data summarized for illustrative purposes in Figure 2B, n = 6). The hNKCC2A displayed an IC_{50} for bumetanide of 4.0 ± 1.0 μM (calculated individually from n = 6).

Inhibitory potency of bumetanide derivatives in the hNKCC2A assay

Only limited samples of bumetanide derivatives were available to us and since only freshly prepared solutions were used for each experiment, a restricted number of drug concentrations could be tested for each compound. Therefore, we decided to test each derivative at 1 and 10 μM. If no clear hNKCC2A inhibition was seen, the concentration was increased to 100 μM. Further increase in concentration was not possible because of the limited solubility of most of the

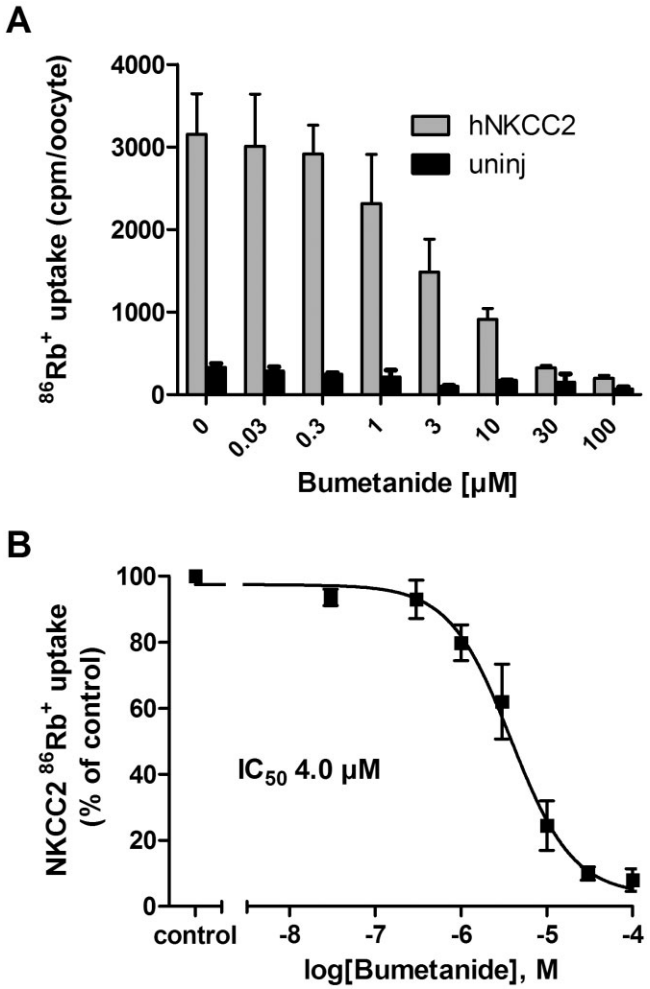


Figure 2

Effect of bumetanide on hNKCC2A-mediated $^{86}Rb^+$ uptake in *Xenopus* oocytes. (A) A representative experiment demonstrating the inhibitory effect of 0.03–100 μM bumetanide on $^{86}Rb^+$ uptake (in cpm, counted for 10 min) in hNKCC2A-expressing oocytes and on batch-matched uninjected oocytes, n = 5–10 oocytes per condition and error bars are SDs. (B) Dose–inhibition curve of bumetanide on hNKCC2A-mediated $^{86}Rb^+$ uptake (corrected for endogenous NKCC contribution in uninjected oocytes) normalized to control (0 μM bumetanide) and averaged across six experiments, with the IC_{50} calculated from each individual experiment prior to averaging.

bumetanide derivatives. If not otherwise indicated, all experiments were repeated at least three times, although for the two drugs (PF-1659 and BUM13), in which an initial experiment with 100 μM yielded no reduction in NKCC2-mediated $^{86}Rb^+$ uptake, the experiment was not repeated (n = 1 for 100 μM). In addition to the three concentrations shown in Figure 3, BUM13 was also tested at 20 μM, indicating no inhibitory effect on hNKCC2A (not illustrated). Representative experiments are shown for the nine derivatives in Figure 3. Seven of the nine bumetanide derivatives inhibited NKCC2 at the concentrations applied, although at widely varying potencies. To allow comparison with bumetanide (Figure 2B) and to correlate to diuretic potency, we estimated IC_{50}s from the concentration–response experiments of each derivative and assigned these eIC_{50} to distinguish these estimates from the IC_{50} obtained with a full data set for bumetanide (see Methods) (Table 1).

For discussion of the inhibitory potency, the bumetanide derivatives were grouped according to the positions (R1, R3, R4, R5) within the bumetanide structure at which side-chain modifications were performed (Table 1 and Figure 1, indicated on graphs in Figure 3). Two compounds were modified in position 1 of the molecule (R1 analogues; PF-2178 and BUM13), three had different substituents in position 3 only (R3 analogues; PF-1712, PF-1659 and PF-1962), three had different substituents in positions 3 and 4 (R3/R4 analogues; HH-562, PF-1573 and PF-1574), and one had different substituents in positions 3 and 5 (R3/R5 analogues; PF-1730). Figure 1 also indicates the lipophilicity (log*P*) of these compounds. As indicated in Figure 1 legend, the acidic dissociation constant, p*K*a, of bumetanide and its derivatives was similar (~3.6) except for PF-2178 (0.7) and BUM13 (7.0).

R1 analogues. In R1 analogues (PF-2178 and BUM13), the carboxylic residue of bumetanide was substituted. PF-2178, in which the carboxylic group was replaced by sulfonic acid, was a less potent hNKCC2A inhibitor than bumetanide (eIC_{50} 7.2 μM; relative potency vs. bumetanide 0.56; Table 1), whereas its diuretic activity in dogs was similar to that of bumetanide (relative diuretic potency 0.925; Table 1), indicating that the carboxylic group is not a prerequisite for high diuretic activity. However, BUM13, in which the carboxylic group was replaced by an anilinomethyl group, did not inhibit hNKCC2A in concentrations up to 100 μM (Figure 3). Its diuretic activity was only about one-tenth of that of bumetanide (Table 1).

R3 analogues. R3 analogues (PF-1712, PF-1659, PF-1962) were substituted in the 3-butylamino side chain of bumetanide. PF-1712, in which this side chain was prolonged to a pentylamino group, did not differ in its hNKCC2A inhibitory potency (eIC_{50} 3.7 μM) from bumetanide, and was only moderately less diuretic than bumetanide (Table 1). In contrast, PF-1659, in which the butylamino side chain was replaced by a 1,3-benzodioxol-5-ylmethylamino group, was devoid of any hNKCC2A inhibitory potency (Figure 3) and almost inactive as a diuretic in dogs (Table 1). PF-1962, in which the butylamino side chain was replaced by a butylthio side chain (Figure 1), displayed around four times higher potency towards NKCC2 inhibition than bumetanide (eIC_{50} 1.1 μM), while the diuretic activity was comparable (Table 1).

R3/R4 analogues. All analogues of this type (HH-562, PF-1574, PF-1573) contained a benzylamino residue instead of the butylamino side chain of bumetanide at position 3 (Figure 1). One compound, HH-562, lacked the phenoxy substituent at R position 4, which was replaced by a chloride atom. These modifications markedly reduced the potency to inhibit hNKCC2A (eIC_{50} 62.6 μM) as well as the diuretic activity compared with bumetanide (Table 1). When the phenoxy substituent of bumetanide was replaced by a 4-chloroanilino group (PF-1573), the inhibitory potency for hNKCC2A was almost doubled (eIC_{50} 2.3 μM) compared with

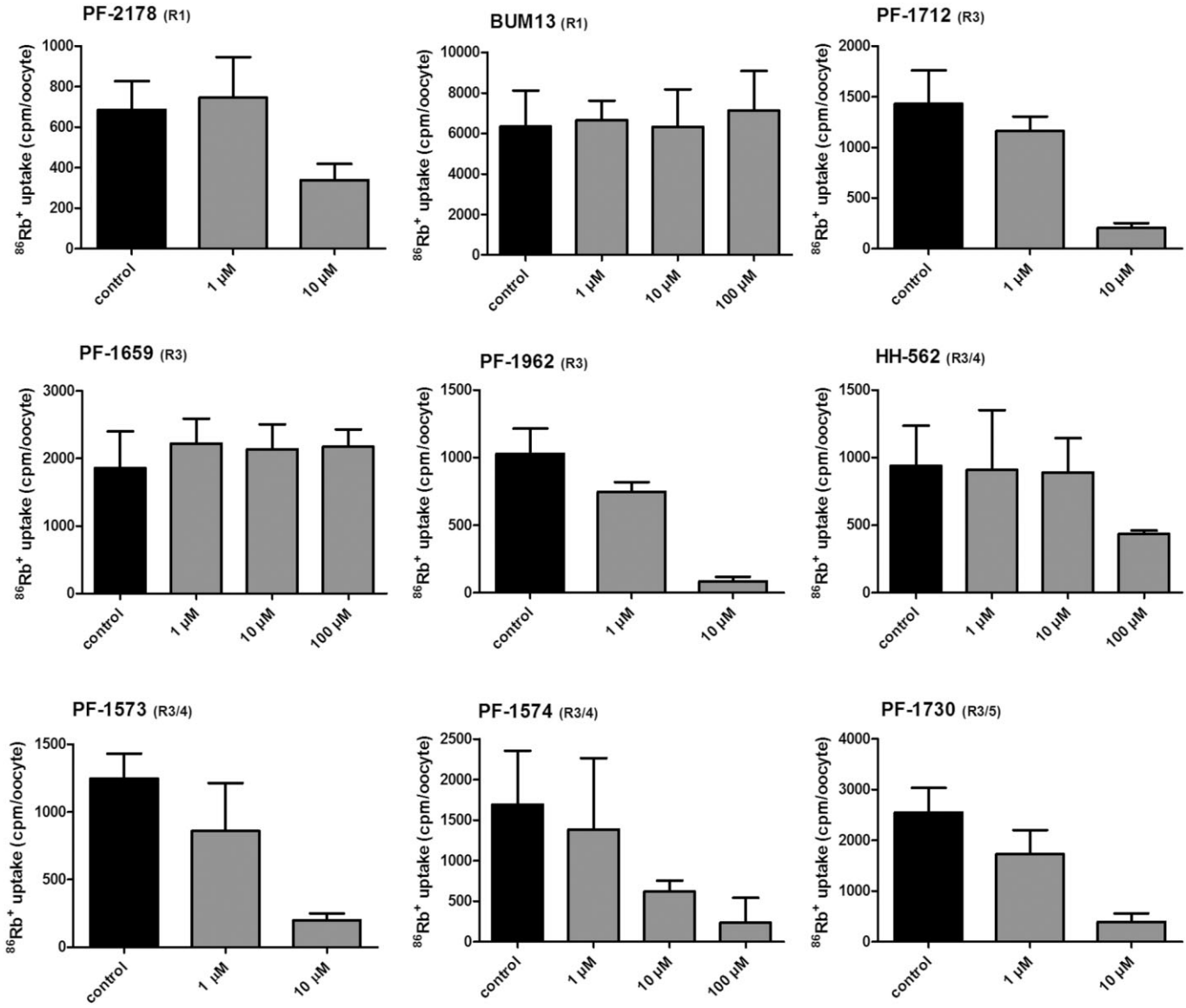


Figure 3

Inhibitory effect of bumetanide derivatives on hNKCC2A activity in the *Xenopus* oocyte assay. $^{86}Rb^{+}$ uptake was measured in the absence or presence of different drug concentrations. Data are shown as a representative experiment (out of at least three identical experiments) with means ± SD from 5 to 15 oocytes per condition in each experiment. For PF-1659 and BUM13, the highest concentration of the compound (100 μM) was only tested once due to lack of inhibitory effect.

that of bumetanide, whereas the diuretic activity in the dog assay was slightly lower (Table 1). When the phenoxy substituent of bumetanide was replaced by a 2,4-dimethylanilino group (PF-1574), both hNKCC2A inhibitory potency (eIC_{50} 12.2 μM) and diuretic activity were considerably reduced (Table 1).

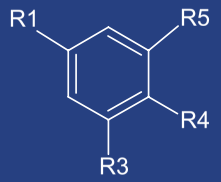
R3/R5 analogues. As with the R3/R4 analogues, the butylamino group in position 3 was replaced by a benzylamino residue; furthermore, the sulfamoyl group in position 5, which is not a prerequisite for the loop diuretic activity of bumetanide (Nielsen and Feit, 1978), was replaced by a methylsulfonyl group, resulting in PF-1730 (Figure 1). This compound was almost twice as potent as bumetanide to inhibit hNKCC2A (eIC_{50} 2.2 μM), but slightly less potent in the dog assay (Table 1).

Diuretic activity in dogs

In order to determine whether the hNKCC2A inhibitory potencies of the various bumetanide derivatives correlated with their *in vivo* diuretic activities, we used previously reported data from the dog assay. The dog assay used to determine the diuretic activity of bumetanide and its derivatives has been described in detail by Ostergaard *et al*. (1972).

Table 1

Diuretic effect of bumetanide derivatives with modifications in R1, R3, R4 or R5 position and inhibitory effect of these derivatives on hNKCC2A



Compound	R1	R3	R4	R5	Diuretic effect in dogs (mL·kg^{-1} or mEq·kg^{-1} per 3 h) Dose (mg·kg^{-1} i.v.)	H_2O (mL·kg^{-1})	Na^+ (mEq·kg^{-1})	K^+ (mEq·kg^{-1})	Cl^- (mEq·kg^{-1})	Relative diuretic potency (based on Na^+ excretion) (bumetanide = 1)	eIC_{50} for hNKCC2A eIC_{50} (μM) ± SEM	Relative to bumetanide (=1)
Control					–	0.93	0.10	0.16	0.08			
Control					–	2	0.19	0.13	0.13			
Bumetanide (PF-1593)	–COOH	$-NH(CH_2)_3CH_3$	$-O-C_6H_5$	$-SO_2NH_2$	0.25	39	4.0	0.84	5.7	1	4.0 ± 1.0	1
					0.1	26	2.4	0.44	3.5			
					0.01	10.0	0.92	0.27	1.4			
	Modification in R1 position											
PF-2178	$-SO_3H$	$-NH(CH_2)_3CH_3$	$-O-C_6H_5$	$-SO_2NH_2$	0.25		3.7			0.925	7.2 ± 2.1	0.56
BUM13	$-CH_2NHC_6H_5$	$-NH(CH_2)_3CH_3$	$-O-C_6H_5$	$-SO_2NH_2$	1	33 (6 h)	4.5 (6 h)	1.15 (6 h)	5.4 (6 h)	0.14	>100	<0.04
	Modification in R3 position											
PF-1712	–COOH	$-NH(CH_2)_4CH_3$	$-O-C_6H_5$	$-SO_2NH_2$	0.25	29	2.9	0.6	4.5	0.725	3.7 ± 1.7	1.08
PF-1659	–COOH	$-NHCH_2C_6H_3$, 3,4(–O–CH_2–O–)	$-O-C_6H_5$	$-SO_2NH_2$	0.25	2	0.1	0.3	0.3	0.025	>100	<0.04
PF-1962	–COOH	$-S(CH_2)_3CH_3$	$-O-C_6H_5$	$-SO_2NH_2$	0.1	25.7	2.5	0.57	3.5	1.04	1.1 ± 0.3	3.8
	Modification in R3 and R4 positions											
HH-562	–COOH	$-NHCH_2C_6H_5$	–Cl	$-SO_2NH_2$	0.25	5	0.5	0.2	0.6	0.125	62.6 ± 18.6	0.06
					10	21	2.0	0.53	2.6			
PF-1573	–COOH	$-NHCH_2C_6H_5$	NHC_6H_5, 4–Cl	$-SO_2NH_2$	0.25	26	2.9	0.6	4	0.725	2.3 ± 1.1	1.7
PF-1574	–COOH	$-NHCH_2C_6H_5$	NHC_6H_5, 2–CH_3, 4–CH_3	$-SO_2NH_2$	0.25	2	0.4	0.4	0.4	0.1	12.2 ± 2.8	0.33
	Modification in R3 and R5 positions											
PF-1730	–COOH	$-NHCH_2C_6H_5$	$-O-C_6H_5$	$-SO_2CH_3$	0.25	21	3.0	0.99	5.1	0.75	2.2 ± 1.1	1.8
					0.1	9	1.1	0.20	1.4			
					1	31	4.8	0.98	5.1			

Compounds were injected i.v. at a dose of 0.25 mg·kg^{-1} (dissolved by means of NaOH) and the urine volume and electrolyte excretion were determined over 3 h (in some experiments over 6 h). Depending on diuretic activity, some compounds were tested at lower or higher doses. Diuretic data shown in Table 1 were taken from the following publications of P.W. Feit and H.-H. Frey's group at Leo Pharma (Ballerup, Denmark): controls, Ostergaard *et al*. (1972), Feit *et al*. (1974) and Feit *et al*. (1970); bumetanide (PF-1593), Ostergaard *et al*. (1972); PF-2178, Palfrey *et al*. (1980); BUM13, Nielsen and Feit (1978); PF-1712, Feit (1971); PF-1659, Feit (1971); PF-1962, Feit *et al*. (1974); HH-562, Feit *et al*. (1970) and Feit (1971); PF-1573, Feit (1971); PF-1574, Feit (1971); PF-1730, Feit and Nielsen (1976).

For calculating the diuretic potency of the derivatives relative to bumetanide (Table 1), the Na^+ excretion measured after 0.25 mg·kg^{-1} was used. In case that a compound was not tested at 0.25 mg·kg^{-1} (BUM13, PF-1962), the diuretic potency of the derivative was compared with that of bumetanide at this dose (0.1 mg·kg^{-1}; PF-1962) or the difference in dosing was taken into account (assuming that the dose–effect relationship is linear) when calculating relative diuretic potency (BUM13, only tested at 1 mg·kg^{-1}).

As shown in Table 1, most bumetanide derivatives exerted a lower diuretic activity than bumetanide in the dog assay. Only PF-1962 and PF-2178 exhibited potency comparable to bumetanide.

Correlation between inhibitory potency of bumetanide derivatives on hNKCC2A activity and their diuretic activity in dogs

To obtain a relation between the diuretic potency of the bumetanide and its nine tested derivatives and their ability to inhibit NKCC2 transport activity, their diuretic potency (relative to that of bumetanide) was plotted as a function of the eIC_{50}s (Figure 4). Non-linear correlation analysis of these two parameters yielded a correlation coefficient (r^2) of 0.817, $P < 0.01$, indicating a significant correlation between the inhibitory potency of these diuretics on the two experimental systems.

Differences in lipophilicity (log*P*; see Figure 1), which may affect the relative membrane permeability of the compounds, were not correlated with IC_{50} values for hNKCC2A ($r^2 = 0.02032$). Furthermore, log*P* was not significantly correlated with diuretic activity in the dog assay ($r^2 = 0.06696$). The same was true for the acidic dissociation constant, p*K*a. The most acidic compound (PF-2178) was only slightly less potent in the dog assay than bumetanide (Table 1).

Discussion

The Na-K-2Cl cotransporter, isoform 2 (*SLC12A1*; NKCC2), is the major salt transport pathway in the apical membrane of the mammalian TAL and the clinical target for loop diuretics such as bumetanide and furosemide (Alvarez-Leefmans, 2012; Markadieu and Delpire, 2014). The sensitivity to bumetanide of the three full-length spliced variants of NKCC2 (A, B, F) has previously been studied in the *Xenopus* oocyte heterologous expression system and all variants

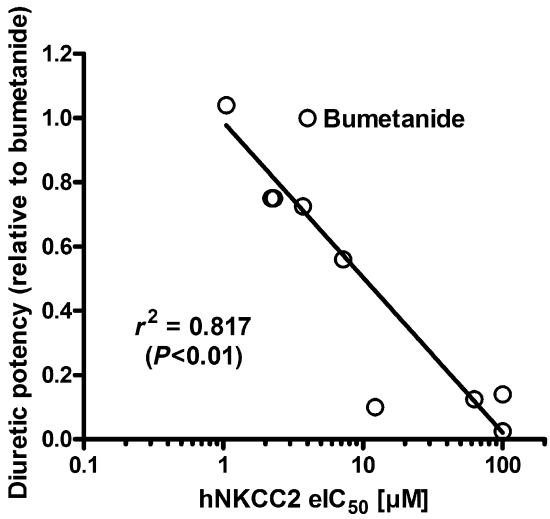


Figure 4

Correlation between log IC_{50} (or eIC_{50}) for inhibition of hNKCC2A in the *Xenopus* oocyte assay and diuretic potency in the dog assay for the 10 compounds used in this study (see Figure 1 and Table 1).

expressed about the same sensitivity to this diuretic drug (Alvarez-Leefmans, 2012). In the present study, the human NKCC2A variant was inhibited by bumetanide with an IC_{50} of 4 μM, which is considerably higher than the 0.54 μM reported by Carota *et al*. (2010) for hNKCC2A expressed in *Xenopus* oocytes but similar to the 2 μM reported for the mouse NKCC2A by Plata *et al*. (2002). In order not to obscure the factual transport limit, and thus skew the IC_{50} obtained for the transport, we determined the NKCC2-mediated transport within the linear part of the time-dependent uptake curve (to prevent time-dependent saturation) and under conditions in which intracellular [Na^+] is expected to be constant due to undisturbed Na^+/K^+-ATPase activity. Plata *et al*. (2002) and Carota *et al*. (2010) included the Na^+/K^+-ATPase inhibitor ouabain in the transport assay, which was carried out with prolonged uptake times (45–60 min), during which the accumulated $[Na^+]_i$ could not be expelled by the blocked Na^+/K^+-ATPase. The NKCC1-mediated accumulation of intracellular [Na^+] would alter the overall net free energy (kJ·mol^{-1}), providing the driving force for NKCC2 activity, and thus decrease the NKCC2 cotransporter activity and possibly shift the IC_{50} of bumetanide to the lower value reported by Carota *et al*. (2010).

To our knowledge, the sensitivity of NKCC2 to bumetanide derivatives has not been reported previously. Overall, we found a significant correlation between inhibition of hNKCC2A in the *Xenopus* oocyte heterologous expression system and diuretic potency in the *in vivo* dog assay. Only three of the nine derivatives, PF-1962, PF-1573 and PF-1730, were more potent as inhibitor of hNKCC2A than bumetanide. However, this higher NKCC2 inhibitory potency did not translate into a higher diuretic activity in dogs. For instance, PF-1730 was almost twice as potent as bumetanide against hNKCC2A, but its relative diuretic potency was only 0.75. One possible explanation is a difference in bumetanide sensitivity between human and dog

NKCC2. Another possibility is that diuretic activity of bumetanide and its derivatives in dogs depends not only on inhibition of NKCC2 but also on carrier-mediated transport from blood into the kidney (Burckhardt, 2012). Bumetanide is 99% negatively charged at pH 7.4, as the acidic dissociation constant (p*K*a) of the carboxylic group is 3.6, whereas the p*K*a of the amino group is 7.7 (Orita *et al.*, 1976). As a consequence of its high ionization rate at pH 7.4 and extensive plasma protein binding (~97–98%), which restrict membrane penetration from blood into tissues by passive diffusion, bumetanide penetrates only poorly into most tissues, including the brain (Löscher *et al.*, 2013; Puskarjov *et al.*, 2014). In contrast, bumetanide rapidly accumulates in kidney and liver (Cohen *et al.*, 1976) as a result of carrier-mediated transport by organic anion transporters (OATs) in the kidney and organic anion-transporting polypeptides (OATPs) in the liver (Petzinger *et al.*, 1996; VanWert *et al.*, 2010; Burckhardt, 2012). The affinity of bumetanide derivatives to OATs responsible for bumetanide uptake into the kidney is not known, but Petzinger *et al.* (1993) studied the interaction of various bumetanide derivatives, including most of the compounds used in the present study, with a transport system for bile acids in isolated rat hepatocytes. No correlation was found between the diuretic potency of bumetanide derivatives and their affinity for the hepatic bile salt transport system (Petzinger *et al.*, 1993).

With respect to the present correlation between the natriuretic effect of bumetanide and its derivatives, as measured *in vivo* in the dog assay system, and their eIC_{50} values in the *in vitro* hNKCC2A expression system, it is possible that an even better correlation would have been obtained if the IC_{50} values had been compared with the renal concentration of each compound that caused a fixed natriuretic response. However, this would require a complete dose–response curve and information on the metabolic fate of each compound. Nevertheless, compounds eliciting weak diuretic responses proved to be poor inhibitors of NKCC2, while compounds promoting active diuresis inhibited NKCC2 at low concentrations (Figure 4 and Table 1). It must be kept in mind that the eIC_{50}s were obtained from only two to four drug concentrations due to our limited access to these derivatives, and that their value could shift slightly if based on a full inhibitory curve.

The nature of the specific substituents at the R3 and R4 positions had a marked effect on the potency of the molecule. Based on these results, it could be stated that a butylthio substituent in R3 is most favourable. Exchange of the phenoxy group for a 4-chloroanilino group yielded better NKCC2 inhibition results, compared with bumetanide. Compounds like PF-1730 with a methylsulfonyl group instead of sulfamoyl group in position 5 should be considered most interesting drug candidates. Furthermore, an anionic function at R1 appears to be essential for activity. Both the active carboxylic acid and the sulfonic acid derivatives are ionized at physiological pH, but when this position is substituted with a non-ionic residue, for example, an anilinomethyl group as in BUM13, inhibitory activity against hNKCC2A was totally lost. Related substituted aryl- or alkyl-amino-methyl derivatives (see Nielsen and Feit, 1978) produce considerable diuresis but may require metabolism to their corresponding benzoic acids before showing diuretic activity. Thus, the finding that BUM13, which lacks an acidic group at R1, still exerts a relatively potent diuretic effect is most likely a consequence of metabolism to bumetanide, which is substantiated by experiments in mice (C. Brandt, K. Töllner and W. Löscher, unpubl. data). For a more extensive discussion of the structure–activity relationships of these compounds as diuretics, the reader is referred to a previous review by Nielsen and Feit (1978).

In addition to structural requirements for hNKCC2A inhibition, it may be possible to explain the differences between the potencies of the analogues in terms of differences in their relative membrane permeability. This impinges on the issue of sidedness of the bumetanide binding site. If as in NKCC1, the high affinity binding sites for bumetanide are on the cytoplasmic side of the translocation cavity (Somasekharan *et al.*, 2012), differences in relative permeability should be considered in addition to differences in chemical structure. Drug lipophilicity is a very important descriptor governing permeation across a biological membrane (Mälkiä *et al.*, 2004). Lipophilicity is generally expressed quantitatively as the $\log_{10}$ of the partitioning of a neutral drug species between n-octanol and water (log*P*) and is the most widely used predictor for drug permeation. For the present compounds, log*P* was neither correlated with their hNKCC2A inhibitory potency in the oocyte expression system nor their diuretic potency in the dog assay. In addition to lipophilicity, passive membrane transport is affected by ionization of the compound in that the passive membrane transport of ions is usually much slower than that of neutral compounds (Mälkiä *et al.*, 2004). Except BUM13, all of the bumetanide derivatives evaluated in this study are highly ionized at plasma pH because of their carboxylic or, in case of PF-2178, sulfonic acid group (Figure 1). Hence, the most acidic compound (PF-2178) was only moderately less potent than bumetanide, which, together with the data from log*P*, indicates that differences in passive membrane permeability play no major role in the differences between the potencies of the analogues observed in this study. Instead, as discussed above, carrier-mediated transport of bumetanide and its derivatives in the kidney is a prerequisite of any diuretic activity, so that we cannot exclude the possibility that differences in such OAT-mediated transport among the derivatives are involved in the differences in their diuretic potency in the dog assay, nor whether putative endogenous expression of OATs in the plasma membrane of *Xenopus* oocytes might affect our data. These oocytes possess a large variety of endogenous membrane transporters (Sobczak *et al.*, 2010) and active organic anion transport has been suggested from taurocholate efflux experiments (Shneider and Moyer, 1993), but we are not aware of any studies to the effect of mapping endogenous expression of OATs or OATPs in *Xenopus oocyte* plasma membranes.

Only one of the nine bumetanide derivatives evaluated in the present study (PF-2178) has previously been tested for inhibition of a NKCC isoform (Palfrey *et al.*, 1980). In this study, a series of diuretically active substituted 3-aminobenzoic acid derivatives and related compounds was investigated on a cyclic AMP-activated Na^+-K^+ cotransport system in avian erythrocytes (Palfrey *et al.*, 1980), most likely representing NKCC1 that is known to be expressed by erythrocytes of different species, including humans (Flatman and

Creanor, 1999; Hannaert *et al.*, 2002; Matskevich *et al.*, 2005; Garay and Alda, 2007). Palfrey *et al.* (1980) reported a good correlation between the diuretic potency of loop diuretics in the dog and their inhibition of cation cotransport in turkey erythrocytes. However, bumetanide was about 20 times more potent than PF-2178 as an inhibitor of cation transport in the avian erythrocytes, whereas the diuretic potency of the two compounds in dogs is almost the same (Palfrey *et al.*, 1980). Therefore, the correlation between diuretic activity and inhibition of avian NKCC1 was poor for some of the tested compounds. In the present study, PF-2178 was only slightly less potent than bumetanide in inhibiting hNKCC2A, indicating that the hNKCC2A expression system used is a useful predictor of diuretic potency of such compounds. Indeed, although bumetanide inhibits NKCC1 and NKCC2 at about the same potency (Alvarez-Leefmans, 2012), putative isoform-specific inhibitory potencies are not known for bumetanide derivatives. Any correlation with diuretic activity should thus relate to the actual renal target of such compounds, the transporter NKCC2.

From a clinical perspective, it would be interesting to search for bumetanide derivatives with high diuretic potency but low or absent inhibitory effects on NKCC1. Bumetanide, particularly when administered at high doses, is associated with a potential risk of ototoxicity, resulting in a temporary or, in some cases, a permanent loss of hearing in patients, including infants (Ward and Heel, 1984; Rybak, 1993; Rybak and Ramkumar, 2007; Pressler *et al.*, 2015). The ototoxicity of bumetanide is markedly increased by concomitant use of aminoglycoside antibiotics such as gentamycin (Rybak and Ramkumar, 2007; Pressler *et al.*, 2015). Inhibition of NKCC1 by bumetanide has been proposed as a cause of deafness by blocking the generation of the endocochlear potential which is necessary for cochlear amplification (Delpire *et al.*, 1999). Thus, compounds that potently inhibit NKCC2, but not NKCC1, would be interesting candidates in the search of loop diuretics without ototoxicity. It remains to be studied whether PF-1712, PF-1962 and PF-1730, which were the most potent hNKCC2A inhibitors found in this study, are less potent inhibitors of hNKCC1 than bumetanide.

On the other hand, bumetanide derivatives with selectivity for NKCC1 versus NKCC2 would be of high clinical interest because of the recent clinical use of bumetanide in treatment of brain disorders, such as neonatal seizures, epilepsy or autism, in which abnormal function of NKCC1 has been implicated (Kahle and Staley, 2008; Kahle *et al.*, 2008; Ben Ari, 2012; Miles *et al.*, 2012; Löscher *et al.*, 2013; Pressler and Mangum, 2013; Kaila *et al.*, 2014; Puskarjov *et al.*, 2014). Use of bumetanide for treatment of brain disorders is associated with problems including poor brain penetration and systemic adverse effects such as diuresis, hypokalemic alkalosis and hearing loss (Löscher *et al.*, 2013; Puskarjov *et al.*, 2014). Thus, a bumetanide derivative with better brain penetration, high affinity for neuronal NKCC1, but lower diuretic activity than bumetanide would be an interesting option (Löscher *et al.*, 2013; Töllner *et al.*, 2014). It remains to be studied whether the structural requirements for inhibition of NKCC1 differ from those of NKCC2.

In conclusion, as shown by the present structure–activity relationships of bumetanide derivatives in the *X. laevis* oocyte expression system, an acidic group at R1 is a requirement for inhibition of NKCC2. We also show that modifications at the R3 or R5 position of bumetanide can lead to compounds which are more potent hNKCC2A inhibitors than the parent drug, which is associated with high-ceiling diuretic activity. Our investigation on bumetanide derivatives has established a close correlation between the inhibition of hNKCC2A in *Xenopus* oocytes and the diuretic potency *in vivo* and allows analysis of the structural requirements for potent inhibition of NKCC2.

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Author contributions

K. L. performed the *in vitro* experiments, analysed and interpreted the data, and critically revised the manuscript. K. T. compiled the *in vivo* experimental data, interpreted the data and critically revised the manuscript. K. R. performed the drug solution experiments, interpreted the data and critically revised the manuscript. P. W. F. substantially contributed to the conception of the work, synthesized most of the bumetanide derivatives, interpreted the data and critically revised the manuscript. T. E. synthesized part of the bumetanide derivatives, calculated and interpreted part of the data, and critically revised the manuscript. N. M. designed the study, interpreted the data and critically revised the manuscript. W. L. designed the study, interpreted the data and wrote the manuscript.

Conflict of interest

The authors have no conflicts to report.

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